Diseases in Asian Aquaculture VI

Editors

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Message from the President, Asian Fisheries Society

It is my great pleasure to address the publication of the proceedings of the Sixth Symposium on Diseases in Asian Aquaculture (DAA VI) organized by the Fish Health Section (FHS) of the Asian Fisheries Society (AFS) to promote high quality scientific research and the exchange of technical information among government and university scientists, diagnosticians, national policy makers and the members of the private sector.

With the theme "aquatic animal health – facing new challenges", the DAA VI was successfully held on 25 - 28 October 2005 in Colombo, Sri Lanka, and participated by more than 180 delegates from Asia and other parts of the world. Twelve keynote presentations, more than 110 oral/poster presentations and a number of pre- and post-symposium meeting and workshops organized jointly with regional and international organizations made DAA VI a big success. On behalf of the AFS, I would like to congratulate the AFS-FHS on holding DAA VI and publishing the proceedings, and would like to thank the great efforts of the organizing team led by Dr. Melba B. Reantaso, the AFS-FHS executive committee, the Local Organizing Committee by Dr. Rohana P. Subasinghe and many Sri Lankan colleagues, all sponsors and supporters, and all participants especially the contributing authors, reviewing experts and editors.

Asia is the leading aquaculture production region in the world, in terms of both volume and value. Health management of aquatic animals has been an important and critical issue to be addressed in the aquaculture industry especially for Asian aquaculture. To achieve the security and safety of farmed aquatic products as well as the sustainable aquaculture development, we need to be constantly vigilant of advances and innovations in fish health management, diagnosis and control.

The FHS has been taking a lead in the health management of aquatic animals in Asia since it was founded in 1989, through encouraging awareness, providing continuing professional service and training to Asian aquaculturists and upgrading their diagnostic skills and control methods towards the sustainable development of Asian aquaculture.

I strongly recommend the DAA VI proceedings to all aquaculture professionals, researchers, students and commercial operators, as it surely provides a great deal of information and a relevant reference on diseases in aquaculture.

May I take this opportunity to state that AFS, together with its sections and branches can make a difference to the aquaculture industry in Asia by working collaboratively together and with other national, regional and international agencies.

Prof. Yang Yi, Ph.D.
President, Ninth Council of the Asian Fisheries Society (2007-2010)
June 2008

Message from the Chairperson, Fish Health Section of the AFS

The Sixth Symposium on Diseases in Asian Aquaculture (DAA VI) with the theme "Aquatic animal health – facing new challenges – was successfully held in Colombo, Sri Lanka. The symposium was officially opened by the Honorable Milroy Fernando, Sri Lanka's Minister of Fisheries and Aquatic Resources, with the lighting of candles, a number of speeches and welcome remarks and ending with a Sri Lankan dance. Another milestone symposium of the Fish Health Section, DAA VI proper was held from 25-28 October 2005; a number of pre- and post-DAA VI activities included the 4th Meeting of NACA's Advisory Group on Aquatic Animal Health (23-24 October), a Training Workshop on Fish Disease Diagnosis and Health Management (23-24 October), and the FAO Expert Workshop on Development of CCRF Health Management Guidelines (1-4 November), making the whole event run through almost three weeks of work and fun.

With more than 180 delegates from 27 countries participating (Australia, Bangladesh, Brunei, Canada, China, Hungary, India, Indonesia, Iran, Israel, Italy, Japan, Korea RO, Malaysia, Mexico, Nepal, New Zealand, Philippines, Norway, Saudi Arabia, Singapore, Sri Lanka, Taiwan, Thailand, U.K., USA and Vietnam), 12 keynote presentations and more than 110 oral/poster presentations all made DAA VI another big success. Six generous sponsors - *Major Sponsor* Farming IntelliGene Tech. Corp., *Sponsors* Intervet, Uni-President Enterprises Corp, and Schering-Plough Animal Health (Aquaculture), and *Supporters* Applied Biosystems and Third World Academy of Sciences - made it possible to sponsor 19 students and young scientists (from Australia, Bangladesh, India, Korea RO, Nepal, Philippines, Sri Lanka, Thailand, Vietnam, USA) a long time tradition of the FHS. The local events organizer – Ace Travel and Conventions Ltd. and the website developer AFFNO, the Local Organizing Committee, the International Scientific Programme Committee, the Sponsorship and Travel Awards Committee, and most especially all DAA VI participants who are members, friends, colleagues, supporters and partners of the FHS – should all be commended for a job well done.

I personally thank the FHS Executive Committee (2002-2005) under the leadership of Dr. Melba B. Reantaso for a job well done and a productive term of office.

On behalf of the FHS Executive Committee (2005-2008) and the members of the FHS-AFS, we are pleased to present the proceedings of the Sixth Symposium on Diseases in Asian Aquaculture (DAA VI). I sincerely thank all the authors, reviewers and editors and all who contributed to making this publication a reality. We hope that this sixth volume of the DAA series will continue to provide further insights on aquatic animal health challenges facing the aquaculture sector and mitigating measures and approaches through original research and review papers in our continued quest for maintaining healthy aquatic food production.

Prof. Takashi Aoki
Chairperson, FHS-AFS Executive Committee (2005-2008)
June 2008

Message from the Editors, DAA VI

A primary requirement for sustaining healthy aquaculture production and responsible product trade is to address health questions with both pro-active and reactive aquatic animal health programmes. The aquaculture sector will continue to be threatened with risks of major disease incursions and newly emerging diseases; and unless appropriate health management measures are continuously put in place and implemented, the government and private sectors will be faced with more costs in terms of production losses and the efforts needed to contain and eradicate diseases, funds that would have been better spent in preventing their entry into the system. It will thus be essential that efforts are channelled towards prevention, on better management practices and on maintaining healthy fish and enhancing biosecurity at all levels of operation. Health management is a shared responsibility, and each stakeholder's contribution is essential to the health management process. The Fish Health Section has a significant role to play in contributing to this process as source of original research and other appropriate health management strategies and through DAA symposia, interaction with fellow researchers/scientists and dissemination and exchange of research and project-related findings.

This sixth volume of the Diseases in Asian Aquaculture series brings together original research and review papers that were presented during the Sixth Symposium on Diseases in Asian Aquaculture (DAA VI) held in Colombo, Sri Lanka from 25-28 October 2005. A total of 45 papers, submitted for inclusion in the proceeding, were sent out to 57 international reviewers. Nine papers were rejected; 36 papers were accepted (with minor and major revisions) forming the contents of this publication.

A voluntary work and a daunting task, we are pleased to be part of this primary publication of our society. We appreciate the opportunity to contribute to this well-established, standardized series (to-date 6 volumes spanning the close to 20 year history of the FHS) that is well known, easily recognized by its distinctive blue and gold volumes, well cited and held in high opinion by fish health workers throughout the world.

We sincerely thank the international expert-reviewers who generously assisted scientists from less developed countries to bring the scientific quality and English usage of their manuscripts up to international standards. Special thanks also goes to Dr. Suppalak, R. Cajilig, Carlos Trabucco and Magda Morales.

We expect that free downloadable copies of all articles will be available at the same time from the FHS website, in support of the 'open archive movement' intended to make science readily available to all, especially for publicly funded research.

We earnestly hope that members and supporters of FHS will continue to dedicate at least one paper once every three years to assist our society and in support of the FHS long standing tradition of producing DAA proceedings.

Melba G. Bondad-Reantaso
C.V. Mohan
Margaret Crumlish
Rohana P. Subasinghe

Biosecurity and Risk Assessment

Maintaining Biosecurity in Aquaculture Systems: A Constraint or a Challenge?

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ABSTRACT

Biosecurity can be defined as the protection of plants, animals (including humans and associated activities) and the wider environment from the unwanted impacts of biological agents including diseases and pests. As a discipline, biosecurity can be applied at various levels. In the context of aquatic animal disease, this can range from managing the health of individual animals, through whole commercial enterprise to national or international biosecurity. The last three decades or so have seen an increase in the farming of aquatic animals worldwide – a situation compounded from a biosecurity perspective by a quantum leap in aquaculture technologies, countries and species new to aquaculture, increased international movement of juvenile animals and broodstock; all in an environment of little knowledge of the health status of source populations and the frequent emergence of new diseases. The end-result of this change has been significant farm level production losses well documented in the scientific and lay literature. The focus on increased farm level biosecurity in recent times has been in direct response to this very real threat. All aquaculture operations rely on trade (commercial exchanges) to some extent. Trade provides stock, genetic material, inputs (such as feeds, vaccines, treatments, etc.) and takes the outputs (product). Aquaculture operations are not isolated from the realities

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of trade and the associated biosecurity risks. This paper describes the various elements that make for good farm level biosecurity and assesses the resourcing needs against net long- and short-term benefits to production. This paper also examines the role that farm biosecurity plays in overall regional or national biosecurity systems, with particular emphasis on the Australian experience. Farm level biosecurity is placed in context with inter- or intra-national disease zoning (and compartmentalisation), national quarantine control and global biosecurity initiatives such as international disease reporting and standards setting. The necessity for on-farm biosecurity as a complement to zoning and the more traditional country quarantine requirements is emphasized.

INTRODUCTION

Historically, international trade was by sea. This trade was slow and was associated with small unit volumes 'man-handled' by stevedores. Quarantine at national borders was relatively easy to maintain, and international trade in aquaculture was also very small (usually eyed ova for restocking). Now, with containers and air-freight, large volumes of agricultural commodities can be moved world-wide in a few hours or days. These commodities, be they live organisms or animal/plant products, can harbour unwanted pests and disease agents. This improved capability in commodity transportation has brought with it the greatly increased risks of spreading pests and pathogens around the world – the increased risk of the globalisation of the world's animal and plant diseases.

This historical change in commodity transportation, the last three decades or so have seen a concurrent increase in the farming of aquatic animals worldwide — a situation compounded by a quantum leap in aquaculture technologies, countries and species new to aquaculture, and increased international movement of juvenile animals and broodstock. For example, well over 200 aquatic animal and plant species were farmed around the world in 2002 (FAO, 2002). Relatively little is known of the health status of most of these species, with frequent emergence of new diseases being reported in the scientific literature (with many incidents going unreported). This is in stark contrast to the handful of familiar and well-studied terrestrial livestock and poultry species farmed around the world, where capability and capacity to manage health is by and large well established.

All aquaculture operations rely on trade to some extent. Most farms are dependent on trade for various inputs such as broodstock, post-larvae/fingerlings and feed. These inputs represent potential pathways by which new pathogens can enter farming systems. In the absence of appropriate regulation, trade (commercial exchange) in high risk commodities has continued with little or no risk management because of commercial pressures, despite the relative lack of knowledge of disease and pest risks, and often without regard to the known risks.

The end-result of this have been severe disease-associated farm level production losses — perhaps none more so than through the well documented spread of white spot disease (WSD) around the world. In 2000, WSD caused estimated global production losses in the order of 200,000 tonnes, valued at approximately US$1 billion (Rosenberry, 2001). The total white spot syndrome virus (WSSV)-associated production loss in Asia between its

first emergence in 1992 and 2001 is an estimated US$4-6 billion (Lightner, 2003). The local and international spread of the causative agent, WSSV has most often been attributed to the movement of live animals, mainly broodstock and post-larval shrimp.

Another example of particular relevance to the region is epizootic ulcerative syndrome (EUS), caused by *Aphanomyces invadans*, considered to have started with the introduction of tilapias into Asia in the 1960s and now spread across Asia (including Australia) and into the USA (Roberts, 2003). The disease is thought to have been introduced into Sri Lanka with imported ornamental fish, a conclusion based primarily on Sri Lanka's island nature, the coincidence of ornamental fish imports form overseas at the time, and its occurrence around 1989 well in advance of its overland spread to India. The focus on increased farm level biosecurity across the aquaculture sector in recent times has been in direct response to this very real threat of disease spread into otherwise free areas, as demonstrated by the WSD and EUS pandemics.

These events are occurring in a global environment of increased public awareness and concerns about biodiversity, resource sustainability and food safety. The World Wildlife Fund, perhaps a moderate among detractors, identifies several environmental and social issues relating to aquaculture, including the following that relate to biosecurity:

⇒potential adverse effects on wild species, including disease transmission
⇒prophylactic use of chemicals, including antibiotics that can harm wildlife and the environment, and may lead to antibiotic resistance.

The subject of aquaculture biosecurity was the subject of a 2001 workshop in Hawaii, sponsored by the US National Oceanic and Atmospheric Administration (NOAA), 'Biosecurity in Aquaculture Production System: Exclusion of Pathogens and Other Undesirables' (Lee and O'Bryen, 2003). The workshop proceedings, published by the World Aquaculture Society (WAS), provide an excellent synopsis of biosecurity issues surrounding aquaculture.

WHAT IS BIOSECURITY?

The literal meaning of biosecurity is 'life protection'. There is no universally accepted definition of 'biosecurity'. Those definitions that do exist are based on needs of specific groups and as such there is no consistency, although there is some overlap. For example, in no particular order of importance:

Biosecurity Australia, the agency responsible for developing Australia's importation policies, does not have a formal definition as yet, but a 'working definition' can be extrapolated from Australia's Quarantine Act:

> *The prevention of the entry, establishment or spread of unwanted pests and infectious disease agents in people, animals, plants or the environment*

New Zealand's equivalent agency, Biosecurity New Zealand, defines biosecurity as:

> *The exclusion, eradication or effective management of risks posed by pests and diseases to the economy, environment and human health. It covers terrestrial, freshwater and marine environments.*

The University of Illinois defines biosecurity as:

> *A process to protect from attack or interference due to biological organisms – that can be applied to yourself, a farm, the state, or our country. [A 'hood-of-the-truck' definition could be shortened to "keeping the bad bugs off the farm"]*

The Wikipedia web encyclopedia states:

> *A biosecurity guarantee attempts to ensure that ecologies sustaining either people or animals are maintained. This may include natural habitats as well as shelter and productive enterprise (especially agriculture) and deals with threats such as biological warfare or epidemics. This is related to the more passive concept of biosafety.*

The Saunder's Comprehensive Veterinary Dictionary defines biosecurity as:

> *Security from transmission of infectious diseases, parasites, and pests*

The US poultry industry's definition is:

> *The cumulative steps taken to keep disease from a farm and to prevent the transmission of disease within an infected farm to neighbouring farms*

In his keynote speech at the 3rd IUCN World Conservation Congress in Bangkok (November 2004), He Changchui, Assistant Director-General and Regional Representative for Asia and the Pacific defined biosecurity as:

> *Management of all biological and environmental risks associated with food and agriculture, including forestry and fisheries. It covers issues related to biosafety, food safety and plant as well as animal health such as recent outbreaks of Avian Influenza…*

The FAO in 'Towards a Food-Secure Asia and Pacific Regional Strategic Framework for Asia and the Pacific' defines biosecurity as:

> *Management of all biological and environmental risks associated with food and agriculture, including forestry and fisheries. It covers issues related to biosafety (reduction of risks associated with the use of products derived from modern biotechnology), food safety and plant as well as animal health...*

The Department of Agriculture – Western Australia in its Cattle Industry Biosecurity Plan – biosecurity is defined as:

> *A set of measures designed to protect a population from transmissible diseases, pests and weeds at national, regional and individual farm levels.*

A Review of Quarantine Systems and Biosecurity Management for the Tasmanian Department of Primary Industries, Water and Environment stated:

> *Biosecurity by its very nature is concerned with addressing risk. Biosecurity is defined as the protection of the economy, environment and health from pests, diseases and weeds.*

An Australian Government Department of Agriculture, Fisheries and Forestry document on 'Marine Biosecurity and Risk Assessment', defined biosecurity as:

> *The activities and strategies concerning protection of native biodiversity. Such activities and strategies encompass barrier control activities such as quarantine efforts, through to post-barrier activities such as monitoring, rapid response (eradication) and longer term control.*

Participants at the 2001 workshop in Hawaii defined biosecurity as:

> *An essential group of tools for the prevention, control and eradication of infectious disease and the preservation of human, animal and environmental health.*

In its broadest sense, biosecurity can be defined as the protection of plants, animals (including humans and associated activities), and the wider environment from the unwanted impacts of biological agents, including diseases and pests. As such, in addition to infectious diseases, 'biosecurity' includes pest issues, genetically modified organisms (GMOs) or other genetic issues, and food safety. It can cover intentional (including illegal) and unintentional actions — biosecurity can even be deal with protective aspects of bioterrorism – *e.g*. the Waihekie Island foot and mouth disease (FMD) hoax in New Zealand.

Given the nature of the symposium presentation on which this paper is based, "diseases in aquaculture", these latter aspects relating to ecological pests, GMOs and bioterrorism are not considered in this presentation although they are significant (perhaps even more significant) issues in their own right.

Within this narrower treatment, the definition of 'biosecurity' can be further narrowed to focus on the exclusion of unwanted pathogens (Lightner, 2003). This paper adopts this more narrow definition, focusing on pathogens and associated infectious diseases of aquatic animals, with 'biosecurity' being the exclusion or keeping out of pathogens from systems that are otherwise free of the pathogens and eradicating pathogens from specified

areas once incursions occur. Aquatic animal health management on the other hand includes aspects of controlling or managing the impacts of pathogens that are effectively 'here to stay'. Biosecurity can therefore be considered a component of aquatic animal health management.

As a discipline, biosecurity can also be applied at various levels. In the context of aquatic animal disease, this can range from managing the health of individual animals, through whole commercial enterprise to national or international biosecurity.

KEY ELEMENTS OF FARM BIOSECURITY

In essence, farm biosecurity has the following three objectives:

⇒ minimising the likelihood of high-risk inputs introducing unwanted pathogens,
⇒ monitoring/surveillance to detect incursions when/if they occur, and
⇒ responding to disease incursions by controlling the spread and eradication of unwanted disease agents.

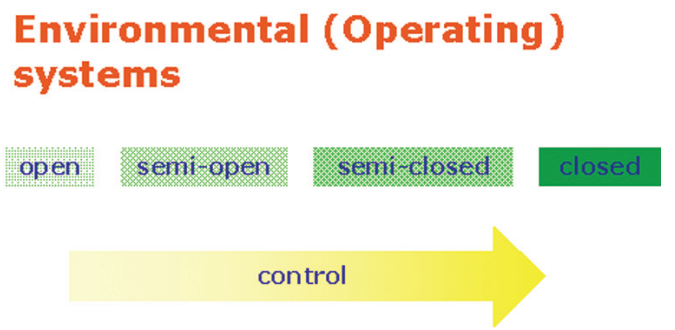
Lee (2003) identified the following key operational elements as needed to achieve these objectives (noting the broader definition of biosecurity as developed by the 2001 Hawaii workshop participants):

⇒ sourcing reliable stocks
⇒ adequate detection/diagnosis
⇒ disinfection and pathogen eradication
⇒ best management practice, and
⇒ practical and acceptable legislation.

Within these elements, a key factor influencing the specific biosecurity measures that could effectively be applied is the degree to which farm stock is (and can be) isolated from the environment — most importantly the level of water exchange. In Australia, national aquatic animal disease emergency response plans have been developed for several pathogens based on farm types categorised into:

⇒ closed, eg recirculation systems
⇒ semi-closed, eg prawn pond culture
⇒ semi-open, eg open-water cage/rope culture, and
⇒ open systems, eg. wild fisheries.

Clearly the level of control that can be applied to closed systems is much greater than for semi-closed and semi-open systems, as depicted below:



Managing risk of pathogen introduction

The most common high risk inputs into aquaculture systems are: (1) stock, e.g. fingerlings/ post-larvae for stocking grow-out ponds, (2) feeds, (3) water, and (4) equipment such as harvest nets, aerators, etc. that may be shared between farms. Some less common inputs include vaccines and instruments such as those used for pearl seeding.

Measures can be taken to minimise the likelihood that live pathogens are present in these farm inputs. For example, stock (including broodstock) can be sourced from populations determined to be free of specific disease agents of concern (specific pathogen free - SPF or specific pathogen resistant - SPR) or populations subject to regular monitoring and known to be of good health status, or be quarantined and tested or treated to reduce the risk. For example, iodophor disinfection of fertilised ova is a standard procedure used all over the world as a precautionary measure against the transmission of many viral, bacterial and fungal diseases (see the OIE Aquatic Animal Health Code Section 5.2; Aquatic Manual Section 1.1).

Feeding of grow-out animals could be restricted to commercial pelletised feeds that are subject to a degree of pathogen inactivation through the heat created by the extrusion process and pellet drying. Fish and crustacean meals used as ingredients in aquaculture feed are also subject to a degree of heat treatment and drying during manufacture. Pelletised or processed feeds are used increasingly in feeding broodstock and all stages through hatchery development. Emerging technologies such as high hydrostatic pressure (HHP) treatment may be of future potential use in minimising feed associated biosecurity risks.

The ability of many aquatic animal pathogens to survive outside a host (sometimes for considerable periods) and to infect a variety of host species means that aquatic animal pathogens can be introduced into aquaculture systems through intake water, either in fomites (including suspended free in the water itself) or in infected/carrier organisms. Intake water may be screened and filtered to varying degrees to exclude or reduce the presence of pathogens, fomites and carrier organisms. Risks associated with water can also be reduced by treatment prior to use, such as by filtration, UV, ozone or chemical treatment (see chapter on disinfection of farms in the OIE Aquatic Animal Health Code – treatment of influent and effluent water). Where practical, underground reserves provide an excellent water source from a biosecurity perspective.

Most farm equipment such as aerators, harvest nets, footwear, and containers and vehicles used to transport live animals can be readily disinfected using chemical treatments, such as sodium hypochlorite, sodium hydroxide and iodophors.

In a summary article on farm-level biosecurity and WSD of shrimp, Mohan *et al.* (2004) provides an excellent general synopsis of measures that are commonly applied, as well as measures proposed, in term of better management practice at the farm level with respect to WSD, the principles of which can be applied in general stock health management (Mohan *et al*., 2004). These measures include pond preparation to eliminate pathogens and carriers, stocking of WSSV PCR-negative post-larvae, screening and disinfecting intake water in reservoirs, as well as reducing intake volumes (moving toward closed systems), and constructing physical barriers to prevent entry of wild crustaceans into production systems (e.g. crab fencing).

An aspect of input control is managing susceptibility of farm stock to pathogens of particular concern. This can potentially be achieved with vaccination (where commercial technology is available), through selection of culture species that are known to be resistant to the pathogen/s of concern or by maintaining good general health of stock through good management practice/husbandry.

Surveillance to detect pathogen incursions

Early detection of a pathogen incursion into a farm system allows for more effective control of the establishment or spread of a pathogen — for more effective response. Surveillance to detect incursions can be either targeted to specific pathogens or more general. For example, for pathogens of particular concern and for which there are diagnostics tests available, animals can be periodically tested, ensuring the number of animals sampled are enough to provide an acceptable level of confidence in detecting the agent were it to be present.

The health status of farm animals can also be monitored more generally through gross and microscopic examination including histology. Both targeted and general surveillance requires a degree of specialised expertise, although much can be done with minimal training. Of note, general surveillance does not need to be very expensive, especially in countries where labour is comparatively cheap.

Response to pathogen incursions

Response plans allow rapid action following detection of a disease incursion into a farm. Response plans essentially detail practical steps that should be taken in the event of an outbreak, saving time because the key decisions (and associated research needs) pertaining to various scenarios would already be made – thus allowing for early action.

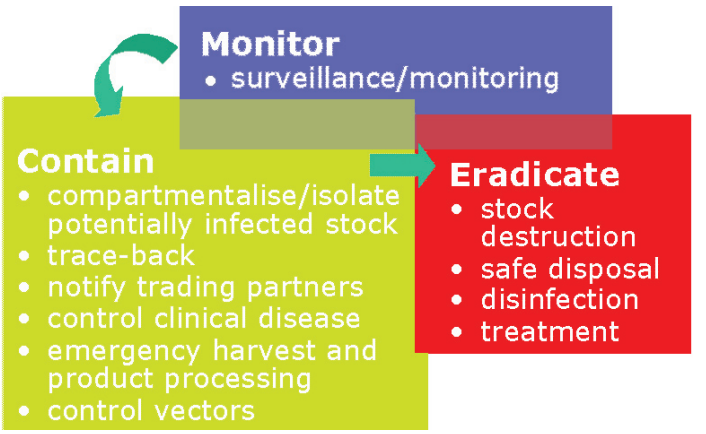
Disease control issues should be taken into account in the site selection and design engineering stages of an aquaculture operation. The location and design of a farm often determine the degree to which various control measures can be applied. For example, building shrimp culture ponds that can be fully drained and dried facilitates relatively cost-effective eradication of many aquatic animal disease agents from those ponds. Equally,

farm layouts can be designed in such a way that when a disease outbreak occurs, the affected animals can be readily isolated, minimising the risk of disease spreading to other parts of the farm or adjacent farms sharing the same water source. With open water cage-culture operations, allowing appropriate distance between cages or groups of cages would have a similar benefit of being able to compartmentalise culture systems.

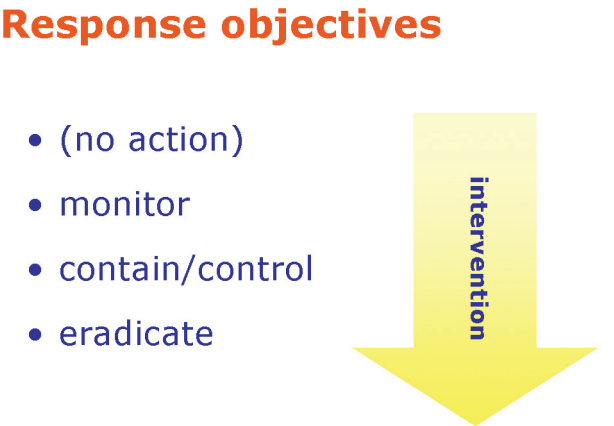
An effective disease response plan has three key aspects:

⇒surveillance/monitoring
⇒containment
⇒eradication

Each of these is associated various activities aimed at ultimately eliminating the pathogen of concern from the farm, e.g., as shown below:



The level of intervention, and therefore cost, is increased for each response objective, as depicted below:



Perhaps of most importance for successful implementation of biosecurity strategies is ensuring that farm managers and staff are aware of the potential risks and educated in the biological principles underpinning biosecurity measures, as well as their individual roles in responding to a disease event. Training programmes are important – strategies need to be developed to ensure that this awareness is not only communicated and understood, but also incorporated into policy/plans so that this capability can be maintained through ongoing training. An example of how governments can assist with such programmes is through the development of regional based resource material. An example of this at the national government level is the Disease Watch and the Field Guide[1].

Cost and Benefits

The cost of implementing biosecurity measures can be significant, particularly for marginal operations where short term profitability is essential to business success. The cost of diagnostic testing as part of surveillance activities or ensuring the absence of farm inputs such as fingerlings/post-larvae can be onerous. For example, the standard cost of a PCR test in Australia is around US$50. Similarly, the cost of filtering or treating intake water can often be prohibitive. The overall cost of biosecurity will, however, depend on the particular circumstances and needs to be looked at on a case by case basis, keeping in mind the range of available measures and that relatively low cost measures such as sourcing post-larvae from sources free of particular pathogens of concern can have tangible biosecurity benefits in most circumstances.

However, benefits are not always tangible. The New Zealand Government, for example, tries to determine threats based on risk to four core asset values (biotic, economic, social and spiritual). In Australia, import risk assessments look at the consequences of disease incursions and examine the likely economic, environmental and social impacts – globally, methodologies for sound decision making are not well established and generally inconsistent and untested. This same struggle to make level headed decisions on the net benefits of implementing biosecurity measures also occur at the farm level.

Clearly, the continued lack of attention to biosecurity by many farmers in the region shows that there is uncertainty in the value of biosecurity measures and that these benefits have not been sufficiently understood to allow a "regret-free"[2] decision. Such decisions are invariably made on relatively short-term commercial grounds (with the objective of early return on investment) and at an individual enterprise level, and in the absence of any regulatory controls.

Despite this relatively short-term view taken at an enterprise level, the significant short and longer term impacts to aquaculture resulting from several key disease problems (such as WSD, Taura Syndrome and Infectious Salmon Anaemia) over the last decade or so, have resulted in slow but increasing attention to biosecurity by industry groups (through codes

[1] Available at: http://www.disease-watch.com/documents/CD/index/body.htm

[2] Regret Theory – where a person anticipates regret if they make a wrong choice, and take this anticipation into consideration when making decisions.

of conduct/practice), and governments (through regulation and extension/education), as well as by international organisations such as the Food and Agriculture Organization of the United Nations (FAO) and World Organisation for Animal Health (OIE), through setting standards and establishing guidelines.

A major driver in this change has been the negative publicity surrounding aquaculture and attempts by those promoting aquaculture to counter accusations of poor or irresponsible practice. This negative press has been related mainly to claims of ecosystems damage but has also included concerns relating to aquatic animal disease[3]. Of particular significance in this regard has been the potential impact of newly introduced diseases on wild aquatic animal populations (for example, see Doubleday, 2001) — it is generally acknowledged that once aquatic animal pathogens enter and establish in natural ecosystems, it is practically impossible to eradicate them.

Of equal if not greater public concern has been with respect to antibiotics (eg chloramphenicol and nitrofurans) and other chemical residues in farmed product for human consumptions (e.g. see the Statement of the Government of Thailand at AquaMarkets 2003, the Manila Conference on Market Access of Aquatic Products[4] or the Centre for Food Safety website[5]) — again driving an industry desire to avoid disease agents, rather than manage diseases chemically.

Risk assessment based resource allocation

The primary objective of commercial aquaculture at the farm level, be it small-scale, family farms or major commercial enterprises, is to make money. As such, all farmers would consider the need for and extent of implementing farm level biosecurity in the context of resources availability. Most if not all recognize the potential business risks associated with disease and allocate some resources toward managing this risk. This resource allocation is, however, rarely based on proper risk assessment. In an environment of finite resources, it is important that resource allocation to disease risk management is commensurate with the biosecurity risk. In this regard it is important not to necessarily equate the pathogen to clinical disease. This error can lead to unwarranted focus on the pathogen, when managing clinical disease may present a more cost-effective option (Hugh and Stoskopf, 1999).

Biosecurity risk in this context is a measure of the likelihood of a disease outbreak in a farm and the short- and long-term negative impacts of the outbreak. Therefore, biosecurity risk assessment at the farm level involves:

[3] See The Aquaculture Disaster – Third World communities fight the 'Blue Revolution' by Martin Khor on the Third World Network website at: http://www.twnside.org.sg/index.htm or World Wildlife Fund website at: http://www.worldwildlife.org/cci/aquaculture.cfm

[4] AquaMarkets (2003): Available at:http://www.enaca.org/modules/wfsection/article.php?page=1&articleid=101

[5] http://www.centerforfoodsafety.org/aquacultur.cfm

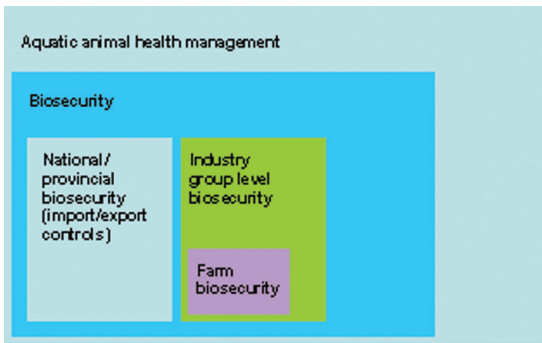
⇒consideration of key disease threats (diseases of concern),
⇒an evaluation of the likelihood that these agents would enter a farm system under existing farm conditions/operating systems — based on an examination of the 'pathways' by which disease agents can enter the farm and become exposed to susceptible host animals, and
⇒an estimation of the overall financial and other losses to the farm if an outbreak of disease did occur.

Based on biosecurity risk assessment, farmers can determine if the risk is such that it is unacceptable from a business perspective and if so, determine the most cost-effective risk management measures. Such a risk assessment approach to managing farm biosecurity would be appropriate to individual farmers, farming groups/cooperatives and government regulators alike.

In considering the need for farm level biosecurity, industry groups and governments in particular need to take into consideration wider and longer term negative impacts. For example, WSD has been associated with substantial losses in shrimp farms during the first few years following introduction of WSSV into systems. Production losses due to WSD are estimated at over 50% in the main shrimp producing state in India (Yap, 2001), between 70-75% in Ecuador (Hill, 2002; Yap 2001), approximately 45% (in 1996) in Bangladesh (Mazid and Banu, 2002), and approximately 80% in Peru (Yap, 2001). Other major shrimp producing countries such as Thailand and China would likely have experienced similar production losses. The significance of these losses may often be lost to individual farmers, but standards setting groups such as industry bodies and government regulators would need to take such impacts into consideration. Similarly, governments and industry groups need to consider the perceived negative impacts of aquaculture with respect to wider issues such as ecosystems damage or human health concerns on chemical residues, issues that are not generally a priority at an individual farm level.

ROLE OF FARM BIOSECURITY IN REGIONAL/NATIONAL AQUATIC ANIMAL HEALTH MANAGEMENT SYSTEMS

Farm biosecurity is a component of aquatic animal biosecurity, together with national level biosecurity associated with import and export controls, and industry group level biosecurity associated with farm cooperatives and codes of practice, as depicted below:



At all levels depicted in the diagram, the objective is to prevent the introduction of unwanted pathogens and thereby avoid associated disease impacts. Ideally, all levels of activity are complementary and together provide an effective overall level of biosecurity against unwanted pathogens.

Aquatic animal biosecurity as a whole can be defined as being a component of overall aquatic animal health management. In Australia, there is in place a national aquatic animal health management strategy (*AQUAPLAN*[6]) that attempts to bring together and coordinate the various elements of aquatic animal health management:

AQUAPLAN 1998-2003

1. International linkages	5. Awareness
2. Quarantine	6. Research and development
3. Surveillance, monitoring and reporting	7. Legislation, policies and jurisdiction
4. Preparedness and response	8. Resources and funding.

In addition to national level biosecurity, there are also international aquatic animal health programs and initiatives that try to promote and harmonise what is being done at national level. With respect to aquatic animals, these include the OIE, the FAO and the Network of Aquaculture Centres in Asia-Pacific (NACA). For example, the OIE sets standards (recognised by the World Trade Organization) for translocation of aquatic animal products, risk analysis and evaluation/recognition of competent authorities, as well as identifying globally significant (notifiable) animal diseases and coordinating the collation and reporting the health status of member countries with respect to these notifiable diseases.

The biological principles underpinning what can be achieved at international, national/provincial and farm levels are the same. As such, all the key elements of farm level biosecurity are mirrored in national and international biosecurity initiatives. The complementarity of international, national and local/farm level biosecurity needs to be stressed. Unilateral national border control on imported aquatic animal commodities for example can only do so much, especially for countries that share water basins or coast lines with neighbouring countries.

WHO HAS RESPONSIBILITY?

In the final analysis, all partners involved in aquaculture have some responsibility with respect to biosecurity; these include:

⇒ Farmers — can apply good biosecurity practice as part of their normal operations
⇒ Industry organisations/cooperatives — can develop biosecurity codes of practice/conduct and facilitate information exchange between farmers, encouraging a

[6] Available at: http://www.daff.gov.au/aquaplan

AQUAPLAN 2005-2010

1. Enhanced integration and scope of aquatic animal health surveillance in Australia
- To identify needs and gaps with respect to surveillance requirements for specific industry sectors.
- To develop cost-effective surveillance systems tailored to address the identified gaps and needs.
- To have a surveillance information system that addresses the deficiencies found in Objectives 1 and 2, which is organised and readily accessible at a national level.
- To improve investigation and reporting of major (wild) fish kills.
- To create a consistent system of aquatic animal disease laboratory diagnosis and reporting across Australia.

2. Harmonisation of approaches to aquatic animal health in Australia
- To harmonise the framework for aquatic animal emergency disease management in Australia.
- To implement a common approach to zoning for disease control and market access.
- To implement a common approach for managing pathogens associated with the translocation of live aquatic animals across Australia.
- To harmonise any new legislative, code of practice or quality assurance approaches as they are initiated in aquaculture.

3. Enhancement of aquatic animal emergency disease preparedness and response framework
- To agree on an approach to the establishment of an aquatic emergency animal disease response agreement for Australian aquaculture industries.
- To ensure the scientific and technical accuracy of *Aquavetplan*.

4. Education and training in the aquatic animal health sector
- To clearly define the current and future needs for aquatic animal health support among Australia's aquaculture industries (established and emerging).
- If required, to modify the current education and training structures to ensure the needs of Objective 1 are met.
- To develop an accreditation and competency scheme for aquatic animal health service providers.
- To provide training in the framework and operational aspects of aquatic animal disease emergency management.

5. Welfare standards for aquaculture
- To develop a scientifically-based and harmonised approach to aquatic animal welfare policies across Australia.
- To increase awareness of aquatic animal welfare issues within industry.
- To assist international standard setting bodies in developing welfare guidelines and standards that are scientifically based.

6. Appropriate use of therapeutics for aquatic animal health management
- To ensure the availability and safe use of therapeutics for cultured aquatic animals in Australia.

7. Aquatic animal health management as part of ecologically sustainable development
- To ensure that market opportunities are not lost due to the use of suboptimal health management practices in aquaculture.
- To raise awareness about disease issues associated with imported live aquatic animals.

corporate view and discouraging secrecy on matters of broader disease concern. (Financiers may also paly a role by requiring farmers to include biosecurity measures into farm operations).

⇒ Local/national governments — can increase awareness through extension, require minimal biosecurity standards at the farm level (e.g. as a part of licensing agreements) and through control of the translocation of high risk commodities within their jurisdictions and from other countries. International organisations — can increase awareness of biosecurity (particularly at the national/provincial government level) and set standards for best biosecurity practice at all levels.

CONSTRAINT OR CHALLENGE

A 'constraint' is a hurdle to achieving an objective. From a farm perspective, the primary objective is profitability – ideally, sustainable profitability. Put in the context of this paper, biosecurity would be a constraint if the cost of implementation on the farm's bottom line was prohibitive. A 'challenge' implies a hurdle that <u>needs</u> to be overcome – the emphasis being on necessity. That is to say, biosecurity rather than being an option, is essential to profitability.

Based on the above definitions, whether biosecurity is a constraint or a challenge is a determination that has to be made on a farm by farm basis, based on the individual circumstances. However, generalisations can be made taking into account what has happened historically and those expected future threats to the industry that are already making significant headlines.

⇒ Is farm biosecurity a 'constraint'? — yes to some, it will cost and marginal operations may well go under.

⇒ Is it a 'challenge'? — yes, it is necessary to the long term financial viability of the aquaculture sector as a whole. Importantly, the long-term down side of not giving serious attention to biosecurity could worsen the already poor public/consumer perceptions of aquaculture, which could result in an even greater (and potentially irreversible) impact on the bottom line of individual farms.

WHAT CAN BE ACHIEVED – A VISION

A common understanding of what biosecurity means, well defined and put in the context of other aspects of aquatic animal/plant health management.

All parties taking collective responsibility in maintaining/improving aquaculture's image in the eyes of consumers and other stakeholders, who are increasingly aware of, and have growing concerns regarding, ecological impacts of aquaculture operations and food safety of farmed product.

Farmers/financiers/industry groups/government extension/academia

Biosecurity established in the psyche of all farmers and becoming a basic cost of doing business, like feed management, stock supply and other routine tracking/traceability operations — in the context of this paper, farm operators who are aware of the potential risks, recognise the limitations of national and international regulatory capability and take greater responsibility for biosecurity at the farm level.

Industry groups

Consistent application biosecurity across industry groups — corporate citizenship.

International organisations

Set of internationally agreed "templates" for achieving biosecure aquaculture facilities/farms. These would cover the engineering factors, husbandry factors, quality assurance programs, the surveillance requirements and the certification for incoming and outgoing stock.

Governments/international organisations

Government regulation at the local level to ensure compliance – and consistent application globally. A system where live animals could be sent with confidence from one biosecure facility to a similar (or lower) standard facility anywhere in the world (where the pathogens that may be present are known) — this is already happening in the areas of food hygiene, shellfish sanitation, and export fish-packing houses; why not for aquaculture?

Biosecurity is a team effort — a shared responsibility. What should be strived for is consistency across the board and cooperation among relevant groups that have clearly defined jurisdictions. But we must have realistic expectations. However, left to itself, it is highly unlikely that industry groups/farmers will or can adhere to a minimum biosecurity standard — it only takes a small portion of a sieve to have a hole for the whole sieve to be useless! In the final analysis, government jurisdiction, be they at national or local level, have a public duty of care to ensure that all farmers comply with a minimum biosecurity standard.

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Pathogen Risk Analysis for Biosecurity and the Management of Live Aquatic Animal Movements[1]

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ABSTRACT

This paper presents a brief introduction to the risk analysis process as applied to the transboundary movement of live aquatic animals and experience from some recent risk analyses, and briefly examines some of the current problems and issues. The use of risk analysis as a practical method to assist developing countries in assessing and minimizing pathogen risks associated with proposed introductions and transfers of live aquatic animals is emphasized. Risk analysis for aquatic animal biosecurity is the process of identifying and estimating the level of risk that is posed by a proposed transboundary movement of aquatic animals, and determining the risk management (sanitary) measures that are needed to reduce the risk to a level acceptable to the importing country. Although this is a relatively new field, and only a few countries have much experience in this area, the risk analysis process need not be overly complicated, particularly for developing country situations where expertise and resources may be limited. Countries must determine the risk analysis methods that are most effective and cost efficient for their

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[1] This paper is dedicated to the memory of Chris Baldock, our good friend, colleague and team member.

particular circumstances, taking into consideration that the process needs to be science-based, systematic, iterative, consistent, timely and transparent. The research community plays an essential role in supporting good risk analysis through conducting basic research on the pathogens, parasites and diseases of the main aquatic species cultured and traded, including studies on their taxonomy, life cycles, host and geographic distributions, pathogenicity and epidemiology. Individual risk analyses may show gaps in knowledge that necessitate well-defined, applied research. Scientists should also serve as experts on risk analysis working groups and provide independent expert review of draft risk analyses. They also have a role to play in educating policy-makers as to the potential adverse socio-economic impacts to national aquaculture, fisheries and aquatic biodiversity that may occur due to epizootic transboundary pathogens.

INTRODUCTION

Live aquatic animals are traded internationally for many reasons, the foremost being for the ornamental fish trade, for aquaculture development and sustainment, and for live food markets. They are also less frequently transported across international borders for the development of capture and sport fisheries, for use as bait, as biological control agents and for research (Arthur, 2004; Subasinghe and Bartley, 2004). They may also be moved unintentionally via a number of mechanisms, such as in the ballast water of ships, via water diversion schemes for hydroelectric power and irrigation, and through the release of unused bait by fishermen.

The international trade in ornamental fishes involves more than 2,000 species (Khan *et al*., 1999; Davenport, 2001), and the movement of hundreds of millions of fish annually. For example, the Ornamental Aquatic Trade Association (OATA) reports that approximately 20 coldwater species, up to 1000 tropical freshwater species, and more than 1000 tropical marine species of ornamental aquatic animal are imported annually into the United Kingdom.[2] Malaysia, which is one of the main exporters of aquarium fishes in Asia, produced some 338 million freshwater ornamental aquatic organisms in 2001, including some 293 million freshwater fish belonging to more than 90 species (Latiff, 2004).

Although the top five exporting countries are Singapore, Hong Kong China, the United States, the Netherlands and Germany (Davenport, 2001), freshwater ornamental fish are raised in many countries around the world, often by relatively small-scale producers. The culture and trade of aquarium fishes is an important source of foreign exchange earnings for some countries. For example, in 2001, Malaysian production of freshwater ornamental aquatic organisms was valued at over 81 million Malaysian Ringgit ($US 21.3 million), a figure which had increased by an average annual rate of 7.5 per cent since 1997. In 2003, Singapore exported ornamental fishes worth more than $US 42.3 million (Ling, Kueh and Poh, 2004).

The aquarium fish trade involves a high amount of transshipment, which often masks the country of origin of individual shipments and species. For example, in 1988, 84.3% of

[2] http:\\www.ornamentalfish.com.

Malaysia's total aquarium fish production was exported via Singapore (Siow and Nagaraj, 1989). Of the US$57 million worth of ornamental fishes exported by Singapore in 2003, the country's 68 ornamental fish farms produced less than half of this total (about 44%) (Ling, Kueh and Poh, 2004).

The tropical aquarium fish industry is characterized by a resistance to regulation, and the complexity of the trade often makes guarantees of disease status difficult or impossible. Diseases of ornamental fishes have not received the detailed attention they deserve; however, the few surveys that have been conducted have shown the presence of a wide variety of pathogens and parasites, some of which are of importance as disease agents of cultured and wild fishes or as human pathogens (e.g., Low, Singh and Tan, 1987; Korting, Fuchs and Michel, 1990; Lumanlan *et al*., 1992; Hedrick and McDowell, 1995; Tampieri *et al.,* 1999; Manfrin *et al.,* 2002). The most serious of these is undoubtedly koi herpes virus disease (KHVD), which has recently caused major losses of common carp cultured for food in Japan and Indonesia (Sano *et al.,* 2004, Sunarto *et al.,* 2004, Iida *et al.,* 2005, Sunarto and Cameron, 2005).

Movements of live aquatic animals for aquaculture development involve the shipment of gametes or fertilized eggs; fry, fingerlings or spat; and of broodstock. The movement of eggs and gametes is recommended by international codes of practice for species introductions (e.g., the International Council for the Exploration of the Sea (ICES), and the European Inland Fisheries Advisory Commission (EIFAC)), as it generally involves a lower risk of pathogen transfer (Turner, 1988; ICES, 2004).

Fry, fingerlings and spat of aquatic animals are frequently moved across international borders, particularly in Asia and Latin America. This trade often involves large numbers of an individual species (e.g., prawn postlarvae, oyster spat). Such movements are characteristic of new industries, those hampered by non-existent or temporarily insufficient national production (e.g., milkfish fry, oyster spat, prawn postlarvae) or industries involving species whose life cycles have not been completed to a commercial level (e.g., groupers, tiger prawn). An example of the magnitude of this trade is given by Hossain (1997), who estimated that in 1995, 50 million nauplii and postlarvae of giant tiger prawn were imported into Bangladesh, primarily from Thailand, India and Myanmar, to support the country's developing prawn culture industry. Such movements can pose an extremely high risk of pathogen transfer and many serious transboundary pathogens are known or suspected to have been introduced to new geographic areas due to the careless movement of aquatic animals for aquaculture development (Bondad-Reantaso, 2004; Bondad-Reantaso *et al.,* 2005).The international movement of broodstock is less frequent and typically involves only a few animals at a time. Such movements are common in commercial aquaculture of species where there is a high industry dependency on wild-caught broodstock (e.g., prawns), for new aquaculture species, and in circumstances where producers avoid delays in aquaculture start-up due to the time needed for maturation of juveniles to broodstock. These movements often involve a high risk of pathogen transfer.

Examples of fish, crustaceans and molluscs traded to supply live food markets include the movement of live oysters from producing countries to consuming countries (e.g., to Europe, North America, South Africa); and the intra-regional trade in Asia involving live finfish and shellfish (e.g., grouper, seabass, shrimp, cockles, etc.) for consumption

in seafood restaurants. Grouper fry, for example, are collected mainly from the wild in developing countries such as Sri Lanka, the Philippines, Thailand and Indonesia (Arthur and Ogawa, 1996). They are shipped to countries such as Singapore, Malaysia and China (Hong Kong) for grow-out in cages. Market-sized fish are then consumed locally or shipped to restaurants in Singapore, Hong Kong, Taiwan and mainland China.

Given the volume of live aquatic animals traded internationally and the diversity of species moved, countries have often found themselves in a difficult position in attempting to find methods that reduce the risk of spreading transboundary pathogens that could seriously impact their domestic aquaculture industries and aquatic biodiversity, while at the same time wanting to increase the benefits of trade in live aquatic species and their products. This is particularly the case for the developing countries in Asia, which often lack the necessary expertise, capacity, policy, legislation and financial resources to adequately manage transboundary disease risks.

THE ROLE OF PATHOGEN RISK ANALYSIS

In the context of aquatic animal health, pathogen risk analysis (also termed "import risk analysis") is a structured process for analyzing the disease risks associated with the international and domestic movements of live aquatic animals and their products. "Risk" is the potential that an unwanted, adverse consequence (a serious disease outbreak) will result from the importation or domestic movement of a living aquatic animal or its product (a "commodity") over a given period of time. "Risk" therefore encompasses or combines the elements of likelihood and impact.

A pathogen risk analysis seeks to answer the following questions:

1) What serious pathogens could the commodity be carrying? (Addressed by the **hazard identification** portion of risk analysis, which answers the general question "What can possibly go wrong?");
2) For each pathogen that could potentially be carried by the commodity, what are the chances that the pathogen will enter the importing country and that susceptible animals in natural waters or aquaculture facilities will be exposed to infection? (Addressed by the **release assessment** and **exposure assessment** portions of **risk assessment**, answering the question "How likely is it to go wrong?");
3) For each pathogen, what are the likely biological and socio-economic impacts of susceptible animals in the importing country becoming exposed to the pathogen? (Addressed by the **consequence assessment**, which answers the question "What would be the likely consequences of it going wrong?");
4) If the importation is permitted without restrictions, then what is the overall risk associated with each pathogen? (Addressed by the **risk estimation** portion of the **risk assessment**);
5) Is the risk determined for each pathogen in the risk assessment acceptable to the importing country? (Addressed by the **risk evaluation** section of **risk management**); and

6) Can the commodity be imported in such a way that the risk is reduced to an acceptable level? (Addressed by the **option evaluation**, **implementation, monitoring and review** portions of risk management, which answer the question "What can be done to reduce either the likelihood or the consequences of it going wrong?") (MacDiarmid, 1997; Rodgers, 2004; Arthur *et al*., 2004a; Murray *et al*., 2004; OIE, 2005).

The strength of risk analysis is that it is provides a clearly defined framework for a structured, repeatable process, thereby removing to a large extent, *ad hoc* and arbitrary decision-making with regards to requests to import aquatic animals and their products. An important feature is that the process can make use of pathway analysis. Through scenario diagrams, the possible routes (pathways) and the individual events or steps in each pathway that need to occur for a given pathway to be successfully completed can be identified. This approach assists by providing a logical process by which the critical risk steps (events) leading to pathogen introduction and establishment in an importing country can be identified. This allows for the probability of each event occurring to estimated, leading to an overall estimate of the probability of a given pathway being completed. Further, pathway analysis can be used to gauge the effectiveness of a risk mitigation measure or combinations of measures, as each measure can be incorporated into the pathway analysis, and the overall risk recalculated to see if the risk has been reduced to an acceptable level. Pathway analysis also allows for sensitivity analysis; that is, it allows for identifying those pathway steps that most influence the final risk estimation for a particular pathogen. This can help in targeting risk mitigation measures, as well as in identifying those areas where information needs are most critical, if highly sensitive pathway steps are associated with a degree of uncertainty or subjectivity.

Another strength is that the risk analysis process is based on science and has transparency; thus points where subjective decisions enter the process can be recognized. The transparency comes both from having a structured and defined process that is understood by all, and also from extensive stakeholder consultation, which is an essential component of any risk analysis. Importantly, risk analysis is an internationally accepted method that permits importing countries the means to protect themselves against exotic diseases while assuring their trading-partner countries that any disease concerns are justified and are not disguised barriers to trade. Finally, risk analysis allows for uncertainty of scientific knowledge; through applying the precautionary approach, importing countries are permitted the time needed to address any important information gaps where research is needed to support sound decision-making.

Risk analysis, of course, is only one of a large number of components in a national aquatic animal health strategy (FAO/NACA, 2000; Arthur *et al.,* 2004a). It cannot function effectively unless other components of the national strategy have also been developed. In addition to appropriate legislation and policy, and the means to implement them, these include capacity in areas such as diagnostics, quarantine and inspection services, disease surveillance and monitoring, reporting, enforcement and contingency planning. One of the major challenges to developing countries is how to develop or have access to these areas of technical capacity.

THE RELATIONSHIP OF PATHOGEN RISK ANALYSIS TO ECOLOGICAL AND GENETIC RISK ANALYSES[3]

Often, an importing country will be confronted with a first request to import a live aquatic animal that is a non-native (exotic) species. In cases where the importation involves the intentional introduction of an exotic species into the natural environment or is for commercial aquaculture development (with perhaps inevitable escape into the wild), the first question that must be asked is "Is the species likely to establish wild populations in the new environment?" and, if so, "What will be the environmental, social and economic impacts of its establishment?" These questions can be explored by conducting an ecological risk analysis, the results of which will provide the Competent Authority with a scientific basis for deciding if an importation should be allowed. Of course, national policy on exotic species will also come into play. Some countries, such as Australia and New Zealand, place a very high priority on protecting natural biodiversity, the integrity of native ecosystems and domestic aquaculture industries from exotic pathogens, and thus treat the introduction of any exotic species with great caution. For various reasons, such as the absence of suitable native species for aquaculture or capture fisheries enhancement, or the prior degradation of native ecosystems, many countries may consider introduction of new species. In the case of requests to import new strains of native or previously introduced aquatic animals, there may be potential for undesirable genetic impacts. In these cases, a genetic risk analysis can be undertaken to help governments evaluate the associated risks (ICES, 2004).

Pathogen risk analysis does not deal with the question of whether or not the introduction of an exotic species, per se, is desirable. Rather, it seeks to answer the question "Does the aquatic animal or its product, as characterized in the commodity description, pose an unacceptably high risk of pathogen introduction?" and if so, "What mitigating measures can be applied that will reduce the risk of pathogen introduction to an acceptable level?"

PATHOGEN RISK ANALYSIS AND INTERNATIONAL TRADE

The past two decades have seen major changes in the patterns of world trade, due primarily to the liberalization of international trade through the General Agreement on Tariffs and Trade (GATT) and the creation of the World Trade Organization (WTO) in 1995. With the adoption of the Agreement on the Application of Sanitary and Phytosanitary Measures (the SPS Agreement) in 1994, WTO member countries are now required to use the risk analysis process as a means to justify any restriction on international trade based on risk to human, animal or plant health that exceeds those measures allowed by international agreement (e.g., those given in the OIE's *Aquatic Animal Health Code*) (WTO, 1994; Rodgers, 2004, Arthur *et al*., 2004a). As a result, risk analysis has become an internationally accepted

[3] The term "pest risk analysis" is widely applied to encompass analysis of the "pestiness" of the commodity species, including any ecological and genetic risks, as well as the potential for introducing any "hitchhiker" species. "Pathogen risk analysis", of course, includes analysis of pertinent aspects of the pathogen's ecology.

standard method for deciding whether trade in a particular commodity (a live aquatic animal or its product) poses a significant risk to human, animal or plant health, and, if so, what measures could be applied to reduce that risk to an acceptable level.

Under the SPS Agreement, the World Organisation for Animal Health (OIE, the Office International des Épizooties) is recognized as the international organization responsible for the development and promotion of international animal health standards, guidelines and recommendations affecting trade in live terrestrial and aquatic animals and their products. The OIE's *Aquatic Animal Health Code* (OIE, 2005), outlines the necessary basic steps in the risk analysis process that should be followed; however, the decisions as to the details of the process are left to individual member countries. The OIE has also established voluntary in-house mechanisms for settling disputes between its 167 Member Countries, avoiding the lengthy and often costly procedures involved in bringing a dispute before the WTO.

Although a veterinary organization, the OIE recognizes that in many cases, particularly in developing countries, the responsibility for aquatic animal health matters (Competent Authority) often lies with the national fisheries agency (Department of Fisheries or similar agency). Thus, the OIE plays a central role in encouraging linkages between the national Competent Authority for aquatic animal health and the national veterinary services. The OIE also plays an important role in assisting countries in the risk analysis process through its international aquatic animal disease reporting system[4] by maintaining, through its collaborating center at the Centre for Environment Fisheries and Aquaculture Science (CEFAS), Weymouth Laboratory, United Kingdom, *The International Database on Aquatic Animal Diseases* (http://www.collabcen.net), and through the OIE *Aquatic Animal Health Code* (OIE, 2005) *and Manual of Diagnostics Tests for Aquatic Animals* (OIE, 2003), as well as publications such as *the Handbook on Import Risk Analysis for Animals and Animal Products* (Murray *et al*., 2004; Murray, 2004).

THE ESSENTIAL OBLIGATIONS OF THE EXPORTING COUNTRY, THE IMPORTING COUNTRY AND THE PROPONENTS OF A PROPOSED IMPORTATION

The risk analysis process is based on the premise that trading-partner countries will deal openly and honestly with each other in providing the information essential to making an accurate risk assessment. For the exporting country, this obligation includes providing the importing country with accurate and timely information, as available, on the national disease status, on the disease status of the specific commodity in question, and on the disease history and status of the particular facility and/or stock under evaluation. The exporting country also has an obligation to provide information to assist the importing country in identifying and evaluating the national Competent Authority, including its diagnostics capability, risk communication procedures, national and provincial legislation and policy, procedures for documenting disease occurrences and archiving of diagnostic, surveillance and monitoring data.

[4] http://www.oie.int/eng/maladies/en_alpha.htm

The importing country has an obligation to deal in a timely and efficient manner with requests to import and, as fundamental components of the risk analysis process, to provide adequate communication with all stakeholders (including the proponents and the exporting country) and transparency of process to ensure trading partners that any sanitary requirements or restrictions placed on trade in the commodity are not disguised barriers to trade.

A good risk analysis begins with a clear and accurate picture of the commodity being proposed for importation. Thus, the proponents of a proposed importation have an obligation is to provide the accurate, comprehensive and timely information that is needed on stock history, disease status, etc., so that the importing country has a detailed and accurate commodity description on which to base its risk analysis.

APPROPRIATE LEVEL OF PROTECTION

A country's appropriate level of protection (ALOP) is the level of protection deemed appropriate by the country establishing a sanitary or phytosanitary measure to protect human, animal or plant life or health within its territory (WTO, 1994). Determining the ALOP is a political decision made at the highest level of government. In making a decision as to what a country's ALOP should be, politicians must weigh many factors, including the importance its citizens place on protecting national biodiversity and natural ecosystems, the availability of species for aquaculture and capture fisheries development, the need for social and economic development, and past trading practices, including those in the plant and livestock sectors. It is important to note that the same ALOP must be consistently applied across all commodities – terrestrial animals, aquatic animals and plants. Figure 1 illustrates the concept of ALOP for a country having a very high ALOP (i.e. a very low risk tolerance or Acceptable Level of Risk, ALOR) using a risk estimation matrix combining the likelihood of pathogen entry and exposure with the consequences resulting from entry and exposure.

The importance of ALOP in the risk analysis process is that it is the standard to which the unmitigated risk is compared to determine if the proposed importation poses a risk that is too high (unacceptable to the importing country), and if necessary, the degree to which the risk must be reduced (mitigated risk) in order for importation to occur. The relationship between ALOP and the amount of risk a country is willing to tolerate (the acceptable level of risk) is inverse – the higher the ALOP, the lower the level of risk that is acceptable. Any description of the ALOP, e.g., as being 'very low' or 'moderate', is only relevant to a risk analyst in the context of the framework or method used to measure risk.

From the above, it is clear that, other than perhaps providing occasional advice to senior government officials, a risk analyst has little, if any, role in determining ALOP and indeed, a risk analyst's personal views on the appropriateness of his/her country's ALOP should not have any bearing on the results of a risk analysis.

Consequences of entry and exposure

Likelihood of entry and exposure	*Negligible impact*	*Very low*	*Low*	*Moderate*	*High*	*Extreme impact*
High likelihood	Negligible risk	**Very low risk**	Low risk	Moderate risk	High risk	Extreme risk
Moderate	Negligible risk	**Very low risk**	Low risk	Moderate risk	High risk	Extreme risk
Low	Negligible risk	Negligible risk	**Very low risk**	Low risk	Moderate risk	High risk
Very low	Negligible risk	Negligible risk	Negligible risk	**Very low risk**	Low risk	Moderate risk
Extremely Low	Negligible risk	Negligible risk	Negligible risk	Negligible risk	**Very low risk**	Low risk
Negligible likelihood	Negligible risk	Negligible risk	Negligible risk	Negligible risk	Negligible risk	**Very low risk**

Figure 1. Illustration of ALOP (band of cells marked "very low risk") for a country having a very high level of protection (very low risk tolerance) (modified from Anon., 2001).

OVERVIEW OF THE RISK ANALYSIS PROCESS

The following sections provide only a brief overview of the risk analysis process. More detailed information and guidelines for conducting pathogen risk analyses can be found in MacDiarmid (1997), FAO/NACA (2000, 2001), AFFA (2001), Murray (2002, 2004), Murray *et al.,* (2004), Arthur *et al.,* (2004a), OIE (2005) and the papers contained in Arthur and Bondad-Reantaso (2004). Figure 2 shows the four main components of the OIE risk analysis process and their interrelationships, while Figure 3 outlines the steps in the risk analysis process.

Deciding if a risk analysis is needed

Upon receiving a request to import a specific commodity, the first question the importing country's Competent Authority will ask is "Is a risk analysis needed?" This initial decision will be made taking into account such factors as the nature of the commodity (including any sanitary measures applied), its source, and past knowledge and experience with the commodity, the importing country's competent authority, the source stock and the proponents. In many cases, a similar or identical request to import may have resulted in a risk analysis that has shown the commodity to represent an acceptable risk, and thus approval can be quickly issued. Occasionally, a previous risk analysis may have shown that the commodity represents an unacceptably high level of risk and that the risk mitigation methods available cannot reduce the risk to an acceptable level, so that the request may be

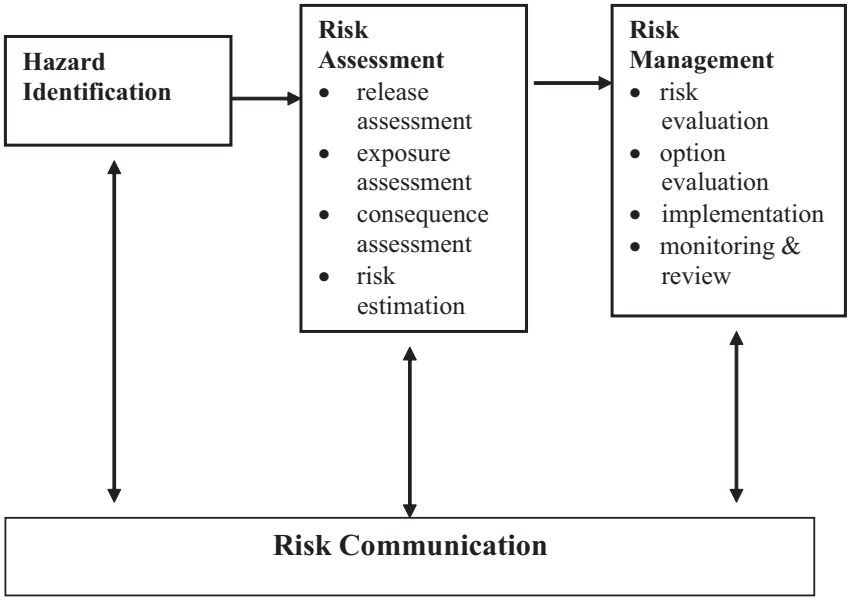

Figure 2. The structure of the OIE risk analysis process (after Murray *et al.*, 2004).

immediately denied. Other requests, such as those involving new species or sources, may require more careful initial evaluation prior to making a decision. In cases where doubt remains as to the level of risk posed by the commodity, a risk analysis is warranted.

Scoping a risk analysis

Having determined that a risk analysis is required, the risk analysis team maintained by the Competent Authority will decide on the type of risk analysis to be conducted and establish a working group with appropriate expertise that will conduct the actual risk analysis. As this decision, and many of those to follow, are based on the commodity description, the importance of a detailed and accurate commodity description cannot be overemphasized. To assist this process, the importing country should provide the exporting country with guidelines and forms for completing a commodity description.

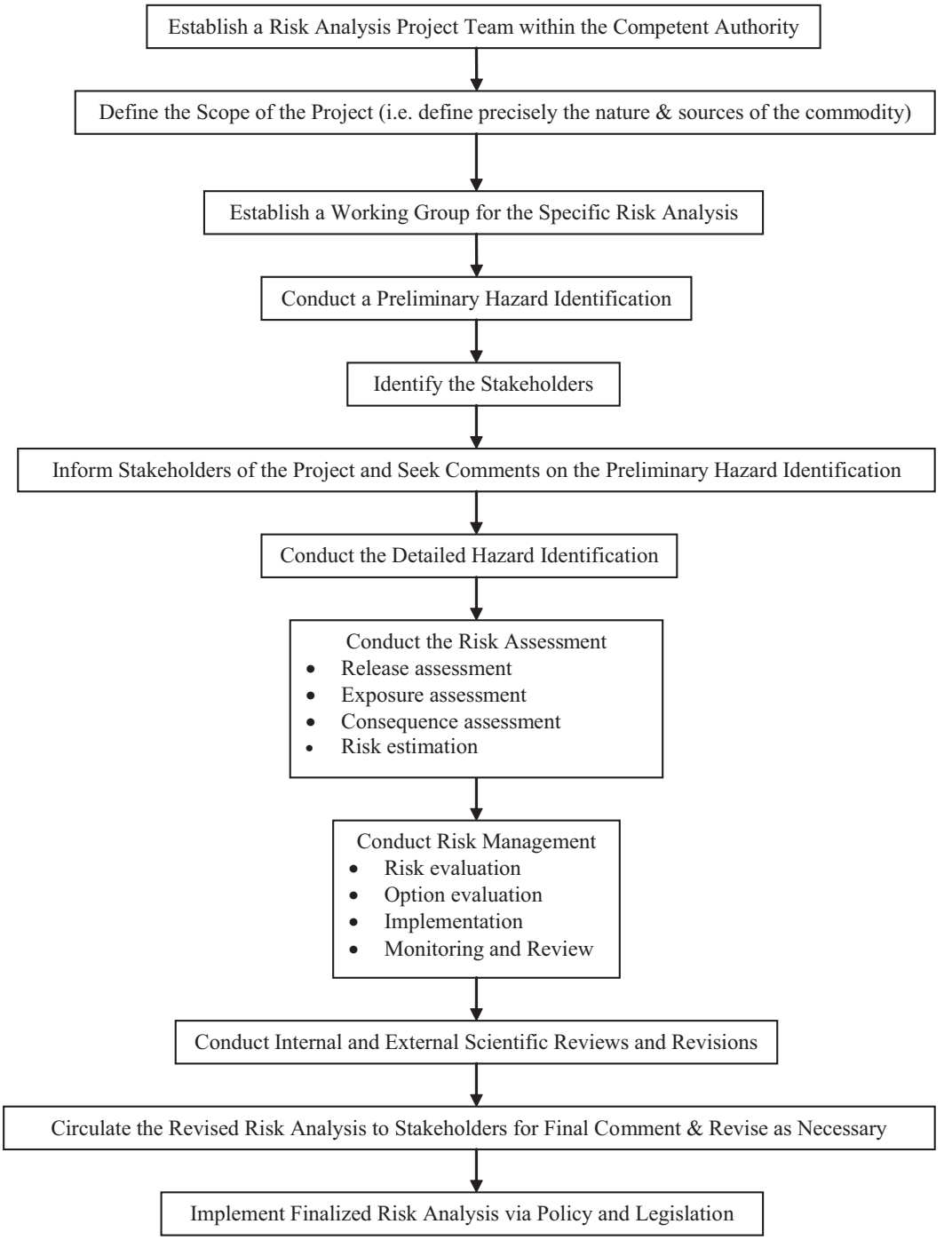

Figure 3. A simplified diagram showing the steps in the Risk Analysis Process (modified from Arthur *et al.*, 2004).

Risk communication

Any risk analysis may involve a large number of agencies, organizations and individuals that have an interest in its outcome. Primary among these will be the proponent, the Competent Authorities in the exporting and importing countries, and the risk analysis team. However, there will be many others with an interest in the outcome, the precise agencies, organizations and individuals varying depending on the commodity being considered and its intended use. Key stakeholders should be identified early in the risk analysis process and methods of advising them and seeking input established. An example of a stakeholders list is presented in Box 1. The importance of good risk communication throughout the entire risk analysis process cannot be overstressed.

Box 1. Example of a list of potential stakeholders for a risk analysis involving the importation of a live marine mollusc for aquaculture development. (from Arthur *et al.*, 2004a)

- Oyster farmers
- Oyster traders
- Restaurant owners
- Fish Vendors
- Consumers
- Aquaculturists
- Seafood processors
- Conservationists
- Concerned international, national and local governments and agencies

Deciding on the type of risk analysis to use

In most cases, where the Competent Authority of the importing country has sufficient expertise, the risk analysis will be conducted using a project team drawn internally (an "in-house" risk analysis), often using independent external reviewers from outside the department to maintain quality control and provide additional insights. However, where sufficient internal expertise is lacking, where the risk analysis is likely to generate considerable controversy and thus exceed available manpower, or where the Competent Authority is also the proponent (and thus a real or perceived conflict of interest exists), the use of outside expertise (e.g., a more extensive risk analysis using consultants or other experts) for all or part of the risk analysis process may be desirable.

Risk assessment methodology may range from the purely qualitative to the purely quantitative. In most cases, particularly for developing countries, a qualitative risk analysis will be simplest, quickest and most cost-effective. As will be discussed further below, in conducting a risk assessment, a pathways analysis approach involving the construction of scenario trees to determine the likelihood of a chain of events occurring (for example, the series of events (pathway steps) that need to occur to result in a given shipment of imported fish being infected with a specific pathogen) is useful and preferred approach. Qualitative risk analyses use qualitative estimates of the likelihood of an event happening (e.g., likelihoods can be categorized as being " low", "medium" or "high"). These estimates are then combined to give an estimate of the likelihood of a given pathway being completed. Similarly, estimates for successful completion of several pathways are combined to give an overall risk estimate for a given scenario, and scenarios are combined to give a total

risk estimate for the individual hazard. Quantitative risk analyses use mathematical probability estimates for events throughout. They can involve a high level of mathematical sophistication and/or computer skills and can be resource intensive in terms of expertise, time and money. However, quantitative risk analyses can help clarify thinking, provide insights into areas where data are lacking, deal with volume of trade issues and improve transparency (Murray, 2002, 2004).

Regardless of the type of risk analysis, a pathways analysis approach to risk assessment and associated construction of pathways diagrams can provide a risk assessment framework that facilitates detailed and transparent examination of the key factors that contribute to the overall risk. While there is a growing body of completed risk analyses for aquatic animals that provide examples of the range of risk analyses from the purely qualitative to the quantitative (see the Annex I of Arthur *et al*., 2004a), there are no universally accepted standards or models. This is probably a good thing, as it allows countries to adapt the risk analysis process to their individual situations, including expertise, manpower and financial resources.

Hazard identification

Hazard identification is the process of determining what pathogens could plausibly be carried by the commodity and then, from this initial list of pathogens, determining those pathogens that pose a serious risk to the importing country. Obviously, the more specific the commodity description and its disease history, and better the knowledge of the disease status of the exporting and importing countries, the easier hazard identification becomes. The most problematic risk analyses involve commodities of unknown health history and status, being exported from countries of poorly known health status to countries of poorly known health status. Unfortunately, this is a situation that often occurs in trade in live aquatic animals and one that may necessitate the application of the precautionary approach. To be identified as a hazard, a pathogen typically:

- must have been reported to infect, or is suspected of being capable of infecting the commodity;
- must cause significant disease outbreaks and associated losses in susceptible populations;
- it must be plausible that the agent might be present in the exporting country; and
- must be exotic to the importing country (or subject to a control/eradication program in the importing country).

Those OIE-listed diseases that may plausibly be carried by the aquatic animal species proposed for importation present a good starting point in constructing a list of pathogens (hazards) for detailed risk assessment. This list should be augmented with additional disease agents based on nationally and regionally reportable diseases, as well as those diseases described in the scientific literature.

Risk assessment

Risk assessment consists of four components: **release assessment, exposure assessment**, **consequence assessment** and **risk estimation**. In release assessment, the biological pathways necessary for an importation activity to "release" (introduce) a hazard into the importing country are defined and the likelihood of that complete process occurring is estimated. Or, more simply stated, release assessment determines the pathways that a pathogen can move with the commodity from the exporting country to the border of the importing country and the likelihood of this occurring. Similarly, exposure assessment determines the pathways by which susceptible populations in the importing country can be exposed to the pathogen and the likelihood of this occurring. An example of a hypothetical exposure pathway is presented below (Figure 4). Consequence assessment identifies the potential biological, environmental and socio-economic consequences expected to result from pathogen introduction, while risk estimation calculates the overall risk posed by the hazard (the unmitigated risk) by combining the likelihood of entry and exposure with the consequences of establishment (Table 1).

Table 1. Example unmitigated risk estimation combining the results of the exposure and consequence assessments for a hypothetical hazard using three qualitative rankings (high, medium and low) (from Arthur *et al*., 2004a).

Likelihood of Entry and Exposure	Consequence of Establishment	Unmitigated Risk Estimate
Low	Low	Low
Low	Medium	Medium
Low	High	Medium
Medium	Low	Medium
Medium	Medium	Medium
Medium	High	High
High	Low	Medium
High	Medium	High
High	High	HIgh

Risk Management

Risk management is the process of identifying, selecting and implementing measures that can be applied to reduce the level of risk. In the **risk evaluation** step of risk management, the unmitigated risk estimate for the hazard is compared with the level of risk acceptable to the importing country (based on the national appropriate level of protection (ALOP)). If the ALOP is met, the importation can be approved without further action. However, if the risk posed by the commodity exceeds that specified by the ALOP, then risk mitigation measures should be considered. During **option evaluation** possible measures to reduce the risk posed by the hazard are identified and evaluated for efficacy and feasibility, and the least restrictive measure(s) found to reduce the risk to an acceptable level are selected. The process is essentially the same at that used during risk assessment, with new scenarios and pathways being constructed that incorporate steps for possible risk mitigation measures

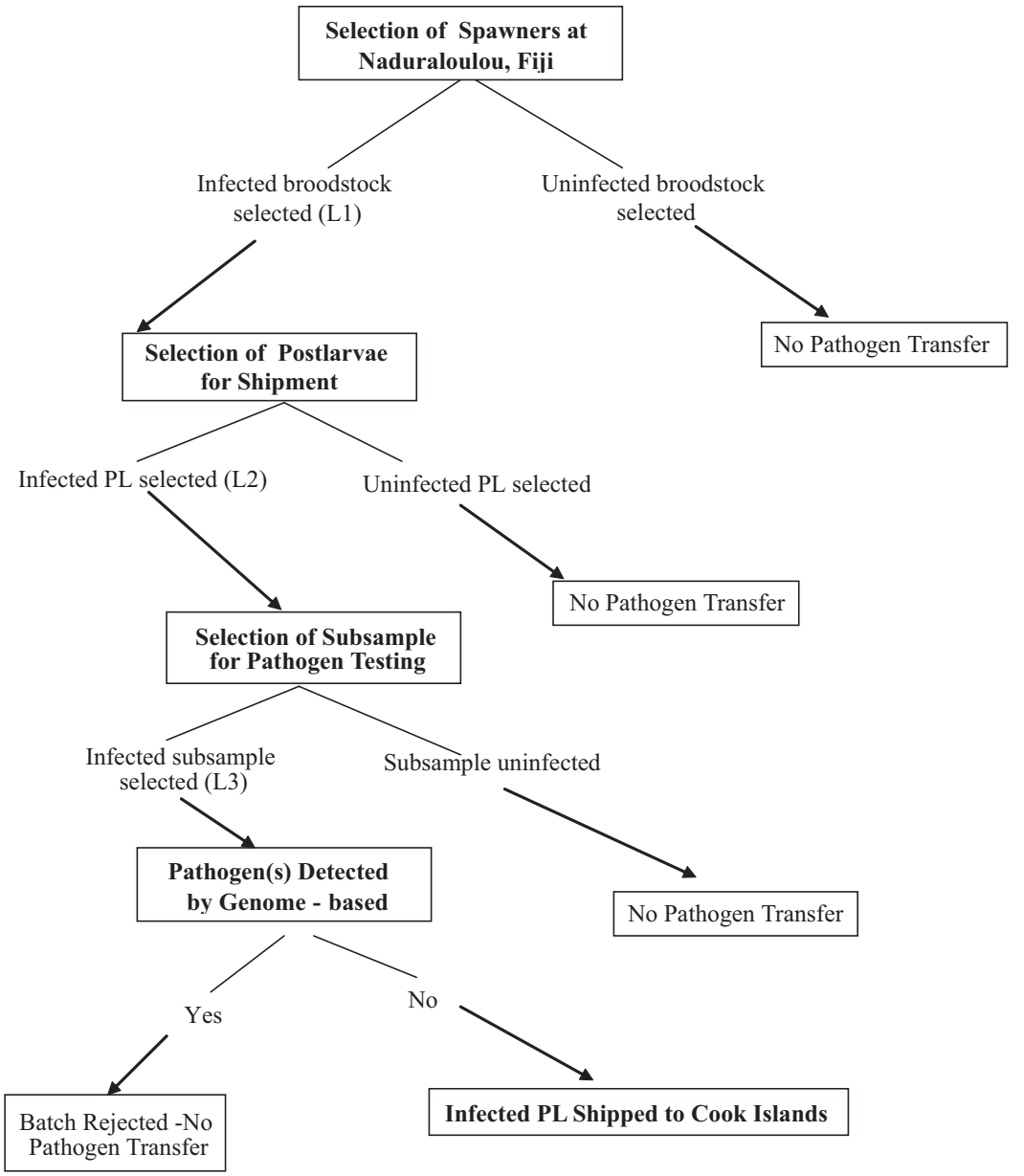

Figure 4. Simplified Pathways Diagram for the release of viral pathogens in *Macrobrachium rosenbergii* postlarvae from Fiji to Cook Islands. Not considered are less probable pathways such as via shipping water or fomites, or failure of the diagnostics tests to detect true positives. In this simplified example, the likelihood that infected PL will be released (LR) is the product of the individual likelihood's = L1 x L2 x L3 x L4. (from Arthur *et al.*, 2004b).

to determine their ability to reduce the overall risk (now the mitigated risk estimate) to an acceptable level. Once effective risk mitigation measures have been identified, the next phase is **implementation, monitoring and review**. In this phase, the requirements for importation, including any mitigation measures, are presented to the Competent Authority of the exporting country (and the proponent) and the importation process is monitored and reviewed by the importing country's Competent Authority to assure that all conditions for importation are met.

Several important principles of the SPS Agreement related to the risk management process are important to keep in mind:

- *Least restrictiveness* - Risk management measures must be applied in the least trade restrictive manner possible. Thus if there are several risk management options that will allow the proponent to meet the importing country's ALOP, the proponent should be able to chose the least burdensome (least trade restrictive) method. .
- *Equivalence of mitigation measures* -The concept of equivalence allows the exporting country the opportunity to prove that its own risk mitigation measures lower the risk to within the importing country's ALOP.
- *Consistency in application* - The importing country must apply the same ALOP (i.e. accept the same level of risk at both external (international) and internal (national) borders; and the ALOP must be applied consistently across the range of commodities in which the country trades, without prejudice as to the country of origin.

THE PRECAUTIONARY APPROACH

The precautionary approach is widely used in fisheries management and elsewhere where governments must take action based on incomplete knowledge. FAO's *Code of Conduct for Responsible Fisheries* (FAO, 1995) states that:

> "States should apply the precautionary approach widely to conservation, management and exploitation of living aquatic resources in order to protect them and preserve the aquatic environment. The absence of adequate scientific information should not be used as a reason for postponing or failing to take conservation and management measures."

Within the context of risk analysis for aquatic animals, a precautionary approach would be that both importing and exporting nations act responsibly and conservatively to avoid the spread of serious pathogens (Arthur *et al.*, 2004a).

The risk analysis process has at least three points where the precautionary approach may come into play (Figure 5):

- throughout the risk analysis process, when "cautious interim measures" are considered necessary to ban or restrict trade until a sound risk analysis can be completed;
- during the pathways scenario portion of the risk assessment process, when sensitivity analysis reveals key information gaps that must be addressed by targeted research; and

- during risk management, when risk mitigation measures are identified to reduce the risk to an acceptable level.

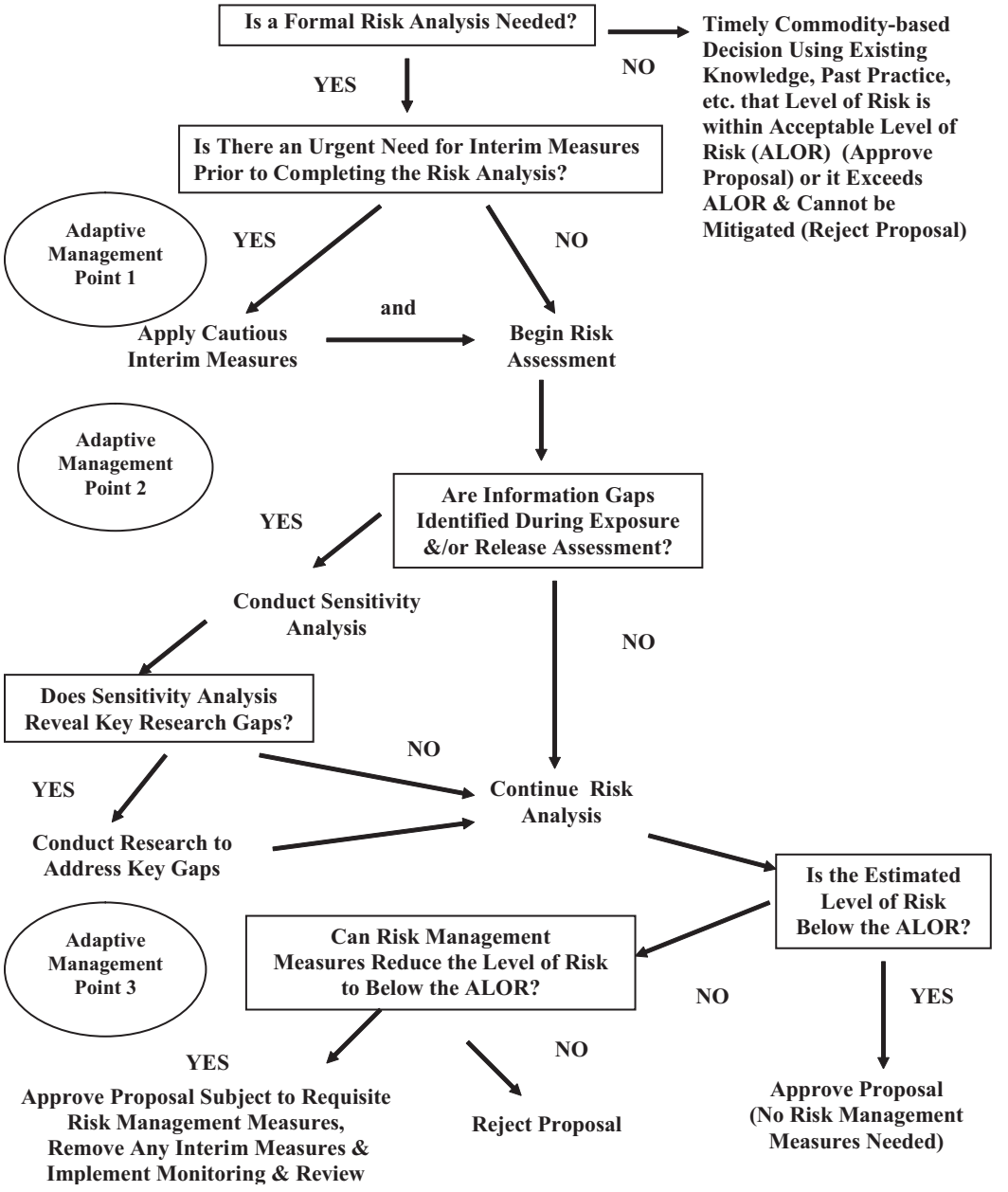

Figure 5. Decision tree for assessment of a proposal to import a commodity showing the use of the precautionary approach through adaptive management.

EXAMPLES OF RECENT RISK ANALYSES FOR AQUATIC ANIMALS

The following section looks briefly at two recent risk analyses conducted by developing countries for the international movement of aquatic species for aquaculture development. The most high profile trade dispute involving risk analyses for an aquatic animal commodity, the Canada-Australia SPS dispute on importations of raw salmon product into Australia that was taken to the World Trade Organization, has been discussed from the perspectives of a risk analyst and a third-party country by Beers (2004) and Amos (2004), respectively. On-line sources for additional risk analyses undertaken by the governments of Australia and New Zealand can be found in Annex I of Arthur *et al.*, (2004a).

Thailand's Risk Analysis for Importation of White Shrimp

Thailand is one of the world's leaders in production of farmed shrimp (estimated production of 300,000 tonnes in 2003; Briggs *et al.*, 2004), and until recently its shrimp aquaculture industry has been based on the culture of the black tiger shrimp (*Penaeus monodon*), a native Asian species. The Thai shrimp culture industry has faced serious problems due to transboundary diseases, environmental degradation and a scarcity of quality broodstock that has affected production capacity. As a result, importation of white shrimp (*Litopenaeus vannamei*) into Thailand for use in aquaculture began in March 2002, and production of this exotic species has increased rapidly, such that during the year 2002-2003, white shrimp contributed an estimated 120,000 tonnes, or approximately 40 percent of the total national shrimp production (Kanchanakhan, 2004). While some importations of broodstock were sourced from specific pathogen free (SPF) stocks in the United States and imported to registered hatcheries under the approval of the Thai Department of Fisheries (DOF), others have involved illegal movement of animals from neighboring countries in the region. As a result of these illegal importations, Thailand experienced it first outbreak of Taura syndrome virus (TSV) in 2003, leading to the drafting of a contingency plan for eradication of the virus (Kanchanakhan, 2004). These developments caused the DOF to become concerned that the existing procedures regulating the importation of postlarvae and broodstock were only partially effective, and would lead to further introductions of TSV and other serious pathogens that could have major negative impacts on the Thai shrimp culture industry.

To determine a course of action, the DOF contracted a group of scientists with expertise in shrimp pathology, shrimp aquaculture, aquatic ecology, economics and trade to prepare a detailed review of legislation, standards and importation regulations for *L. vannamei* in Thailand and abroad, to estimate the various positive and negative impacts of importation and cultivation of this species in Thailand and to provide a summary and recommendations. In a second study also funded by the DOF, the Centex Shrimp, Mahidol University and the Aquatic Animal Health Center, Prince Songkla University conducted a study on the impact of white shrimp importation into Thailand and a pathogen risk analysis, respectively, which included as part of its hazard identification, a survey of the viral and bacterial diseases present in Thai hatcheries and in both domestically produced and imported white shrimp broodstock. The risk analysis also included susceptibility testing of various native crustaceans to viral pathogens using extracts taken from infected white shrimp. Based on

these studies, the risk analysis concluded that there was a high risk posed by imported white shrimp due to Taura syndrome virus (TSV), white spot syndrome virus (WSSV) and infectious hypodermal and haematopoietic necrosis virus (IHHNV).

The DOF then convened a series of public meetings at which the experts presented facts and options on the pros and cons of banning further introductions and of permitting continued importations, although with more stringent controls, so that the policy makers and stakeholders could decide what to do. The DOF's goal was clearly to "clean up" a pre-existing situation that was considered "risky", primarily from the standpoint of possible pathogen introductions, and it was generally understood that the goal was to introduce new regulations that would legalize importations under conditions that would reduce the associated risks due to pathogens to an acceptable level.

The recommendations presented by the experts have been partially implemented, including:

- Organizing a DOF/industry group to travel to the United States to visit producers of specific pathogen free (SPF) *L. vannamei* and to select from these, a list of acceptable sources for permitted importation of broodstock to Thailand with limited quarantine procedures. The current list includes six producers whose broodstock are all believed traceable to the SPF stocks developed by the Oceanic Institute's program (the Oceanic Institute sells only to US producers). This has now been in effect for over one year.
- Making all other sources illegal.

Recommendations made to DOF (that have not yet been implemented) also included the following:

- The period of continuous importation from the approved producers was to last only for approximately one year, until local nucleus breeding centers (NBC) could be set up with more strictly quarantined stocks.
- Once this had been achieved, all continuous imports are to stop and any new introductions are to follow the same strict quarantine protocol leading to establishment of local NBC only.
- Postlarvae and broodstock originating from the local NBC are to be accompanied by certification documents that will be backed up by DNA microsatellite records.
- Policing is to be carried out by random checks at all levels of production and any shrimp not conforming to accepted microsatellites are to be immediately destroyed according to the Animal Epidemic Law.

DOF intends to follow this program once the biosecure hatcheries and farms are established and functioning (S. Chinabut, AAHRI, pers. comm.).

So far, two private companies are dedicated to setting up local NBC for *L. vannamei.* As the details of their operations are not open to the public, it is unknown how much actual progress they have achieved towards setting up and operating local NBC with genetic selection programs. Thus, although the approach followed by the DOF to this difficult problem is quite promising, more time will be needed before its success or failure will be known.

The SPC's ecological and pathogen risk analysis for the importation of giant river prawn from Fiji to the Cook Islands

The Secretariat of the Pacific Community (SPC), an intergovernmental agency assisting Pacific Island Countries and Territories (PICTs) with headquarters in Noumea, New Caledonia, has recently begun assisting its member countries to develop risk analysis methods for the importation of live aquatic animals for aquaculture development. As part of this initiative, in 2004, the SPC commissioned risk analyses for the transboundary movements of blue shrimp (*Litopenaeus stylirostris*) and giant river prawn (*Macrobrachium rosenbergii*). The terms of reference were to conduct ecological and pathogen risk analyses for these proposed importations that could serve as models for subsequent risk analyses for other commodities whose importation was being contemplated by SPC member countries (see Arthur et al., 2004b; Bondad-Reantaso *et al.*, 2004). This paper presents a brief case history of the risk analysis for the importation of giant river prawn from Fiji to the Cook Islands.

A project team was formed consisting of two Australian experts on crustacean ecology (P.B. Mather and D. Hurwood), a local aquatic ecologist (E.R. Lovell), and two experts on aquatic animal pathology and risk analysis (J.R. Arthur and M.G. Bondad-Reantaso). On-site visits to the proposed source hatchery in Fiji and the site of the proposed aquaculture facility at Rarotonga, Cook Islands were made and meetings with the proponent and key staff of the Ministry of Marine Resources (MMR) in the Cook Islands, and staff of the source hatchery and the Ministry of Fisheries and Forestry (MFF) in Fiji, as well as with other potential stakeholders, such as the veterinary services, Chief Veterinary Officer, airport quarantine officers and environmental services.

The proponent of this proposed importation was a private entrepreneur who wished to establish a backyard integrated hydroponic production facility for giant river prawn to serve the local tourist market in the Cook Islands. In making arrangements for importation, he was assisted by the MMR. The commodity to be imported was postlarval *M. rosenbergii* originating from the MFF Aquaculture Center at Naduruloulou, Fiji. In this instance, the commodity description was prepared by the consultant with the full cooperation and assistance of the proponent and the staff of the supplying hatchery.

The ecological risk analysis was based on a literature review of the species life cycle and ecology, history in the importing and receiving countries, and ecological impacts in other countries. In broad terms, the assessment examined:

- the risk of escape,
- the potential for *M. rosenbergii* to establish sustaining local populations,
- the potential for widespread dispersal, and
- the possible effects on native species should a population of *M. rosenbergii* become established in the wild.

Results from the literature review were summarized and tabulated using a modification of the method promoted by the *ICES Code of Practice on the Introductions and Transfers of Marine Organisms 2003*. Additionally, a slightly modified version of the decision model proposed by Kohler (1992) for the *Environmental risk management of introduced*

aquatic organisms in aquaculture was used as a decision-making tool to assess the level of risk relative to the potential benefits of introduction. A major modification to the ICES procedure was to assume that escapes from aquaculture facilities in the Cook Islands would inevitably occur and rather than rejecting the importation based on the likelihood of escape (as called for in the ICES protocol), to look at the probability of escaped animals establishing populations in the wild and the likely impacts, both negative and positive, that would occur.

The pathogen risk analysis was more problematic due to the large number of uncertainties involved. These included the poorly known health status of the stock of origin, the poorly known health history of the facility of origin, the poorly known pathogen status of the country of origin, and the poorly known pathogen status of the importing country.

Due to the absence of any information on the diseases of *M. rosenbergii* in the proposed stock of origin, or in populations of wild and cultured crustaceans in general in either Fiji or the Cook Islands, the preliminary hazard identification included all pathogens and parasites reported from this species throughout its world-wide distribution. The criteria for consideration during preliminary hazard identification was thus the following:

- The potential hazard must be an identifiable biological agent or a disease believed to be produced by a single (as yet unidentified) biological agent (thus generalized syndromes were not considered).
- The agent must have been recorded from *Macrobrachium rosenbergii.* Pathogens reported for any life cycle stage and any geographical locality were included.

From the list of over 60 potential pathogens identified during the preliminary hazard identification, one crustacean virus listed as reportable to the OIE and one other disease associated with concurrent infections by two non-OIE listed viruses were identified as requiring further consideration:

- White spot syndrome virus (WSSV)
- White tail disease (WTD), due to *Macrobrachium rosenbergii* nodavirus (MrNV) and/or Extra small virus (XSV)

The criteria used to select a pathogen for further consideration in the detailed hazard identification were:

- The pathogen was reported to infect, or suspected of being capable of infecting postlarval *M. rosenbergii*;
- It was obligate pathogen (i.e., it is not a ubiquitous free-living organism capable of becoming an opportunistic pathogen of *M. rosenbergii* under certain environmental or culture conditions);
- It causes significant disease outbreaks and associated losses in populations of *M. rosenbergii* or, if not a significant pathogen of *M. rosenbergii*, it causes serious disease outbreaks in populations of other species of aquatic organisms; and
- It was plausible that the agent might be present in populations of *M. rosenbergii* in Fiji.

Based on past practices and trading partners, it was recommended that the Cook Islands adopt an appropriate level of protection (ALOP) that is "very conservative", and a risk tolerance (acceptable level of risk) that is "very low".

Both the pathogen and ecological risk analyses were characterized by a high level of uncertainty. For the former, this was due to an absence of information on the health history and current health status of the Fijian stock of *Macrobrachium* to be introduced, and the general lack of any aquatic animal health information for both Fiji and the Cook Islands; while for the latter, it was due to a general lack of information on the ecology of *M. rosenbergii* and of follow up studies from previous introductions of this species to other countries. It was concluded that if the Cook Islands wished to act very conservatively, it could apply the precautionary approach until such a time as data on health status of the parent stock and on important ecological issues, such as potential interactions of *M. rosenbergii* with native *Macrobrachium* spp. were obtained.

Although there is a general paucity of country-specific and species-specific data to support the analyses, the ecological risk analysis suggested that the benefits of introduction appeared to outweigh the potential negative effects.

The pathogen risk analysis concluded that the proposed introduction could be accomplished within the recommended ALOP if appropriate disease mitigation measures were adopted to minimize the risk of the imported postlarvae (PL) being infected with whitespot syndrome virus (WSSV) and white tail disease (WTD). These included that:

- statistically appropriate samples of the PL to be introduced would be tested for WSSV using the methods specified by the Office International des Épizooties, and for *Macrobrachium rosenbergii* nodavirus (MrNV) and extra small virus (XSV) using the genome-based methods cited in the report;
- no animals would be removed from the receiving facility without prior permission from the Ministry of Marine Resources (MMR);
- the operator would keep detailed records of mortalities and would report any occurrences of serious disease outbreak or mortality to MMR; and
- a contingency plan would be developed requiring that in the event of serious disease outbreak or mortality, all animals would be destroyed and disposed of using an approved sanitary method, and the facility fully disinfected.

The main lesson learned from this risk analysis is that it is very difficult to assess risks when there is a high level of uncertainty present in many key areas. In such cases, general precautionary and sanitary methods such as those outlined by the OIE, ICES and other regional and international bodies are recommended. The analysis also revealed the need for basic diagnostic capability and the development of history of stock health status of cultured species in the exporting country.

While a similar case study for the risk analysis for importation of blue shrimp from Brunei to Fiji (Bondad-Reantaso *et al.*, 2004) will not be presented here, it is worth noting that the uncertainties encountered by the risk analysis team were quite similar, that importations that appeared to be highly risky had already occurred and were continuing, and that the supplier of the postlarvae in the exporting country and the Competent Authority were

not forthcoming in supplying essential information such that preparing a commodity description was very difficult. One recommendation of the risk analysis was that such lack of responsiveness on the part of the exporting country and/or supplier should be considered sufficient reason to deny the request to import, particularly, as in the present case, where other sources of postlarvae of known health history were readily available.

SOME ISSUES RELATED TO RISK ANALYSES AND THE RISK ANALYSIS PROCESS

Within the context of the Sanitary and Phytosanitary Agreement (SPS), risk analysis for aquatic animals has evolved from the past experience with terrestrial animals and plants, historically well established and studied agricultural commodities. As a result, certain issues such as how to deal with rapidly emerging diseases, the importance of trade in wild-caught commodities, the more frequent occurrence of uncertainties and knowledge gaps, and the use of the precautionary approach have greater relevance in aquatic animal risk assessment. There is an urgent need for standards-setting bodies such as the OIE and the FAO to address these and other issues. This section looks briefly at some of the issues and problems encountered by the authors during the risk analyses that they have conducted and suggests some possible solutions.

Problems with the Risk Analysis Process

- *How to apply the concept of Appropriate Level of Protection (ALOP)* - An important and still unresolved question is "How should an importing country apply the concept of ALOP?" Currently, with respect to a particular pathogen, a risk analyst would apply the ALOP in terms of the risk posed by a specific commodity. However, should the total risk associated with a given pathogen be measured in terms of the sum of risks presented by all commodities that are capable of introducing that pathogen into a country? A related issue, particularly in quantitative risk analyses, is how to take into account the volume of trade in the commodity or commodities, which of course, can greatly increase the risk of pathogen introduction.
- *Consistency in applying ALOP.* Another important question is "How can national governments ensure that the ALOP is consistently applied, both for risk analyses involving commodities of a similar nature (e.g., aquatic animals, aquatic animal products) and across widely differing commodities (e.g., across risk analyses conducted for aquatic animals, terrestrial animals and plants)?"
- *Consistency in applying the Precautionary Approach* - As with ALOP, how countries apply the precautionary approach, both for commodities of similar nature and across commodities, can be quite subjective. Developing countries, in particular, need scientifically based guidance and examples of what is reasonable precaution, and what is not.
- *Standardization of risk assessment methodologies*. At least at the country level, and preferably among countries conducting frequent trade in aquatic animals, there is a need to standardize risk assessment methods. There are no "fundamental truths" with respect to risk assessment outcomes, but rather all determinations are dependent

on the declared (ALOP), and this can only be appreciated in the context of the assessment methodology applied. Thus, standardization and consistent application of methodology is perhaps the most important aspect of good risk assessment and decision-making. A risk analysis template should be developed that countries can adopt and, if necessary, adapt to their individual situations. Compared to risk assessments for plant and terrestrial animal commodities, those for pathogens of aquatic animals are often very subjective, due to associated environmental complexities, the wide range of pathogen and host species involved and the many unknowns that must be taken into account. Technical guidelines to assist risk analysts in minimizing the level of subjectivity in their risk analyses are thus needed.

Problems with Individual Risk Analyses

- *Failure of trading partners to respond adequately to requests for information.* The reluctance of the proponents of a proposed importation or of the Competent Authority of the exporting country to cooperate fully with requests for information should immediately raise a warning flag with the Competent Authority of the importing country. Such lack of cooperation should be sufficient reason to deny importation. The importing country may recommend that the proponents seek another source for the commodity.
- *Undefined national ALOP* many developing countries have no formally stated ALOP or level of acceptable risk. In order to facilitate risk analysis across all sectors and commodities and to advise trading partners, such countries should begin the process of defining a national ALOP.
- *Inaccurate/inadequate information provided in the commodity description. In most cases, it is the proponent's responsibility to develop the commodity description.* However, the importing country must provide the proponents with detailed instructions on the information required (typically, via guidelines and standardized forms for commodity description), and then review the description and return it to the proponents for any corrections or elaboration. The Competent Authority should make it clear from the very beginning that further action is contingent upon approval of an acceptable commodity description.
- *Inadequate coordination/communication between national agencies.* Few countries have good coordination mechanism between the various "stakeholder" agencies (biosecurity, quarantine, environment, fisheries, veterinary services, human health, customs, legal services, etc.) whose mandates touch on the importation of aquatic animals and their products. This lack of coordination and communication can make it difficult to conduct risk analyses in an efficient and timely manner.
- *Inadequate external review.* The importance of good external scientific review cannot be overstressed. Such review lends credence to the risk analysis process and will assure trading partners and other stakeholders that the conclusions of the risk analysis are well supported.
- *Potential conflicts of interest.* A potential conflict of interest arises when the proponent and the Competent Authority of the importing country are one and the

same. In such cases, to avoid real and apparent conflict of interest, and the appearance of "rubber stamping", transparency is highly important, and the risk analysis should be conducted and/or reviewed by independent outside experts.

- *Lack of scientific information.* Lack on scientific information on pathogens affecting a species, their pathogenicities, life cycles, distributions etc. can create a high level of uncertainty such that the results of an importation cannot be predicted. Where this occurs, the precautionary approach should be applied and research into critical areas conducted.
- *Improperly constituted risk analysis team.* Occasionally a poor quality risk analysis has resulted due to an improperly constituted risk analysis team. Good risk analysis requires appropriate expertise, including specialist knowledge on pathogens, on the commodity, and on conducting risk analyses, and more general knowledge on the local receiving environment. Thus the risk analysis team should typically include both specialists and general fisheries biologists having local knowledge.
- *Inordinate haste.* Private aquaculturists, national and state government agencies and internationally funded projects for aquaculture and fisheries enhancement that involve the use of exotic species or strains of aquatic animals often do not take into consideration the need to allow adequate time and resources for risk analysis and the risk mitigation measures (such as diagnostics testing and extended quarantine periods) that may be required. There is thus a tendency in developing countries for importation to occur without adequate risk assessment and disease prevention, and for questions of ecological and/or pathogen risk to be raised only after importation has already occurred. This of course drastically reduces the possibility of preventing adverse ecological and pathogen impacts from occurring and may make the risk analysis process a rather futile one.
- *Research loopholes.* National fisheries agencies and staff at universities have often imported aquatic species for research or trial in aquaculture and fisheries development projects. Despite the perception that these importations are for research and therefore represent "little risk", the exotic organisms often end up being released into the natural environment. Requests for importations for research purposes need to be more closely monitored. Ecological and pathogen risk analyses should be mandatory for those requests that are likely to result in release into natural environments, while approval of those involving requests for animals strictly for use in laboratory research should require that all stock be kept under stringent quarantine isolation and that they be destroyed at the end of the study.

DEVELOPING COUNTRIES AND RISK ANALYSIS

Although some the risk analyses undertaken by developed nations (particularly those few high profile trade disputes involving aquatic animals and their products that have reached the World Trade Organization) appear to be highly complex, developing countries should not be intimidated, as the risk analysis process is both highly flexible and readily adapted to developing country situations.

A key issue for developing countries is how to find the expertise and financial resources needed to conduct a risk analysis. However, there are many sources of support and assistance. International and bilateral donor agencies such as the Food and Agriculture Organization of the United Nations (FAO), the Network of Aquaculture Centres in Asia-Pacific (NACA), and the World Animal Health Organisation (OIE) have provided assistance to developing countries in Asia and Latin America understanding the risk analysis process and in developing appropriate national expertise. In the South Pacific, the Secretariat of the Pacific Community is playing a lead role by assisting its member countries to develop risk analysis capacity and to undertake risk analyses for the most urgent proposals to introduce exotic species. Further assistance by these and other agencies in training and capacity building will certainly be made available as the need arises.

Developing countries can also place much of the responsibility for conducting and/or funding many of the risk assessment activities on the would-be importers. This can include the hiring of private consultants to undertake the risk assessment. As risk analyses are conducted, a growing level of national and regional expertise and experience will be developed that can be tapped by developing countries.

CONCLUSIONS

Clearly, the most cost-effective way for many developing countries to conduct risk analyses is through combining national expertise with the risk analysis expertise available in neighboring countries through regional approaches to shared problems. This includes the sharing of databases and other sources of information, and particularly for introductions involving shared waterways, the sharing of risk analysis approaches and associated costs.

Small countries, such as the Pacific Island Countries and Territories (PICTs), typically have small populations, and thus very limited manpower and specialized expertise within their Competent Authorities. As the number of risk analyses such countries are likely to undertake is quite limited, it makes little sense for individual countries to attempt to develop extensive expertise and capacity for aquatic animal risk analysis. A better approach is for each country to have one or two staff members that have a general familiarity with the risk analysis process and an in-depth knowledge of local aquaculture, fisheries, environmental and socio-economic issues, etc. When a specific risk analysis is needed, these staff can be assisted by appropriate specialists, including outside consultants. The SPC is currently developing a regional project to assist member countries in developing this basic knowledge and capacity for risk analysis and is undertaking ecological and pathogen risk analyses on several key exotic species whose importation is of broad interest to member countries. The project will also promote linkages between risk analysts in the various PICTS, provide training, establish shared databases on pathogens, develop shared approaches and promote linkages and communication between Competent Authorities in the plant, livestock and aquatic animal sectors and between aquatic animal risk analysts and experts concerned with the broader issue of invasive aquatic species.

As previously mentioned, risk analyses involving trade between developing countries often involve a great deal of uncertainty in many key areas, making accurate risk estimation difficult. A key reason for this is the general lack of basic knowledge on the

ecology and pathogens of aquatic animals in developing countries. There is thus a need to establish the appropriate research capacity and to conduct targeted studies. For example, research to support aquaculture biosecurity should focus on the pathways of pathogen spread, methods for inactivation of infectivity, "barrier" vaccination strategies, etc. Epidemiological research should include investigation of biological factors (identification of at-risk populations, hazards, pathways, pattern of spread, incubation period, nature of the pathogen); risk factors; interventions and methodologies (e.g., surveillance techniques, disease outbreak modeling, use of geographic information systems), etc. Risk analysis information/knowledge requirements should be given high priority. Essential research areas, for example, include pathogen studies, information on trade and most importantly, biological pathways for the introduction (release assessment), establishment (exposure assessment) and spread (consequence assessment) of a pathogen. Other important areas of research include studies on host susceptibility; modes of transmission; infectivity, virulence and stability; intermediate hosts and vectors; and effects of processing, storage and transport. For newly emerging diseases as well as some diseases in poorly studied aquatic animal species, basic studies on their pathology and methods for rapid and accurate diagnosis (including standardization, validation and inter-calibration) are essential to facilitate accurate risk assessment and biosecurity management. Increased surveillance of wild fish to detect significant disease problems at an early stage will also be required.

Developing countries such as those in the South Pacific and in Southeast Asia should consider a regional effort to establish hatcheries and stocks of known health history (ideally, specific pathogen free (SPF) stocks) for the most frequently traded species (e.g., ,tilapia, marine shrimp, giant freshwater prawn, oysters, etc.). There is little justification for importing countries to continue to accept the risks inherent in importing live aquatic animals of uncertain health status.

Finally, in aquatic animal risk analysis, Murphy's Law *"If anything can go wrong, it will."* and its corollary *"Left to themselves, things tend to go from bad to worse"* apply. Occasionally, and despite the best risk analysis and risk mitigation measures, serious pathogens will be introduced and cause major disease problems. This is because of limitations in diagnostics techniques, the existence of cryptic pathogens, and the ability of "benign organisms" (normally non-pathogenic parasites, bacteria, viruses, fungi, etc.) to become pathogenic when introduced to new hosts and environments. Therefore, good disease surveillance, monitoring and reporting and well-designed emergency plans are essential back ups to detect and contain or eradicate new diseases.

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International Trade in Aquatic Animals – A Risk to Aquatic Animal Health Status?

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ABSTRACT

The movement of live aquatic animals, genetic material and aquatic animal products carries an inherent risk of moving aquatic animal pathogens contained in those commodities. International trade is of particular concern because of the large volumes of live animals and products moved, the large distances covered, and the risk that entire countries hitherto free of a particular pathogen may become infected. International trade provides significant economic and other benefits to those directly involved in the trade, as well as to governments and to the public in both importing and exporting countries. It is therefore realistic to accept that such trade will always take place, and that there will always be an associated risk of spread of aquatic animal pathogens. It is also realistic to accept that once an aquatic animal pathogen has become established in a new location, it is very difficult, if not impossible, to completely eradicate it (although the effects of clinical disease can be mitigated). The old adage of "prevention is better than cure" certainly holds true. This paper suggests that the approach to managing aquatic animal disease risks associated with international movement of live aquatic animals, genetic material and aquatic animal products should be similar to a HACCP approach. Potential risk management measures can then be identified in advance for each individual step in the process of international movement of such commodities. International standards and guidelines describe such measures, both preventative (for example, certification and import risk analysis) and reactive ones (for example, contingency plans), and a range of tools are available at national, provincial, local and farm levels. All parties involved in

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international trade must recognise the need for vertical (farm, local, provincial, national, international) and horizontal (farm-to-farm, local-to-local, etc.) integration, application, and agreement regarding such measures, to ensure a continuous chain. Implementation is a responsibility that must be shared among all parties involved.

INTRODUCTION

The movement of live aquatic animals, genetic material and aquatic animal products carries an inherent risk of transferring aquatic animal pathogens. International trade is of particular concern because of the large volumes of live aquatic animals and their products moved (e.g., in 2002, about 38% – or more than 50 million tonnes live weight equivalent – of world fish production was traded internationally [FAO, 2004]), the large distances covered, and the risk that entire countries hitherto free of a particular disease may lose that status. Analysis of the disease risks associated with the translocation (including international movement) of live aquatic animals and their products has been the subject of conferences, training courses and workshops (Arthur *et al.,* 2004; Rodgers, 2001). Moreover, guidance is provided in international reference documents (OIE, 2006a, 2004a, b; Arthur *et al*., 2004; FAO/NACA, 2001, 2000).

International trade provides significant economic and other benefits to those directly involved in the trade, as well as to governments and to the public, in importing and exporting countries. It is therefore realistic to accept that such trade will continue, with associated risk of spread of aquatic animal pathogens.

More than 90% of the 50 million tonnes (live weight equivalent of world fish production) that are internationally traded is dead product (FAO, 2004). Despite this large volume, there is a paucity of scientific data on sampling strategies for product, diagnostic tests for product, or information on pathogen survival in product. Some product is internationally traded for use as feed for aquatic animals, providing a possible pathway for pathogen transfer, yet the safety of such aquatic meals for aquatic animals is not well understood or documented.

The risk of pathogen transfer is generally considered greater for movement of live aquatic animals than for movement of dead product; this paper therefore focuses on strategies to reduce the risks associated with international trade in live aquatic animals, but the authors suggest that a consistent approach should be taken for dead product.

The paper explores the options available to reduce the risk to an acceptable level of residual risk so that trade may continue relatively safely. The concepts of vertical (farm, local, provincial, national, international) and horizontal (farm-to-farm, local-to-local, etc.) integration, application, and agreement regarding such measures are explained in detail.

AQUATIC ANIMAL HEALTH RISKS IN INTERNATIONAL MOVEMENT OF LIVE AQUATIC ANIMALS

There is an inherent health risk associated with the movement of any animal (including aquatic animals), plant and human being, because any living organism harbours infectious organisms. Compared to the movement of terrestrial animals, the risk may be bigger in aquatics for the following reasons:

- Infections in aquatic animals are frequently sub-clinical, *i.e*. they would not be directly noticed before, during and immediately after the movement of that animal. This may be due to the different nature of aquatic host species in terms of, for example, unspecific defence as well as specific immune systems: host species have been able to develop despite the ubiquitous presence of infectious organisms in the aquatic environment and few means for the host animal to escape.
- The contribution of aquaculture to global supplies of fish, molluscs and crustaceans has grown from 3.9% in 1970 to 29.9% in 2002, with over 220 different farmed aquatic animal and plant species reported in 2002 (FAO, 2004). It is not surprising, then, that new pathogens continue to emerge (Bondad-Reantaso *et al.,* 2005; Murray and Peeler, 2005) and laboratory tests are not readily available, let alone standardised and validated (OIE, 2006b).
- There are multifactorial disease syndromes where several infectious agents are implicated; however, it is not known which ones are necessary *versus* which ones are sufficient to cause the disease. Examples are midcrop mortality syndrome (MCMS) of prawns in Australia (Cowley *et al*., 2005; Owens *et al.,* 2003, 1998; Spann *et al*., 1997), *Penaeus monodon* slow growth syndrome in Thailand (Chayaburakul *et al.,* 2004) and white tail disease of the giant freshwater prawn *Macrobrachium rosenbergii* in the French West Indies, China (People's Republic, PR) and India (Bonami *et al.,* 2005). To compound these problems, some viruses have been implicated in more than one disease syndrome, for example, gill-associated virus (GAV) in MCMS as well as in peripheral neuropathy and retinopathy in prawns (Callinan and Jiang, 2003; Callinan *et al.,* 2003), and some have been found at high prevalence in apparently healthy animals, for example GAV (Walker *et al.,* 2001) and Mourilyan virus (Cowley *et al.,* 2005).
- Unlike livestock, aquatic animal species are usually not domesticated, and there is comparatively little information available on their biological requirements as well as on their disease background, yet an increasing number of aquatic animal species that are native to a particular region are being developed for aquaculture (often by taking broodstock from the wild) and, when proven successful in that region, are translocated to other regions of the world as promising prospects for aquaculture (Bondad-Reantaso *et al*., 2005; Briggs *et al*., 2004).
- Disease control options are very limited (for example, there are few efficacious, commercially available vaccines, and there are very few drugs registered for use in aquatic animals).

International trade is of particular concern, because:

- The disease may spread to countries hitherto free of that disease.

- The long period of travel and associated stress can weaken the host animals' defence system and so increase both the likelihood of infection and the infectious load during transport. The traded animals may be clinically healthy before transport but may experience transport stress so that sub-clinical infection leads to disease outbreak in the entire consignment. This may only become apparent well after arrival, making tracing-back difficult.
- The traded animals may be of a species that is new to the importing country and hence immunologically naïve to resident aquatic animal pathogens. The traded animals may succumb to infection with such resident pathogens once they have been released into their new environment.
- Susceptible animals in the importing country may have never been confronted with aquatic animal pathogens carried by the imported animals, *i.e*. they are immunologically naïve and may succumb to clinical disease.
- In many cases it will not be known whether aquatic animal species in the importing country are susceptible to the pathogens that imported animals may carry, or whether the imported animals are susceptible to resident pathogens, before such un-intended "field trials" are conducted.

The disease risk inherent in the translocation (including international movement) of live aquatic animals has been well documented (Bondad-Reantaso *et al.,* 2005). Some of these movements are regarded as having caused not just localised outbreaks but even pandemics, for example, furunculosis, crayfish plague and epizootic ulcerative syndrome (Roberts, 2003). Given that the risks are well known by now, especially because of past experience and devastating socio-economic consequences (Bondad-Reantaso *et al.,* 2005), it is perhaps surprising that live aquatic animals are still being traded in a way that continues to lead to rapid international spread of disease. Examples of such spread in the Asian region in recent years are koi herpesvirus disease (KHVD) and Taura syndrome.

KHVD was first reported in Israel and United States in 1998. In the Asia-Pacific region, infection with koi herpesvirus (KHV) has been reported from China PR, Hong Kong SAR China, Indonesia, Japan, Republic of Korea, Philippines, Singapore, Taipei China and Thailand (OIE, 2005a,b; NACA/FAO, 2005a,b). KHV may have a wider than currently reported distribution because – typical of a herpesvirus – it may be present in apparently healthy fish, and the level and extent of current surveillance may be inadequate to detect such infections.

Taura syndrome was first reported in shrimp farms in Ecuador in 1991-1992 and spread throughout the Americas through shipments of infected post-larvae and broodstock, causing mass mortality of cultured shrimp. The Asia-Pacific Regional Quarterly Aquatic Animal Disease reports (until June 2005) show that Taura syndrome has been reported from Indonesia, Malaysia (suspected), Myanmar, Taipei China, Thailand and Vietnam (OIE, 2005a,c; NACA/FAO, 2005a,b). China PR reported the first occurrence in its territory of Taura syndrome in shrimp farms where a high mortality rate was observed in Hainan, Guanxi and Guangdong provinces in April, May and June 2003 (FAO/OIE/WHO, 2004).

TO TRADE OR NOT TO TRADE?

International trade in live aquatic animals (including eggs and gametes) and dead aquatic animal product creates economic and social benefits:

- Importers can obtain and sell a product that is not available, or only at a higher cost, on the domestic market. This leads to personal income for the importer as well as tax revenue for the importing country.
- Exporters make earnings from the sale of animals and products. Again, such sales create personal income for the exporter and tax revenue for the exporting country.
- The community of the importing country obtains access to a desired product.

As long as there is a financial gain to be made through international trade in aquatic animals and their products, such trade will take place (legally or illegally) and inevitably pose a risk to aquatic animal health, including to aquatic animals in the importing country. There are many possible ramifications, including:

- production losses in aquaculture
- food shortage
- unemployment
- loss of biodiversity (if wild animals are affected)
- loss of domestic consumer confidence (in the safety of seafood)
- loss of health status (loss of "free" status for a certain disease) and resultant loss of export market access
- establishment of the introduced pathogen.

The resulting question then is not "To trade or not to trade?", but what can be done to minimise the outlined risks.

It is realistic to accept that once an aquatic animal pathogen established in a new location, it is very difficult, if not impossible, to eradicate it (although the effects of clinical disease can be mitigated). The old adage of "prevention is better than cure" certainly holds true.

This paper suggests that the approach to managing aquatic animal disease risks associated with international movement of live aquatic animals, genetic material and aquatic animal products should be similar to a HACCP approach: potential risk management measures can then be identified in advance for each individual step in the process of international movement of such commodities. International standards and guidelines describe such measures, both preventative (for example, certification and import risk analysis) and reactive ones (for example, contingency plans), and a range of tools are available at national, provincial, local and farm level. All parties involved in international trade must recognise the need for vertical (farm, local, provincial, national, international) and horizontal (farm-to-farm, local-to-local, etc.) integration, application, and agreement regarding such measures, to ensure a continuous chain. Implementation is a responsibility that must be shared among all parties involved.

MANAGING AQUATIC ANIMAL HEALTH RISKS IN INTERNATIONAL TRADE

There is no magic wand that will simply remove all risk. As has been shown above, trade will happen, legally or illegally, and therefore prohibiting trade is not an option that is 100% effective. The other extreme – doing nothing at all and simply accepting all risk – may on the surface appeal because it initially does not incur any cost. However, it will in the worst case scenario lead to all diseases occurring everywhere. This, in turn, could not only lead to wide-spread and significant production (and income) losses, consequential loss on productivity because of the need for treatment of affected animals, and consumer concern and loss of consumer confidence in seafood (for example, because of residues from the treatment). It could also lead to reduction of biodiversity and loss of social amenities such as recreational fishing. In addition, "doing nothing" is rarely a politically acceptable decision.

The solution therefore lies in the middle, that is, in reducing the risk to an acceptable level of residual risk so that some trade may continue relatively safely.

MANAGING RISK – A SHARED RESPONSIBILITY

Scarfe (2003) describes biosecurity in aquaculture production as a program for protecting cultured or managed populations of aquatic organisms from harmful effects of introduced diseases, a description easily expanded to biosecurity in fisheries. He suggests that to be maximally effective, frameworks for aquatic animal biosecurity need to *inter alia* adhere to the principle of vertical (local, state, national, international) and horizontal (local to local, state to state, etc.) integration, application, and agreement (standardisation and harmonisation).

"Vertical integration, application, and agreement" includes that there needs to be a chain of sanitary measures to prevent or reduce the impact of aquatic animal disease introduction, and that this chain needs to be uninterrupted and logical in the way its elements are connected. For example, the World Trade Organization (WTO) in its Agreement on the Application of Sanitary and Phytosanitary Measures (the SPS Agreement) sets out the basic rules for food safety and animal and plant health standards, but it does not develop those standards. Rather, for animal (including aquatic animal) health and zoonoses, it recognises the standards developed by the World Organisation for Animal Health (OIE) as a reference within the SPS Agreement. The OIE, in turn, does not develop standards in isolation but through a formal process that involves Specialist Commissions, international experts, and all its Member Countries. That way, countries have the opportunity to influence the development of international animal (including aquatic animal) health standards that they are subsequently encouraged to apply when developing their national SPS measures.

The SPS Agreement encourages governments to establish national SPS measures consistent with these international standards, guidelines and recommendations. This process is often referred to as "harmonisation" (see "horizontal integration, application, and agreement" – Scarfe [2003]). The recommendations in the OIE standards make reference only to the animal (including aquatic animal) health situation in the exporting country, and assume

that either the disease is not present in the importing country or is the subject of a control or eradication programme. Therefore, when determining its import measures, an importing country should do so in a way that is consistent with the principle of national treatment[1] and the other provisions of the WTO SPS Agreement.

Whilst governments carry some responsibility for ensuring market access for their industries as well as protecting them from the introduction of pests and diseases, the biosecurity "chain" will only be effective when the elements at the other end are also intact and functional. Thus, fishers and aquaculturists, too, need to act responsibly. Whether they produce fish for aquaculture, for recreational fishing, for human consumption, for ornamental purposes, or for feeding other fish, it is crucial that they learn to understand disease as a calculable business risk, not as something vague that will never happen to them. Like their governments who put national measures in place to manage the risks of introduction of aquatic animal pathogens with imports from other countries, farmers need to know how to minimise the risk of disease introduction to their enterprises. For example, rather than purchasing low-cost post-larvae, fry or fingerlings, farmers should put pressure on hatcheries and request that sanitary measures are put in place and that those hatcheries' produce is certified before purchase. Similarly, like a government that will combat disease outbreaks of national significance, farmers need to know what to do in an outbreak situation, for example, early notification of authorities and generic disease control measures.

MANAGING RISK – THE TOOLS

An important characteristic of any trans-boundary animal disease is the speed with which it can spread to other farms, villages, districts, the entire country, and even beyond that country's borders. In the aquatic world, the situation is worse: aquatic pathogens spread quickly through waterways that know no political boundaries, so that the first neighbouring farm or river system to which the disease spreads from the index case may already be located in another country, adding international spread to an already complex situation. The problem therefore needs to be tackled on all levels, *i.e.* at the farming level, the local, provincial, national and international level.

A variety of tools for aquatic animal health risk management are available. These tools are for managing risk at international, national, provincial, local and farm levels.

The SPS Agreement

The Agreement on the Application of Sanitary and Phytosanitary Measures (the "SPS Agreement") entered into force with the establishment of the WTO on 1 January 1995 (WTO, 1998). The SPS Agreement sets out the basic rules for food safety and animal

[1] A WTO Member that has a disease in a part of its territory may not impose sanitary measures that result in a higher level of protection for imports compared to the measures applied domestically to manage the disease within the country.

and plant health standards. "Sanitary and phytosanitary measures" are defined in the SPS Agreement as follows:

"… any measures applied:

(a) to protect animal or plant life or health within the territory of the Member from risks arising from the entry, establishment or spread of pests, diseases, disease-carrying organisms or disease-causing organisms;
(b) to protect human or animal life or health within the territory of the Member from risks arising from additives, contaminants, toxins or disease-causing organisms in foods, beverages or feedstuffs;
(c) to protect human life or health within the territory of the Member from risks arising from diseases carried by animals, plants or products thereof, or from the entry, establishment or spread of pests; or
(d) to prevent or limit other damage within the territory of the Member from the entry, establishment or spread of pests."

These include sanitary and phytosanitary measures taken to protect the health of fish and wild fauna, as well as of forests and wild flora.

Sanitary and phytosanitary measures, by their very nature, may result in restrictions on trade. The basic aim of the SPS Agreement is to maintain the sovereign right of any government to provide the level of health protection it deems appropriate, but to ensure that these sovereign rights are not misused for protectionist purposes and do not result in unnecessary barriers to international trade.

Members are encouraged to use international standards, guidelines and recommendations where they exist. However, Members may use measures which result in higher standards if there is scientific justification. They can also set higher standards based on appropriate assessment of risks so long as the approach is consistent, not arbitrary.

The OIE and its standards

The World Organisation for Animal Health (Office International des Epizooties, OIE) has the core mandate to improve animal health in the world. The OIE develops normative documents relating to rules that its Member Countries can use to protect themselves from diseases without setting up unjustified sanitary barriers. The main normative works produced by the OIE for aquatic animals are the *Aquatic Animal Health Code* (*Aquatic Code*) and the *Manual of Diagnostic Tests for Aquatic Animals* (*Aquatic Manual*). These aquatic standards are prepared by the Aquatic Animal Health Standards Commission (in brief, Aquatic Animals Commission), one of the OIE's four Specialist Commissions, with the assistance of internationally renowned experts. The standards are finally adopted by the OIE International Committee at the annual General Assembly of all Delegates of OIE Member Countries. The value of the OIE standards is therefore twofold:

- The measures published in the OIE standards are the result of consensus among the veterinary authorities of OIE Member Countries.
- The OIE standards constitute a reference within the SPS Agreement as international standards for animal health and zoonoses.

The OIE Aquatic Animal Health Code

The aim of the *Aquatic Code* is to assure the sanitary safety of international trade in aquatic animals (fish, molluscs and crustaceans) and their products. This is achieved through the detailing of health measures to be used by the veterinary or other competent authorities of importing and exporting countries so that the transfer of agents pathogenic for animals or humans is minimised but unjustified sanitary barriers are avoided.

The *Aquatic Code* is updated regularly, and a new edition is published each year, both in hard copy and on-line (see www.oie.int/eng/normes/en_acode.htm)

General provisions that OIE Member Countries can adopt to prevent and control aquatic animal disease

The provisions listed below are of a general nature, that is, they do not relate to specific diseases.

- Section 1.1 of Part 1 of the *Aquatic Code* provides contextual definitions of the terms or expressions used.
- Section 1.2 describes "Notification systems", commencing with the statements that "Countries shall make available to other countries, through the OIE, whatever information is necessary to minimise the spread of aquatic animal diseases and their aetiological agents and to assist in achieving better world-wide control of these diseases." This important "ground rule" is followed by detailed reporting requirements for OIE Member Countries. These rules specify the events in which immediate notification (within 24 hours) of the OIE is required and suggest that countries also provide information on the measures taken to prevent the spread of diseases, including possible quarantine measures and restrictions on the movement of aquatic animals, aquatic animal products, biological products and other miscellaneous objects that could by their nature be responsible for transmission of disease. The overall purpose of these provisions is transparency about the animal health situation worldwide. While the necessity for such transparency is particularly obvious in disease emergencies, this section of the *Aquatic Code* points out that the presence of an infectious agent, even in the absence of clinical disease, should also be reported. Section 1.2 then presents the disease listing and disease notification criteria, and the diseases listed by the OIE.
- Section 1.3 presents "Obligations and ethics in international trade", which includes information on certification procedures. The aquatic animal health situation in the exporting country, in the transit country or countries and in the importing country should be considered before determining the requirements that have to be met for trade. This Chapter then continues with "Responsibilities of the importing country", for example, that the import requirements included in the international aquatic animal health certificate should assure that commodities introduced into the importing country comply with the national level of protection, and that importing countries should restrict their requirements to those justified for such level of protection. If these are stricter than the OIE standards, guidelines and recommendations, then they should be based on an import risk analysis. There also "Responsibilities of the exporting country", notably that it is prepared to supply certain information to

importing countries on request. Responsibilities in case of an incident occurring after importation are also laid down. The second Chapter in Section 1.3 specifies certification procedures.

- Section 1.4 describes the principles of conducting risk analyses and provides detailed guidelines for each step, *i.e*. hazard analysis, risk assessment, risk management and risk communication. Two further Chapters in this section provide guidance on the evaluation of competent authorities and on zoning.
- Section 1.5 is about "Import/export procedures" and describes aquatic animal health measures applicable before departure, during transit, and on arrival. Some of these are very detailed and technical requirements; for example, disinfection of transporters, treatment of transportation water, and discharge of infected water (Chapter 1.5.1), whilst others are more general requirements to be met before and at departure (Chapter 1.5.2), for example, that each country should only authorise the exportation from its territory of live aquatic animals and aquatic animal products that are correctly identified, and inspected according to the procedures outlined in the *Aquatic Code* and *Aquatic Manual*. Chapter 1.5.5 describes aquatic animal health measures applicable on arrival, for example, that an importing country should only accept into its territory, live aquatic animals that have been subjected to examination by a member of the personnel of the Competent Authority of the exporting country or a certifying official approved by the importing country, and that are accompanied by an international aquatic animal health certificate.
- Sections 1.6 and 1.7 provide guidelines for "Contingency plans" and for "Fallowing" in aquaculture, respectively.
- Part 3 of the *Aquatic Code* contains recommendations for "Blood sampling and vaccination" and "Inactivation of pathogens". In addition, model international aquatic animal health certificates are provided in Part 4.

Recommendations applicable to specific diseases

The recommendations in each of the chapters in Part 2 of the *Aquatic Code* are designed to minimise the risk of specific diseases being introduced and established in the importing country, taking into account the nature of the commodity and the aquatic animal health status of the exporting country. This means that, correctly applied, the recommendations ensure that the intended importation can take place with an optimal level of animal health security, incorporating the latest scientific findings and available techniques.

At the General Session in 2006, the OIE International Committee adopted the ninth edition of the *Aquatic Code* including most disease chapters in a new format. There are two key aspects of this new format:

- Surveillance for declaration of freedom from disease
  Chapter 1.1.4 of the *Aquatic Manual* contains general requirements for surveillance for declaration of freedom from infection with a listed disease. These requirements differ, depending on the previous infection status and take into account, for example, historical freedom and absence of susceptible species. Where targeted surveillance is necessary, it needs to be underpinned with scientifically based, disease-specific surveys, rather than following a rigid schedule of testing 150 animals regardless

of the epidemiological situation, the disease biology and diagnostic test sensitivity and specificity. This constitutes a graded, risk-based approach. For example, where there are no susceptible species, there is no need for targeted surveillance, but basic biosecurity conditions need to be in place. These "basic biosecurity conditions" are defined in the *Aquatic Code*. They require, *inter alia*, that an "early detection system" is in place, which is also defined and which must include "veterinarians or aquatic animal health specialists trained in recognising and reporting suspicious disease occurrence".

- Commodities
  There are commodities for which, when authorising import or transit, Competent Authorities should not require any conditions relating to the disease in question, regardless of the status of the exporting country for that disease. This concept was introduced to provide better guidance to OIE Member Countries on commodities that can be traded safely and also to better reflect the realities of trade, for example the fact that more than 90% of the 50 million tonnes live weight equivalent of fisheries produce that were traded internationally in 2002 were in processed form (FAO, 2004).
  For all other commodities, a key aspect of the new disease chapters, which is consistent with the SPS Agreement, is that importing countries should not simply reject a commodity because it is deemed "too risky", but they should assess the risk and try to reduce it to an acceptable level. To aid this process, the new chapters provide guidance on disease-specific risk management measures. These depend on the status of the exporting country for that disease but also take into account the intended end-use for the traded commodity (for example, release into aquaculture, or for direct human consumption).

FAO/NACA Regional Technical Guidelines

The spread of aquatic animal pathogens has directly led to serious disease outbreaks in the Asia-Pacific region, impacting on aquaculture productivity, livelihoods, trade, and national economies. Such problems have also indirectly impacted on the trade of aquatic animal products within Asia and between Asia and major trading partners. Some of the most serious problems faced by the aquaculture sector in the Asia-Pacific region are those pathogens and diseases introduced and spread through movements of hatchery produced stocks, new species for aquaculture, and the ornamental fish trade; examples are white spot disease, Taura syndrome, and KHVD. More recent examples of economic losses due to spread of aquatic animal diseases include the following: carp mortalities in Indonesia, with estimated losses of 50 billion Indonesian rupiahs, approximately US$5.5 million (NACA/ACIAR, 2002); losses due to KHVD in Japan, estimated to be 150 million yen, approximately US$1.4 million (ISID, 2003a); and abalone mortalities in Taipei China, estimated to be 400 million Taipei China dollars, approximately US$11.4 million (ISID, 2003b).

Through cooperation of FAO, OIE and NACA and with the aid of additional regional and international expertise, guiding principles for responsible movement of aquatic animals and aquatic animal health management were established. The guiding principles

in the "Asia Regional Technical Guidelines on Health Management and the Responsible Movement of Live Aquatic Animals" were adopted by 21 governments in the Asian region in 2000. Within Asia, the Technical Guidelines and their associated implementation plan, the Beijing Consensus and Implementation Strategy, provide the basic framework and guidance for national and regional efforts in reducing the risks of diseases due to trans-boundary movement of live aquatic animals and place emphasis on the concept of "phased implementation based on national needs" (FAO/NACA, 2000). There is strong endorsement by many regional, inter-governmental, and global organisations, and a shared commitment from national governments to support its implementation.

National policies and programs

One of the first countries to develop a national strategic plan for aquatic animal health was Australia. The plan was developed under the oversight of a joint industry-government committee and launched in December 1999 by the Australian Government Minister as "AQUAPLAN: Australia's National Strategic Plan for Aquatic Animal Health 1998-2003" (Commonwealth of Australia, 1999).

AQUAPLAN 1998-2003 delivered a range of tangible outputs that not only increased awareness about aquatic animal health, but also significantly enhanced Australia's capability to be prepared for, and respond to, aquatic animal disease emergencies. Standard diagnostic techniques for aquatic animal diseases, a series of emergency preparedness and response plans, training resources and disease simulation exercises were some of those outputs. *AQUAPLAN – A Five Year Review* (Commonwealth of Australia 2002) found that considerable progress had been made under *AQUAPLAN 1998-2003*, that it had delivered significant benefits to the industry and that its integrated approach was required for Australia to remain competitive. The review also noted that several priority areas within aquatic animal health remained to be addressed. The second plan – *AQUAPLAN 2005-2010* – was again jointly developed by governments and private industry sectors and launched by the Australian Government in July 2005 (Commonwealth of Australia, 2005).

Australia is not the only country that has invested into a national aquatic animal health strategy. Bondad-Reantaso *et al.,* (2005) present examples of other countries' economic investments in aquatic animal health through national strategies, disease control programmes, or research.

Import risk analyses have been conducted in several countries and are publicly available, for examples see Peeler and Thrush (2004), Peeler *et al.* (2004), Biosecurity Australia (2003), Diggles (2002), AQIS (1999a,b), EPA [United States of America] (1999), Biosecurity Authority [New Zealand] (1999) and Stone *et al.* (1997).

Farm level tools

It is well known that all cultured aquatic animal species can harbour infectious organisms that can be transferred to other regions and countries through international movement of those animals or their products. Despite the existence of the various codes, protocols, guidelines and manuals mentioned above, disease outbreaks continue to happen in new locations, as mentioned above for KHVD and Taura syndrome.

Disease outbreaks in wild aquatic populations may go unnoticed, depending on how remote the location is, *i.e.* how likely it is that there are humans to notice such an event. However, disease outbreaks (or early signs thereof) in farms would be observed fairly early, that is at a time when some intervention may be possible to mitigate the effects of the disease. The role of the farmer is therefore very important as he or she is the basic unit of the aquaculture industry and shares the responsibility for ensuring that the introduction and dispersion of pathogens is kept to a minimum. If individual farmers do not have the relevant knowledge, skills, resources or willingness, then the health of their animals (and possible that of their neighbours' animals) will be at risk.

Good farm management practices are important, and farmers need to pay special attention to biosecurity measures such as screening potential wild vectors before introducing them to the population to be cultured, and disinfecting or quarantining post-larvae, fry or fingerlings before introducing them into the aquaculture system. Complying with these rules will minimize the risk of infection. It is also crucial that farmers must notify disease outbreaks – or even suspicion of an infectious event – to other farms that may be situated on the same water supply or may have received stock from the affected farm directly or from a common supplier. The local fish or animal health authorities must also be notified. Farmers must understand the importance of providing this information, for example, so that a surveillance system can be put in place to assess and manage the risks of disease transfer associated with the trade of aquatic animals (within the country and internationally). Countries with little resources can request the assistance of international organisations.

An excellent example of an extension manual for farmers has recently been published by the Indian Marine Products Export Development Authority (MPEDA/NACA, 2003). This extension manual summarises farm level risk factors and practical management practices that can be used to reduce risks of shrimp disease outbreaks and improve farm production.

CHALLENGES AND OUTLOOK

As the above sections have shown, there are many tools available at farm, local, provincial, national and international levels to minimise the risk of international spread of aquatic animal pathogens. Why, then, do we still see international spread of aquatic animal diseases?

Of course, there is no one simple reason, but it is obvious that if we view effective biosecurity as a chain of sanitary measures to prevent or reduce the impact of aquatic animal disease introduction and spread, any missing or weak link in the chain will jeopardise the outcome, no matter at what level – farm, local, provincial, national or international – the weak member of the chain is located.

At the farm level, there can be complacency ("it won't happen to me"), or unwillingness to change traditional practices, or a genuine lack of understanding. In addition, there may be reluctance to invest resources into prevention of disease, which is often more of an insurance-like investment where returns are not obvious; buying cheaper post-larvae might have more immediate appeal.

At the local, provincial and national levels, authorities may not be sufficiently resourced to provide the required aquatic animal health services. Where regulations exist, there may be insufficient means to enforce compliance. Where governments do not compensate farmers for certain costs brought about by disease outbreaks, there is little incentive for the farmer to conscientiously report a disease outbreak to the authorities.

At an international level, there is acknowledgement that the drafting of globally applicable standards is inherently difficult, and that complicated or "over-the-top" standards have little chance of being applied. However, the need to develop such standards through a thorough, consultative process puts the onus not just on the drafting commissions but also on the countries that are subsequently expected to apply those standards.

The research community, too, is a part of the chain. There are many areas where scientific data are not available, for example, the effectiveness of inactivation of aquatic pathogens through commercial processing.

Finally, it is realistic to accept that trade will continue to occur, and despite all precautions, diseases will continue to be spread internationally. However, the risk of this happening can be reduced, and the effects can be mitigated, if all parts of the chain of trade, from producer to international organisations, accept their responsibility to cooperate in providing an unbroken chain of biosecurity.

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Recent Technological Advancements on Aquatic Animal Health and Their Contributions Toward Reducing Disease Risks - a Review

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ABSTRACT

Advances in biotechnology have made a significant impact in reducing disease risk for aquaculture. Numerous rapid methods have been developed for the detection of pathogens in fish, shellfish, molluscs and their environment though immuno- and molecular diagnostics. As such methods become more reliable and more widely used their impact will continue to grow. Vaccination has also been extremely successful in reducing the disease risk in fish, but, biological, scientific and technical restrictions still prevent the production of commercial vaccines for all economically significant fish diseases. Current innovative approaches to vaccine development are using information about the sequence of pathogen genomes, gene function and derived products. In addition, novel vaccine identification methods are being devised using combinations of various techniques such as genomics, proteomics, knockout technologies and epitope mapping. Technological advancements are also being made in the detection of pathogens. Methods such as immunochromatography-based Rapid Kits and muliplex testing using the Bio-Plex Protein Array System and micro-array technology are bringing a new dimension fish health control.

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INTRODUCTION

The Food and Agriculture Organization of the United Nations (FAO) has estimated that the annual demand for seafood will outstrip the capacity of wild fisheries by 55 million tones by the year 2025 (FAO, 2002). This presents a major challenge for the aquaculture industry and the application of modern technology to enhance production and health of aquatic species offers great potential to meet this demand. This paper aims to highlight those technologies that have made important contributions to the improvement of aquatic animal health. It will review recent/current technological advancements and their contributions towards reducing disease risks, focusing on rapid detection of pathogens and vaccine development.

Disease is a major constraint to global aquaculture production. For example, significant costs from individual diseases amount to US$1 billion per year for yellowhead virus (YHV)/white spot syndrome virus (WSSV) in Asian cultured shrimp (Briggs *et al*., 2004). Other national losses due to specific diseases include US$33 million lost due to infectious salmon anaemia virus (ISAV) in salmon during the 1998/1999 epidemic in Scotland (Hastings *et al.,* 1999); US$31 million lost due to *Marteilia refringens* and *Bonamia ostreae* in oysters from France between 1980 and 1983 (Grizel and Héral, 1991), US$15 million due to losses caused by suspected KHV in carp in Israel, and abalone mortalities of unknown origin in Taiwan cost US$11.5 million (Bondad-Reantaso and Subasinghe, 2005). Many factors have contributed to increased losses due to disease, including greater globalization, further intensification, introduction of new species, expansion in the ornamental fish trade, stocking with cultured species, interaction between wild and farmed populations, lack of bio security, emerging diseases, misperception and mis-use of pathogen-free stocks, climate change and other human mediated activities. In terrestrial farming, global production is based on a limited number of animal species while over 230 different species are cultured in aquaculture systems. The industry has expanded, intensified and diversified over last 30 years. It is also based heavily on movements of live animals and products (*e.g.* broodstock, seed and feed). Thus, there is an increased risk of disease. Any successful health management programme should monitor the health status of fish, identify and manage risks to fish health, reduce exposure to or spread of pathogens and manage the use of antibiotics/chemicals.

HOW CAN NEW TECHNOLOGIES REDUCE DISEASE RISK?

A variety of technologies, in particular biotechnology, have already made an impact in reducing disease risk and many novel methods will contribute in the future (Adams and Thompson, 2006). Improved nutrition, use of probiotics, improved disease resistance, quality control of water, seed and feed, use of immunostimulants, rapid detection of pathogens and the use of affordable vaccines have all assisted in health control in aquaculture. The success of vaccination in reducing the risk of furunculosis in salmon in Scotland and Norway is an excellent example of technology having made a significant impact. This is turn led to a reduction of the use of antibiotics (Markestad and Grave, 1997) that has been sustained, and productivity has increased as a result of vaccination (Gudding *et al*., 1999). There is also a downward trend in the use of antibiotics in Japanese

aquaculture as there is increased acceptance in the use of vaccines. This clearly is desirable as increase in antibiotic resistance through over-use of chemotherapeutants is of concern with a variety of bacterial fish pathogens. The development of rapid testing methods has also made a substantial impact in reducing disease risk, and as these become more reliable and more widely used their impact will continue to grow. Traditional methods such as histopathology, bacteriology, virology, parasitology and mycology also continue to be used effectively. These are appropriate for the identification of common, easily cultured pathogens but for many pathogens this may be expensive, time consuming (*e.g.* lag phase for the culture of some bacteria, slow growth and contamination), require access to high levels of expertise, and may not lead to a definitive diagnosis even when bacteriology and virology are supported by histological evidence. Thus rapid methods can both complement and enhance traditional methods.

RAPID DETECTION OF PATHOGENS IN FISH, SHELLFISH, MOLLUSCS AND THEIR ENVIRONMENT

Rapid detection of pathogens is useful in a variety of situations e.g., in clinically infected animals, in sub-clinically infected animals and in the environment. Although immunodiagnostics, molecular diagnostics and multiplex technologies are all valuable rapid methods for the detection of pathogens in fish, shellfish and molluscs not all these technologies are equally well suited to all samples. There are differences in sensitivities and specificities for each method and in the type of samples that can be used (e.g. formalin fixed, fresh, tissue, blood, water). It should also be noted that for many of the rapid methods live and dead pathogens cannot be distinguished, therefore, the inclusion of enrichment methods and the use of live/dead kits are useful supplementary methods (Vatsos *et al*., 2003). Interpretation of results needs to be carefully considered with all the other clinical evidence, including histology and attempted culture of the pathogen.

Immunodiagnostics

A large number of methods have been developed for immunodiagnostics and these are used routinely in many laboratories for the detection of fish and shellfish pathogens. Such methods include agglutination (slide/latex); fluorescent antibody test (FAT/IFAT); immunohistochemistry (IHC); enzyme linked immunosorbent assay (ELISA); and blot (dot-blot/dip-stick/western blot) (Adams 1999; Adams 2004; Adams *et al*., 1995, Miahle *et al*., 1995). Selection of the antibody-based method depends on a variety of factors since each method has its merits and disadvantages For example IHC is very simple and is ideal for use with fixed tissues (Adams and Marin de Mateo, 1994), as shown in Figure 1 for *Renibacterium salmoninarum*, the pathogen that causes bacterial kidney disease (BKD). On the other hand, IFAT can be completed more rapidly than IHC. IFAT is extremely sensitive and ideal for use in the detection of viruses, in tissue or following tissue culture, as shown in Fig 2 for infectious salmon anemia virus (ISAV), but requires a fluorescent microscope to read the results (Adams *et al.,* 1995). This method was used successfully by Miles *et al*. (2003) for the detection of *Aphanomyces invadans*, the causative agent of epizootic ulcerative syndrome. The ELISA can be used in a variety of formats, both for the

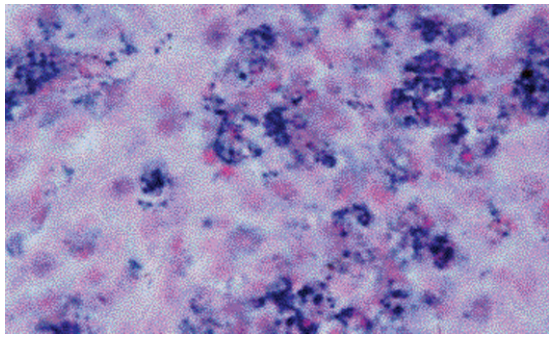

Figure 1. Detection of *Renibacterium salmoninarum* in fixed kidney tissue by IHC. (Photograph courtesy of Dr K.D. Thompson, University of Stirling).

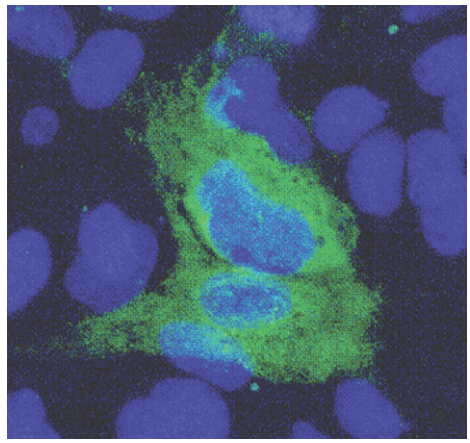

Figure 2. Detection of ISAV in SHK-1 infected cells by IFAT (Photograph courtesy of Dr K.D. Thompson, University of Stirling).

detection of pathogen and for serology (detection of antibodies to the pathogen) and has the advantage of high throughput, automated equipment is available, and it is quantitative. (Adams, 1992; Adams, 2004; Adams and Thompson 1990; Rose *et al.*, 1989; Cochennec *et al*., 1992; Boulot *et al*., 1989). It is, however, more complex than IHC and IFAT.

Immunochromatography and multiplex tests are now being developed and show great potential for the future (Adams and Thompson, 2006). Immunodiagnostic methods have not been widely used for the detection of shrimp and mollusc pathogens, although there are some reports (Lightner and Redman, 1998; Cochennec *et al*., 1992; Romestand and Bonami, 2003). However, with the development of Rapid Kits (immunochromatography/ lateral flow), which are simple to use, sensitive and inexpensive, this may change in the future. Commercial Rapid Kits are already available to detect WSSV in shrimp and ISAV in fish.

Any antibody-based test is only as good as the antibody used in it, and a standard protocol and reliable source of standard specific antibody is crucial. Antibody probes can be

produced in a number of ways, including polyclonal antibodies (prepared in rabbits, sheep or goats), monoclonal antibodies (prepared using hybridoma technology; Harlow and Lane, 1988), phage display antibodies or antibody fragments (McCafferty *et al*., 1990). Polyclonal antibodies can be very useful tools for the detection of pathogens (Adams, 2004; Steiropoulos *et al*., 2002). However, serum contains many different types of antibodies and mixed populations of antibodies can create problems in some immunological techniques. Monoclonal antibodies on the other hand are homogeneous and are of a defined specificity. Many have been developed for use in aquaculture (Adams *et al*., 1995; Adams, 1999; Adams and Thompson, 2006), some of which are now commercial available. Knowledge of the specificity of antibodies, whether they are polyclonal or monoclonal, is vital for the reliability of any antibody-based test. This is at two levels: (1) confirming that the antibody reacts with all isolates of that pathogen species e.g., from different geographical locations; and (2) confirming that the antibody does not cross react with other pathogens e.g., with other pathogens or micro-organisms that are likely to be in the aquatic environment. Phage display technology can also be used for the production of antibodies or antibody fragments, although few phage display antibodies for use in aquaculture have been developed (Zhang *et al*., 2004; Zhang *et al*., 2006). Phages are virus particles that infect bacteria and in this approach a foreign gene sequence is spliced into the gene of one of the phage coat proteins. The foreign peptide is then displayed on the phage surface.

Although some antibody-based methods can be very sensitive and carrier status can be detected (Rose *et al*., 1989), such technology can be limited in sensitivity when environmental samples are used, *e.g.,* water samples (Vatsos *et al*., 2002), and molecular methods are ideal in this situation.

Molecular technologies

Molecular technologies are also widely used for the detection of fish pathogens (Adams and Thompson, 2006; Cunningham, 2004; Karunasagar *et al*.,1997; Wilson and Carson, 2003). They have been successfully utilized for the detection and identification of low levels of aquatic pathogens. Such methods are also particularly useful for micro-organisms that are difficult to culture, may exist in a dormant state,, are involved in zoonosis, or in the elucidation of pathogen life cycles. In addition, molecular methods can be used for the identification to pathogens to species level (Puttinaowarat *et al.,* 2000) and in epidemiology for the identification of individual strains and differentiating closely related strains (Cowley *et al.,* 1999). Because of the general unavailability of the traditional pathogen isolation methods and immunodiagnostics for molluscs and crustaceans, molecular techniques have increasingly been used (Berthe *et al.,* 1999; Lightner, 1996; Lightner and Redman, 1998). The DNA-based methods such as polymerase chain reaction (PCR) are extremely sensitive. However, false positive and false negative results can cause problems due to contamination or inhibition (Morris *et al.*, 2002). Real-time PCR (closed tube to reduce contamination) and Nucleic Acid Sequence Based Amplification (NASBA) are alternatives that reduce this risk and offer high sample throughput (Overturf *et al.,* 2001; Starkey, *et al.*, 2004). Some of the most common PCR-based technologies used for the detection of pathogens are nested PCR, random amplification of polymorphic DNA (RAPD), reverse transcriptase-PCR (RT-PCR), reverse cross blot PCR (rcb-PCR) and RT-PCR enzyme hybridisation assay (Cunningham, 2004; Puttinaowarat *et al.*, 2000; Wilson

and Carson, 2003). *In situ* hybridisation is also widely used in the detection of shrimp viruses (Lightner, 1996; Lightner and Redman, 1998; Tang and Lightner, 1999; Tang *et al*., 2005) and confirmation of mollusc parasites (Stokes and Burreson, 1995; Le Roux *et al*., 1999; Cochennec *et al*., 2000; Carnegie *et al*., 2003). Colony hybridisation has also been used sucessfully for the rapid identification of *Vibrio anguillarum* in fish (Aoki *et al*., 1989) and has the advantage of detecting both pathogenic and environmental strains (Powell and Loutit, 2004).

Sensitivity, specificity, accuracy and reproducibility are all important factors in the development and subsequent use of molecular (and other methods). Increased sensitivity may be compromised by a reduction in reproducibility, as was shown by Starkey *et al.* (2005) when real time PCR and NASBA were compared for the detection of the salmon virus ISAV. Although NASBA proved to be 10 times more sensitive in detecting ISAV in clinical samples, the reproducibility of the assay was less than for real-time PCR.

PCR has been widely applied to the detection of shrimp viruses so that the risk of disease can be controlled (Corsin *et al*., 2001; **Lightner,** 2005; Mannivanan *et al*., 2002; Pantoja, 2005; Thakur *et al.,* 2002). This includes screening of broodstock, larvae and post larvae in the hatchery and before stocking (Lo *et al*., 1998). PCR is also used for identifying carriers, checking water and sediment for viral contamination and monitoring health of shrimp in growout ponds (Hossain *et al*., 2001, 2004). Several risk factors as well as the pathogen are needed for disease to occur (Thrusfield, 1986). Epidemiological data has been reported for WSSV indicating that the presence of the virus does not necessarily result in white spot disease (WSD). It has been shown that if the risk is minimised then the disease can be avoided or reduced. Thus, successful shrimp crops can be harvested when WSSV and other shrimp viruses are present at low viral prevalence (Umesha *et al*., 2006), when stress is reduced or when the virus is detected early enough, and outbreaks can be prevented despite the presence of WSSV. It has been reported that sick and dying shrimp lead to rapid progression of WSD and there is increasing evidence that the ingestion of sick or dying shrimp is the major mode of transmission. Treatment of WSD is not an option so early detection is vital. A quick response and damage control are required to prevent spread of the disease. Thus, early detection of sick/dying shrimp (monitoring numbers), use of pond side diagnostics and safe disposal of dying shrimp reduce the impact of WSD (Mohan *et al*., 2002). Many commercial kits are available (e.g. PCR, DNA dot blot, DNA *in situ,* immunoblot, IHC).

Serology as a screening tool for disease control

Serology is an alternative approach to pathogen detection. This technology enables detection of the host response to the pathogen (*e.g*. sero-conversion). Detection of specific antibodies in the serum of animals is recognised as a useful indicator of previous exposure to pathogens and such methods are regularly used in clinical and veterinary medicine (Fournier and Raoult, 2003; Palmer-Densmore *et al.,* 1998; Yuce *et al.,* 2001). They are capable of indicating infection before it is possible to detect the pathogen by culture or other methods and they have the advantage of being non-destructive. Serology can also be applied to the detection of pathogen-specific antibodies in fish. This may be in fish

suspected of having been exposed to specific pathogens, e.g., in broodstock screening for exposure to viruses or following vaccination to monitor immune response. The enzyme linked immunosorbent assay (ELISA) is well suited to large scale screening and this can be performed in any species of fish when an anti-fish species antibody is available. This opens up the possibility of vaccine potency testing using serology and perhaps vaccine efficacy testing in some cases when live pathogen challenge is unreliable or not available. This would not only reduce number of fish used in testing but would cut costs significantly for vaccine producers.

Application of rapid detection technologies in reducing risk of disease

The application of antibody-based methods (immunodiagnostics and serology) and molecular technologies can be extremely useful for epidemiological studies. For example, such research on ISAV highlighted mechanisms by which the incidence of ISA might be reduced. These included the screening of broodfish for ISAV carriage, screening of smolt before sea launching, stamping out of net pens before sea launching and the removal of wild salmonids around marine farms with ISA before emptying the affected farms. In addition, it was recommended that infected farms should be surrounded by nets to prevent a close contact between infected salmon and wild salmon (Richards *et al.,* 2005).

Novel technologies for pathogen detection

A number of new technologies are being developed for the rapid detection of pathogens and monitoring host responses. These include immunochromatography (*e.g.* lateral flow technology) and muliplex testing using the Bio-Plex Protein Array System or microarray technologies (Adams and Thompson, 2006). Lateral Flow is simple methodology enabling accurate (high sensitivity, specificity), simple, easy to use (2 steps, no instrument required) testing that is also economic (time/labour saving).

The Protein Array System (Luminex) theoretically offers simultaneous quantitative analysis of up to 100 different biomolecules from a single drop of sample in an integrated, 96-well formatted system. These methods are in their infancy with few published articles, mainly focusing on the detection of cytokines (Dupont, 2005; Giavedoni, 2005).

Vaccine technologies

Vaccination is the action in which a host organism is exposed to organic (biological) molecules that allow the host to mount a specific immune reaction through which it has a better capability to fight subsequent infections of a specific pathogen when compared to genetically similar non-vaccinated hosts. It has also been shown to be cost effective and has led to the reduction in use of antibiotics. In Norway, for example, antibiotic use has decreased from 47 to approximately one ton annually (Markestad and Grave, 1997).

A wide range of commercial vaccines is available against bacterial and viral pathogens and many new vaccines are under development. Most target salmon and trout and there are expanding opportunities in marine fish (Thompson and Adams, 2004).

Traditionally, the organic molecules used for vaccination are directly derived from the pathogen in question. The most straightforward approach is to culture the pathogen whereafter it is inactivated and presented to the host. At present, vaccines containing more than ten bacterial pathogens and five viral pathogens are produced based on such inactivated antigens (Sommerset *et al*., 2005). Alternatively, the pathogen is not inactivated but chemically or genetically weakened so as to survive only for a limited period in the host where it induces a specific immune response yet without causing disease and mortality. Such vaccines are generally described as "live" vaccines and there is concern that the attenuated strain may back-mutate and revert to the virulent wild type (Benmansour and de Kinkelin, 1997). Due to environmental and control concerns in most countries, only two bacterial (*Edwardsiella ictaluri* and *Flexibacter columnarae* for Channel catfish in the USA) and one viral vaccine (koi herpesvirus for carp, in Israel) are presently commercially available.

Vaccines for fish can be administered through three separate application strategies namely through injection (intramuscular or intra-peritoneally), through immersion (bath or dip-vaccination) or orally.

CHALLENGES AND PITFALLS

The above general outline theoretically allows the formulation of vaccines and vaccination application methods for all diseases and all pathogens as well as for all hosts and their live stages, but this is not so. Biological, scientific and technical restrictions presently still prevent the generation or commercialization of vaccines for all economically significant fish diseases. Individual fish typically have a low production cost as compared to other farmed homoeothermic animals. This low intrinsic value of the individual host only allows for a low affordable cost factor to protect the animal. Vaccines must therefore be made at a low sales and application cost. The production of specific carp vaccines typically illustrates the discrepancy between an ultra-low host cost and a fairly high expected vaccine cost for a vaccine containing koi herpesvirus (KHV), *F. columnarae* and motile aeromonads. Of course this is not true for koi carp.

Intensively cultured fish are typically susceptible to a wide range of pathogens. In general terms, any cultured species, irrespective of the environmental, climatological or geographical conditions, suffers from at least six major pathogens. Logically farmers want to protect their stocks against all the prevailing pathogens for the same relatively low cost. In addition, culture conditions and vaccination application costs presently necessitate that only a single vaccination application is affordable. For example, the salmonid market presently uses heptavalent vaccines containing *Vibrio (Listonella) anguillarum* serotypes O1 and O2, *V. salmonicida, Moritella viscosa, Aeromonas salmonicida* and infectious pancreatic necrosis virus antigen (IPNV). However, additional antigens against infectious salmon anaemia virus (ISAV), pancreas diseases virus (PDV) and viral haemorragic septicaemia virus (VHSV) and infectious hematopoietic necrosis virus (IHNV) are needed for the Northern Hemisphere.

Mass culture methods of pathogens have been established for a wide variety of pathogens yet, for some pathogens, *i.e*., several viral agents, some (facultative) intracellular bacterial pathogens and most metazoan (parasites) pathogens, the mass culture methods are not yet available. When mass culture methods are not available it is virtually impossible to produce antigens for vaccine formulation through classical means.

Some pathogens can be cultured but the resulting organisms do not display the antigen required in sufficient amount or in an appropriate form to induce a protective immune response. Essential antigens might only be produced in the complex host environment and not in an artificial culture medium. In addition, antigens might be hidden in the organism and only become "available" to the immune system when processed and expressed in conjunction with host immune cells. In addition, antigens might be weak, i.e., they do not elicit a sufficiently high immune response on their own to protect the host for the duration of its live.

The production of antigens might be feasible on a large scale but the live stage of the host might be such that the application of the vaccine in advance of the naturally occurring infection (vaccination window) does not allow for effective vaccination. This can either be because the available window occurs before the fish is immunological mature or the available live stage of the fish does not allow for the application method presently required to obtain protection, i.e. protection is obtained through injection but the fish are too small to inject (e.g. nodavirus (VNN) infections in larval fish). Moreover, not all antigens can simply be combined into a single formulation. In mammals, two different cell-mediated immunological pathways exist: typically described as the TH_1 and TH_2 reaction pathways. While these pathways have not yet been confirmed in fish, there is some evidence that the basic building blocks do exist which could cause interference between antigens depending on separate pathways. This, in practice, can lead to antigenic competition and the loss of the required immune reaction.

BIOTECHNOLOGY AND VACCINE DEVELOPMENT

The future development of fish vaccines will originate from different scientific fields; genetics, immunology, physiology and chemistry. Over the last few years tremendous advances have been made to sequence bacterial, viral and metazoan genomes. This combined with the ever increasing knowledge on gene function and their derived proteins will allow novel approaches for those pathogens for which classical culture is not (yet) feasible or doesn't yield sufficient antigen. A series of "new" methodologies have become available based on genome information (Leong *et al.,* 1997; Smith, 2000).

Genetic-based technologies

Recombinant technology

The identification of the protective antigen from a pathogen combined with the gene sequence that codes for this antigen presently allows insertion of the gene into a "production" host. This production host can subsequently be cultured on a large scale

from which the protective antigen is then purified from the production host or the medium and used in vaccine formulation. Although this technology is well established, future developments will likely lead to new expression systems (production hosts) in which yield, glycolysation of the proteins and restoration of the tertiary structure are increased. Recombinant expression systems have the main advantage that specific antigens can be produced when the actual host is difficult to produce or when culture systems are not available. An IPNV vaccine based on a recombinant expressed viral protein has been developed (Frost and Ness, 1997) and has been on the market for several years for use in salmon.

Vector technology

Vector technology is largely similar to recombinant technology but utilizes mostly viral production hosts and the entire host with the expressed antigen is used as vaccine antigen. This approach has, in some cases, the advantage that small proteins or peptides are expressed together with a set of production host antigens which augment the induced immune reaction or steer the immune reaction towards a more favorable immune pathway. Vector vaccines additionally can be used as live vaccines.

Construction of live mutants

Knowledge of the pathogen's genome can be used to delete certain genes or to insert non-functional gene sequences into certain genes resulting in an infective organism which is, however, unable to replicate repeatedly inside the host. The resulting mutant is then used as crippled pathogen which induces a protective immune response yet does not cause disease. The advantage of this approach is that the insertions or deletions are well defined and can be made such that reversion to virulence of the pathogen is virtually impossible. However, allowing the use of live vaccine strains for fish remains a matter of concern for most governments, mainly because the lack of spread of the vaccine strain through water effluents is difficult to verify. Whenever a live vaccine is used there is always concern that the attenuated strain may back-mutate and revert to the virulent wild type (Benmansour and de Kinkelin, 1997).

DNA vaccination

DNA vaccination is the process in which naked-DNA coding for a required antigen, and not the final antigen itself, is presented to the animal to be protected. This naked DNA, usually presented as a plasmid, is translated by the host cell into the immunogenic protein and expressed on the cell surface. The presence of a pathogen antigen in conjunction with host cell surface molecules will potentially trigger an effective immune response against the antigen. DNA vaccines for fish have been shown to be effective when based on DNA-sequences encoding rhabdovirus glycoproteins (Lorenzen and La Patra, 2005) and presently a first such vaccine is licensed in Canada against IHNV.

Vaccine antigen identification techniques

In order to be able to produce recombinant subunit antigens, or to make vector vaccines or DNA vaccines for a specific antigen, the protective antigen(s) of a certain pathogen must be identified. This is now being done by (combinations) of various techniques. In genomics, the DNA sequence of the genome of the pathogen is determined and mined for

information. By performing a detailed bioinformatics analysis of the genome sequence, one can identify the antigens that are expected to be surface-exposed. This can be validated by proteomics, *i.e.*, the analysis of the individual proteins of a pathogen in 2D gels, followed by either reaction with immune sera and/or mass spectrometry. This whole process is called reverse vaccinology and this process is most appropriate for bacteria and parasites. Other methods being used in reverse vaccinology are knockout technologies, which indicate whether a certain antigen is essential or important for survival of the pathogen in the host. Examples are: (1) RNA interference, where expression of certain genes is blocked by double -stranded RNA, (2) *In Vivo* Expression Technologies (IVET) and (3) Signature Tagged Mutagenesis. The information obtained with several of these techniques is then combined with the existing literature data to prioritize vaccine candidate antigens for cloning, heterologous recombinant expression and vaccine efficacy testing.

Epitope mapping has also recently been used to identify potential vaccine antigens for nodavirus (Costa, 2005). In this technology, B-cell epitopes (those parts of the capsid or envelope proteins that are specifically recognised by the binding sites of antibodies) are identified (van Regenmortel, 1996). These antibody footprints (14-20 amino acids) are identified by synthesising overlapping peptides representing the parts of the virus proteins. These peptides are then coupled to individual identifiable beads in the Pepscan System and then antibodies from the host fish (following challenge with the pathogen) are reacted with the beads to identify with which peptide (s) binding takes place (Costa, 2005). This technology (Luminex) is flexible and can also be used for the detection of pathogens and other analytes by binding antibodies to the beads instead of peptides (see multiplex technologies for the rapid detection of pathogens).

Immunology-based vaccine technologies

Adjuvant technologies

The word adjuvant is derived from the Latin word *adjuvare* = to help. Adjuvants are therefore chemical or biochemical compounds which help an antigen to elicit a protective immune response. In fish vaccinology, adjuvants are currently widely used in the salmon industry and virtually all salmon are injected with oil-adjuvanted vaccines. Typically antigens, contained in a watery suspension, are incorporated in oil to form a water-in-oil emulsion. Although the mode of action for such oil-adjuvanted vaccines is not fully understood, clear evidence is available to illustrate that some pathogens or the antigens thereof, require the oil-adjuvant to induce protection.

Advances in fish immunology will most likely allow the use of more specific adjuvants. These specific adjuvants would include molecules such as interleukins and heat shock proteins. The discovery that immune responses can be manipulated towards the desired pathway through the administration of specific chemical patterns common in bacteria and viruses but largely absent in phylogenitically higher animals allows for adjuvant design to be tailored to a specific antigen. The use of CpG DNA motifs is an example of the use of the pattern recognition of the host's immune cells and was has been shown to induce protection against IPNV infections in salmon (Jorgensen *et al*., 2003).

Chemistry–physiology based vaccine technologies

Oral vaccines

Oral vaccination strategies are preferred for their ease in application. However, oral application of vaccines characteristically generates low protection levels and usually a relatively short duration of protection. Two factors are believed to contribute to this low response: the partial degradation of the antigens in the gastro-intestinal tract and the low transfer rate of the antigens from the intestinal lumen to the immune reactive cells. Recent advances in delivery systems have led to systems that counteract these negative effects considerably and thereby allow presentation of the antigen to the immune cells. Successful oral delivery of a recombinant subunit vaccine has recently beenreported (Lin *et al*., 2005) in which *Artemia* sp. nauplii were encapsulated with recombinant bacteria containing the antigen.

Synthetic vaccines

Many antigens are based on polysaccharide antigens. Most molecular biology methods yield protein antigens. However, for many pathogens the induction of a protective immune response requires polysaccharide antigens. Advances made in carbohydrate chemistry might allow the *in vitro* construction of large quantities of specific polysaccharides which, when administered, induce a protective response. Far less attention has been given to the production of synthetic vaccines but this may changes in the future.

CONCLUSIONS AND FUTURE PROSPECTS

Biotechnology is likely to allow vaccine development against pathogens for which, until now no methods are available to economically produce sufficient amounts of antigen. It should be realized, however, that most of the above cited developments are still in their infancy and challenges towards multi-component vaccines are yet to be addressed. Furthermore, advanced technologies require substantial research and production costs. These costs will ultimately be translated into the vaccine cost price which presently makes the use of vaccines derived from such antigens only likely for high valued fish species.

Biotechnology is also enabling the development of a variety of novel technologies for the detection of pathogens. Although many of these are still in their early stage of development, they hold the potential to bring diagnostics in aquaculture forward to a new level to assist in disease risk control.

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Aquatic Animal Health Professional Certification Schemes: Some Options for the Asian Fish Health Community

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ABSTRACT

Many governments are under increasing pressure from international trading partners to provide better information on the health status of exported aquatic animal commodities, particularly live animals. A central factor in this situation involves the training and certification of various links in the Competent Authority chain-of-command, including aquatic health service providers, aquatic pathogen detection laboratories, aquatic species pathologists, veterinarians and personnel who facilitate the endorsement of aquatic animal certificates of inspection. Standardized methods to ensure the competence of those people charged with providing and interpreting information on the health status of aquatic animals and/or their products for export is imperative to maintain the confidence of international trading partners. A thorough and logical certification protocol for aquatic animal health personnel would lend additional credibility and assuredness/peace of mind to the importer/consumer in the quality of the product and the production of health certificates of instantly recognizable meaning. Existing professional certification programs for aquatic animal health providers will be reviewed. These programs could provide guidance as a Professional Standards establishing mechanism for veterinarians and non-veterinary personnel in Asia who currently provide aquatic animal health services. This paper proposes a professional standards mechanism, developed through NACA and the Asian Fisheries Society-Fish Health Section (AFS-FHS), to promote and authorize a certification protocol for aquatic animal health providers in Asia as highly useful in improving this situation.

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BACKGROUND

Aquaculture is an important and expanding industry within the region; however, losses due to disease are often a primary constraint to aquaculture growth, with potential effects on both the socio-economic development in many countries that rely heavily on aquaculture for domestic consumption in rural communities, and/or foreign currency income through export markets. The impacts of disease are particularly problematic to small-scale farmers, who represent the majority of aquaculture production in many rural communities in Asia and worldwide. The reasons for this differential impact are multifactorial, but inequities in access to aquatic animal medicine/husbandry services, education and inaccurate information are core issues.

Aquaculture extension services for farmers in Asia are currently provided by personnel with varying levels of education and experience in aquatic animal health, including private individuals, governmental and non-governmental organizations, feed company representatives and others. This chain of information and services provided to the importer/customer from the farmer, through local and regional laboratories and governmental regulatory institutions, is currently fragmented at best. Updated guidelines (OIE, 2007) for facilitating the safe international movement of live aquatic animals have made the aquatic animal disease status of exporting and importing countries much more important from a regulatory standpoint. As such, there is increasing pressure from trading partners to provide better and more standardized information on the health status of exported aquatic animal commodities. Additionally, the globalization of seafood trade and the increasing awareness and demand of consumers for safe and high quality food have put food safety and quality assurance high in public awareness, and made those a priority for many governments. Such concerns also include the use of drugs and other chemicals in aquaculture, and the presence of human pathogens in seafood.

Although the field of aquatic animal health has rapidly developed in the past few years, standardized methods to ensure the competence of those people charged with providing and interpreting information on the health status of aquatic animals and/or their products for export have lagged in development. A thorough and logical certification protocol for aquatic animal health personnel would lend additional credibility and assuredness/peace of mind to the importer/consumer in the quality of the product and the production of health certificates of instantly recognizable meaning. Similar types of certification are practiced in many professional fields as a way of setting standards/baseline for professional competence.

In order to effectively implement such a certification program, national responsibility for aquatic animal health control needs to be clearly assigned, or better defined in cases where jurisdiction is unclear. The responsible authorities for aquatic animal health management vary from country to country, but such mandates may derive from the ministries or departments responsible for fisheries, agriculture and/or livestock, not the veterinary authorities. To this end, designation of a Competent Authority (CA) specifically for aquatic animal health issues is essential. Subsequent levels of authority for aquatic species, as mentioned in following sections, should devolve from the national level. Regardless of any delegated CA, and the actual aquatic animal health service providers

who may be involved, the need exists to have open and effective communication among the health workers, the CA and the governmental competent veterinary authority that has responsibility for reporting aquatic animal pathogens to the World Animal Health Organisation (OIE).

This need was emphasized at the 23rd Conference of the OIE Regional Commission for Asia, the Far East and Oceania, held in Noumea, New Caledonia in 2003, where a report was presented by the Commission "to demonstrate the current low level of interest in aquatic animal health matters shown by Veterinary Authorities in some Member Countries, deficiencies in communications between Veterinary Authorities and other Competent Authorities involved in aquatic animal health, and inaccuracies and inconsistencies in aquatic animal disease reporting to the OIE by veterinary authorities".

CURRENT RURAL AQUATIC ANIMAL HEALTH SERVICES IN ASIA

The majority of Asian aquaculture production comes from rural small-scale farms in which disease problems are often thought to be attributable to poor husbandry. In many cases the picture is more complicated, as disease problems arose and continue to exist due to unsafe and unregulated commerce of diseased aquatic animals – both locally and internationally. Economic factors, access to information and access to "safe" alternatives also likely play a role. The spread of many shrimp viruses (and recently of koi herpes virus in fish) are the plausible results of this combination of factors.

Extension services currently offered to rural Asian farmers are provided by many types of governmental and non-governmental organizations, utilizing a heterogeneous mix of personnel with varying levels of education and experience. Often the representative from a feed company is the main (or only) person assisting the farmers with disease and husbandry issues, particularly for more commercial aquaculture such as shrimp farming. Recent literature has acknowledged deficiencies in this approach, including a lack of knowledgeable and experienced extension personnel trained in aquatic animal pathology and appropriate disease control measures; logically, this situation can lead to decreased levels of confidence by many importing countries in the certification of aquatic animal health status.

AQUATIC ANIMAL HEALTH CARE CERTIFICATIONS

The certification of aquatic animal personnel in Asia is not a new idea. The need for this has been repeatedly emphasized at recent meetings and workshops. However, the needs of small-scale farmers/individuals/communities engaged in aquaculture may be substantially different from those of large commercial production facilities. Small scale rural farmers require the same or better disease control, preventative measures and extension services than larger producers, but do not receive them because they are (apparently) less commercially attractive as individual operations due to their size and scale of operation. Larger facilities might need consultation on vertically-integrated biosecurity encompassing feed production, infrastructural components, vehicular movements, distribution, personnel, and compliance with sanitary requirements of product processing, as well as disease

control advice. In contrast, small-entity rural farmers might be better served with a basic level of biosecurity awareness and husbandry techniques. This could include concepts of animal density and environmental stress-related disease prevention. The level of education required to provide this range of service may thus indicate the need for a tiered certification of aquatic animal health providers.

The idea for certifying Asian aquatic animal health personnel can be modeled after the currently-implemented three-tiered approach to pathogen detections /surveillance, based on sophistication of the provider and the diagnostic tools available. Tier 1 involves gross examination and basic microscopy for assessment of gross clinical presentations involving parasites or external fungal infections. Tier 2 involves more sophistication such as microbiological techniques including bacteriological culture and simple serologic tests. Tier 3 involves tools such as cell culture and molecular techniques for virus detection. The goal is to evolve from Tier 1 to Tier 3 by building competence of staff and infrastructure. Other tiers for assessing nutritional status, genetics, environmental factors and other factors could be added over time.

As in human and veterinary medicine, where primary health care may be provided by non-physicians and non-veterinarians who are certified in specialized fields, a similar approach may work for rural Asian aquatic animal health providers. In this case, 'primary care' refers to accessible medicine that is delivered by generalists rather than specialists, entails relatively low technology, places emphasis on prevention and tends to be less costly. By training and certifying primary and secondary aquatic animal health providers, the needs of the rural farmer, industry, government and importing countries can be met, while making extension work more effective. This type of certification system might improve some of the gaps in aquatic animal health extension services in Asia.

U.S. ANIMAL HEALTH CERTIFICATION PROGRAMS

There have been substantial changes in the way aquatic animal health services have been provided over the last few decades in the United States. Few veterinarians in 1975 were available, experienced or interested in aquatic animals beyond mammals in zoological facilities. Diseases of aquatic animals were the mainly the purview of parasitologists and microbiologists. Veterinary medical education in the U.S. and internationally has largely been focused on the diseases of important terrestrial mammals including swine, bovine, equine, feline and canine species, and poultry. As the needs of the U.S. aquaculture industry are evolving to require more advanced and experienced aquatic animal health professionals, the veterinary community has also evolved to meet those needs, but there are still relatively few experienced and available U.S. practitioners whose practice is solely devoted to aquatic animal medicine. However, many veterinarians increasingly incorporate aquatic animal medicine into their existing overall practice.

As an example of a model certification protocol, the U.S. Department of Agriculture, Animal and Plant Health Inspection Service (USDA APHIS), administers the National Veterinary Accreditation Program (NVAP). This is a voluntary program that certifies private veterinary practitioners to:

- issue Federal regulatory health certifications for animal movement and export
- conduct extensive disease surveillance and monitoring
- be aware of and execute the notification protocols for reportable, zoonotic and emerging diseases
- safeguard U.S. animal health and control animal diseases

Benefits of the NVAP program include:

- training of a sufficient number of veterinarians to perform activities for regulatory work in a manner that is consistent with international and interstate trade requirements
- standardized accreditation procedures and requirements allowing for more uniform administration of USDA programs

Basic requirements for the NVAP:

- successful completion of a Doctorate of Veterinary Medicine;
- licensure to practice veterinary medicine in the State in which the veterinarian wishes to perform accredited duties; and,
- completion of an orientation program, which includes information on:
  1. Federal and specific state animal health regulations
  2. Interstate movement requirements for animals
  3. Import and export requirements for animals
  4. USDA animal disease eradication and control programs
  5. Laboratory support in confirming disease diagnoses
  6. Ethical/Professional responsibilities of an accredited veterinarian

The above criteria apply to veterinarians engaged in the health care of all types of farmed animals. However, with some modifications, the model is adaptable to veterinarians dealing with aquatic species.

Under the developing US National Aquatic Animal Health Program (NAAHP), provisions will be eventually be made among three Federal agencies, designated as co-Competent Authorities, for the training and certification of personnel involved with the exportation of farmed, resource and wild-caught aquatic animals. Finfish and shellfish health inspections, for example, will be conducted by individuals (inspectors) with appropriate education, training, equipment and facilities. It is important that any aquatic animal inspector be an impartial third party who has no potential of personal gain dependent upon the outcome of the inspection. The initial and continued success of this program will rely on the professional integrity and faithful execution of the duties of accredited veterinarians and other aquatic animal personnel.

AMERICAN FISHERIES SOCIETY/FISH HEALTH SECTION FISHERIES PROFESSIONAL CERTIFICATION

The American Fisheries Society (AFS)/Fish Health Section has developed educational and experience requirements to certify individuals in a tiered Fisheries Professional certification program. Although this program does not hold any Federal or otherwise official status,

the U.S. Department of Interior, Fish and Wildlife Services and the Department of Commerce, National Oceanographic and Atmospheric Administration acknowledge AFS Certified Fish Health Pathologists and Fish Health Inspectors as competent individuals in the field of aquatic animal health for finfish species under their respective jurisdictions. Some State agencies in the U.S. legally recognize individuals with AFS certification and provide increased compensation as an incentive to obtain and retain the certification. This certification program has been a successful attempt to raise the professional profile of AFS professionals by encouraging its members to stay current with advances in the field, and through requiring periodic re-certification to maintain their status.

NATIONAL ASSOCIATION OF TESTING AUTHORITIES (NATA), AUSTRALASIA

The NATA, which certifies member laboratories in Australasia to ISO/IEC 17025 standards, has recently recognized the qualification of an "aquatic diagnostician". This designation is defined as:

"A veterinary pathologist specializing in fish pathology who has satisfactorily completed the Australian College of Veterinary Scientists (ACVSc) Aquatic Animal Chapter examinations or equivalent course of research post graduate qualification that included a large component of aquatic animal pathology.

OR

A non-veterinarian who has completed an approved graduate education program in fish pathology; and has completed a minimum of 3 years professional level full-time fish health work experience including a substantial pathology/histopathology component; and is authorized to make a diagnosis under the relevant state legislation in the jurisdiction in which the laboratory operates.

Examples of post graduate qualification could include a higher degree or doctorate of philosophy in a relevant discipline with demonstrated experience in diagnostic techniques. Three years full-time work experience (minimum 0.75 FTE) is in line with the requirements of the American Fisheries Society (who register both veterinary and non-veterinary people as "fish pathologists" after examination)."

In Australia, there is no professional body similar to the American Fisheries Society which registers non-veterinarian 'fish pathologists' in a similar way to a professional veterinary Board certification process for veterinarians with specialized post-graduate training. This has limited the application of the above "aquatic diagnostic" definition, since veterinarians generally object to non-veterinarians receiving any official recognition for diagnosing or treating animal diseases. Moves to increase professional opportunities for aquatic animal practitioners in Australia have centered on the development of the Aquatic Animal Health Chapter of the ACVSc, which now offers membership by examination for registered veterinarians. However, most veterinarians, although authorized by virtue of their position to sign health certificates and make diagnoses for a wide spectrum of aquatic diseases affecting many species, often have only finfish- or salmonid-centric experiences.

INTERNATIONAL RECOGNITION

One of the issues which Asian countries (and others) will likely encounter is that the veterinary qualifications of an individual are not transferable to or recognized in other countries regardless of the country from which the veterinary degree was obtained, or the professional status and experience of the individual. The building of stronger formal and informal veterinary professional ties between countries, particularly ones with adjoining or shared water resources, could eventually allow for cross-recognition of veterinary and non-veterinary aquatic animal health qualifications.

RECOMMENDATIONS

One important achievement for Asia would be the establishment of an Asian equivalent of the American Fisheries Society certification approach, with membership achieved through specific required training and examination. Such a registration process would act as a professional standards-setting mechanism for non-veterinarian professionals in Asia who currently provide health and management services, including pathogen detections, environmental assessments and modifications, nutritional advice, provision of therapeutic options (but generally not prescribing therapeutants restricted to veterinary prescription), and advice for many other factors affecting aquatic animals.

Professional standards that could be developed for certifying personnel who want to provide aquatic animal health services are currently being drafted for review by the Network of Aquaculture Centres of Asian-Pacific (NACA) and the Asian Fisheries Society-Fish Health Section (AFS-FHS). These standards might offer farmers the opportunity to identify and choose from a wider pool of qualified individuals than currently exists; and would lend additional credibility and assurance to importers and consumers regarding the quality of the product**.** Such standards would also facilitate the issuance of valid certificates of inspection for aquatic animals. Ultimately, individual countries may need to adopt, modify or vary these suggestions to suit their own particular situations and resources.

With all this said, individual certification is only one part of the picture. There is a need also to put in place a system for training of the certified people. This is not a small task, but could be done based on a system of recognized and "certified" training centers. This topic is thoroughly covered in Subasinghe (1995).

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Diseases of Finfish

A Primary Study on Oral Vaccination with Liposomes Entrapping Koi Herpesvirus (KHV) Antigens Against KHV Infection in Carp

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ABSTRACT

"Koi herpesvirus (KHV)" is an emerging piscine herpesvirus that only infects koi carp *Cyprinus carpio koi* and common carp *C. carpio carpio* causing mass mortalities with severe economic losses worldwide since 1998. Therefore, protection of carp with KHV vaccines is urgently needed. We developed an improved liposome-vaccine containing KHV antigens within the liposomal membrane compartment for oral vaccination to carp. Carp immunized by 3 day oral administrations of the liposome-KHV vaccine showed 77% survival against a challenge with $10^{1.3}$ $TCID_{50}$ /100 μL of KHV while unvaccinated control fish showed 10% survival. The relative percent survival (RPS) was 74%. This demonstrated that oral immunization with the liposome-KHV vaccine was efficacious against KHV infection in carp.

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INTRODUCTION

Koi herpesvirus (KHV) is an emergent piscine herpesvirus that only infects koi carp *Cyprinus carpio koi* and common carp *C. carpio carpio* causing mass mortalities with severe economic losses in Japan and other parts of the world such as Israel, the USA, Germany, Netherlands, Taiwan, Indonesia and South Africa since 1998 (Hedrick *et al*., 2000; Gray *et al.,* 2002; Ronen *et al*., 2003; Perelberg *et al*., 2003; Sano *et al*., 2004; Schlotfeldt, 2004; Haenen *et al*., 2004). Protection of carp with KHV vaccines is much needed. In order to prevent KHV infection, a live vaccine using attenuated KHV has been developed for the intramuscular injection (Ronen *et al*., 2003). Vaccination by injection is time-consuming and labor intensive, and subjects the fish to severe stress by handling. Oral vaccination, in which vaccine is mixed with food, is a more practical method because it requires little labor and causes no stress to fish. Our goal was to develop an efficacious oral vaccine to protect carp against KHV infection. In oral vaccination, the uptake of antigens is expected to occur in the intestine. Previous study has shown that fishes took up both crude lipids and proteins in the posterior intestine (Miyazaki and Fujiwara, 1988). The nature of lipid uptake in the intestine is common in juvenile as well as grow-out fishes. This finding suggests that antigen contained in lipid particles can be used not only to deliver antigens to the posterior intestine but also facilitate their uptake. Liposomes are known to be easily taken up into cells (Matyas *et al*., 2003; Kamps and Scherphof, 2004). We have developed improved liposomes that can exist as suspension in water, so that it can be mixed with moist pellet-diets or soaked into dry pellet-diet for field application (Yoshimura *et al*., 2004).

In the present study, we formulated a liposome-vaccine entrapping formalin-killed KHV antigens and examined its ability to stimulate immunity in common carp by oral administration. We evaluated both antibody titers in orally vaccinated fish and protection against experimental infection with KHV.

MATERIALS AND METHODS

KHV and liposome-vaccine preparation

KHV (isolate: NYKK0411) was originally isolated from a diseased koi carp in Japan in 2004 and cultured using KF-1 cell (koi fin cell, given by Dr. Hedrick, University of California, USA) according to Hedrick *et al*. (2000). After fragmentation of all of KHV-infected cells, the viruses were inactivated by adding formalin in 0.3% (v/v) final concentration for 48 h at 24^{o}C. The mixture was centrifuged at 3,000×g, for 15 min and ultrafiltrated (450 nm) to remove cellular debris. After ultra-centrifugation (200.000xg for 60 min), the resulted pellet of KHV was resuspended in phosphate-buffered saline (PBS, pH 7.2), and the washing was repeated three times to remove the formaldehyde. The recovered formalin-killed KHV was suspended in 1 ml PBS. The formalin-killed KHV suspension and live KHV were used for liposome-vaccine preparation and challenge tests in carp, respectively.

Liposomes containing KHV antigens were produced using the procedure described below. A chloroform solution containing phosphatidylserine, phosphatidylcholine and cholesterole at a molar ratio of 1:10:5 (total 16 μmol) was evaporated in a tube to produce phospholipid thin films on the internal surface of the tube). One ml of the formalin-killed KHV suspension (corresponding to a protein concentration of 10 g) was presonicated with a probe type sonicator SONIFIER 250 (Branson) and was added to the tube with lipid films and agitated for 30 sec. The lipid and protein solution was resonicated several times. The resulting semi-transparent solution was centrifuged at 750xg for 5 min at 4°C. The supernatant contained liposomes fused KHV antigens in the liposomal membrane compartment and were about 5-10 m in diameter with a confocal fluorescence microscope (Carl Zeiss) (data not shown). The provided liposomes were used as the liposome-KHV vaccine.

Vaccination, serum antibody assay and challenge tests

All common carp used in the experiment were offspring of the same parent fish. The fish were randomly divided into 7 groups: 4 vaccinated groups and 3 unvaccinated control groups (n=5, 25-30 g body weight). They were held in tanks (25 °C, 30 l) with a water filtrate system. The liposome-KHV vaccine was completely absorbed within dry pellet-diet and given to fish in vaccinated groups for 3 days at a 24-hr interval. Each fish daily received a total of 20 μL of the liposome-KHV vaccine. After third administration of the liposome-KHV vaccine, fish were nursed for the following 21 days with normal dry pellets. Two vaccinated fish jumped out of their tanks and died. The control groups received dry pellets absorbed the same amount of PBS without liposomes for 3 days and normal dry pellets for the following 21 days. To evaluate the serum KHV-antibody titers on day 22, the blood was taken from one group of vaccinated fish (5 fish) and one group of control (5 fish) while fish were anesthetized with 1.5% carbamic acid ethyl esthyl. The sera were processed in a routine way. KHV neutralization assay was performed using a 96-well tissue culture plate method with KF-1 cells and KHV ($10^{1.25}TCID_{50}$/50μL), and neutralization titers of the anti-sera were determined.

To determine the efficacy of the liposome-KHV vaccine, all fish must be challenged at the same dose of live KHV. We established a novel challenge method based on the mode of KHV natural infection which occurred in a pond, i.e., KHV invaded respiratory epithelial cells of gills. This finding has been confirmed by other researchers (Hedrick *et al*., 2000; Gray *et al.,* 2002; Perelberg *et al*., 2003; Sano *et al*., 2004; Schlotfeldt *et al*., 2004; Haenen *et al*., 2004). We inoculated KHV by putting the virus suspension on the gill surface while fish were anesthetized with 1.5% carbamic acid ethyl esthyl. The fish were wrapped with wet papers and kept in the air for 5 min to complete KHV adhesion on gill cells. Fish were then returned to the experimental tanks. To determine the challenge dose of live KHV, we applied this method and obtained data determining success of the artificial infection with KHV. Mortality was correlated to inoculated level of KHV: 100% mortality at $10^{3.3}TCID_{50}$/100μL, 80% at $10^{2.3}TCID_{50}$/100μL and 40% mortality at $10^{1.3}TCID_{50}$/100μL. The moribund fish and fish just after death showed definite changes of gill tissues. We determined the challenge dose at $10^{2.3}TCID_{50}$/100μL of KHV. In the challenge trials, a total

of 13 vaccinated fish and 10 unvaccinated fish were inoculated with KHV by dropping a total of the 100μL KHV suspension on both sides of the gills through the gill cavity with a 1 ml syringe. The fish were returned in water and observed the onset of KHV infection and mortality at 21-22°C for 21days.

Polymerase chain reaction (PCR) assay for KHV

Polymerase chain reaction (PCR) for detection of a KHV DNA fragment was performed according to Gray *et al*. (2002). PCR assay was performed using DNA extracts from tissues of the gill and kidney, which was a representative of visceral organs in all of moribund and survived fish in both vaccinated and unvaccinated groups.

RESULTS

Orally-vaccinated fish showed high levels of the antigen specific antibody ($2^{5.75}\pm2^{5.48}$; mean±SEM), which was significantly ($P<0.01$) higher than that of unvaccinated group ($2^{3.59}\pm2^{2.09}$).

In challenge trials, unvaccinated control groups showed 90 % mortality (4/5, 5/5 fish) within days 7-16 post challenge (Fig. 1). Moribund fish and fish just after death showed dark-body coloration and many swollen cells of gill epithelia. Most fish of the vaccinated groups were weakened showing dark-body coloration but only 23% mortality (0/5, 1/4 and 2/4 fish) occurred within days 7-9 post challenge. The remaining fish fully recovered several days later. The moribund fish displayed many swollen cells in the gill epithelium

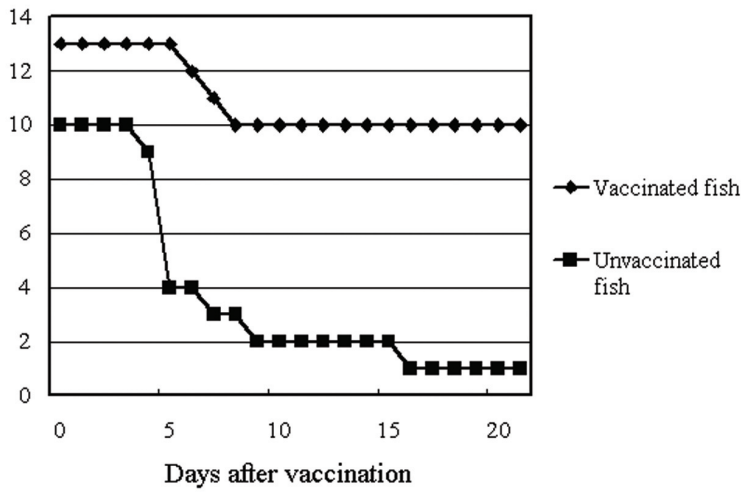

Figure 1. Mortality graph of the vaccinated koi (□ n=13) and unvaccinated carp (■ n=10) after challenge with live KHV at $10^{1.3}$ $TCID_{50}$/100mL on day 22 post-vaccine administration. For the following 21days, vaccinated carp show a 77% survival rate while unvaccinated carp showed a 90% mortality rate. The average relative percent survival was 74%.

while all of survivors showed no abnormal cells in gills. The resulting relative percent survival (RPS) was 74%. In the PCR assay, the putative PCR amplicons were derived from DNA extracts from the gills and kidney of moribund fish in both unvaccinated and vaccinated groups. On the other hand, except for one fish, PCR resulted negative on the gills and kidney of 9 survivors in vaccinated groups (data not shown).

DISCUSSION

In the present study, we developed an improved liposome-vaccine entrapping KHV-antigens, which could exist as suspension in water and be soaked into dry pellet-diet. Oral administration of the liposome-KHV vaccine for 3 days revealed that it could induce high titer of the specific KHV antibody. Challenge trials resulted in high survival of vaccinated fish (RSP 74%), indicating that vaccinated fish were protected against KHV. Based on the PCR results on survivors, all except one, vaccinated fish were protected from KHV infection. Even if they allowed KHV to infect cells of gill epithelia just after inoculation, they recovered from KHV infection. Thus, oral vaccination with the liposome-KHV vaccine indicated the induction of immunity against KHV infection in carp.

CONCLUSIONS

Three conclusions can be drawn from this study: (a) an improved liposome-KHV vaccine was developed, (b) it was effective in oral vaccination and induction of antibody against KHV infection and (c) it will be a breakthrough in mass vaccination for farmed fishes.

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Infection Experiments with *Aphanomyces invadans* in Advanced Fingerlings of Four Different Carp Species

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ABSTRACT

Using artificial infection tests with *Aphanomyces invadans*, the etiological agent of epizootic ulcerative syndrome (EUS), the present investigation examined the disease susceptibility and inflammatory response of advanced fingerlings of four different carp species, *i.e.*, three species of Indian major carps (catla, rohu and mrigal) and common carp averaging 12.1±1.8, 11.4±1.1, 12.7±1.5 and 10.2±0.96 cm in body length, respectively. The findings of disease susceptibility experiments indicated that over an experimental period of 12 days, there was 100% mortality with severe gross lesion development in Indian major carps, whereas in common carp, neither any mortality nor any gross visible lesions were observed. Inflammatory response studies demonstrated that the injected zoospores were able to germinate in the muscles of all the four experimentally infected carp species. Only in Indian major carps, the germinated hyphae were able to massively proliferate and induce extensive necrotic lesions in the large areas of myotome. In the common carp, the lesion area was confined to the line of injection and with time course the lesion area appeared to be healed with regenerated muscle fibres. Thus, the findings inferred that advanced fingerlings of Indian major carps are highly susceptible to EUS. Therefore, in the EUS season, the cultured populations of Indian major carps, which are in this age group, are likely to be at high risk.

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INTRODUCTION

Epizootic ulcerative syndrome (EUS) is one of the most destructive disease of both fresh and brackish water farmed and wild fish which caused major fish losses in many countries for over last three decades (Baldock *et al*., 2005). The disease is caused by an oomycete fungus, *Aphanomyces invadans* (Mohan and Shankar, 1995; Lilley *et al*., 1998; Thompson *et al*., 1999; Johnson *et al*., 2004). More than 100 fish species are reported to be affected by it (Lilley *et al*., 1998) and until recently, EUS remains an important issue in the carp culture ponds (Ahmed and Hoque, 1999; Lilley *et al*., 2002; Khan and Lilley, 2002; Islam *et al*., 2003; Nandeesha and Karim, 2006) particularly during the winter months. Fingerlings of Indian major carps (IMC) suffering from heavy mortalities during natural outbreaks (Roberts *et al*., 1989; Chinabut and Roberts, 1999; Khan and Lilley, 2002) and artificial infection experiments (Mohan, 2002) have been reported. Interestingly, during EUS outbreaks in several southern (Vishwanath *et al*., 1997a, b, 1998; Jayaraman, 1991) and northeastern states of India (Kumar *et al*., 1991), IMC in many water bodies had been observed to be unaffected. High temperature in south India has been suggested as one possible factor responsible for the increased resistance of IMC to *A. invadans* infection (Roberts *et al*., 1994). However, the temperature theory alone may not support some of the observations made in northeastern states of India, where temperature was ideal for EUS outbreaks. The possibility of age or size influencing the susceptibility of IMC to EUS was suggested by Lilley *et al*., (1998) and Chinabut and Roberts (1999). However, no artificial infection studies have been undertaken on IMC to confirm this observation. Hence, using artificial infection tests with *A. invadans*, the present study examined the susceptibility of four-month old advanced fingerlings (higher age/size group of fingerlings) of IMC (catla, *Catla catla*; rohu, *Labeo rohita* and mrigal, *Cirrhinus cirrhosus)* to EUS in south India, and for comparison, corresponding age groups of EUS resistant common carp, *Cyprinus carpio* (Wada *et al*., 1996; Lilley *et al*., 1998; Kurata *et al*., 2000) was used in the artificial infection trials.

MATERIALS AND METHODS

Fish and experimental systems

Fifteen-day old IMC and common carp fry were procured from Karnataka State Government Bhadra reservoir project fish hatchery. Cement cisterns (50 m^2 area; 15 cm soil bed) were used to rear the fish in the College of Fisheries fish farm facilities, Mangalore, India, for four months. Prior to stocking, the cisterns were drained, dried for a week, filled with freshwater to a depth of 0.5 m, and fertilized with agricultural lime ($CaCO_3$) and cow dung, respectively. After one week, the water level was increased to 0.9 m, and each fish species was stocked separately at a density of 100/50 m^2. The fish were fed with rice bran and ground nut oil cake mixture (1:1) at the rate of 10% of their body weight. Fifty percent of the water was changed with open well water once in 15 days and re-fertilization was done with cow dung.

Preparation of fungal spores

Suspension of motile secondary zoospores of *A. invadans* (strain B99C provided by J.H. Lilley) were prepared as described by Lilley *et al*. (1998). Briefly, three agar blocks (3×3 mm in size) of actively growing mycelium were placed in a petri dish containing glucose-peptone-yeast (GPY) broth and incubated for four days at 20°C. After four days, the nutrient agar from the resulting fungal mat was washed out by sequential transfer through five petri dishes containing autoclaved pond water (APW) and mats were kept in a petri dish containing 25 ml of (APW) at 20°C. After about 12 hr, the motile secondary zoospores were collected and number of zoospores in the suspension was counted ($6x10^4$ spores per ml) using haemocytometer.

Challenge with *A. invadans* spore

Forty advanced fingerlings each of catla, rohu, mrigal and common carp (CC) (averaging 12.1±1.8, 11.4±1.1, 12.7±1.5 and 10.2±0.96 cm, respectively) were used for challenge test. All the fish species were divided into four groups such as disease susceptibility group, sequential inflammatory response study group and two control groups (one for each study) having equal numbers of fish. The experimental fish were injected intramuscularly (into the left flank of fish just below the middle of dorsal fin region) with 0.1 ml of spore suspension ($6x10^4$ spores per ml) of *A. invadans* (strain B99C) as described by Chinabut *et al*., (1995). The control fish groups were treated with 0.1 ml autoclaved pond water at the same time. After injection, each species of experimental and control groups were kept separately in 500 l capacity fiberglass tubs containing 400 l water. Aeration was maintained with replenishment of 50% of water daily, and water temperature of the experimental tanks ranged from 26 to 29°C as measured twice daily in the morning and evening. For disease susceptibility studies, the fish mortality pattern was recorded daily up to 12 days post challenge and specific causes of mortalities confirmed by histology and reisolation of *A. invadans* from muscle tissue as described by Lilley *et al*., (1998). For sequential inflammatory response studies, one fish each from experimental and control groups were sampled at every alternate day (till all the experimental fish had died or completion of experimental period of 12 days which ever was earlier).

Histopathological analysis

After gross (eye) observation, lesion area was excised and fixed in 10% neutral buffered formalin. All the histopathological analysis was carried out as described by Chinabut and Roberts (1999). Samples were embedded in paraffin wax, sectioned at 5 µm and stained with haematoxylin and eosin (H&E). Selected slides were stained with Grocott's methenamine silver nitrate for demonstration of fungal hyphae.

RESULTS

There was 100% mortality in the case of catla, rohu and mrigal and the mortality had started after 6 days in catla, 8 days in rohu and 9 days in mrigal. There was 100% mortality after 10 days in catla and rohu and 11 days in mrigal; there was no mortality in the case of common carp and also in control group of fish. The detailed cumulative mortality pattern of advanced fingerlings of catla, rohu and mrigal recorded over a period of 12 days is presented in Table 1. At the time of morbidity or mortality, 100% of the fish had severe swollen hemorrhagic areas. Histopathological observations of the moribund Indian major carps indicated massive proliferation of fungal hyphae in the lesion area (Figure 1), and severe myonecrosis in large areas of myotome and the severity was so high that virtually no normal muscle fibres were observed in the lesion area (Figure 2) and around most of the hyphae there were no inflammatory cells. On the other hand, none of the common carp had developed any gross visible lesion or mortality at the end of experimental period of 12 days, but mycotic granulomatous lesions were observed histologically (Figure 3). The sequential inflammatory response studies indicated that in the case of Indian major carps, the sequence of progression of infection was similar as demonstrated by Mohan (2002) for fingerlings. Briefly, after one day of injection of zoospores, few fungal hyphae penetrating the muscle fibers were observed in the lesion area but no inflammatory cellular responses were found around the hyphae. After two days of injection, many hyphae were observed in the lesion area of the injected side. Four days after injection, the mycotic lesion had occupied both injected and non-injected sides. Six to nine days after injection, injected and non -injected sides and almost all the internal organs were extensively occupied by the mycotic lesions and there was massive proliferation of hyphae in the lesion area and there was extensive myonecrosis in large areas of myotome. These extensive pathological changes were always associated with gross visible lesions (*i.e.* severe swollen hemorrhagic areas) and morbidity or mortality.

Table 1. Cumulative percentage mortality of advanced fingerlings of four different species of carp (catla, rohu, mrigal and common carp) injected with zoospores of Aphanomyces invadans over a period of 12 days.

Days post challenge	Cumulative percentage mortality of advanced fingerlings of four different species of carp				
	Catla	Rohu	Mrigal	Common carp	Control
4					
5	0				
6	10				
7	50	0			
8	80	40	0		
9	90	90	60		
10	100	100	90		
11	100	100	100		
12	100	100	100	0	0

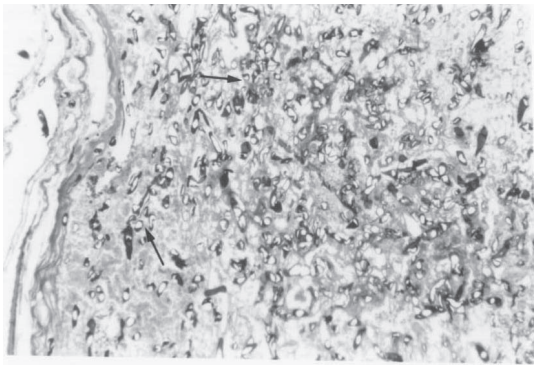

Figure 1. Mycotic lesion area in catla after 6 days of post injection (dpi) showing massive proliferation of hyphae (arrows) (Grocotts – H&E, x100).

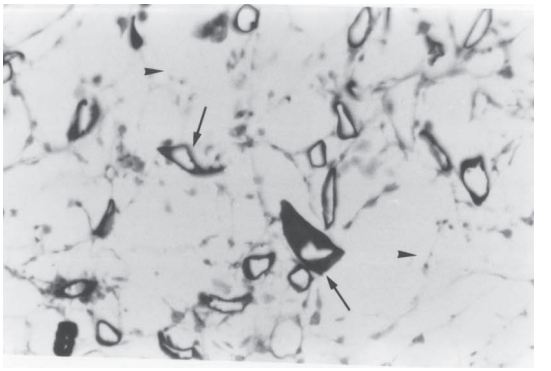

Figure 2. Extensive liquefaction of muscle fibers (arrow heads) and hyphae without any inflammatory cells around (arrow) in the lesion area of rohu at 8 dpi (Grocotts – H&E, x400).

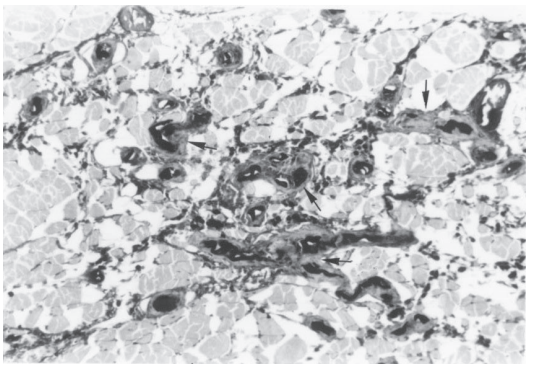

Figure 3. Well developed encapsulatory response by the epithelioid cells around the hyphae (arrows) and adjacent normal muscle fibers in the mycotic lesion area of common carp (CC) at 12 dpi (Grocotts – H&E, x200).

Between the three species of Indian major carps, the sequence of events (following challenge with zoospores of *A. invadans*), *i.e.*, penetration of muscle fibres by the germinated fungal hyphae, degeneration of those muscle fibres, infiltration of inflammatory cells to the lesion area, development of inflammatory foci of macrophages and/or epithelioid cell granulomata at the central part of lesion area, increase in fungal hyphae number in the lesion area and frequent penetration of fungal hyphae to the adjacent muscle fibres, necrosis of those muscle fibres and increase in overall mycotic lesion area with respect to days of post injection etc. were also similar. But, with respect to time course of these events, there was little difference between the species i.e. it was faster in catla and comparatively slower in rohu and mrigal. The mortality pattern of the susceptibility experiment was also reflected in the similar manner.

On the other hand in the case of common carp (CC), there was distinct difference in terms of inflammatory response when compared to IMC and their lower age groups, *i.e.*, fingerlings. In the case of CC, at 4 dpi, there was extensive infiltration of inflammatory cells into the lesion (Figure 4) and after 8 dpi, there were well developed epithelioid cell granulomata (Figure 5) and the lesion area appeared to be healed with regenerated muscle fibres. At the end of experimental period of 12 days, in the case of CC, the lesion area appeared completely healed (Figure 6) and the biological activity of the fungal hyphae appeared suppressed.

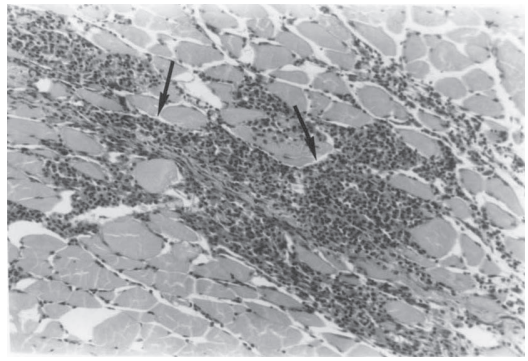

Figure 4. Mycotic lesion areas in CC at 4 dpi, showing extensive infiltration of inflammatory cells (H&E, x200).

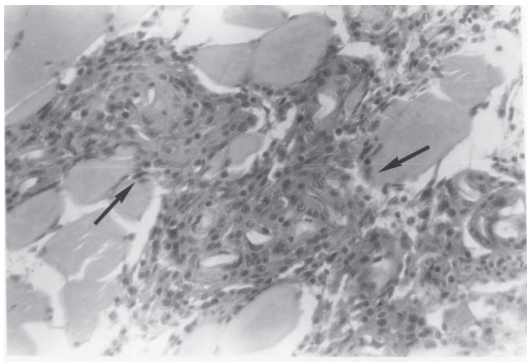

Figure 5. Well developed epithelioid cell granulomata consisting of several layers of epithelioid cells in CC at 8dpi (arrows) (H&E, x 400).

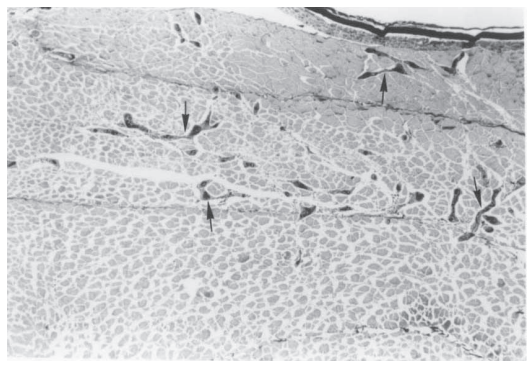

Figure 6. Lesion areas in CC at 12 dpi, appearing to be healed with well developed regenerated muscle fibers and injection site showing many fungal hyphae (arrows) (Grocotts–H&E, x40).

DISCUSSION

Mortalities due to artificial infection with *A. invadans*, in the case of advanced fingerlings of IMC have not been previously reported. However, in the present study, consistently in all of the three species of IMC, mortalities were observed in 100% of the fish and the gross observational results were supported by the histopathological observations. Therefore, it was considered that the advanced fingerlings of IMC could not resist against *A. invadans* infection whereas the corresponding age groups of common carp could do that.

However, in both groups of fish (*i.e.* IMC and CC), the injected spores had germinated but only in the case of IMC, the germinated hyphae were able to massively proliferate resulting in extensive necrotic pathology in large areas of myotome and almost all the internal organs and was reflected in the form of severe gross lesions, that in turn caused severe mortality. On the other hand, in the CC, the germinated hyphae were not able to proliferate and with time course the lesion area was healed. But, mycotic granulomatous lesions were observed histologically. Khan *et al*., (1998) had reported that injection of spores might circumvent the normal means of protection in some resistant fish and in their artificial infection studies, they had observed low mortality of tilapia, even though tilapia is considered as one of the resistant species to EUS (Lilley *et al*., 1998).

In the present study, since, same concentration of spores (from the same batch of spore suspension) were injected to all the fish, it was assumed that some of local and systemic factors might be providing an appropriate environment for multiplication of the hyphae in the IMC. While the effect in the case of CC, it would have been the opposite. Kurata *et al*., (2000) has reported that CC serum has fungicidal activities. Hatai (1980) opined that growth of *A. piscicida (=A.invadans)* would decrease if the fungus were exposed to an environment unconducive for its growth, which supports such an argument. Further, similar to our findings, Wada *et al*., (1996) reported that in ayu (a species susceptible to EUS), the number of hyphae were significantly more than that in CC (species resistant to EUS). In addition, the number of hyphae in ayu significantly increased as the infection progressed. Comparison of the degree of inflammatory cellular infiltration between IMC and CC indicated that in CC, there was very extensive infiltration of inflammatory cells in the lesion area and the inflammatory cells encapsulated almost all the hyphae. On the contrary, in the case of IMC, around most of the hyphae no inflammatory cells were found. Therefore, it was assumed that, the extensive inflammatory cells might be one of the factors in preventing the spread of hyphae to neighboring tissues and further proliferation, in the resistant fish group. Thompson *et al.,* (1999), through *in vitro* studies, have indicated that macrophages were getting clumped around the growing hyphal tips. Therefore, it appears that a similar phenomenon might be occurring in *in vivo* conditions to prevent the spread of hyphae in the case of CC. Since in IMC, around most of the hyphae, no inflammatory cells were observed, in those fish species, the hyphae might have migrated, unopposed, causing extensive myonecrosis in the myotome area due to release of proteolysins. Hence, it was considered that advanced fingerlings of IMC are highly susceptible to *A. invadans* infection. This was supported by field level studies (with a pathology-based diagnosis) in Bangladesh, where it has been reported that the major carps are the most significantly affected farmed fish and once an outbreak occurs in a carp pond, EUS can damage the entire crop (Khan and Lilley, 2002; Lilley *et al*., 2002).

However, in extensive (observational) studies, during EUS outbreaks, in Karnataka (South India), it was found that IMCs present in many water bodies were not affected (Vishwanath *et al*., 1997a, b, 1998). Similarly, in Tamil Nadu, IMCs present in culture ponds were also reported to be unaffected during EUS outbreaks (Jayaraman, 1991). Roberts *et al*., (1994) consider temperature in south India to be high enough for the IMCs to resist the fungus. Chinabut and Roberts (1999) feel that there is an anomaly in relation to geography in that in southern India, IMCs appear resistant but not in the north. Interestingly, in several northeastern states, even during low temperature periods, IMCs were found to be mildly affected or resistant during EUS outbreaks (Kumar *et al.*, 1991). Based on the present experimental study findings, it may be assumed that fish in those affected water bodies could be of still higher age groups. Therefore, artificial infection studies should be undertaken on still higher age groups of IMCs to confirm this hypothesis.

CONCLUSION

From the present study, it is clear that advanced fingerlings of IMCs are highly susceptible to *A. invadans* infection. Therefore, in the EUS season, the cultured population of IMCs, which are in this age group and/or lower age groups, are likely to be at high risk.

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KHV, CNGV, or CyHV-3, Which is the Koi/Carp Killer?

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ABSTRACT

Carp interstitial nephritis and gill necrosis virus (CNGV) is the cause of a lethal disease that afflicts koi and carps. Four genes of this virus are similar to those of Cyprinid herpesvirus 1 (CyHV-1) and Cyprinid herpesvirus 2 (CyHV-2) and so it was suggested that it be named CyHV-3. The virus induces gill necrosis and interstitial nephritis as early as 4-6 days post infection. The virus propagates well in the intestine and kidney and infected fish droppings contain high amounts of infectious virus. Thus, fish faeces is an appropriate source for non-invasive diagnosis by virus isolation in cell culture, PCR and ELISA tests. Strategies for controlling viral infection in fish are of limited use. Preventing introduction of CNGV into aquaculture facilities is desirable, but is not fail-safe. Live attenuated virus was developed in Israel and found to be an efficient and economic prophylactic vaccine. Morphologically CNGV resembles herpesvirus, but its genome size and its DNA sequences are different from herpesviridae members. Moreover, CNGV possesses genes resembling poxvirus rather than herpesvirus, indicating that additional information is required before classifying this virus.

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INTRODUCTION

Common carp (*Cyprinus carpio carpio*) is a fish species that is widely cultivated for human consumption, with 1.5 million metric tons harvested annually, principally by China, but also in other Asian countries, as well as several European countries (www.fao.org). Unlike the common carp, the subspecies koi (*Cyprinus carpio koi*) is a colorful ornamental fish, the focus of a hobby whose enthusiasts keep koi in backyard ponds and large display aquaria for personal pleasure and/or competitive showing.

A lethal viral carp disease was detected in the United Kingdom in 1996, but the first scientific reports did not appear until 1998, when Ariav *et al.* (1998) described the disease following major outbreaks in several carp farms along the Israeli coast. The disease was not restricted to the United Kingdom and Israel, and appeared in Germany with mass mortality (Bretzinger *et al*. 1999; Hoffman, 2000) and then in many countries all over the world (Crane, Sano and Komar, 2004; Haenen *et al.* 2004).

Although the morphology, the genome size and about 20% of the genomic sequences (see below) have been described, the International Committee on Virus Taxonomy has not yet determined the nomenclature of this virus. The virus has been designated by various names, first as koi herpesvirus (KHV), according to its morphological manifestation (Hedrick ett al. 2000), then as carp interstitial nephritis and gill necrosis virus (CNGV), according to the pathogenic effects it induces in fish (Pikarsky *et al*., 2004; Ronen *et al*., 2003). Recently it was shown that CNGV (KHV) possesses several genes whose sequences resemble those of CyHV-1 and CyHV-2 and that the CyHV-1 genome is approximately 295 kbp, similar in size to CNGV (KHV) (Hutonan *et al*., 2005; Ronen *et al*., 2003; Waltzek *et al*., 2005). These findings led to the suggestion that CNGV be designated cyprinid herpesvirus 3 (CyHV-3), recognising its unique characteristics (Waltzek *et al*., 2005).

THE DISEASE CAUSED BY CNGV

The disease caused by CNGV is seasonal, appearing in spring and autumn when the water temperature in open-air ponds is 17 to 28°C. It is highly contagious, spreads from infected to healthy fish sharing the same pond, and can induce 80 to 100% mortality rates. Mortality occurs within 6 to 22 days post infection (dpi), peaking at between 8 and 12 dpi (Perelberg *et al.*, 2003). Fish exposed to the virus at 20 to 24°C for 3 days and then transferred to a non-permissive temperature survived the disease (Perelberg *et al*., 2005; Perelberg *et al*., 2003; Ronen *et al*., 2003). On the other hand, many of the fish held at 13°C for 30 days developed the disease following a temperature shift up to 22 to 24°C. Persistence of the virus in the fish is limited, since fish transferred to the permissive temperature after 64 days at 13°C did not die (Gilad *et al*., 2003). Although the disease is highly contagious and extremely virulent, morbidity and mortality are restricted to koi and common carp (*Cyprinus carpio*) populations

(Bretzinger *et al*., 1999; Perelberg *et al*., 2003; Walster, 1999). The virus does not induce disease in other cyprinid or non-cyprinid species, and these virus-exposed fish do not

transfer disease to naïve carps, suggesting that species other than *Cyprinus carpio* are not carriers of CNGV (Perelberg *et al*., 2003; A. Perelberg, unpublished data).

Sick fish were apathetic and gathered close to the water surface, suffering from suffocation. Gill necrosis appeared as early as 3 dpi, coupled with an increase in the levels of external parasites and bacteria (Ariav *et al*., 1998). The skin showed a lack of lustre, with pale patches and increased mucus secretions (Haenen *et al*., 2004; Hedrick *et al*., 2000; Perelberg *et al*., 2005; Perelberg *et al*., 2003; Bergmann, unpublished data).

We examined the pathobiology of this disease in carp by using immunohistochemistry and PCR. We found large amounts of virus in the kidneys of sick fish and smaller amounts in liver and brain. Following the onset of disease a rapid increase in the viral load in the kidneys was detected by using both immunofluorescence and semiquantitative PCR. Histological analyses of fish at various times after infection revealed signs of interstitial nephritis as early as 2 dpi, which increased in severity up to 10 dpi. There was severe gill disease evidenced by loss of villi with accompanying inflammation in the gill rakers (Pikarsky *et al*., 2004).

Identification and characterization of CNGV

Hedrick and coworkers first isolated the virus after exposure of cultured KF-1 cells with cell extracts prepared from the organs of sick fish (Hedrick *et al*., 2000). Establishment of primary cultures of koi fin cells (KFC) and carp fin cells (CFC) allowed us to isolate the etiologic agent in Israel (Perelberg *et al.*, 2003; Ronen *et al.*, 2003). The isolated virus from sick carp was confirmed as the etiologic agent for this disease, based on the following data: (i) the virus was successfully isolated from infected fish but not from naïve specimens; (ii) inoculation of the virus propagated in KFC culture into naïve fish induced a similar disease; (iii) co-cultivation of kidney cells taken from specimens with the induced disease, but not from the mock-infected fish, yielded a similar virus, which propagated in KFC cultures; (iv) three cycles of transferring the virus between sick fish and cultured CFC ("ping-pong" technique) were successfully applied; (v) cloned virus isolated in tissue culture induced the same disease in fish; (vi) rabbit sera prepared against purified virus interacted specifically with tissues from experimentally infected fish as well as with sick fish from ponds; and (vii) viral DNA was identified in infected KFC and in sick fish but not in naive fish (Hutoran *et al*., 2005; Perelberg *et al*., 2003; Ronen *et al*., 2003). This initial identification of CNGV facilitated diagnosis of the disease by infection of KFC, PCR, and immunological methods.

Electron microscopy of negative-stained particles showed an icosahedral morphology with an average core diameter of 100 to 110 nm, resembling the core of herpesvirus (Hedrick *et al*., 2000; Hutoran *et al.*, 2005; Roizman, 1990). Thin sections of a purified virus pellet revealed enveloped particles with a thread-like structure (tegument) on the surface of the core. The cores bear atypically non-symmetrical electron-dense regions, which probably contain the viral genome (20) (Figure 1). The virus has a buoyant density of 1.16 g/ml and bands at 37 to 39% (wt/vol) sucrose following centrifugation in a sucrose gradient (Hutoran *et al.*, 2005). CNGV survived in pond water for 4 h at temperatures of around 22°C and probably survives for a longer period in droppings and pond mud (Crane, Sano and Komar, 2004; Dishon *et al.*, 2005; Hutoran *et al.*, 2005; Perelberg *et al.*, 2003).

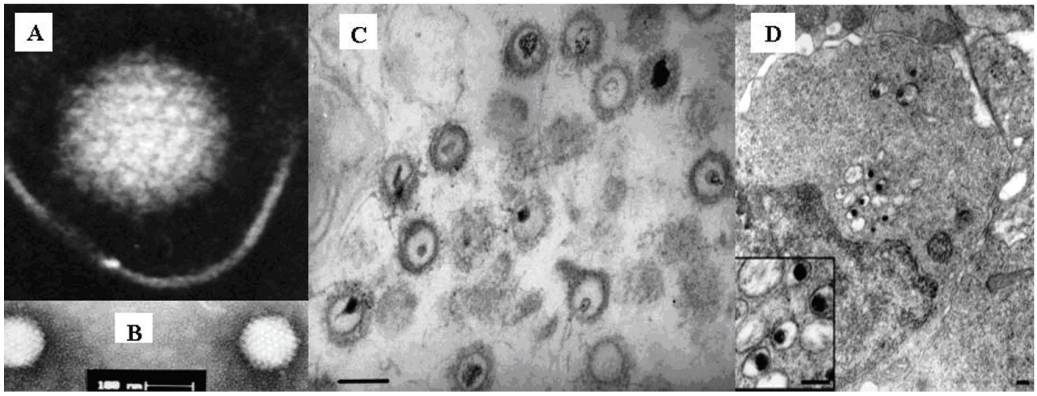

Figure 1. Electron micrographs of CNGV. CNGV harvested from infected KFC was purified by sucrose gradient and negatively stained with 2% phosphotungstate (panels A and B). Particle size was in the 96-105 nm range, with an average diameter of 103 nm (bar represents 0.1 μm). Thin sections (panel C) were made for ultrastructural analysis by transmission electron microscopy (TEM). Purified virus pellets were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate and stained with uranyl acetate and lead citrate. Panel D shows the ultrastructural appearance of CNGV particles in infected kidney 8 dpi. This cell harbors several cytoplasmic viral particles with round electron-dense cores (magnified in the insert). Bars represent 200 nm.

However, as a guideline for virus inactivation, infectivity is abolished after 2 days at 35°C (Perelberg *et al*., 2003) or after 30 min at 60°C (Crane, Sano and Komar, 2004). The virus is inactivated at pH values of below 3 and above 11, and it is also readily inactivated in chloroform, 25% ether, or 0.1% Triton X-100 (Crane, Sano and Komar, 2004; Hutoran *et al*., 2005).

Viral DNA

The CNGV genome is a large linear dsDNA of 270-295 kbp (Waltzek *et al*., 2005), as determined by pulsed-field gel electrophoresis (Hutoran *et al*., 2005; Waltzek *et al*., 2005) and is larger than those of vaccinia virus, herpes simplex virus type 1 (Figure 2), and all known members of the *Herpesviridae* except CyHV-1 (40). At present only ~20% of the viral sequences are present in GenBank, revealing that most of the viral genome (~80%) consists of DNA with no similarity to any sequences available in the databases. Moreover, the viral genome bears DNA sequences divergent from those of all other known avian and mammalian herpesviruses. Small genomic fragments of 16 to 45 bp show similarity mainly to members of the *Herpesviridae*, *Adenoviridae*, *Poxviridae*, and *Baculoviridae* families (Hutoran *et al*., 2005). One example is the DNA sequence of the CNGV thymidine kinase (TK) gene (accession #AJ535112) (Waltzek *et al*., 2005), of which only a 21-bp DNA fragment is similar to the Shope fibroma virus (a poxvirus) TK gene. However, recent studies found that the DNA sequences of the CNGV DNA polymerase, major capsid protein, helicase, and intercapsomeric triplex protein genes and of an additional open reading frame encoding an unidentified protein resemble those of CyHV-1 and CyHV-2 (Waltzek *et al*., 2005).

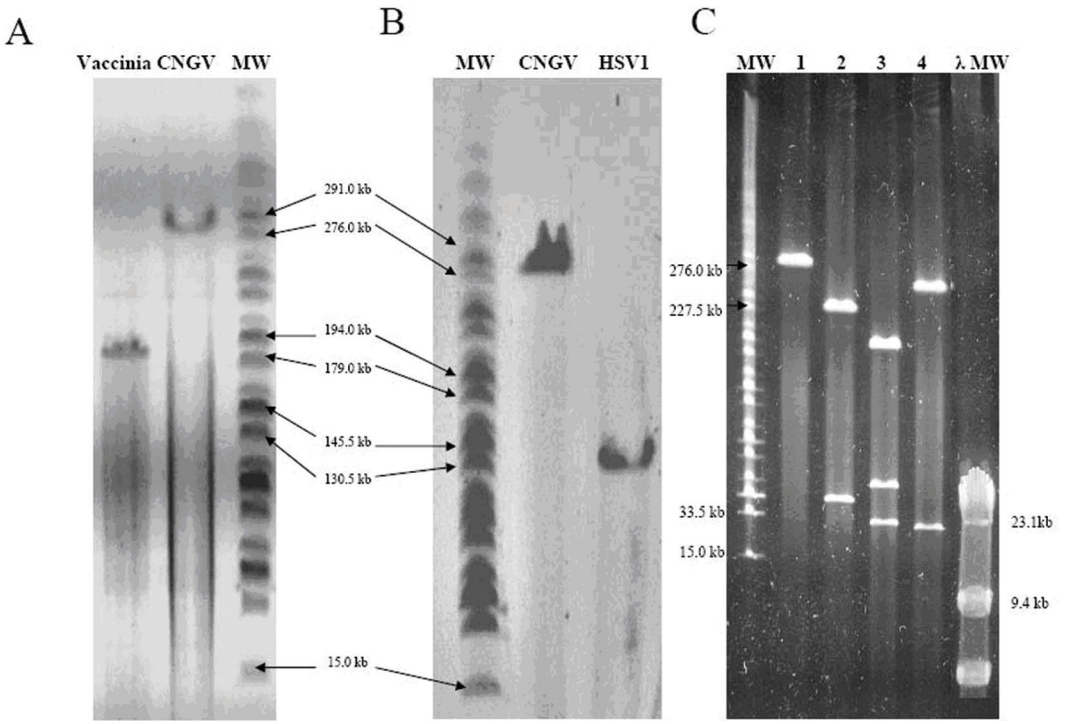

Figure 2. Analysis of CNGV DNA by pulsed-field gel electrophoresis (PFGE). Viral DNAs were obtained from purified viruses after incubation with proteinase K and SDS and phenol extraction. (A) Comparison of CNGV DNA and vaccinia virus DNA. (B) Comparison of CNGV DNA and HSV-1 DNA. (C) CNGV DNA cleaved with PmeI, PacI, and SwaI restriction enzymes. The molecular weight markers (MW) used were HindIII-cleaved λ phage DNA and a MidRange I PFGE marker (New England Biolabs).

Diagnosis of CNGV

The rapid dissemination of the virus in the fish body prompted us to develop efficient, rapid diagnostic means to identify the virus. Early diagnosis of the disease is extremely important to farmers, hobbyists, and veterinary authorities, allowing them to take appropriate precautions to prevent spread of the disease. It also allows hobbyists to save their fish by elevating the temperature and farmers to undertake early marketing of their fish. Several diagnostic techniques are currently in use, including isolation of the virus in cultured cells, histological and immunohistochemical methods, electron microscopy, enzyme-linked immunosorbent assay (ELISA), PCR, and *in situ* hybridization. These methods were described in detail by Haenen *et al*. (2004) and Pokorova *et al*. (2005).

The early appearance (3 to 5 dpi) of viral particles in carp droppings allows non-invasive detection of CNGV using PCR, ELISA, and infection of cultured cells (Dishon *et al*., 2005) (Figure 3). It is of great interest that virus present in sick fish droppings are infectious to fish, indicating that the virus is transmitted via faeces. It is not yet known whether infectious virus is preserved for long periods in the pool-bottom mud.

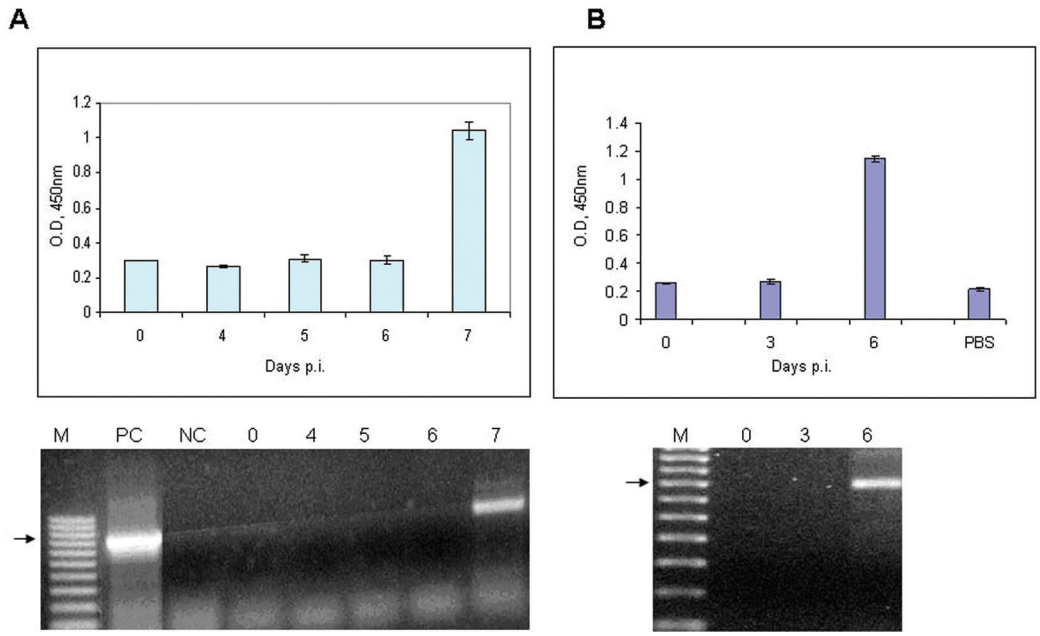

Figure 3. Detection of CNGV in fish-droppings by ELISA and PCR. Two fish (A and B) were kept separately, each in a tank, and their droppings were collected on the indicated days and frozen at -700C until processing. Each sample was assessed by ELISA and PCR. M=EZ load 100 bp molecular ruler (BIORAD). PC=positive control - purified CNGV DNA. NC=negative control – no template added. Arrow indicates the specific CNGV amplification product (see Materials and Methods). Data are shown as the mean OD values of wells ± the standard error of the mean.

Although most of the diagnostic methods are in use, they are time consuming and laborious and require specialized equipment. Recently, a "one-step ELISA kit" was developed (A.D. Thompson, pers. comm.), and a loop-mediated isothermal DNA amplification method was described by Gunimaladevi *et al.* (2004). and by Soliman and El-Matbouli (36). Both techniques are easy to use as rapid field diagnostic tools. It would be very helpful if these two new techniques were adapted to test for the presence of viral components in fish droppings. Detection of pre-vaccinated fish and/or fish surviving the disease following exposure to CNGV is crucial for veterinary authorities. Identification of viral DNA is not always successful, while detection of anti-CNGV antibodies in the fish serum by ELISA is relatively straightforward (Perelberg *et al.*, 2005; Ronen *et al.*, 2003). Unfortunately, a suitable kit for detecting anti-CNGV antibodies is not yet available.

Development of an efficient vaccine against CNGV

Currently, immunization of carp by injection of inactivated virus is ineffective. Live, attenuated vaccines potentially have many advantages in aquaculture (Benmansour and de Kinkelin, 1997). In general, live vaccine stimulates all phases of the immune system, resulting in balanced systemic and local responses involving both humoral and cellular

branches of the immune system. The advantages of using live attenuated virus vaccine are especially prominent in fish, where heat-inactivated virus is poorly immunogenic and large amounts of protein are required for achieving an efficient and durable immune response(Marsden *et al*., 1998; Marsden *et al*., 1996). With the use of live attenuated virus, the chance that reverted mutated virus will appear and threaten immunized populations is present but small. Experiments to achieve a nonpathogenic attenuated virus have been carried out in our laboratory since 2003 (Perelberg *et al.*, 2005; Ronen *et al*., 2003). The attenuated virus was isolated following serial transfer of the Israeli CNGV isolate in KFC. Viruses harvested after 20 passages in culture induced the disease in a small percentage of naïve fingerlings following injection or bathing (Perelberg *et al.*, 2005; Ronen *et al.*, 2003). It can be postulated, therefore, that the genetic alterations that accumulated in both the viral and host cell genomes facilitated the isolation of an attenuated virus. The attenuated virus was cloned in tissue culture in order to avoid undesired recombination, complementation, and reversion to a pathogenic virus. Several cloned viruses were UV irradiated and then re-cloned in order to insert additional mutations into the viral genome. This procedure was repeated twice. Currently, the selected attenuated virus clone does not induce lethal disease, and efficiently protects immunized fish against challenge infection (Perelberg *et al.*, 2005; Ronen *et al.*, 2003). Carp are very susceptible to pathogenic and attenuated viruses, and a short immersion of fish in water containing virus is sufficient for infection. The infection of fish with pathogenic and attenuated viruses is temperature restricted; fish held at the non-permissive temperature immediately following infection were not affected by the pathogenic virus and were not rendered resistant to the disease. The attenuated virus must propagate in the host fish in order to induce protection against the wild-type virus. Like the pathogenic virus, which induces the disease only at the permissive temperature, the attenuated virus requires the appropriate temperature to confer protection. Efficient protection is achieved by immersing the fish in water containing attenuated virus (10 to 100 PFU/ml) for 40 min, followed by incubation at the permissive temperature for an additional 48 to 72 hr (Perelberg *et al.*, 2005).

Protection against CNGV is associated with elevation of specific antibodies against the virus. The CNGV-specific antibody titer rises at 7 dpi and peaks at 21 dpi (Ronen *et al.*, 2003). Similar kinetics of antibody production was found in fingerlings immunized with the attenuated virus. The levels of anti-CNGV antibodies remained high in fish injected with either the pathogenic or the attenuated virus during the entire test period of 56 days (Fig. 4). These results point to a correlation between the survival rate and increased titers of anti-CNGV antibodies in the infected fish. The fish with naturally acquired immunity in ponds remain resistant for a long time (6 to 12 months). At present, we know that vaccination with the live attenuated virus confers resistance to a challenge infection for at least 8 months (unpublished data).

CNGV phylogenetics

Herpesviruses have complex and characteristic structures consisting of both symmetrical and nonsymmetrical components (Homa and Brown, 1997; Steven and Spear, 1997). The spherical virion comprises the core, capsid, tegument, and envelope (Nathanson and Murphy, 1997). The core consists of the viral genome, which is packaged as a single linear

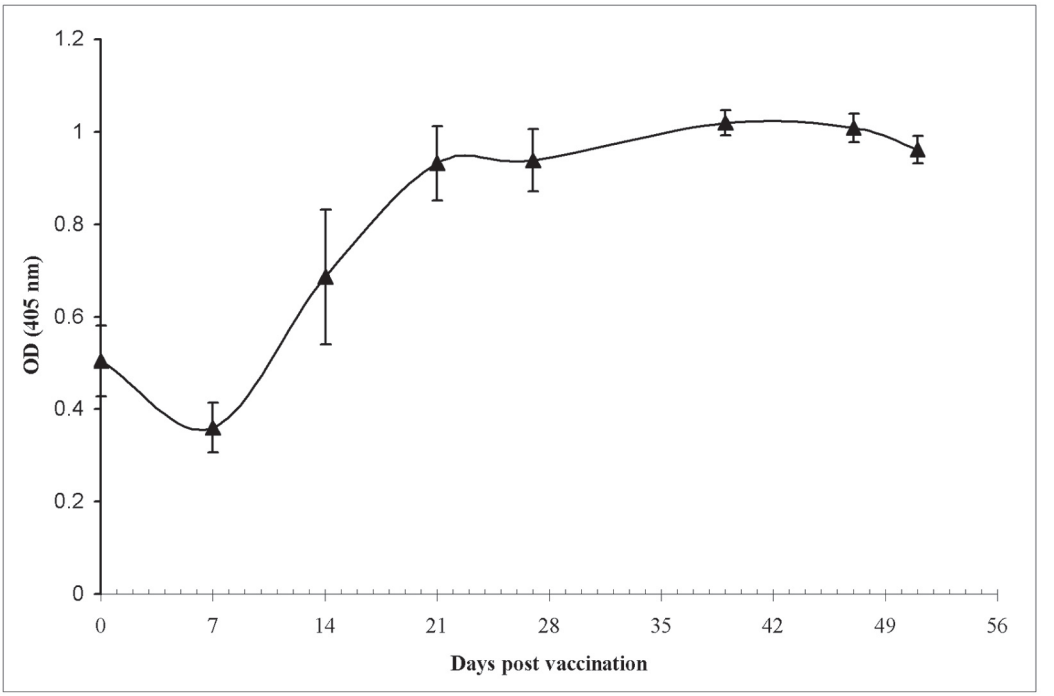

Figure 4. Induction of anti-CNGV antibodies in vaccinated fish. Fish (n=5 fish, average weight = 50 gr.) were immunized by intra-peritoneal injection of CNGV-P26 and thereafter, sera samples were collected, diluted 1:100 and analyzed by ELISA. Each point represents the average of 5 fish and the Error bars indicate ±S.E.

double-stranded DNA (dsDNA) molecule ranging from 125 to 240 kbp in size. Thus, the use of "herpes" in the CyHV may require either changing the definition of the herpesvirus family to include viruses with a genome larger than 240 kbp, or classifying these viruses with a large genome into a specific group. We and others estimate that about 80% of the CNGV genome is divergent from all the sequences available in the databases, making the classification of this virus very difficult.

Mammalian and avian herpesviruses are highly adapted to their hosts, and lethal infection occurs only in fetuses, in immuno-suppressed organisms, or following infection of an alternative host. Herpesviruses establish lifelong latent infection, a feature which is assumed to be the hallmark of all herpesviruses (Van Regenmortel *et al*., 2000). In contrast, infection with CNGV causes an acute disease with a higher than 90% mortality of both fry and adult carp. Assuming CNGV to be truly a member of the *Herpesviridae* family implies that it persists in the host as a latent virus. Solving the question of latency will contribute not only to the phylogenic classification of this virus, but also to understanding its epidemiology and improving prophylactic measures to prevent the spread of the disease.

The number of open reading frames in the viral genome and the number of proteins assembled in the virion are not yet known. Rough estimation made by sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of purified virions approximated the

number of proteins to be between 31 (Pokorova *et al*., 2005) and 80 (unpublished data). However, due to the large genomic size of this virus, the number of proteins expressed by CNGV may be even larger. Recent data showed that the CNGV genome allows 183 to 185 open reading frames (T. Aoki, pers. comm.).

Our early studies (Hutoran *et al.*, 2005) suggested that the DNA sequence of CNGV is divergent from all viral genomes available in the databases. However, following partial DNA sequencing of CyHV-1 and CyHV-2 genomes, it was demonstrated that CNGV and these viruses bear four similar genes (Waltzek *et al.*, 2005).

Comparison of the translation products of the viral DNA sequences to those available in the databases by using the BLAST X program showed that the major capsid protein, DNA polymerase, helicase and intercapsomeric triplex genes have some similarity to the proteins of anguillid herpesvirus 1 (Lee, Kobayashi and Miyazaki, 1999), ictalurid herpesvirus 1 (Hutoran *et al.*, 2005), ranid herpesvirus 1 (Davison *et al*., 1999), and salmonid herpesvirus 1 (Davison, 1998). Based on this similarity, Waltzek *et al.* (2005) suggested that these viruses can be included in a new group of aquatic herpes-like viruses. In agreement with this view, Rijsewijk *et al.* (2005) demonstrated by CyHV-3 (KHV) and AngHV-1 are closely related according to the sequences of their DNA polymerase proteins. Although the amino acid sequences of these two viruses are similar to those of ranid herpesvirus 1 and ictalurid herpesvirus 1, it has been suggested that they be categorized as a separate group (Rijsewijk *et al.*, 2005).

Although four genes of CNGV and CyHVs are similar to each other, there was no apparent homology with other DNA viruses. Providing additional CyHV-1, CyHV-2, and CNGV DNA sequences to GenBank will assist in the phylogenic classification of CNGV, which will be instrumental in developing strategies for controlling the lethal disease caused by CNGV. It is conceivable that fish viruses that evolve in distinct ecological environments are genetically highly divergent from each other. In this case it would be expected that CNGV, which thrives in captivity and is restricted to limited species of *Cyprinus*, bears only slight similarity to other viruses.

Importantly, the CNGV sequence encodes three enzymes involved in the processing of nucleotides for DNA synthesis, i.e., ribonucleotide reductase, thymidylate kinase (TmpK), and thymidine kinase (TK), which are similar to those encoded by the poxvirus genome (Table 1). It should be emphasized that B22R encoded by CNGV is a protein found exclusively in poxviruses. B22R and TmpK have not yet been found in herpesvirus (Meyer *et al*., 2005; Smith, Carlos and Chan, 1989). It would be of great interest to determine whether CyHV-1 and CyHV-2 code for these three proteins as well. Several other CNGV polypeptides show similarity to proteins derived from ictalurid herpesvirus 1, African swine fever virus, and shrimp white spot virus (Hutoran *et al.*, 2005). Iyer *et al*. (2001) showed that African swine fever virus, poxviruses, iridoviruses, and phycodnaviruses share a common evolutionary origin but have no direct evolutionary relationship to herpesviruses. Based on these analyses, we speculate that either CNGV evolved by intensive incorporation (probably by recombination) of genetic fragments derived from poxviruses and other DNA viruses or the CyHVs group descended from a unique common

Table 1. Comparison of CNGV to representatives of Pox viruses and other large DNA viruses. Four CNGV DNA sequences were analyzed using translating BLAST (http://www.ncbi.nlm.nih.gov/BLAST). The search was carried out against the Viruses subclass of the NR database. Representatives from different virus families are presented in the table. Identity (%) is the number of amino acids with identity/total number of amino acids of the protein; Similarity (%) is the number of conserved amino acids/total number of amino acids; Gaps (%) are numbers of gaps/total number of amino acids. a The assessed fragment does not represent the sequences of complete protein.

Gene and Acc#	Family	Genus	Acc#	Identity (%)	Similarity (%)	Gaps (%)
B22R like gene (AY661550)	Poxviridae	Fowlpox virus	CAE52648	78/262 (29%)[a]	133/262 (50%)	8/262 (3%)[a]
Thymidylate kinase (DQ118125)	Poxviridae	Canarypox virus	NP_955193	106/216 (49%)	143/216 (66%)	8/216 (3%)
	Nimaviridae	Shrimp white spot syndrome virus	AAK77840 TK-dTMP	82/191 (42%)	117/191 (61%)	1/191 (0%)
	Iridoviridae	Chilo iridescent virus	AAK82112	72/176 (40%)	117/176 (66%)	3/176 (1%)
	Asfarviridae	African swine fever virus	CAA79604	57/192 (29%)	89/192 (46%)	8/192 (4%)
Ribonucleotide reductase (AY786308)	Poxviridae	Monkeypox virus	AAU01269	447/803 (55%)	580/803 (72%)	35/803 (4%)
	Baculoviridae	Spodoptera litura nucleopolyhedrovirus	AAL01709	415/763 (54%)	555/763 (72%)	13/763 (1%)
	Phycodnaviridae	Feldmannia irregularis virus a	AAR26844	394/765 (51%)	529/765 (69%)	12/765 (1%)
	Mimivirus	Acanthamoeba polyphaga mimivirus	YP_142667	346/733 (47%)	489/733 (66%)	12/733 (1%)
	Nimaviridae	Shrimp white spot syndrome virus	AAL89096	379/845 (44%)	538/845 (63%)	49/845 (5%)
	Asfarviridae	African swine fever virus	P42491	285/737 (38%)	415/737 (56%)	64/737 (8%)
	Herpesviridae	Ostreid herpesvirus 1	YP_024594	263/741 (35%)	415/741 (56%)	58/741 (7%)
Thymidine kinase (AJ535112)	Poxviridae	Cowpox virus	AAF21104	71/171 (41%)	114/171 (66%)	3/171 (1%)
	Mimivirus	Acanthamoeba polyphaga mimivirus	YP_142612	60/181 (33%)	102/181 (56%)	14/181 (7%)
	Nimaviridae	Shrimp white spot syndrome virus	AAG40728	69/178 (38%)	100/178 (56%)	8/178 (4%)
	Asfarviridae	African swine fever virus	AAQ07945	52/177 (29%)	81/177 (45%)	10/177 (5%)

ancestor differing from the mammalian, avian, and reptile viruses, as well as from the clade of ostreid herpesvirus (Davison *et al.*, 2005). If the latter possibility proves to be true, it would be logical to include the CyHVs as a separate family under the umbrella of the order *Herpesvirales* (McGeoch, Rixon and Davison, 2006).

CONCLUSION

In conclusion, the morphology of CNGV is similar to that of the *Herpesviridae*. However, the genome of the isolated virus is composed of a 295 kbp linear dsDNA molecule, larger than that of the other *Herpesviridae* members and bearing highly divergent DNA sequences that encode polypeptides resembling those of other large dsDNA viruses. Analysis of the DNA and amino acid sequences of this high-molecular-weight viral genome revealed that CNGV could be a novel virus bearing a mosaic genome including genetic elements derived from phylogenetically distant DNA viruses.

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Enhancement of Immune Responses in Indian Carp, *Catla catla*, Following Administration of Levamisole by Immersion

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ABSTRACT

Effects of short term administration of levamisole by immersion on immune responses of the cultured food fish, *Catla catla* were investigated. Sub-adults of *C. catla* were given two hr bath in 1.25 or 2.5 mg/L levamisole solutions and the immune responses were assessed on 14, 21, 28, 42 and 56 days after the treatment in comparison to the controls. Results revealed that leucocrit levels, total leucocyte counts, abundance of leucocytes, total phagocytic activity, phagocytic index, myeloperoxidase activity and oxygen radical production by phagocytes were increased significantly in levamisole treated fish in comparison to the controls. No significant differences in the degree of immuno-stimulation were seen between the fish groups exposed to the two concentrations of levamisole tested. For both exposure levels, most of the parameters tested were greatly elevated on 42 days post exposure to levamisole. Challenge experiments showed that levamisole treated fingerlings of *C. catla* co-habitated with gold fish which had been infected with *Ichthyophthirius* and *Dactylogyrus* exhibited lower infestation levels and mortalities in comparison to the control fish. Levamisole treated *C. catla* which had been challenged with *Aeromonas hydrophila* displayed comparatively less mortalities compared to the controls. The results support the potential use of levamisole as an immunostimulant in culturing this fish.

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INTRODUCTION

Levamisole is an antihelthic used for the treatment of nematode infections in man and animals (Treves–Brown, 2000). Levamisole has been reported to be an effective immunostimulant for common carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*), gilthead seabream (*Sparus aurata*), and Atlantic salmon (*Salmo salar*) (Siwicki, 1987; 1989; Mulero *et al*., 1998; Sakai,1999; Findlay and Munday, 2000).

Catla catla (Catla), an Indian carp is a commercial edible fish species cultured in the South Asian region. It has been used for stocking reservoirs and in polyculture systems (Pillay, 1990). *Catla catla* is one of the exotic fish species currently used in culture based fishery in inland reservoirs in Sri Lanka. Catla stocks are maintained in freshwater fish breeding stations of the National Aquaculture Development Authority (NAQDA) of Sri Lanka for continuous seed production for culture systems. However these fish maintained in the breeding stations are highly susceptible to parasitic and bacterial infections (Balasuriya, 1987; Subasinghe, 1992; Wimalawickrama and Pathiratne, 2005).

The aim of the present study was to evaluate the potential of using levamisole as a short-term bath treatment to increase the resistance of Catla to diseases and stress. Specific objectives of the study were to assess the concentration response and time course effects of levamisole treatment through immersion route on selected components of the immune system of Catla and to evaluate the responses of levamisole treated Catla to the experimental bacterial and parasitic challenge. The concentrations of levamisole used in the present study were based on the study carried out by Findlay and Munday (2000) with Atlantic salmon.

MATERIALS AND METHODS

Fish

Catla sub-adults and fingerlings were obtained from the Udawalawa Fish Breeding Station, NAQDA, Sri Lanka. Fish were maintained in outdoor cement tanks filled with aged tap water with continuous aeration under the natural photoperiod for 30 days. During the acclimation period, fish were fed daily with commercial fish food pellets (Prima, Colombo, Sri Lanka). Sub-adult stage of the fish was used in the haematological and serological tests whereas the fingerling stage was used in the experimental challenge tests. The fish used in this study were free of gross lesions or parasitic infections externally and considered as apparently healthy individuals.

Levamisole treatment

Samples of sub-adults of Catla (18-26 cm in total length and 160-210 g in body weight) were bathed in glass aquaria containing 1.25 mg/L or 2.5 mg/L levamisole (Sigma, MO, USA) in aged tap water for two hr. Comparable size fish which were introduced to glass aquaria containing only aged tap water at same biological loads, served as controls. After two hr of exposure, the control fish (aged tap water) and the fish exposed to levamisole

(1.25 mg/L or 2.5 mg/L) were transferred to outdoor cement tanks filled with continuously aerated aged tap water. As the two levamisole treatments could not be tested concurrently due to practical constraints, the two concentrations were tested at two stages. Hence two comparable control groups were used for the two treatments. Fish were provided with commercial food pellets daily at 2% of the body weight. Half of the water in each of the tanks was exchanged with fresh aged tap water every four days. At pre-determined time points (14, 28, 42 and 56 days) after the treatment, levamisole treated and control sub-adults of Catla were killed by pithing to assess the immunomodulatory effects of the treatments based on haematological and serological tests. The blood samples were collected from the fish by bleeding caudal vein. Fish were not tested for the natural immune response prior to the experiment. The responses of the levamisole treated fish were compared with those of comparable controls at the predetered time points. The sub-adult stage was used for haematological and serological tests as adequate blood samples could be obtained.

To evaluate the responses of levamisole treated Catla to the experimental challenge, fingerlings (5.5-6 cm in total length and 5-10 g in body weight) were exposed to 1.25 mg/L levamisole in glass aquaria for two hr before conducting bacterial and parasitic challenge tests after 14 days of the treatment. Comparable sized fingerlings were also transferred to glass aquaria containing only aged water concurrently and maintained in these aquaria at comparable densities for two hr. These fish were considered as controls.

Haematocrit and leucocyte counts

For deteration of the haematocrit and leucocrit levels, blood samples were taken into heparinized capillary tubes and centrifuged in the haematocrit centrifuge. Heamatocrit value of each sample was measured using the haematocrit gauge. Height of the leucocyte column was measured under a light microscope using a micrometer scale in order to determine the leucocrit level. Total leucocyte count was detered using Shaw's solutions as dilution fluids following the method of Hesser (1960). Blood smears of the fish were prepared, fixed in 100% methanol and were stained with Wright-Giemsa stain. Different types of leucocytes were identified as described by Hibiya (1982).

Phagocytosis assays

Phagocytic cells were detected using *Staphylococcus aureus* (Sigma, MO, USA) as described by Anderson and Siwicki (1995). A sample (0.1 mL) of blood was placed in a microtiter plate well, 0.1 mL of *Staphylococcus aureus* 1×10^7 cells suspended in phosphate buffered saline pH 7.2, was added and then mixed well. The bacteria–blood solution was incubated for 20 at room temperature. Five µL of this solution was taken on to a clean glass slide and a smear was prepared. The smear was air dried, then fixed with ethanol (95%) for 5 min and air dried. Then the smear was stained with Giemsa stain for 10 min. The two smears were made from each fish. The total of 100 neutrophils and monocytes from each smear were observed under the light microscope and the number of phagocytizing cells and the number of bacteria engulfed by the phagocyte were counted. Phagocytic activity and phagocytic index were calculated as follows: Phagocytic activity equals the number

of phagocytizing cells divided by the total number of phagocytes counted. Phagocytic index is expressed as the total number of bacteria engulfed by the phagocytes, divided by the total number of phagocytes containing engulfed bacteria.

Nitroblue tetrazolium assay and Myeloperoxidase activity

Production of oxygen radicals from phagocytes in the blood was measured using nitroblue tetrazolium (NBT) dye as described by Anderson and Siwicki (1995). A sample (0.1 mL) of heparinized blood was placed in to a microtiter plate well and equal amount of 0.2% NBT (Sigma, MO, USA) was added, the NBT-blood cell suspension was incubated for 30 min at room temperature. A sample (0.05 mL) of the NBT-blood cell suspension was taken out and added to a glass tube containing 1.0 mL of N,N-dimethylformamide solution. Then the mixture was centrifuged for 5 min at 3000 g. The supernatant was taken into a glass cuvette and absorbance was read at 540 nm using a spectrophotometer.

Myeloperoxidase activity was detected using commercially available kits (The peroxidase kit, catalog no. 391- A, Sigma, MO, USA). The positive cells were counted as described by Anderson and Siwicki (1995).

Lysozyme assay

Lysozyme activity of blood serum was detered as described by Anderson and Siwicki (1995) with some modifications. Blood serum was prepared by centrifuging the blood at 3000g for 5 min. Serum (0.1 mL) was placed in test tubes and 0.9 mL of a 0.75 mg/mL *Micrococcus lysodeikticus* (Sigma, MO, USA) suspension in phosphate buffered saline, pH 6.2 was added and mixed well. The absorbance was measured at 450 nm by a spectrophotometer at 1 min intervals for 10 min after mixing with bacteria and rate of change of absorbance calculated. Lysozyme activities were calculated using hen egg white lysozyme (Sigma, MO, USA) as a standard.

Total protein and total immunoglobulin in plasma

Total protein content in blood plasma was determined using Peterson's modifications of the micro-Lowry method using a protein assay kit (Sigma Diagnostics, P 5656, Sigma, MO, USA). The protein concentrations were determined using a calibration curve prepared using bovine serum albumin as the standard. For the determination of the immunoglobulin in the plasma, immunoglobulins were separated from the plasma by precipitation with polyethylene glycol as described by Anderson and Siwicki, (1995). Plasma (0.1 mL) was placed in plastic serum vial and 0.1 mL of 12% polyethylene glycol was added and incubated at room temperature for 2 hr under constant mixing. After incubation, the solutions were centrifuged at 7000 g for 10 min. The protein content in the supernatant was detered using protein assay kit. The total immunoglobulin content was detered by subtracting the protein content in the supernatant from the total protein content in the plasma.

Challenge test by cohabitating test fish with parasitic infested fish

Fingerlings of Catla which were treated with 1.25 mg/L levamisole were subjected to a parasitic challenge by cohabiting the fish with a sample of gold fish *Carasscius auratus* infested with *Ichthyopthirius* sp and *Dactylogyrus* sp in glass aquaria. As significant concentration specific difference with respect to the degree of immunostimulation was not clearly seen between the two exposure levels of levamisole, it was decided to use 1.25 mg/L levamisole for challenge tests. After the introduction of parasite infested fish, mortality and abnormal signs of fish were observed daily and parasitological survey was carried out at as described by Kabata (1985) on 7, 14, 21, 28, 35, 42 and 49 days after levamisole treatment.

Challenge test with bacteria

Fingerlings of Catla which were treated with 1.25 mg/L levamisole were subjected to a bacterial challenge. A virulent strain of *Aeromonas hydrophila* (donated by Mr. C. Hettiarachchi, Confifi Aquaculture Pvt., Ltd., Sri Lanka), that has been isolated from characteristic lesions in an ornamental fish *Trichogaster leeri* (pearl gouramy), was used in the challenge tests. Fingerlings of control Catla (n=30 individuals) and Catla treated with levamisole (n = 30 individuals) were injected intraperitoneally (i.p.) with *Aeromonas hydrophila* using sterile syringes at a dose of 0.1 mL, containing 10^9 Colony Forg Units in 0.85% NaCl per fish. Fish were observed for 49 days after the i.p. injection.

Statistical analysis

Haematological and serological data are presented as mean ± standard error of the mean for 6 fish per group. Data were analyzed using one- way analysis of variance (ANOVA). Where differences are significant, differences among the mean parameters of fish exposed to different levamisole concentrations were compared using Tukey's test. The intensities of parasitic infestations were analyzed using non-parametric Mann-Whitney U test (Zar, 1999). The accepted level of significance was $P \leq 0.05$.

RESULTS

All control and levamisole treated sub-adults of *C. catla* survived during the experimental period. No significant difference was found between levamisole treated fish and respective control fish in relation to the haematocrit levels in the blood (results not shown, $P > 0.05$). Leucocrit levels and the total leucocyte counts of levamisole treated fish were significantly higher than that of the respective control fish on 14 days of post exposure onwards. Maximum increase in these parameters occurred on 42 days of post exposure to levamisole (Figure 1). The leucocrit levels and leucocyte count in the blood increased by nearly two folds on 42 days of levamisole post exposure compared to the respective controls. However, no concentration specific significant difference was detected at each time point ($P > 0.05$).

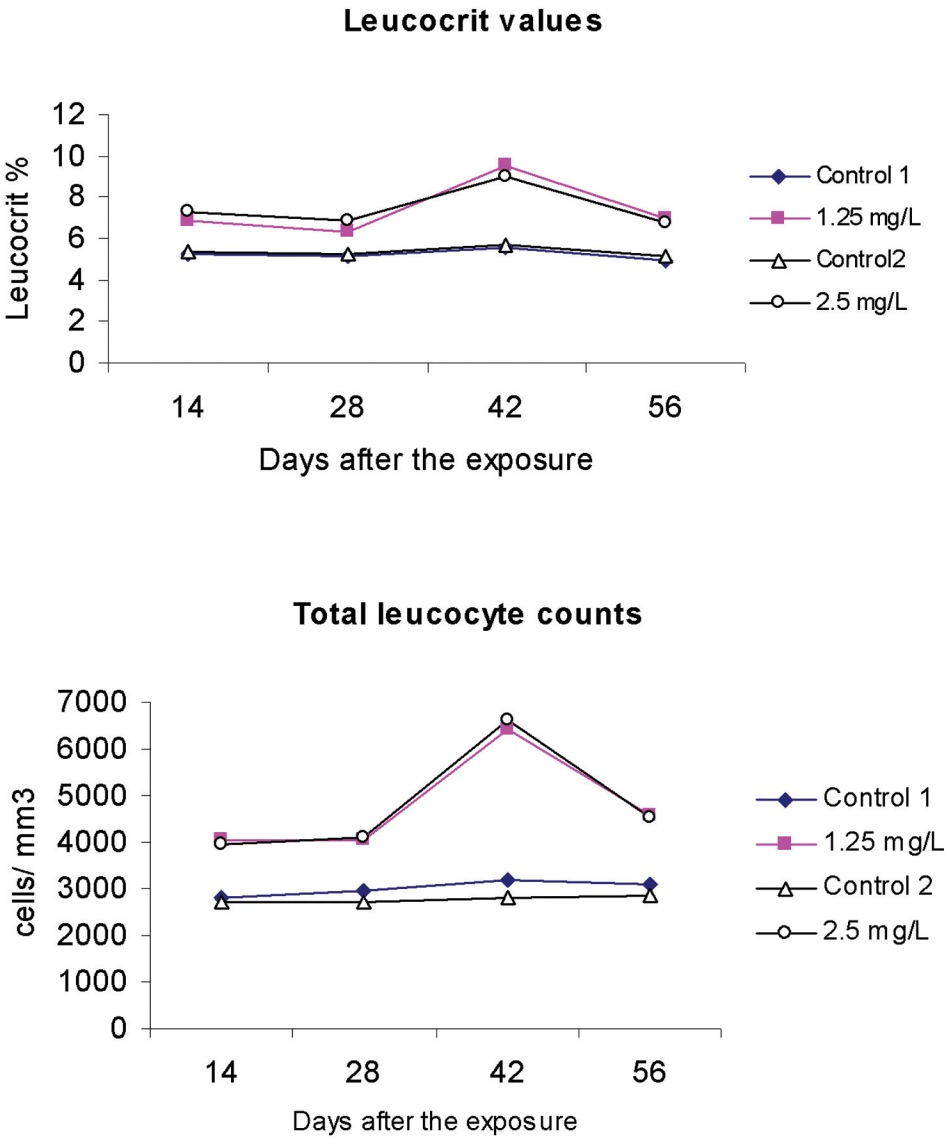

Figure 1. Time-course elevation pattern in the mean leucocrit levels and total leucocyte counts in the blood of *Catla catla* following levamisole exposure (1.25 mg/ L or 2.5 mg/L for 2 hrs) through immersion route. At each time point, the parameters in the blood of levamisole treated fish were significantly different from those of the controls (ANOVA, Tukey's test, $P < 0.05$)

Percentages and absolute numbers of populations of neutrophils and monocytes in the blood of fish increased significantly after levamisole treatment. Maximum increase in these parameters occurred on 42 days of post exposure to levamisole. On the 42nd day of the post immersion to 1.25 and 2.5 mg/L levamisole, abundance of neutrophils increased by 2.5 and 3.2 folds respectively whereas populations of monocytes in the blood of levamisole treated fish increased by 2.7 and 3.6 folds compared to the respective controls (Table 1).

Populations of neutrophils in the blood of levamisole treated fish remained increased by nearly two folds even on the 56th day of post levamisole administration. Even though percentage abundance of leucocytes in the blood of levamisole treated fish decreased in comparison to the controls (results not shown), absolute numbers of lymphocytes in the blood of fish treated with 2.5 mg/L levamisole were significantly increased during the study period (1.6–2.6 folds) compared to the respective controls (Table 1). However, a significant increase in the abundance of lymphocytes in the blood of fish treated with 1.25 mg/L levamisole was seen only on 42nd and 56th day of post treatment.

Table 1. Concentration–response and time-course effects of levamisole adistration through immersion route on different leucocyte populations (in cells mm^{-3}) in the blood of sub-adults of *Catla catla*.

Leucocyte type and fish group	Days after levamisole treatment			
	14 days	28 days	42 days	56 days
Neutrophils				
Control 1	1368 ± 103[a]	1469 ± 82[a]	1599 ± 156[a]	1546 ± 119[a]
Levamisole 1.25 mg/L	2323 ± 260[b]	2435 ± 123[b]	3926 ± 318[b]	2605 ± 207[b]
Control 2	1276 ± 230[a]	1258 ± 200[a]	1273 ± 94[a]	1362 ± 202[a]
Levamisole 2.5mg/L	2459 ± 170[b]	2584 ± 166[b]	4073 ± 528[b]	2308 ± 204[b]
Monocytes				
Control 1	185 ± 60[a]	186 ± 43[a]	240 ± 63[a]	207 ± 64[a]
Levamisole 1.25 mg/L	389 ± 83[b]	378 ± 59[b]	652 ± 63[b]	369 ± 56[b]
Control 2	138 ± 30[a]	167 ± 72[a]	216 ± 39[a]	176 ± 64[a]
Levamisole 2.5mg/L	410 ± 28[b]	407 ± 93[b]	776 ± 137[b]	398 ± 59[b]
Lymphocytes				
Control 1	1342 ± 135[a]	1286 ± 114[a]	1369 ± 143[a]	1388 ± 188[a]
Levamisole 1.25 mg/L	1314 ± 225[a]	1160 ± 194[a]	2008 ± 507[b]	1880 ± 50[b]
Control 2	1274 ± 112[a]	1226 ± 114[a]	1298 ± 169[a]	1273 ± 86[a]
Levamisole 2.5mg/L	2005 ± 168[b]	2116 ± 72[b]	3317 ± 177[b]	2204 ± 303[b]

Fish were exposed to levamisole at 1.25 mg/L or 2.5 mg/L for 2 h as immersion and different types of leucocytes were evaluated at different times of post exposure. Results are expressed as mean ± SEM for 6 fish per group. For a specific type of lymphocyte, the data in a column with different superscripts are significantly different from each other (ANOVA, Tukey's test, $P < 0.05$)

Total phagocytic activity, phagocytic index, NBT activity and myeloperoxidase activity in the blood of fish exposed to levamisole are compared with the respective controls in Figure 2. Total phagocytic activity and phagocytic index of fish exposed to different concentrations of levamisole were significantly higher than that of the control fish. NBT activity and myeloperoxidase activity in the blood of fish exposed to levamisole were also significantly higher than that of the respective control values at each time point. Time course pattern showed that levamisole induced elevation of NBT activity in the blood of fish were maximum on 42 days of post treatment. There were no concentration related significantly difference between the fish exposed to 1.25 mg/L and 2.5 mg/L levamisole with respect to the above four parameters tested.

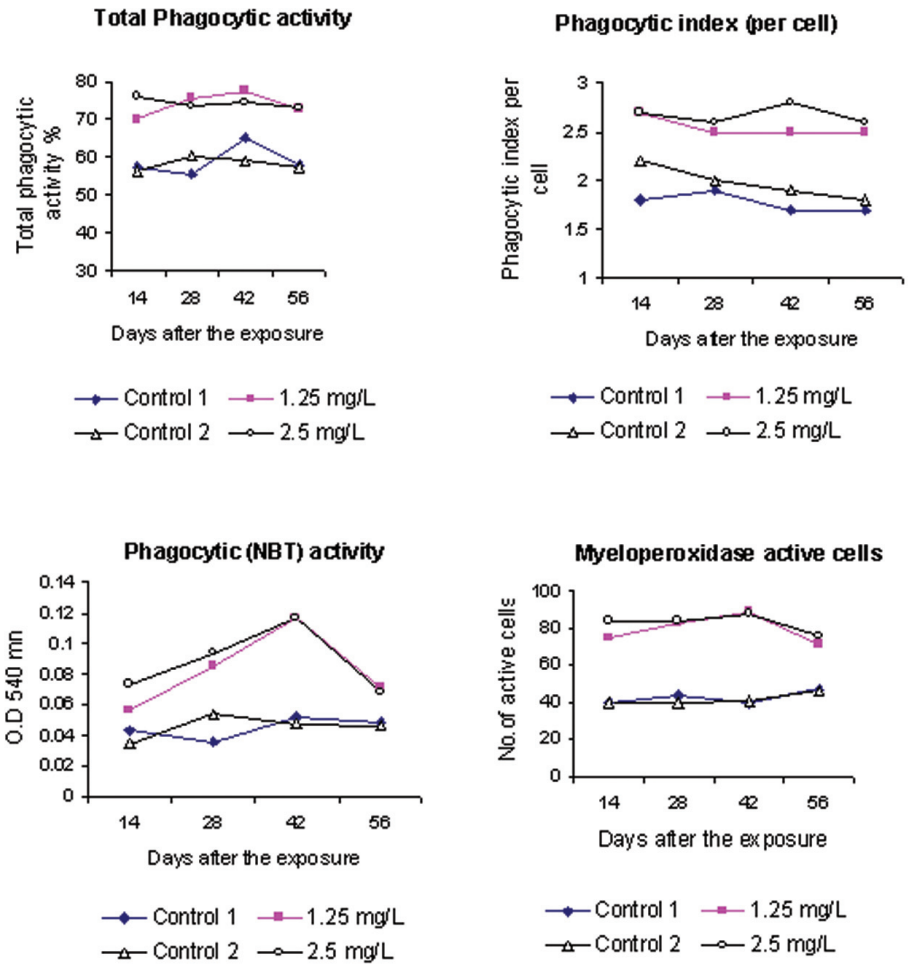

Figure 2. Time course elevation pattern in the total phagocytic activity, phagocytic index, NBT activity and myeloperoxidase activity (mean values) in the blood of *Catla catla* following levamisole exposure (1.25 mg/L or 2.5 mg/L for 2 hr) through immersion route. At each time point, the parameters in the blood of levamisole treated fish were significantly different from those of the controls (ANOVA, Tukey's test, $P < 0.05$).

Lysozyme activity in the blood serum of the fish exposed to both concentrations of levamisole was significantly higher only on 28th day of post exposure in comparison to the controls. No significant difference was found between levamisole treated fish and control fish during the other study periods probably due to large individual variations (Table 2). Total protein levels in the circulating blood of fish exposed to levamisole were significantly higher than that of the control values (Figure 3). Time course stimulation pattern showed that levamisole induced elevation of protein levels in circulating blood of fish persist at least 56 days post exposure. Maximum increase in the blood protein levels was seen on the 42nd day of the post exposure. However, immunoglobin level in the blood of fish exposed to levamisole was significantly not different from the control fish (Figure 3).

Table 2. Concentration–response and time-course effects of levamisole adistration through immersion route on serum lysozyme activities (in µg//ml serum) of sub-adults of *Catla catla*.

Fish	Days after exposure			
	14 days	28 days	42 days	56 days
Control 1	4.8 ± 1.2[a]	2.7 ± 0.4[a]	3.8 ± 1.3[a]	2.3 ± 1.2[a]
Levamisole 1.25 mg/L	4.0 ± 1.0[a]	5.1 ± 0.9[b]	4.3 ± 1.4[a]	2.4 ± 0.7[a]
Control 2	2.5 ± 1.6[a]	2.7 ± 0.5[a]	4.2 ± 1.7[a]	2.6 ± 0.3[a]
Levamisole 2.5mg/L	3.6 ± 1.9[a]	4.8 ± 1.3[b]	4.8 ± 1.5[a]	3.2 ± 0.3[a]

Fish were exposed to levamisole at 1.25 mg/L or 2.5 mg/L for 2 hr as immersion and serum lysozyme levels were evaluated at different times of post exposure. Results are expressed as mean ± SEM for 6 fish per group. The data in a column with different superscripts are significantly different from each other (ANOVA, Tukeys' test, P > 0.05).

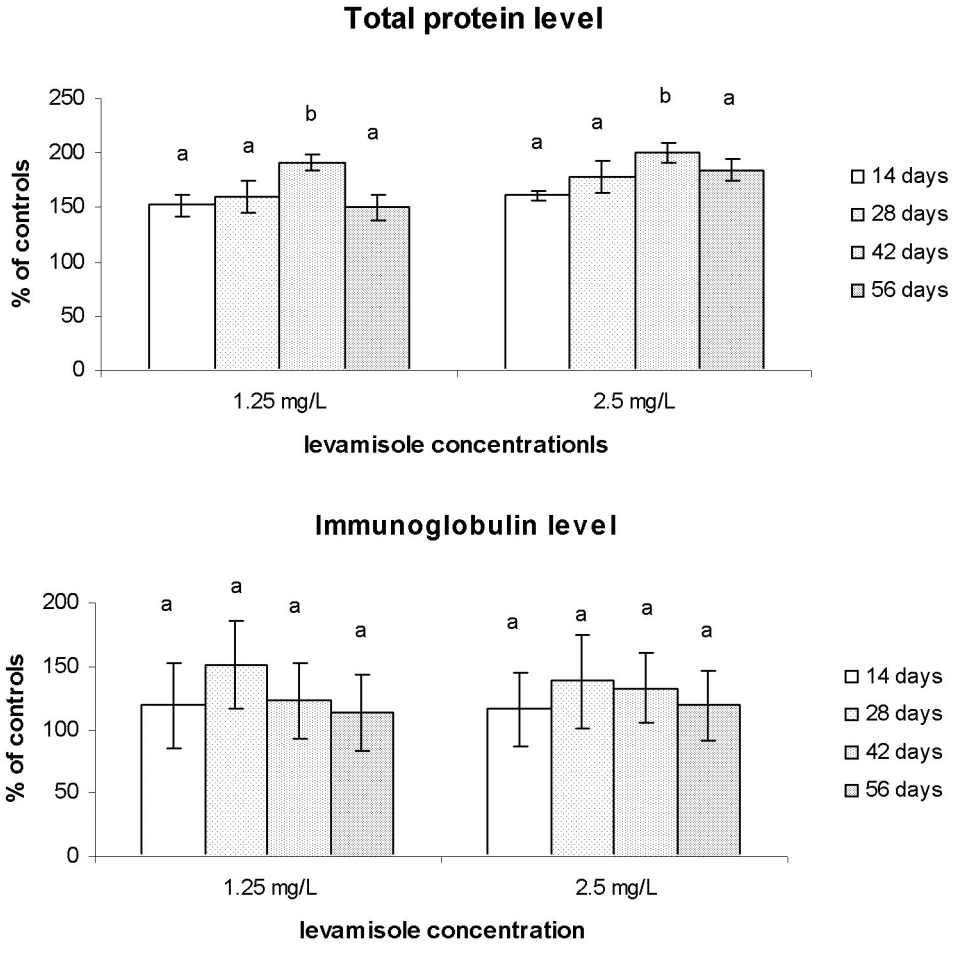

Figure 3. Effects of adistration of levamisole (1.25 mg/L and 2.5 mg/L for 2 hr). through immersion route on total protein levels and immunoglobulin levels in *Catla catla.* For each concentration, bars with the different letters are significantly different from each other (ANOVA, Tukey's test, P <0.05)

Occurrence of *Ichthyophthirius* and *Dactylogyrus* on fingerlings of Catla cohabitated with the gold fish infested with *Ichthyophthirius* sp. and *Dactylogyrus* sp. are presented in Table 3. Numbers of parasites on the gill filaments were higher in control fish cohabitated with parasites in comparison to the fish treated with levamisole. The cumulative mortality of the control fish cohabitated with gold fish was 7% where as none of the fish treated with levamisole died during the study period. The results of the challenge experiment show that cumulative mortality of control fingerlings challenged with *Aeromonas hydrophila* were higher than that of the levamisole treated fish (Figure 4). No mortality in the control fish or levamisole treated fish which were not challenged with the bacteria (negative control groups) was observed during this period. In the control fish challenged with the bacteria, injected site turned into a reddish patch within two days of the challenge and it was spread to a larger area with time. The injection site of the fish treated with levamisole was completely healed by 8 days.

Table 3. Occurrence of parasites in the gills of *Catla catla* after cohabitation with gold fish infested with *Ichthyophthirius* sp and *Dactylogyrus* sp.

Fish	Intensity of infestation						
	7 days	14 days	21 days	28 days	35 days	42 days	49 days
Ichthyophthirius sp							
Control fish	7-8	10-14	7-9	9-11	9-10	7-10	1-12
Levamisole treated fish	0-2	0	0	0	0	0-1	0
Dactylogyrus sp.							
Control fish							
	21-26*	24-28*	29-31*	34-38*	30-36*	34-41*	3-34*
Levamisole treated fish							
	5-6	3-5	1-4	1-2	3-4	1-2	2-4

Results are presented as ranges (n=30 fish per group).
* Significantly different from levamisole (1.25 mg/L for 2 hr) treated fish cohabitated with gold fish infested with parasites (Mann-Whitney U test, $\propto = 0.05$)

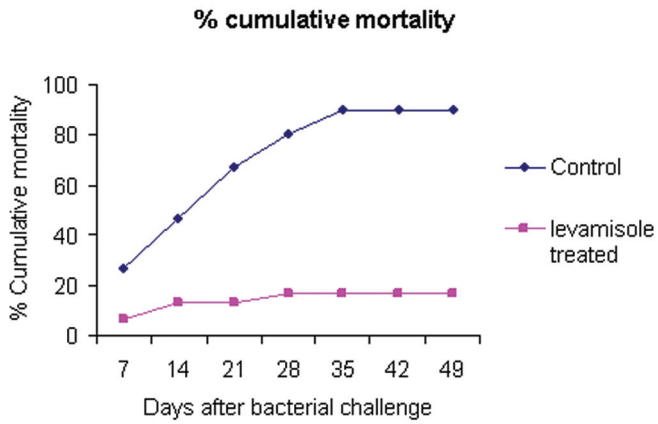

Figure 4. Mortality of fingerlings of *Catla catla* challenged with *Aeromonas hydrophila.*

DISCUSSION

The use of immunostimulants in fish culture is opening new opportunities to improve fish health and prevent losses due to diseases. Levamisole has been shown to act as an immunostimulant in number of fish species: by oral administration or i.p. injection for *Cyprinus carpio* (Siwicki, 1987; 1989; Sakai, 1999) by oral administration for *Sparus aurata* (Mulero *et al.,*1998) and for *Labeo rohita* (Wijendra and Pathiratne, 2004), by immersion for *Salmo salar* (Findlay and Munday, 2000) and by immersion and injection for *Oncorhynchus mykiss* (Anderson *et al.,* 1995). The present study demonstrated immunostimulatory properties of levamisole administered through immersion route on an Indian carp, *C. catla.*

Findlay and Munday (2000) used 2.5 mg/L levamisole as a 2 hr bath treatment for *Salmo salar* to increase immunostimulation. In this study, levamisole was tested as a 2 hr bath treatment at two different concentrations (1.25 and 2.5 mg/L) to assess concentration–response and time-course effects on the immune system of Catla. The results revealed that both concentrations of levamisole could enhance the immune system especially some components of the non specific immune system. Leucocrit levels, total leucocyte counts, abundance of neutrophils, monocytes and lymphocytes, total phagocytic activity, phagocytic index, NBT activity, myeloperoxidase activity and total protein level in the blood were increased significantly in levamisole treated fish in comparison to the control fish on 14-56 days post exposure to levamisole. It appears that proliferation of leucocytes increases several days after exposure to levamisole. No significant concentration specific differences was found between 1.25 and 2.5 mg/L levamisole treatments with respect to above parameters except the time course stimulation pattern of lymphocyte populations. Time-course pattern of immune stimulation revealed that enhanced immune responses persist at least 56 days whereas for most of the parameters tested, maximum response was observed at 42 days post administration.

Transiently lower haematocrit values have been shown in gilthead seabream (*Sparus aurata*) fed diets containing higher levels (125-500 mg/kg body weight) of levamisole (Mulero *et al.,* 1998). In the present study, no significant difference was found between levamisole treated Catla and respective control fish for haematocrit values in the blood corroborating the findings of Anderson *et al.,* (1995), Findlay and Munday (2000) and Wijendra and Pathiratne (2004) for *Oncorhynchus mykiss, Salmo salar,* and *Labeo rohita* respectively. The haematocrit levels serve as a general indicator of fish health. It is often used as a confirmation that an immunostimulant is not disturbing the profile of erythrocytes. Thus, levamisole does not appear to modify the erythrocyte levels in the blood of the fish.

Elevated leucocyte numbers, enhanced phagocytic acitivity, and myeloperoxidase activity in neutrophils and elevated lysozyme levels have been reported in *Cyprinus carpio* following levamisole injections. Oral adistration of levamisole has also increased the number of leucocytes, lysozyme levels in the serum and the phagocytic index and NBT reduction by phagocytes in carp (Siwicki, 1987; 1989). In this study, treatment of fish with levamisole bath at 1.25 and 2.5 mg/L for 2 hr significantly increased leucocrit levels, total leucocytes, populations of neutrophils, monocytes and lymphocytes in the blood of Catla nearly by 2-3 folds during the study period. Monocytes and neutrophils which are

the main cells of the non-specific defence system, are phagocytic and capable of killing a variety of pathogens including bacteria. The primary function of fish lymphocytes seems to be to act as the cells of specific immune system via antibody production (Evelyn, 2002). Hence enhancement of immune responses could be expected in Catla treated with 1.25 and 2.5 mg/L levamisole through its stimulatory effects on different leucocyte populations.

Fish phagocytes have potent bactericidal and larvicidal activity and thus, presumably possess both intracellular, and extra cellular killing mechanisms (Secombes, 1996). In the present study, total phagocytic activity, phagocytic index, NBT activity and myeloperoxidase activity in Catla treated with 1.25 and 2.5 mg/L levamisole were significantly higher in comparison to the respective control fish at least 56 days post-levamisole treatment. In seabream, *Sparus aurata* leucocyte functions including phagocytosis have also been enhanced by dietary intake of high levels of levamisole (Mulero *et al.,* 1998). It is known that oxygen free radicals are produced by fish phagocytes during the respiratory burst (Evelyn, 2002). The production of oxidative radicals (detected by NBT) was enhanced in *Cyprinus carpio* fed with levamisole (Siwicki, 1989). In the present study, NBT activity in the blood of Catla also rose after the levamisole treatment through immersion route.

Lysozyme is found in a wide range of vertebrates including fish and is one of the defensive factors against invasion by microorganisms (Evelyn, 2002). Lysozyme is an important enzyme in blood that actively lyses bacteria. Findlay and Munday (2000) found that bath treatment of fish with levamisole can induce increased activities of both mucus and serum lysozyme of Atlantic salmon, *Salmo salar*. In the present study, even though significantly elevated levels of serum lysozyme were found on 28 days of post exposure, there was no significant difference between levamisole treated fish and respective control fish with respect to the serum lysozyme levels in the other time periods. Immunoglobulins are a major humoral component of the specific immune system. In the present study, total immunoglobin levels of levamisole treated fish were slightly increased in comparison to the control fish but the difference was not statistically significant probably due to large individual variations. Nevertheless, total protein level in the blood of levamisole treated fish were significantly higher than that of the respective control fish.

Challenge experiments have shown that levamisole increased protection against pathogenic bacteria in carp and trout specially through activation of the non-specific defense mechanisms (Anderson *et al.,* 1995; Sakai, 1999). In the present study, Catla not treated with levamisole (controls) and subsequently cohabitated with fish infested with *Ichthyophthirius* and *Dactylogyrus* exhibited significantly high intensity of parasitic infestation than the fish treated with levamisole. The cumulative mortality of the control fish cohabitated with gold fish was 7% where as none of the fish treated with levamisole died during the study period. The results also showed that levamisole treated Catla had developed some resistance to *Aeromonas hydrophila* bacterial challenge. Survival of the levamisole treated fish experimentally challenged with *Aeromonas hydrophila* was higher in comparison to the control fish challenged with the bacteria. Infection experiments showed the *in vivo* immune responses of levamisole treated Catla to *Ichthyophthirius, Dactylogyrus* and *Aeromonas hydrophila.* Levamisole bath used in the present study (1.25 mg/L for 2 hr) was effective enough to provide Catla a considerable resistance to parasitic and bacterial challenge.

CONCLUSIONS

The results revealed that administration of levamisole (1.25 or 2.5 mg/L for two hr) through immersion route is a potential method in Catla culture for enhancing the resistance of fish to diseases and stress. Both concentrations of levamisole could enhanced Leucocrit levels, total leucocyte counts, abundance of neutrophils, monocytes and lymphocytes, total phagocytic activity, phagocytic index, NBT activity, myeloperoxidase activity and total protein level in the blood on 14 - 56 days post exposure to levamisole. Time-course pattern of immune stimulation revealed that enhanced immune responses persist at least 56 days whereas for most of the parameters tested, maximum response was observed at 42 days post administration. As significant concentration specific difference with respect to the degree of immunostimulation was not clearly seen between the two exposure levels of levamisole, it would be economically more advantageous to use 1.25 mg/L levamisole for *Catla catla* culture for potential immunostimulation.

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Spring Viremia of Carp in the United States of America: Evaluation of Current Diagnostics

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ABSTRACT

Accurate diagnosis is essential for prevention and avoiding the spread of foreign animal diseases. Spring viremia of carp (SVC), which was considered an exotic disease in the US, was first detected in April 2002. The United States Department of Agriculture (USDA) responded immediately to prevent the spread of the disease. Depopulation of all infected and exposed fish followed by quarantine and a surveillance program were initiated in each facility. There are several diagnostic tests that are available for the detection of SVC virus (SVCV); however, the tests have not been validated. *in vitro* sensitivity and specificity for several SVCV diagnostic tests were evaluated. Serial dilutions of SVCV virus as low as 10^1 $TCID_{50}$/mL were used to evaluate the *in vitro* sensitivity of SVCV diagnostic tests. *In vitro* specificity was evaluated with serial dilutions of five different strains of

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fish rhabdoviruses using the SVCV diagnostic tests. The study showed that a minimum viral titer of 10^5 $TCID_{50}$/mL is required to detect the virus in cell culture within 48 hours. Molecular and serological based techniques proved to be more sensitive and were able to detect the virus in less time than virus culture. Among these tests, reverse transcriptase-polymerase chain reaction (RT-PCR) was the most sensitive and detected the virus at 10^1 $TCID_{50}$/mL with specific primers designed for the US isolate. With regards to specificity, RT-PCR and enzyme linked immunosorbent assay (ELISA) assays did not detect any fish rhabdovirus other than SVCV. Although *in vivo* studies are pending, the preliminary findings of this study indicate that RT-PCR could be recommended as a test of choice for rapid and accurate diagnosis of a US SVCV isolate.

INTRODUCTION

Outbreaks of spring viremia in carp have been recognized by European carp farmers for centuries. The disease is associated with a rhabdovirus, spring viremia of carp virus (SVCV) or *Rhabdovirus carpio*, which is readily transmitted in the feces of infected fish. The virus is environmentally stable and is possibly transferred between surface waters and aquaculture farms by waterfowl, fish parasites, and fomites (Fijan *et al.,* 1971; Ahne *et al.,* 2002). External clinical signs of the disease include an extended abdomen, exophthalmia and an inflamed vent. Internally, the fish exhibit ascites and petechial hemorrhages of the internal wall of the swimbladder. The disease is generally associated with high mortality; often approaching 70% in young fish (Ahne *et al.,* 2002). Consequently, introduction of the virus into a commercial hatchery or aquaculture facility can have profound economic effects.

In April 2002, SVC was reported for the first time in the US in one of the largest koi farms in the United States of America (US) with operations consisting of approximately 202 ponds spanning the states of North Carolina (NC) and Virginia (VA). The World Organisation for Animal Health (OIE) confirmed the presence of SVCV in the affected fish. As SVC is considered a foreign animal disease, State quarantines were placed on the facilities upon confirmation of the disease. A joint request from States and industry was made to the United States Department of Agriculture-Animal and Plant Health Inspection Service (USDA-APHIS) for assistance with depopulation, cleaning, disinfection and indemnity. In March of 2003, APHIS received approval to implement an SVC control and indemnity program using emergency funding. A depopulation, cleaning and disinfection effort of this magnitude had not previously been undertaken at a fish farm. The logistics of carrying out the eradication effort required input from several State and Federal agencies as well as input from SVC experts both within and outside of the United States. Depopulation efforts commenced in early July of 2003 and the State quarantines on the facility were released by the end of October 2003 following completion of cleaning and disinfection. In addition to efforts put forth at the infected premise, APHIS instituted a nationwide voluntary surveillance. No positive cases have been identified through the surveillance program. Following the NC/VA outbreak, episodes of mass mortalities of wild common carp were reported in Wisconsin in the spring of 2002 (Goodwin *et al*., 2002, Marcquenski

et al., 2003, Dikkeboom *et al*., 2004). In July of 2004, SVC was also reported in a backyard pond in Washington State and in August of 2004, a commercial farm in Missouri was also diagnosed with SVC. APHIS depopulated, cleaned and disinfected these premises and also conducted epidemiologic investigations to try and determine the source of infection.

Since 2002, the presence of SVCV in the US has been extensively investigated in both wild and farmed cyprinid populations. A variety of serology and PCR-based assays have been developed to assist veterinarians and fish health professionals document the presence of SVCV. The OIE recommended diagnostic tests for SVCV include virus culture, reverse transcriptase-polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA), immunofluorescent antibody technique (IFAT). However, detection of SVCV by PCR or serologic detection of SVCV antibody must be confirmed by virus culture. Each of these tests has its own merits and deficiencies, varying in cost, labor and training requirements, sensitivity, specificity, and diagnostic predictive value. In this study, we measured and compared the *in vitro* accuracy of currently available diagnostic techniques for SVCV. Our aim in this study was to identify the most appropriate currently available diagnostic techniques for detection of SVCV and where appropriate, refine them and enhance their accuracy.

MATERIALS AND METHODS

Virus Culture

Epithelioma papulosum cyprini (EPC) cells were maintained at 20°C in Leibovitz-15 medium supplemented with 10% fetal bovine serum (FBS). An isolate of SVCV from the NC/VA outbreak (referred to as the US isolate hereafter) was obtained from the Center for Environment, Fisheries, and Aquaculture Science (CEFAS), Weymouth, England (OIE Reference Laboratory for SVC). The viral supernatant was filtered using a 0.2-micrometer filter and propagated in EPC cells and purified. Briefly, EPC cells were infected with SVCV and after cytopathic effect (CPE) was observed, the cells were scraped into the medium and the crude virus was clarified by centrifuging at 5000 × *g* for 30 min at 4°C. The supernatant was aspirated and loaded carefully on a 26% sucrose cushion and subjected to ultracentrifugation at 120 000 *g* for 2 hr at 4°C. Finally, the virus pellet was resuspended in 500 µl of HO buffer (0.01 M Tris-Hcl, 0.25 M Nacl, 0.01M β-mecaptoehatnol), titrated by the method of Reed and Muench (1938) by 50% tissue culture infective dose ($TCID_{50}$) endpoint analyses and stored at –20°C until use. The titrated stock virus was serially diluted to yield 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 $TCID_{50}$/mL to perform the sensitivity assays.

To test the specificity of the assays, five fish rhabdoviruses including American and European strains of infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV), and pike fry rhabdovirus (PFRV) were used (provided by Dr. G. Kurath, USGS). The viruses were grown in EPC cells cultured in L-15 media at 15°C, purified and titrated as noted above. Viruses were serially diluted as noted above, but based on the results of the sensitivity studies only 10^3, 10^4, 10^5 and 10^6 $TCID_{50}$/mL were used for the *in vitro* specificity studies

Sensitivity of Virus Culture

Cells (EPC) grown in 25cm^2 flasks were infected in triplicate with 100 µl of serial dilutions of SVCV and incubated at 20^{o}C. The flasks were monitored for cytopathic effect every 24 hrs (OIE, 2003).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Viral RNA was extracted from undiluted and serially diluted viral supernatants from infected and uninfected EPC cells (negative control) using QIAamp viral RNA kit (Qiagen, MD) following the manufacturer's protocol. The extracted viral RNA and negative control RNA were subjected to one step RT-PCR reaction (Qiagen). Two sets of primers were designed based on the OIE manual (2003) and Stone *et al*., (2003) to amplify the 714 bp fragment of the SVCV glycoprotein (G) (Table 1). A degenerate primer set and a primer set specific for the US isolate were designed. The primer set specific for US isolate was designed based on the nucleotide sequence of the G protein (Gene bank accession number: AY527273). Using viral RNA as a template, RT was performed at 50°C for 30 min followed by 95°C for 15 min. The cDNA was then amplified by 30 and 40 cycles of 95°C for 1 min, 55 °C for 1min, 72 °C for 1 min, and a final extension step of 72°C for 10 min. The RT-PCR products were visualized after electrophoresis on a 1% agarose gel stained with SYBR green.

Table 1. Oligonucleotides used for testing the sensitivity and specificity of the RT-PCR diagnostic test for SVCV. Orientation of virus-specific sequence of the primer is shown as sense (+) or antisense (-).

Nucleotide Sequence	Orientation	Designation	Expected size (bp)
SVCV			
Degenerate Primer			
TCTTGGAGCCAAATAGCTCAGGTC	+	SVCVF1	718
AGATGGTATGGACCCCAATACATCACGCAC	-	SVCVR2	
US isolate specific			
AGATGGTACGGACCCCAATATATAACCC	+	SVCVNCF	718
TCTTGGGGCCAAATAACTCAAATCC	-	SVCVNCR	
IHNV			
TCAAGGGGGGAGTCCTCGA	+	IHNVF	786
CACCGTACTTTGCTGCTAC	-	IHNVR	
VHSV			
GGGGACCCCAGACTGT	+	VHSVF	811
TCTCTGTCACCTTGATCC	-	VHSVR	
Semi-nested Primer for SVCV and PFRV			
CTGGGGTTTCCACCTACCAGTTGC	+	PFRnestF	606

For specificity assays, viral RNA from undiluted and serially diluted fish rhabdoviruses (mentioned above) and the negative control were extracted and subjected to RT-PCR following the above protocol. Primers specific for IHNV and VHSV were designed based on the OIE manual and SVCV primers were employed for PFRV (Stone *et al*., 2003). Semi-nested RT-PCR based on Stone *et al*. (2003) was also performed on SVCV and PFRV to detect any cross reactivity that is believed to exist between these two viruses. The SVCV reverse primer from the above RT-PCR assay was used along with the forward primer that was designed based on Stone *et al.* (2003). The template from the previous RT-PCR reaction was then used to perform the semi-nested PCR assay with similar amplification conditions described above.

Enzyme Linked Immunosorbent Assay (ELISA)

The ELISA assay was performed following the protocol of the CEFAS laboratory. Briefly, 96 well plates (in triplicate) were coated with 100 µl of anti SVCV γ-globulin at a concentration of 5 µg/mL in carbonate buffer and incubated overnight at 20°C. Following incubation, the plates were washed thrice with a buffer containing phosphate buffered saline and thiomersal (PBS-Thio). The plates were then blocked with 5% fish skin gelatin (FSG) at 37°C for 45 min. Using PBS+0.05%Tween 20 (PBST) buffer the plates were washed thrice. The plates were than loaded with 100 µl of undiluted and serially diluted SVCV viral samples (US isolate) along with a negative control (uninfected EPC cell supernatant) and incubated at 37°C for 30 min. Viral dilutions were made using the extraction buffer (PBST+Nonidet P-40). The plates were then rinsed with PBST followed by 3 washes. One hundred microlitres of a biotinylated anti SVCV IgG at a dilution of 1:1000 in PBST/FSG was then added to each well on the plate and incubated at 37°C for 30 min. The plates were washed as in the previous step and then 100 µl of ExtrAvidin horse radish peroxidase (HRPO) conjugate (Sigma MO, USA) diluted to 1:1000 in PBST/FSG was added to each well followed by incubation at 37°C for 30 min. Following washing as in the previous step, the plates were drained thoroughly by tapping the plates on absorbent paper. The substrate buffer (phosphate-citrate buffer with sodium perborate (Sigma) was made up according to the manufacturer's protocol and one tablet of tetramethylbenzidine dihydrochloride (TMB, Sigma) was added. This solution (100 µl) was then added to each sample well, plus two additional wells (blanks) and incubated at room temperature for 10 minutes. Once the positive controls showed a strong blue color the reaction was stopped using 25 µl of 10% H_2SO_4. The color was read in a microplate reader (ELX-800, Bio-Tek Instruments, VT, USA) at 450 nm (standard protocol from the CEFAS, laboratory). The mean optical density (OD) [absorbance value (A450)] after subtracting the blank value was recorded for each viral sample as well as negative controls using the KcJunior software (Bio-Tek Instruments).

Undiluted and diluted fish rhabdoviruses (mentioned above) and a negative control of either no EPC cells or EPC cells were subjected to ELISA under similar conditions as described above to determine the specificity of the assay. The assay with no EPC cells as a negative control used serial dilutions of all viruses while the one with EPC cells used only undiluted virus of all fish rhabdoviruses.

Indirect Fluorescent Antibody Technique (IFAT)

The IFAT test was conducted in 24-well plates following the protocol from the CEFAS laboratory. Fresh monolayers of EPC cells (24 hr old) were passaged into 24 well plates (in triplicate) and incubated for 3 hours at 20°C. Following incubation, cells were infected with undiluted and serially diluted viral supernatants of SVCV (US isolate) and incubated overnight at 20°C. Uninfected wells served as negative controls. One mL of PBS was then carefully added to each well and incubated at room temperature for 1 min. The buffer (PBS) was then carefully removed using a pipette and 0.5 mL of freshly made 80% aqueous acetone was added to each well and incubated at room temperature for 20 min. The acetone was removed and the plates were air-dried. For staining the plates, the anti SVCV γ-globulin, at a dilution of 1:300 in PBST, was added (200 µl) following addition and immediate removal of 0.5 mL of PBST to the air-dried wells. Following a 30 min incubation at 37°C, the antibody was removed and washed by adding 1 mL of PBST to each well and incubated at room temperature on an orbital shaker at 250 rpm for 10 min. The wash liquid was poured out and the wash was repeated once. The plate was tapped gently to remove excess fluid from the plate and 200 µl of 1:1000 dilution of goat anti-rabbit FITC conjugate (Sigma) was added to each well and incubated at 37°C for 30 min. The conjugate was removed after incubation and washed twice as in the previous step. After the final wash was removed, 200 µl of distilled water was added to each well and read immediately on an inverted fluorescent microscope.

For the specificity experiment, the test was carried out as described above with undiluted and serially diluted fish rhabdoviruses (as mentioned above).

Data Analysis

In-vitro sensitivity of each test was determined as the lowest concentration detectable by each test while *in-vitro* specificity was recorded as a categorical (dichotomous) variable, either detection or no detection of other fish rhabdoviruses for each test. For ELISA, the absorbance values shown are the mean of three plates with two wells per plate for each sample. Descriptive statistics were calculated using Microsoft Excel (Microsoft Corporation, Seattle, Washington). Minitab (Minitab Inc., State College, Pennsylvania) was used to conduct a one-sided Mann-Whitney-U test to compare assay results with the respective control (Armitage and Berry, 2002). A p-value $< .05$ was considered statistically significant.

RESULTS

Virus Culture

Monolayers of EPC cells in 25cm^2 were infected with different dilutions of SVCV and monitored every 24 hrs for the appearance of CPE. Cells showed extensive CPE within 48 hrs in the undiluted (10^6 $TCID_{50}$/mL) and 10^5 $TCID_{50}$/mL SVCV infected flasks. CPE

progressed in the other dilutions and complete CPE could be noticed only after 7 days post infection in the lowest dilution (10^1TCID$_{50}$/mL). Negative control cells (uninfected EPC cells) did not show signs of CPE.

RT-PCR

Viral RNA extracted from several dilutions of SVCV was subjected to RT-PCR following the OIE protocol with two sets of primers. A specific product of 714 bp was recorded with both the primers. With the degenerate primer set, SVCV could be detected up to 10^5 TCID$_{50}$/mL with 30 cycles of cDNA amplification (data not shown). Upon increasing the amplification cycles to 40 cycles, SVCV could be detected even at 10^4 TCID$_{50}$/mL (Figure 1). A tremendous increase in the capability of RT-PCR to detect SVCV was recorded when the primer set designed based on the nucleotide sequence of the G protein of the US isolate was used. The virus could be detected even at the lowest dilution of 10^1 TCID$_{50}$/mL while the negative control (RNA extracted from uninfected EPC cells) was not amplified (Figure 2).

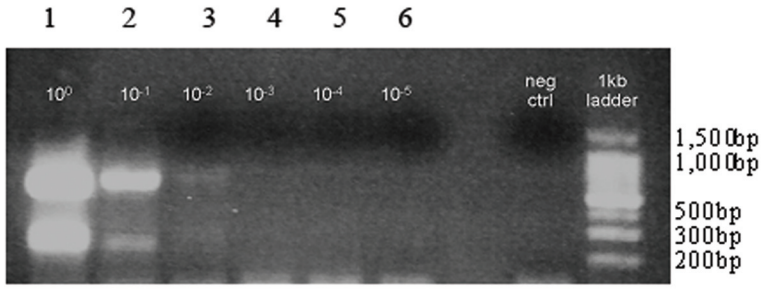

Figure 1. *In vitro* sensitivity analysis of the SVCV RT-PCR diagnostic test with degenerate primers. RT-PCR was performed on serial dilutions of SVCV ranging between 10^6 TCID$_{50}$/mL to 10^1 TCID$_{50}$/mL (lanes 1-6).

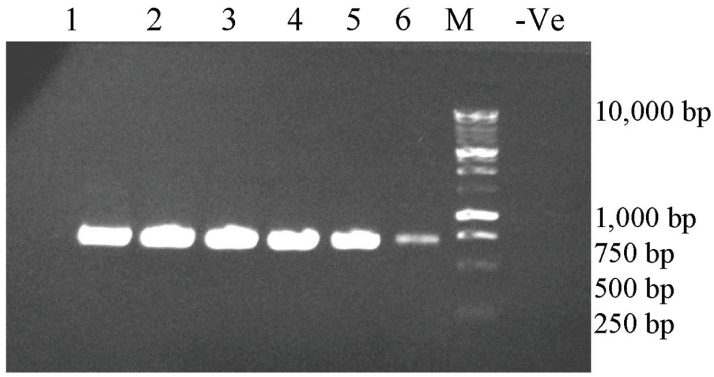

Figure 2. *In vitro* sensitivity analysis of SVCV RT-PCR diagnostic test with US isolate specific primers. Serial dilutions of SVCV ranging between 10^6 TCID$_{50}$/mL to 10^1 TCID$_{50}$/mL (lanes 1-6) were made and subjected to RT-PCR.

Several fish rhabdoviruses were tested concurrently with SVCV virus to compare the specificity of the assay. It was found that both primer sets could detect only the US isolate of SVCV (Figure 3). However, when virus specific primers for IHNV, VHSV and PFRV were employed, RT-PCR products specific for IHNV (786 bp product) and VHSV (811 bp) were amplified (Figure 4). The SVCV primers based on Stone et al., (2003), which were used for PFRV, did not yield any product (Figure 4). A semi-nested RT-PCR was also conducted with SVCV and PFRV using the US isolate specific reverse primer and a new forward primer (PFRnesF). However, no products could be generated for either of the viruses (Figure 4).

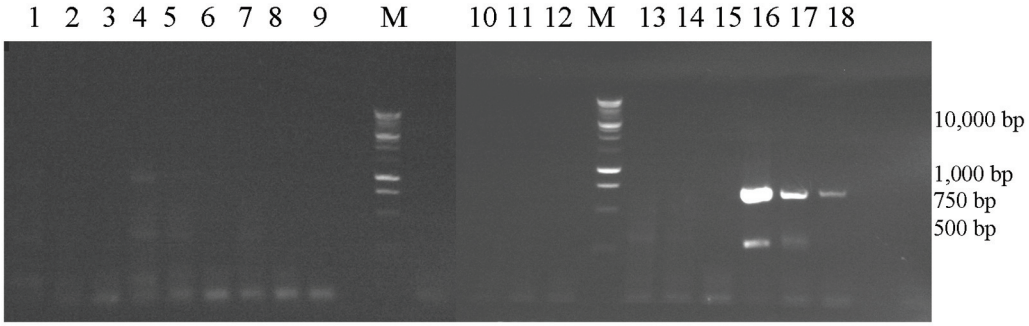

Figure 3. *In vitro* specificity analyses of SVCV RT-PCR assay using different strains of fish rhabdoviruses. A two fold serial dilution of 5 strains of fish rhabdoviruses and SVCV primers based on the OIE manual were used. Lanes 1-3: IHNV (European strain), 4-6: IHNV (American strain), 7-9: VHSV (European strain), 10-12: VHSV (American strain), 13-15: PFRV, 16-18: SVCV.

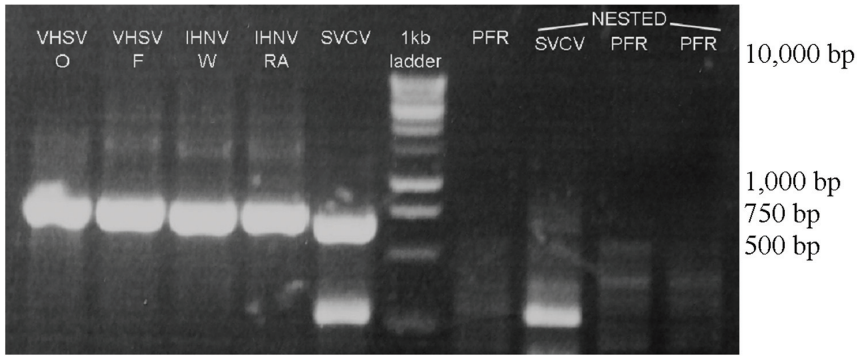

Figure 4. Virus specific RT-PCR for different fish rhabdoviruses using primers specific to IHNV, VHSV and SVCV. The primers used for PFRV were the same as for SVCV. Semi-nested RT-PCR was employed to test for cross reactivity. No nested-RT PCR products could be generated.

ELISA

Serially diluted SVCV (US isolate) was tested in an ELISA based on a CEFAS laboratory protocol. It was found that there was a significant difference ($p<.05$) in absorbance at 10^6

$TCID_{50}$/mL (undiluted), 10^5 $TCID_{50}$/mL, and 10^4 $TCID_{50}$/mL dilutions compared to the negative control (Figure 5). No significant absorbance values could be recorded in the lower dilutions.

Specificity assays for ELISA showed a significant difference ($p<.05$) in absorbance of all viruses at the undiluted concentration compared to the negative control without EPC cells (Figure 6). In this experiment, a significant difference in absorbance for SVCV was only

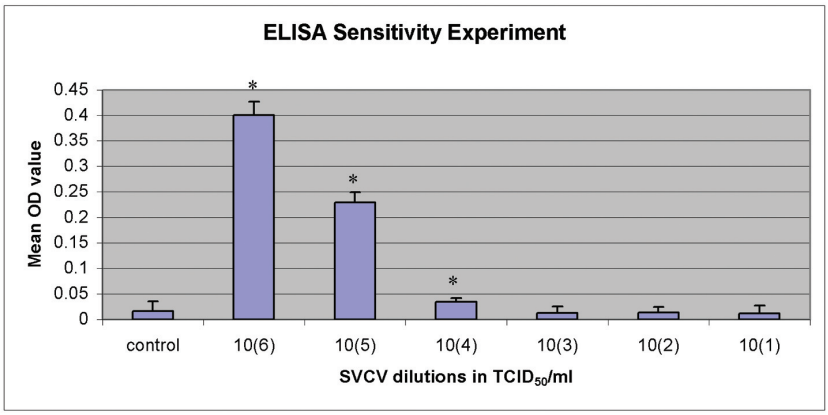

Figure 5. *In vitro* sensitivity analysis of the SVCV ELISA diagnostic test. ELISA was performed on serially diluted SVCV using anti-serum raised against the virus. The OD was read at 450nm and the values shown are the mean values for three plates with two wells each per plate. Significance at the 0.05 level using a one-sided Mann-Whitney test is indicated by *.

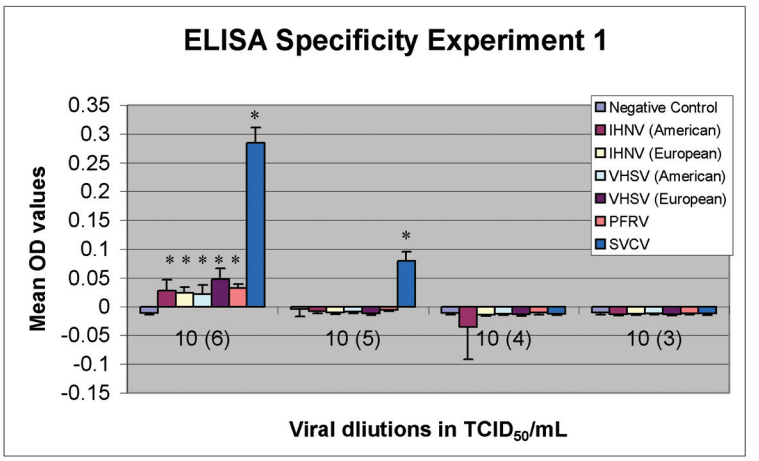

Figure 6. *In vitro* specificity analysis of SVCV ELISA diagnostic test. Serially diluted SVCV, IHNV, VHSV and PFRV were subjected to ELISA using anti-serum raised against the virus. The OD was read at 450nm and the values shown are the mean values from three plates with two wells each per plate. Negative control did not contain EPC cells. Significance at the 0.05 level using a one-sided Mann-Whitney test is indicated by *.

seen at 10^6 $TCID_{50}$/mL (undiluted) and 10^5 $TCID_{50}$/mL. In another experiment, when EPC cells were included as the negative control, only the absorbance of the undiluted US isolate of SVCV differed significantly ($p<.05$) from other fish rhabdoviruses and the negative control (Figure 7).

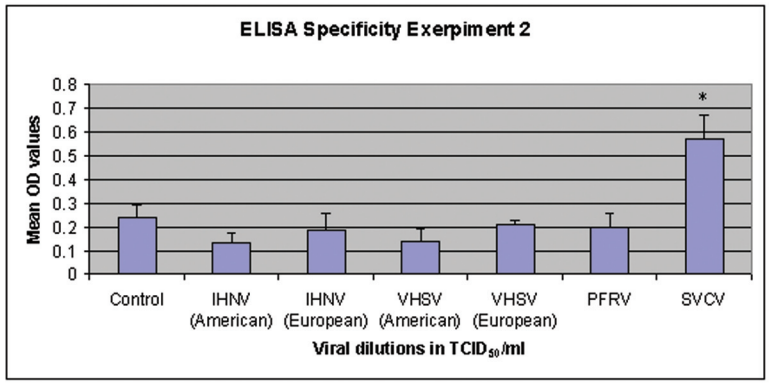

Figure 7. *In vitro* specificity analysis of SVCV ELISA diagnostic test. Undiluted SVCV, IHNV, VHSV and PFRV were subjected to ELISA using anti-serum raised against the virus. The OD was read at 450nm and the values shown are the mean values from three plates with two wells each per plate. Negative control contained EPC cells. Significance at the 0.05 level using a one-sided Mann-Whitney test is indicated by *.

IFAT

The ability of IFAT to detect SVCV at different dilutions of SVCV (US isolate) was carried out following the CEFAS laboratory protocol. The test showed detection of the virus at 10^6 and 10^5 $TCID_{50}$/mL dilutions. No detectable fluorescence could be detected in lower dilutions. The test was repeated three times and consistent results were obtained.

The specificity assay for IFAT using different fish rhabdoviruses were not conclusive. Although we were able to detect only SVCV in the first experiment, we were not able to reproduce the results and cross reaction with other rhabdoviruses was recorded in subsequent experiments (data not shown).

DISCUSSION

Rapid detection of a pathogen is an essential component of all surveillance programs aimed at preventing the introduction of a foreign animal (transboundary) disease. The more rapidly the presence of the pathogen is identified, the more rapidly infected populations can be treated or depopulated. Spring viremia of carp virus is a serious pathogen of carp, which was recently introduced into the United States. (Goodwin, 2002). Rapid and accurate detection of the virus is vital to efforts to prevent the further introductions and spread of the virus. In addition to virus culture, several diagnostic tests have been developed to detect SVCV. However, OIE recommends that the detection of virus by any of the serological

or PCR based methods be confirmed by virus culture. Based on our studies, virus culture, although very sensitive, can take up to 7 days for detection of very low virus levels. Consequently, it does not meet the standard of necessity for rapid detection. A seven-day delay could have serious economic consequences for a producer that waits to take control measures to prevent further mortality. We compared virus culture with other alternative assays available to detect SVCV. Of the assays we evaluated, RT-PCR appears to offer great promise for the rapid accurate detection of SVCV. The US isolate from NC/VA outbreak was detected even at very low levels (10^1 $TCID_{50}$/mL), comparable to virus concentrations detectable by virus culture. However, the time required to conduct RT-PCR analysis of a sample is markedly less than the time required to obtain culture results. Similar sensitivity was reported by Koutna *et al*. (2003). They tested a combination of RT-PCR and nested PCR for SVCV on several clinical samples and reported a sensitivity of 10^{-1} $TCID_{50}$/mL. Our results for the US isolate showed similar sensitivity and hence can be recommended as a method of choice to confirm the presence of US isolates of SVCV. Results also showed that the sensitivity of OIE recommended RT-PCR for SVCV can be increased by one log by increasing the amplification cycles to 40 instead of 30. Also, the fact that the NC specific primer was more sensitive compared to the degenerate primer suggests that the US isolate from NC is different from the reference strain. This observation is in agreement with the recent study by Dikkeboom *et al.* (2004) who reported that the US isolates of NC and Cedar Lakes, WI are more closely related to an Asian strain than the European reference strain. A follow up test of this primer for its applicability to all US isolates would be useful. Efforts are in progress in our laboratory to procure all the isolates needed to conduct these tests.

Specificity was excellent for *in vitro* RT-PCR, as neither the RT-PCR nor the nested PCR detected any fish rhabdovirus other than SVCV (Figure 4). Although, it is reported that the PCR is non-specific and detects PFRV-like viruses in most cases (Stone *et al*., 2003), no such cross reactivity was seen in this study. However, according to Stone (personal communication) the semi-nested RT PCR product can be seen after 30 PCR cycles but disappears upon increasing the number of cycles. In our study, we used 40 PCR cycles in all assays, which could be one of the reasons we did not detect any semi-nested PCR products. This suggestion will be incorporated into follow up tests and *in vivo* studies. Although our RT-PCR assay shows high *In vitro* sensitivity and specificity and no cross reactivity with PFRV, it is premature to believe that our primer is highly specific for SVCV before *in vivo* studies are performed.

The serological based methods like ELISA and IFAT could also detect SVCV. However, the sensitivity was low compared to RT-PCR. Although these tests are routinely practiced to confirm the presence of SVCV and are recommended by OIE, they are time consuming, cumbersome, and often difficult to replicate. Additional studies aimed at optimizing these techniques are needed to overcome the drawbacks associated with these tests.

In summary, the results from our study clearly indicate that RT-PCR is fast, accurate, reproducible, and could definitely be recommended as a method of choice for diagnosing SVCV. The method can be completed in less than 24 hrs as compared to other serological based techniques or virus culture. Thus RT-PCR provides significant saving in time, cost and materials. These observations need to be further validated by conducting studies using

naturally or artificially infected fish. Virus culture is currently required by OIE guidelines to confirm the results of PCR-based assays because of concerns about detecting nonviable viral RNA. The RT-PCR assay could be used as a screening tool to identify SVCV, which could then initiate response activities such as quarantine and strict biosecurity protocols. Due to the possibility of RT-PCR detecting nonviable virus, all depopulation events can be deferred until the results of virus isolation is confirmed. Uniform OIE approved test guidelines and test conditions would enhance confidence in test RT-PCR results, and with this standardization the results of this assay could be considered definitive indication that the virus is present. Protocol standardization and a standard procedure for quality assurance assessment of individual laboratories should be developed to ensure diagnostic consistency between laboratories.

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Monoclonal Antibody-based Detection of *Aphanomyces invadans* for Surveillance and Prediction of Epizootic Ulcerative Syndrome (EUS) Outbreak in Fish

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ABSTRACT

Epizootic Ulcerative Syndrome (EUS) has caused large scale mortality of fresh and brackish water fishes in several parts of South-East Asia including India since 1980s. EUS susceptible fishes such as *Mugil* spp., *Glossogobius* sp. *Sillago* sp., and *Platycephalus* sp. were collected from previously EUS affected brackish waters of South Canara and Udupi districts of Karnataka, India from June 2003 to June 2005. Tissues collected from grossly healthy and ulcerated fishes were screened for the presence of *Aphanomyces invadans* using a monoclonal antibody (MAb) based immunodot. Ulcers were only observed in fishes during the months of August and September each year, coinciding with low water temperature and low salinity. All the ulcerated fishes were found to be positive for *A. invandans* by immunodot. The immunodot could detect the fungus in grossly healthy fish in June two months before appearance of the ulcers in August. The immunodot could also detect the fungus in fishes with healed ulcers during late October and early November. Overall, among the grossly healthy fishes collected from June to November 20% of *Mugil* spp, 2% of *Glossogobius* sp, 10% of *Sillago* sp., and 4% of *Platycephalus* sp. were positive by immunodot. The study indicates that the immunodot could be used for early detection of *A. invadans* and to predict EUS outbreaks at least two months in advance of a disease episode.

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INTRODUCTION

Ever since its first report in 1972 in Australia, epizootic ulcerative syndrome (EUS), a destructive ulcerative disease caused by *Aphanomyces invadans,* has resulted in severe mortalities both in freshwater and in brackishwater fishes throughout the South East Asia (Kenzie and Hall., 1976; Hatai., 1994; Robert *et al.,* 1994b). In India, the first outbreak of EUS occurred in May 1988 in the northeast states and later the disease spread to Arunachal Pradesh in late 1988, Orissa and Bihar in 1989, Maharashtra, Uttar Pradesh, Andra Pradesh and Madhya Pradesh in 1990 and finally to Haryana, Kerala and Karnataka in 1991 (Kumar *et al*., 1991; Das and Das, 1993; Mohan and Shankar, 1994). Most recently EUS was confirmed in fish from Punjab Province in April 1996 and from Sindh Province in January 1998 (DFID, 1998). It has been reported that more than 100 fish species are affected by the disease (Frerichs., 1988; Lilley *et al.,* 1999). Susceptibility of *Mugil* spp. (mullets)*, Platycephalus* sp. (flatheads)*, Sillago* sp. (sillago)*, Glossogobius* sp. (goby) and *Terapon* sp. (therapon) to EUS in brackish water systems in India is well documented (Vishwanath *et al.,* 1997b, 1998). Severe chronic granulomatous mycosis has been consistently seen as the diagnostic pathology in all the EUS affected fishes from different parts of the world (Roberts., 1993, 1994a, b; AAHRI/IOA/NACA, 1997; Mohan and Shankar., 1994; Khan *et al.,* 1999). It is now well established that the surface ulcers and the fish mortality are because of the invasive activity of the fungus *A. invandans* (Vishwanath *et al.,* 1998). In addition to the presence of the causative agent *A. invadans*, several abiotic factors such as low water temperature, rapid change in salinity and dissolved oxygen have been reported to promote the disease (Phillips and Keddie, 1990; Virgona, 1992; Fraser *et al*., 1992). An ulcer is a non-specific clinical lesion, which may be caused by many different agents (Robert *et al.,* 1986). Not all ulcers are EUS related, because they do not occur in epizootic proportions or are not seasonal in nature (Shankar and Mohan, 2002). In light of this, the development of specific diagnosis for EUS is important to avoid confusion with other occasional and serious ulcerative conditions. Histopathology which can detect the fungus in advanced stages of the disease condition is tedious and time consuming. Our laboratory has developed a monoclonal antibody (MAb) based immunodot for specific and rapid detection of *A. invadans* (Gayathri *et al.,* 2004). In the present investigation, the immunodot was used to detect *A. invadans* for surveillance and prediction of EUS outbreaks in fishes from three brackish water bodies of Karnataka, India.

MATERIALS AND METHODS

Sample Collection

Three brackishwater bodies, Nethravati and Mulky estuaries in South Canara and Kundapur estuary in Udupi districts of Karnataka, India were selected for the study. EUS outbreaks were reported earlier from these water bodies. Ten EUS susceptible fish each belonging to *Mugil* sp.*, Platycephalus* sp.*, Sillago* sp. and *Glossogobius* sp. were collected once a month from the above water bodies from June 2003 to June 2005. However, due to the abundance of ulcerated fish during the month of August and September of each year, samples were collected twice a month for close monitoring. The sample consisting of both apparently

healthy and ulcerated fish was brought to the laboratory on ice and muscle tissue extracted for use in the immunodot. Water temperature and salinity were recorded to correlate the relation between incidence of EUS and environmental factors.

Preparation of fish tissue for the immunodot

Muscle tissue was taken from around the ulcerated site on the fishes, while the muscle tissue was randomly taken from 4 to 5 places of the body of apparently healthy fish. The muscle tissue was macerated in TNE buffer (0.02M Tris, 0.2M NaCl, HCl and 1mM EDTA, pH 7.4) at 1:10 w/v by using a pestle and mortar. The homogenate was allowed to settle for 5 min and the supernatant used in the immunodot assay.

Immunodot assay

The immunodot was carried out according to Gayathri *et al.* (2004) with slight modifications. Three µl of tissue homogenate prepared as above from the ulcerated and grossly healthy fish was dotted onto the nitrocellulose paper (pore size 0.2µm, Bio Rad). Semi-purified *A. invadans* was used as a positive control and tissue homogenate from a healthy fish, previously confirmed as negative for the fungus by histopathology and immunodot, used as negative control. The dots were air dried for 5 min. The dotted paper was blocked with 2 ml of 3% BSA (Bovine Serum Albumin, Merck, Mumbai) in PBS (Phosphate Buffer Saline) for 30 min followed by washing with PBS Tween-20. Later the paper was treated with 2ml of MAb C14 (IgM isotype, recognising epitopes on 43, 37, 29, 23, and 19 KD proteins of *A. invadans*) for 90 min. Pooled culture supernatant of the C14 clone from several batches of culture was used to maintain uniform level of the MAb throughout the study. The MAb was poured off and the paper was washed with PBS Tween-20. Then rabbit-anti-mouse IgG horseradish peroxidase (Genei, Bangalore) in 3% BSA (1:200) in PBS was added and incubated for 30 min. After washing 3 times with PBS Tween-20, substrate (4- Chloro-1-napthol/ H_2O_2) was added and a purple/ blue colored dot developed which was recorded as positive.

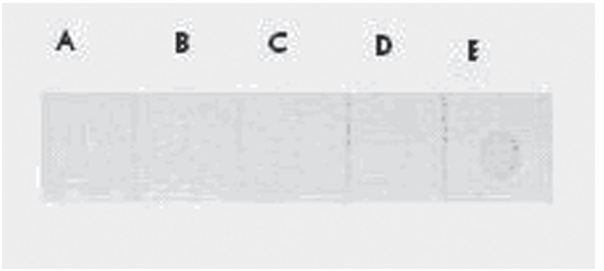
Reaction of the MAb in the immunodot with other ulcer causing aquatic pathogens such as *Aeromonas hydrophila*, *Bacillus* sp., *Saprolegnia* sp. and *Achlya* sp. was checked. These organisms were grown in the laboratory using suitable culture media for each organism. The colonies of the fungi were collected and homogenized with liquid nitrogen and suspended in PBS. Bacterial colonies were suspended in PBS. Three µl (2.5µg) antigen was dotted onto the nitrocellulose paper and examined for the MAb reaction by immunodot as above.

RESULTS

Specific reaction of the MAb with *A. invadans* in the immunodot is depicted in Figure 1. The MAb did not react with other ulcer- causing organisms such as *A. hydrophila*, *Bacillus* sp., *Saprolegnia* sp. and *Achyla* sp. In all the three brackish water bodies, EUS- outbreaks in fish, with visible ulcers were noticed during August and September each year coinciding with low water temperature and low salinity (Table 1). The immunodot could detect

Table 1. Detection and surveillance of *A. invadans* in fish by immunodot in Netravathi, Mulky and Kundapura estuary from June 2003 to June 2005.

Months	Netravathi estuary				Mulky estuary				Kundapura estuary			
	Water temperature (^{0}C)	Salinity (ppt)	Occurrence of EUS ulcer	Result of immunodot	Water temperature (^{0}C)	Salinity (ppt)	Occurrence of EUS ulcer	Result of immunodot	Water temperature (^{0}C)	Salinity (ppt)	Occurrence of EUS ulcer	Result of immunodot
June 2003	28.00	2.26	-	+	28.90	2. 76	-	+	29.20	3.54	-	+
July	28.80	0.00	-	+	29.80	0.96	-	+	28.70	0.78	-	+
Aug.-I	28.50	0.00	+	+	28.30	014	+	+	28.20	0.22	+	+
Aug. II	28.30	0.72	+	+	28.70	0.00	+	+	28.50	0.00	+	+
Sep. -I	28.90	1.62	+	+	28.90	0.70	+	+	28.80	0.85	+	+
Sep. - II	29.00	1.78	+	+	29.00	0.92	+	+	29.10	0.80	+	+
Oct.	28.90	2.18	-	+	30.20	2.46	-	+	29.60	2.37	-	+
Nov.	29.60	4.45	-	+	29.30	4.92	-	+	29.90	4.12	-	+
Dec.	30.30	12.17	-	-	29.80	12.64	-	-	29.50	13.86	-	-
Jan. 2004	30.70	17.86	-	-	30.20	15.24	-	-	30.30	18.13	-	-
Feb.	30.50	22.26	-	-	30.50	20.63	-	-	30.80	21.86	-	-
Mar.	31.20	25.18	-	-	31.30	28.60	-	-	31.00	26.74	-	-
April	31.80	27.68	-	-	31.40	29.34	-	-	31.20	29.65	-	-
May	32.20	30.20	-	-	31.90	30.40	-	-	31.70	30.92	-	-
June	28.30	2.26	-	+	28.70	4.32	-	+	28.90	4.10	-	+
July	28.40	0.00	-	+	28.90	0.45	-	+	28.20	0.58	-	+
Aug -I	28.60	0.00	+	+	28.50	0.00	+	+	28.00	0.00	+	+
Aug. II	28.20	0.00	+	+	28.20	0.00	+	+	28.30	0.00	+	+
Sep. I	29.00	1.30	+	+	28.80	0.52	+	+	28.50	0.93	+	+
Sep. II	28.70	1.25	+	+	28.60	0.72	+	+	28.20	0.65	+	+
Oct	29.80	2.20	-	+	29.60	2.86	-	+	28.80	3.12	-	+
Nov.	29.60	4.60	-	+	30.10	3.93	-	+	29.30	5.54	-	+
Dec.	30.50	13.00	-	-	30.80	14.17	-	-	29.90	15.14	-	-
Jan 2005	30.60	18.30	-	-	30.40	16.92	-	-	30.30	19.24	-	-
Feb	31.00	22.90	-	-	31.20	23.10	-	-	30.90	24.52	-	-
Mar.	31.50	26.00	-	-	31.70	27.30	-	-	31.00	27.36	-	-
April	31.80	28.00	-	-	32.00	29.23	-	-	32.00	30.20	-	-
May	32.40	30.60	-	-	32.20	30.80	-	-	32.60	30.80	-	-
June	28.80	3.20	-	+	28.70	3.93	-	+	29.20	5.10	-	+



A: *A. hydrophila*, B: *Bacillus* sp., C: *Achyla* sp., D: *Saprolegnia* sp., E: *A. invadans*

Figure 1. Immunodot of *A.invadans* with other ulcer causing agents, *A. hydrophila*, *Bacillus* sp., *Achyla* sp., and *Saprolegnia* sp.

A. invadans in all the ulcerated fishes in the three water bodies (Figure 2a). However, interestingly fishes collected during June and July of each year were apparently healthy, and immunodot could consistently detect the fungus in a good number of them (Figure 2b). EUS outbreaks were over by September in all the three water bodies, but immunodot could detect the fungus in the apparently healthy fishes during October and early November (Figure 2b). However, fishes collected from December to May of each year were consistently negative by immunodot (Figure 2c). Overall, among the grossly healthy fish tested from June to November by the dot, 20% of *Mugil* spp., 2% of *Glossogobius* sp, 10% of *Sillago* sp., and 4% of *Platycephalus* sp. were positive for the fungus (Figures 3a,b,c;, Table 2).

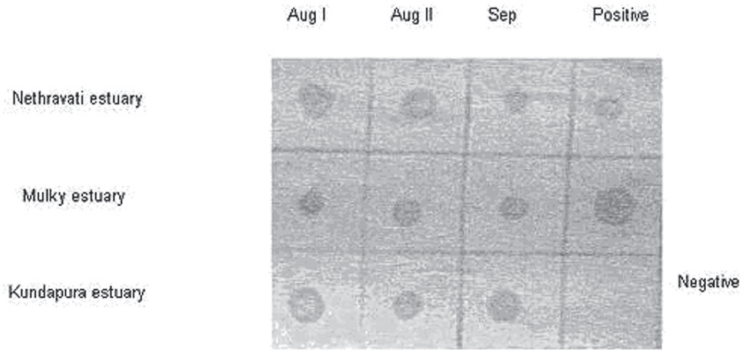


Figure 2a. Immunodot of fishes collected during August and September.

Figure 2b. Immunodot of fish sample without ulcer during June to November.

Figure 2c. Immunodot of fish sample (without ulcer) collected from December to May.

Table 2. Immunodot detection of *A. invadans* in individual fish species during June to November of 2003, 2004 and June 2005.

				Netravati estuary		Mulky estuary		Kundapur estuary	
Year	**Months**	**Fish type**	**No. of fish sampled**	**No. of fish + without ulcer**	**No. of fish + with ulcer**	**No. of fish + without ulcer**	**No. of fish + with ulcer**	**No. of fish + without ulcer**	**No. of fish + with ulcer**
2003	June	Mullets	10	2	-	3	-	2	-
		Goby	10	0	-	0	-	0	-
		Sillago	10	2	-	1	-	2	-
		Flat heads	10	1	-	2	-	1	-
	July	Mullets	10	3	-	2	-	4	-
		Goby	10	1	-	0	-	0	-
		Sillago	10	3	-	3	-	2	-
		Flat heads	10	2	-	2	-	0	-
	Aug.- I	Mullets	10	1	5	1	6	2	5
		Goby	10	0	2	1	1	0	2
		Sillago	10	2	3	1	3	1	3
		Flat heads	10	1	2	0	2	0	3
	Aug.- II	Mullets	10	0	7	1	6	1	5
		Goby	10	0	2	0	2	0	2
		Sillago	10	1	2	0	3	1	3
		Flat heads	10	0	3	1	3	0	2
	Sep. I	Mullets	10	1	5	1	4	0	4
		Goby	10	0	1	0	1	0	2
		Sillago	10	0	3	0	4	1	3
		Flat heads	10	0	3	1	3	0	2
	Sep - II	Mullets	10	2	3	1	4	1	4
		Goby	10	0	1	0	1	0	1
		Sillago	10	1	3	0	3	2	2
		Flat heads	10	0	2	1	2	1	2
	Oct.	Mullets	10	4	-	3	-	3	-
		Goby	10	1	-	1	-	1	-
		Sillago	10	3	-	3	-	4	-
		Flat heads	10	2	-	2	-	1	-
	Nov.	Mullets	10	3	-	2	-	3	-
		Goby	10	0	-	0	-	0	-
		Sillago	10	2	-	1	-	2	-
		Flat heads	10	0	-	0	-	1	-

Table 2. (continued)

2004	June	Mullets	10	3	-	3	-	2	-
		Goby	10	0	-	0	-	0	-
		Sillago	10	2	-	2	-	2	-
		Flat heads	10	1	-	2	-	1	-
	July	Mullets	10	4	-	4	-	4	-
		Goby	10	1	-	0	-	0	-
		Sillago	10	3	-	2	-	2	-
		Flat heads	10	2	-	1	-	0	-
	Aug.- I	Mullets	10	2	6	3	6	2	6
		Goby	10	0	2	1	1	0	2
		Sillago	10	2	3	1	3	1	3
		Flat heads	10	1	2	1	2	0	3
	Aug.- II	Mullets	10	0	6	1	7	1	7
		Goby	10	0	2	0	2	0	2
		Sillago	10	2	3	0	3	2	3
		Flat heads	10	1	3	1	3	0	2
	Sep. I	Mullets	10	0	5	2	6	3	4
		Goby	10	0	1	0	1	0	2
		Sillago	10	1	3	0	4	1	3
		Flat heads	10	1	3	1	3	0	2
	Sep - II	Mullets	10	3	3	1	4	1	4
		Goby	10	0	1	0	1	0	1
		Sillago	10	2	3	2	3	1	2
		Flat heads	10	0	2	1	2	2	2
	Oct.	Mullets	10	4	-	3	-	4	-
		Goby	10	1	-	1	-	1	-
		Sillago	10	3	-	3	-	2	-
		Flat heads	10	2	-	2	-	1	-
	Nov.	Mullets	10	3	-	3	-	3	-
		Goby	10	0	-	0	-	0	-
		Sillago	10	2	-	3	-	2	-
		Flat heads	10	0	-	0	-	1	-
2005	June	Mullets	10	2	-	4	-	3	-
		Goby	10	0	-	0	-	0	-
		Sillago	10	3	-	2	-	2	-
		Flat heads	10	1	-	1	-	1	-

(-): No ulcers found on fish

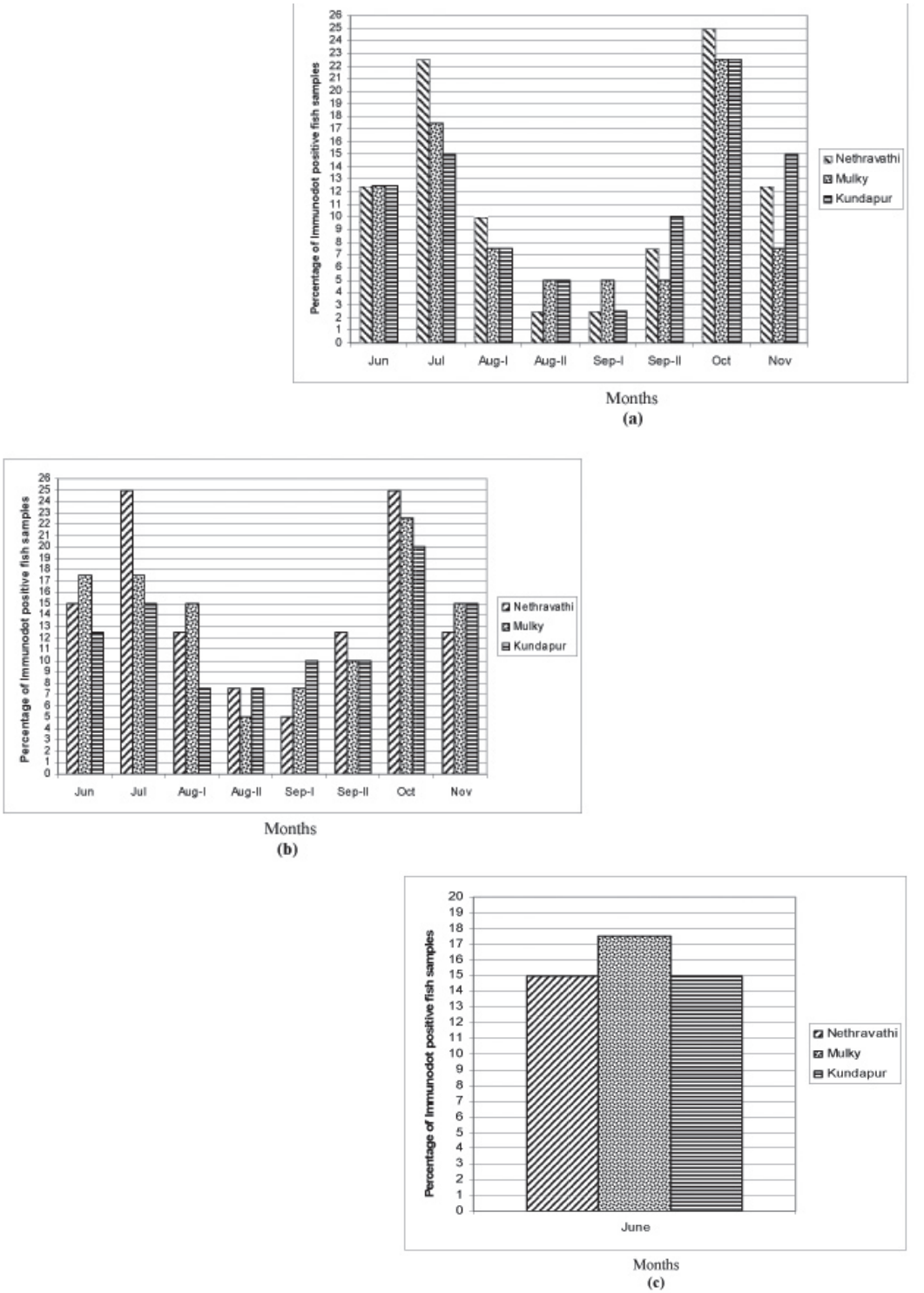


Figure 3. Immunodot detection of *A. invadans* in grossly healthy fish (pooled result of individual species) collected during June to November of 2003 (Figure 3a, 2004; Figure 3b) and in June 2005 (Figure 3c).

DISCUSSION

EUS outbreaks in fish, with typical clinical signs were recorded during August and September of each year in all the three brackish water bodies. During these months salinity and water temperature were low due to monsoon rains which favored development of EUS ulcers, which has been very well documented earlier in India (Mohan and Shankar, 1994; Vishwanath *et al.,* 1997a) and elsewhere (Lilley *et al.,* 1998). The dot detected the EUS fungus, *A. invadans,* in all the ulcerated fishes found during August and September. However, the dot could detect the fungus two months in advance in June in apparently healthy fish, without visible ulcers. It is well known that the fungus penetration into fish takes place at very early stage of the disease, where there may not be significant external clinical signs (Vishwanath *et al.,* 1997a). It indicates that the fungus after infection of fish in early June persisted for two months until favorable conditions such as low water temperature and low salinity are available for its rapid growth which causes ulcer. Lowering of water temperature and salinity may lower the innate defense competence of the fish to EUS ((Vishwanath *et al.,* 1997b; Chinabut and Roberts, 1999) and thus aggravate the ulcer condition. Furthermore, immunodot could also be used to detect the fungus in healing or healed fish during October and November. This shows the persistence of the fungal hyphae in fish tissue for at least two months after the disease outbreak. Conventionally, the fungus infection in EUS outbreak has been detected and confirmed by histopathology, characterized by presence of invasive fungal hyphae with granulomas. However, the fungus could not be detected in grossly healthy fish tissue by histopathology. Specific and sensitive detection of *A. invadans* in fish by DNA hybridization (Blazer *et al.,* 2002) and PCR (Panywachira *et al*., 1999) have been standardized, which however are sophisticated and time consuming. The MAb based immunodot is specific, simple and rapid requiring only 3 h to perform. The dot could detect the fungus in grossly healthy fish in June, two months before disease outbreaks in August, and in November, two months after EUS outbreaks. The MAb based immunodot has a detection sensitivity of 45-90 ng of the fungus protein. Therefore, the immunodot could be very useful for early and low level detection of the fungus ideal for prediction of EUS outbreak. Further, sensitivity of the immunodot is being evaluated with histopathology from the samples taken from experimental infections.

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Occurrence of Epizootic Ulcerative Syndrome in Pond Fish of Kapilvastu District of Nepal

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ABSTRACT

An interview-based questionnaire survey of 30 fish farmers randomly selected in Kapilvastu District of Nepal was carried out to study the prevalence of epizootic ulcerative syndrome (EUS) in pond fish. The survey was carried out during the EUS season (November 2004 to March 2005). At each pond site, 100 fish were examined for lesion, and one fish of each species with lesions was sampled for histological diagnosis. A fishpond was classified as affected with EUS if one or more fish of any species had a positive diagnosis based on the presence of characteristic mycotic granuloma in histological sections. A descriptive statistical tool-SPSS was used to analyze socio-demographic and pond characteristics. Out of 30 ponds examined for lesions, fish in 6 ponds (20 %) were confirmed to be EUS positive. In total, 3000 pond fish were examined and 291 samples with lesions were collected and processed in the histopathological unit of the Central Fisheries Laboratory, Balaju, and Teaching Hospital, Maharajgang, Kathmandu. Of 291 samples, 143 were confirmed to be EUS positive. A total of 17 species with lesions were sampled for histology. Among them, 13 fish species (two cultured and 11 wild) were confirmed as EUS-positive. However, the four cultured species, *i.e.* common carp, silver carp, grass carp and bighead carp had non-EUS lesions. Results indicate that ponds with high relative risks of EUS were characterized by the presence of wild fish, entry of flood water, and connection with paddy fields. Ponds that were not drained, limed and shared contaminated nets had high risk of getting EUS.

Dahail, S.P., Shrestha, M.K., Pradhan S.K. and Jha, D.K. 2008. Occurrence of epizootic ulcerative syndrome in pond fish of Kapilvastu District of Nepal, pp. 169-178. *In* Bondad-Reantaso, M.G., Mohan, C.V., Crumlish, M. and Subasinghe, R.P. (eds.). Diseases in Asian Aquaculture VI. Fish Health Section, Asian Fisheries Society, Manila, Philippines. 505 pp.

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INTRODUCTION

Epizootic ulcerative syndrome (EUS) was first officially recognized as a major problem at the FAO Expert Consultation in Bangkok in 1985 (Lilley *et al*., 1998). The use of the term "syndrome" highlights the complexity of the condition, which involves the interaction of a specific monoclonal fungus, a wide variety of environmental factors and a range of secondary invading pathogens, which differ with each outbreak. It is now recognized to be synonymous with the condition mycotic granuloma (MG) first described from Japan in 1971 and red spot disease (RSD) described from Australia in 1972 (Chinabut and Roberts, 1999). Epizootic ulcerative syndrome (EUS) is a seasonal epizootic condition of great importance in wild and farmed freshwater and estuarine fish (OIE, 2003). It was first reported in farmed ayu (*Plecoglossus altivelis*) in Japan in 1971 (Egusa and Masuda, 1971). It was later reported in estuarine fish, particularly grey mullet (*Mugil cephalus*) in eastern Australa in 1972 (McKenzie and Hall, 1976). The outbreak has extended its range through Papua New Guinea into SoutheEast and south Asia, and recently into west Asia, where it has now reached Pakistan (Lilley *et al*., 1998; Tonguthai, 1985). The disease with severe ulceration and causing heavy mortality of fishes from small and large water bodies has been a major concern almost all over the countries of the Asia Pacific region since 1972. So far no fish disease has been as virulent and menacing as EUS in fish (Chinabut, 1994).

Aquaculture is potentially an important sector of agriculture in Nepal (Pradhan and Shrestha, 1996). Since 1989, EUS has been considered the most serious disease affecting freshwater fish in Nepal (Shrestha, 1990). The initial outbreak of EUS in Nepal was reported in February 1989 from the eastern part of Nepal (Shrestha, 1994). The occurrence of EUS has been reported from Terai, mid-hills, and valleys, *e.g.* Pokhara and Kathmandu (Dahal, 2002; Dahal, 2003). However, the trend of severity in general seems comparatively lesser than in 1990s. Kapilvastu District was selected for the purpose of the present study, as this district was one of the EUS-affected in the country (DoFD, 2002).

The broad objective of this study was to assess the occurrence of EUS in pond fish in Kapilvastu District of Nepal; specific objectives were to identify fish species susceptible to EUS, to confirm the occurrence of EUS by histopathological diagnosis and to assess the relative risk of EUS outbreak with the pond management practices.

MATERIALS AND METHODS

Selection of study area

A list of village and the number of ponds in each village was obtained from the fisheries profile of the District Agriculture Development Office (DADO) in Kapilvastu in September 2004. Each pond was numbered chronologically. Out of 405 fish farmers in Kapilvastu District, 30 fish farmers with a pond was selected, in October 2004, using simple random sampling method. But if for some reasons, when we visited the farm, the farmer was not there, or did not co-operate, or the pond didn't contain susceptible fish, a nearby pond was selected.

Sources of information and data collection

The respondent fish farmers were the major source of primary data. The data were collected through interviews, observations, fish sampling, water quality testing, informal group discussions and key informant surveys. Technical reports, articles, books, proceedings, research report, etc. published by different institution were the major source of secondary data.

Field survey

The actual field survey was carried out from November 2004 to March 2005, recognized as EUS-season in Nepal. On arrival at the sampling pond site, information was gathered from the farmer and this was followed by measurement of water quality parameters. After the completion of the water quality measurement, at least 100 susceptible fish from each pond site were examined for EUS-lesions. At least one fish of each species recorded with lesions at each site was sampled for histology. During the study period of five months, the randomly selected pond was visited five times with monthly interval to observe for the occurrence of EUS in farmed fish.

Descriptive statistical tool

A descriptive statistical tool such as percentage and frequency distribution was used in analyzing fish pond management, socio-demographic and farm characters, which included condition of the pond dike, pre-stocking, stocking and post-stocking management, of the randomly selected ponds

Fish sample collection

Fishermen were used to net the pond and catch the fish. 100 fish were randomly selected from the hapas and examined individually for the presence of external lesions. One fish of each species with visible lesions was sampled for histopathology. Fish with lesion was killed and two pieces of muscle of size 1 cm^3 each taken from the lesion and surrounding muscle for histology study. Sample were fixed immediately in cold 10% formalin and labeled. Each sample was put in a separate container.

Water quality analysis

Four important water quality parameters, *i.e.* temperature, pH, dissolved oxygen and transparency, were measured monthly *in situ* using portable water analysis kit (Hack Kit).

Histopathological analysis

Processing of formalin fixed tissues was carried out at Central Fisheries Laloratory, Balaju, Kathmandu and Histopathological laboratory, Teaching Hospital, Maharajganj, Kathmandu. The processing as well as H&E staining procedure described by Chinabut and Roberts (1999) was followed.

Method and techniques of data analysis

Data were analyzed with the help of Statistical Packages for Social Sciences (SPSS) computer software package.

RESULTS

A total of 3000 fish samples were examined of which 291 samples with external lesions were processed for histopathology. Of the 291 fish with external lesions, 143 fish were confirmed as EUS-positive, based on the presence of mycotic granulomas. Of the total 17 fish species examined, 13 species (two cultured and 11 wild) were confirmed to be EUS-positive. The Chinese carps and common carp with external lesions similar to EUS were negative for EUS. A total of 752 wild fish found in ponds along with cultured species, were examined for lesions. Of these, 156 with external lesions were sampled for histology and 67 were confirmed as EUS- positive. The average prevalence of EUS in wild fish species was 8.3 percent with a range of 4.8 to 11.5 percent (Table 1). Photographs of EUS affected wild fish species are given in Figure 1a and b.

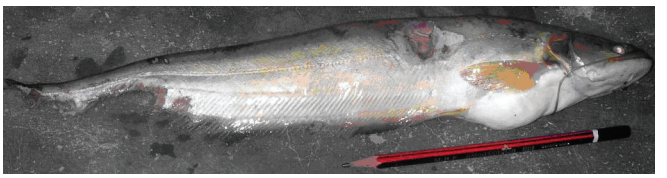
a. Naini (*Cirrhinus mrigala*) b. Rohu (*Labeo rohita*)

Figure 1 (a,b). Photographs of cultured fish species showing typical EUS lesions.

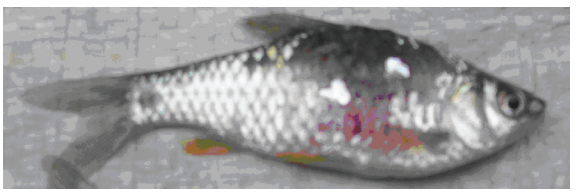
Table 1. Prevalence of EUS in wild fish species.

S.N.	Fish species	No. of fish examined			Confirmed cases (EUS-positive)	Percent infection of EUS
		Total	Healthy	With lesions		
1.	Tengri (*Mystus tengara*)	53	45	8	5	9.4
2.	Pothia (*Puntius ticto*)	55	35	20	5	9.0
3.	Darahi (*Puntius sarana*)	65	55	10	6	9.2
4.	Pothia (*Puntius chola*)	50	40	10	5	10.0
5.	Kabai/kotri (*Anabas testudineus)*	160	140	20	12	7.5
6.	Garahi (*Channa punctatus*)	139	104	39	16	11.5
7.	Buhari (*Wallago attu*)	12	07	05	01	8.3
8.	Mungri (*Clarias batrachus*)	51	39	12	5	5.8
9.	Dhebari (*Nandus nandus*)	60	48	12	5	8.3
10.	Gainchi (*Macrognathus aral*)	42	37	05	2	4.8
11.	Chelwa (*Salmostoma bacaila)*	65	50	15	5	7.7
	Total	752	600	156	67	Mean: 8.3

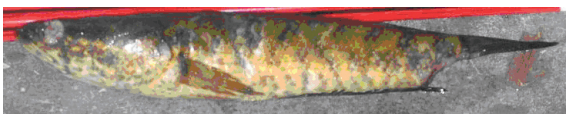
Amongst the cultured fish species, rohu and naini (major carps) were found to be affected by EUS. The total number of major carps examined was 704; of these rohu and naini were 312 and 392, respectively. The average occurrence of EUS was 9.9 and 11.5 in rohu and naini, respectively (Table 2). Photographs of EUS affected cultured fish species are given in Figure 2.



a. Buhari (*Wallago attu*)



b. Pothia (*Puntius ticto*)



c. Hile (*Channa punctatus*)

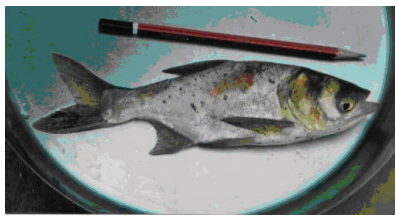
Figure 2 (a-c). Photographs of wild fish species showing typical EUS lesions, Kapilvastu (2005).

Table 2. Prevalence of EUS in cultivated fish species.

S.N.	Fish species	No. of fish examined			Confirmed cases (EUS-positive)	Percent infection of EUS
		Total	Healthy	With lesions		
1.	Rohu (*Labeo rohita*)	312	273	39	31	9.9
2.	Naini (*Cyprinus mrigala*)	392	347	45	45	11.5
	Total	704	620	84	76	Mean: 10.7

Cultured common carp and three Chinese carps were found with clinical signs (open dermal ulcers) similar to EUS. However, histopathological examination did not reveal the presence of mycotic granulomas in any of the samples (Table 3). Histopathologically, EUS affected fish showed the presence of distinctive MG in affected tissues caused by the oomycete fungus *Aphanomyces invadans* (Lilley *et al.*, 1998). Photographs of cultured fish species showing clinical signs similar to EUS, but negative for MG are given in Figure 3.

a. Common carp (*Cyprinus carpio*)



b. Bighead carp

c. Silver carp

Figure 3 (a-c). Photographs of cultured fish species showing clinical signs similar to EUS but negative for MG.

Table 3. Details of cultured fish species with clinical signs similar to EUS but negative for mycotic granulomas, the diagnostic feature of EUS.

SN	Fish species	No. of fish examined		
		Total	Healthy	With lesions
1.	Common carp (*Cyprinus carpio*)	397	379	18
2.	Silver carp (*Hypophthalmichthys molitrix*)	507	495	12
3.	Grass carp (*Ctenopharyngodon idella*)	291	275	16
4.	Bighead carp (*Aristichthys nobilis*)	349	340	09
	Total	1544	1489	55

The study showed that 36.4% of ponds in Kapilvastu District do not have permanent source of water and such ponds depended for water on rainfall. Ponds that received water from the canal connected to the rice field, river and reservoir and wetland showed relatively high risk of EUS while the occurrence of EUS was not observed in ponds that had received water only from rainfall or underground source. The study showed that the majority (60%) of the fishpond was not dried and 40 percent of the pond dried during the previous year. About 27.8 percent of EUS occurrence was confirmed in ponds that had not dried in the previous year. Majority (70%) of the fish growers in Kapilvastu district do not apply lime during pond drying and post stocking management. The occurrence of EUS was observed mostly in ponds that did not apply lime during the previous year. All of the EUS positive confirmed cases were collected from the ponds that were not limed before.

DISCUSSION

Occurrence of EUS in wild and cultured fishes in Kapilvastu District was confirmed through histopathological studies. Of the total of 17 fish species collected with EUS like lesions, only 13 (2 cultured and 11 wild species) were confirmed positive for EUS based on the presence of mycotic granulomas, the diagnostic characteristic of EUS. Amongst the commercially important cultivated species, Chinese carps (silver carp, bighead carp and grass carp) and common carp were observed with EUS like lesions, but did not have MG. Hatai (1994) had observed that Chinese carp and common carp were not affected during EUS outbreaks in Japan. Mechanisms of resistance in these species have been described by Wada et al. (1996). The study showed that ponds containing more wild fish were at high risk of EUS occurrence. Lilley *et al*. (1998) reported more than 100 wild species as susceptible to EUS in Asia Pacific region.

The present study showed that 36.4% of ponds in Kapilvastu district do not have permanent source of water and such ponds depended for water on rainfall. About 26.6 percent of ponds received water from the canal connected to the rice field while 20.0 percent received water from canal connected to river. Ponds that received water coming through rice field and river/ditch had showed high relative risk of EUS while the occurrences of this disease was less in ponds that had received water only from underground source. Ahmed and Rab (1995) indicated that wild fish in natural bodies might be the source of infection, and methods of excluding of wild fish and other potential carriers from pond are likely to be effective in reducing occurrence of EUS (Jha and Shrestha, 2003).

The information gathered showed that there is more chance of EUS occurring in culture ponds containing wild fish. There was also higher relative risk of EUS occurring on farmed fish when pond embankments were not high enough to prevent incoming water. Similarly, ponds that were repeatedly flooded that year also showed a higher relative risk. Fish farms directly connected to water bodies that allowed the entry of wild fishes also showed a higher relative risk of EUS.

In this study, 17 fish species were examined. Of these, 13 species were histopathologically confirmed as EUS positive, while common carp (*Cyprinus carpio*), silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Aristichthys nobilis*) and grass carp (*Ctenopharyngodon idella*) were negative for EUS. It has been reported that EUS has no effect on common carp (Hatai and Egusa, 1978). Therefore, these species are considered as EUS resistant. Some scientists have commented that the severely affected species in natural outbreak are generally bottom dwellers (Llobrera and Gacutan, 1987). This comment seems true to some extent with the present study findings.

Failure to drain and lime ponds prior to stocking has a high risk of outbreak of EUS (Mohan and Shankar, 1994; Jha, 2002). In the present study, ponds that had not been dried and limed, showed higher prevalence of EUS. Pond watercolor indicating high levels of phytoplankton or zooplankton had no effect on EUS outbreak. Khan *et al.* (1999) opined that the vulnerability to the occurrence of EUS might be high in ponds having frequent contact of animals, people and other materials as compared to closed ponds. Most (90%) of the fish farmers did not remove the upper layer of pond bottom after draining pond. Old ponds that have heavy deposit of mud (humus) at the bottom were found vulnerable to the occurrence of EUS.

CONCLUSION

EUS was confirmed in Kapilvastu District. Only two out of seven cultured species were affected by EUS indicating that cultured species are less susceptible than indigenous/wild species. Eleven wild species were found affected by EUS indicating that indigenous species are more vulnerable to EUS. Wild fish in the natural bodies might act as the source of infection. Ponds with more wild fishes were at high risk of EUS. Removal of all susceptible species from the pond and obtaining water from EUS free sources might help to avoid EUS. Drying out and liming of pond prior to fish stocking might prevent the outbreak of EUS. Pond management practices such as repair of pond dike condition to prevent the entry of wild fishes along with flood-waters into the pond; mud removal from the pond bottom; application of lime during post stocking management; restriction on washing and cattle bath in the pond appear to be helpful in avoiding the occurrence of EUS. Stocking of less number of susceptible species and substituting resistant fishes such as Chinese carps and common carp having similar feeding niches might be helpful to avoid EUS in culture ponds.

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Tetrahymenosis, Columnaris Disease and Motile Aeromonad Septicaemia in Golden Perch, *Macquaria ambigua* (Richardson), from Australia

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ABSTRACT

Golden perch cultured in earthen ponds in northern Australia suffered severe mortalities in two successive winters from infections with *Tetrahymena corlissi* and motile aeromonads. Skin lesions were initially detected as small red pinpoint lesions that rapidly increased in size to large round, deep skin ulcers with hyperaemic margins within 3 to 4 days. Laboratory trials of chemotherapeutics done *in vitro* on fish infected with *T. corlissi*, included potassium permanganate, malachite green, methylene blue and salt, formalin and salt, and copper sulphate, and were ineffective in killing *Tetrahymena corlissi.* The aetiology and progress of the disease suggests that golden perch was naïve to the local strain of *T. corlisii*, increasing its pathogenicity and speed of ulceration, as there was poor immune response to initial invasion by *T. corlissii* through dermal tissue. Infection with *T. corlissii* responded well to treatment with Emytryl (400 mg/g dimetridazole), a systemic protozooicide, administered orally at 30mg/kg/fish/day. This drug was effective within 3 days of treatment as mortalities ceased in ponds, and healing of ulcers was observed in fish in tanks.

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INTRODUCTION

The disease status of golden perch has been well documented (Rowland and Ingram, 1991), due to their widespread use in fishery enhancement programs. However, to date, there has been no report of *Tetrahymena sp*. infestation in this species. *Tetrahymena spp.* are ubiquitous free-living hymenostome ciliates, that cause disease in a wide variety of fish, crustaceans, amphibians and turbellarians (Hoffman *et al.*, 1975; Wright, 1981; Lom, 1984; Ferguson *et al.*, 1987; Lom and Dyková, 1992; Edgerton *et al.*, 1996; Ponpornpisit *et al.*, 2000). *Tetrahymena corlissi* recorded primarily as a parasite of freshwater tropical fish and amphibians, is histophagous, and can destroy surface tissue, invading the skin, skeletal muscle and internal organs, causing mass mortalities in aquarium fishes (Lom and Dyková, 1992; Imai *et al.*, 2000; Wakita *et al.*, 2002). *T. corlissi* has been associated with gross clinical signs of epidermal sloughing, raised scales, skin ulceration and lesions, and skeletal muscle inflammation and necrosis. Clinical disease is rapid in onset and often fatal (Lom and Dyková, 1992; Imai *et al.*, 2000; Wakita *et al.*, 2002).

Motile aermonads are gram negative bacteria, ubiquitous in aquatic ecosystems. These bacteria are routinely isolated as both normal flora and as primary or secondary pathogens from sick or moribund fish. Motile aeromonad septicaemia may cause acute or chronic infections in many different species of freshwater fish. The bacteria responsible (*Aeromonas caviae*, *A. hydrophila* and *A. sobria*) all produce extracellular enzymes and toxins causing cell lysis and necrosis (Roberts, 1993). The disease is strongly correlated with stress, overcrowding, poor hygiene and stress-mediated immunosuppression (Toranzo *et al.*, 1987; Roberts, 1993; Thune *et al.*, 1993). Previous motile aeromonad septicaemia (*A. sobria*) of golden perch at the Freshwater Fisheries and Aquaculture Centre (FFAC) had symptoms of petechial haemorrhage on the ventral surfaces of the fish, disoriented swimming behaviour, and septicaemia leading to death in 4-5 days (unpublished observations).

Columnaris disease caused by *Flavobacterium columnare* has been documented worldwide in over 36 species of freshwater fishes including cultured species, barramundi *Lates calcarifer* (Bloch), channel catfish *Ictalurus punctatus* (Rafinesque), tilapia *Oreochromis* sp. and ornamental fish including goldfish *Carassius auratus* (L.) [Carson *et al.*, 1993; Soltani *et al.*, 1996; Plumb, 1999]. Gross lesions typically include gill necrosis, skin ulceration, jaw erosion and fin and tail rot to varying degrees (Ullrich, 1992; Clayton *et al*., 1998). Columnaris disease at FFAC is usually manifested as a saddleback lesion across the body, usually posterior to the second dorsal fin (Mosig, 2002). Outbreaks of columnaris disease are associated with environmental factors including low water temperatures, crowding, high organic loads, handling, poor nutrition and stress (Chowdhury and Wakabayashi, 1991; Wakabayashi, 1991; Carson *et al.*, 1993; Soltani and Burke, 1994; Altinok and Grizzle, 2001; Shoemaker *et al.*, 2003).

In two successive winters of 2001 and 2002, golden perch grow out trials at FFAC were devastated from mass mortalities of fish from infections with *Tetrahymena*, motile aeromonad septicaemia and columnaris disease. This paper describes the gross, histology, microbiology and parasitology findings from golden perch affected by these diseases.

Results from chemicals used in ponds and *in vitro* trials in tanks against *Tetrahymena corlissi* are also presented.

METHODS AND MATERIALS

Growth Trials

Growth trials were conducted on golden perch, in 2000/2001 and 2001/2002. Two groups (A and B) were grown in 2000/2001, and one group (C) in 2001/2002. All ponds used in the growth trials were 350 m^2, with a volume of 230 m^3, lined with polyethylene, and fully netted and fenced to exclude predators. Ponds were aerated with submerged perforated polyethylene pipes. Aspirators provided supplementary aeration and mixing, as required during pond chemotherapy treatments. Pond preparation prior to stocking included removal of organic matter, liming with agricultural lime at 1.2 tonnes/ha and dry-out for one month. Incoming water was filtered with 500µm screens. Water quality parameters in ponds and in tanks measured daily included pH, pond water temperature, and dissolved oxygen. All dead fish and sick fish were removed from ponds daily and recorded. All fish were fed daily to satiation with a sinking barramundi grower diet (Nutreco Pty. Ltd., Rosny Park, Tasmania) (43% protein, 15% lipid, 22% carbohydrate, 11% ash). Feed trays were used to monitor feed consumption.

Group A: In March 2000 two different strains of fingerling golden perch (Murray-Darling strain and Fitzroy River strain) were purchased from a commercial hatchery (30-50 mm TL). The two strains were weaned in tanks before transfer to ponds in May. Water lettuce (*Pistia stratiotes*) was introduced from local ponds in December 2000 for algal control and to provide fish with shelter. In April 2001, fish were graded and moved into 4 new ponds (Table 1). Fish were again graded in April 2001 and moved into newly prepared ponds, along with the water lettuce. Water was sourced from a ground water well. Maximum biomass in each pond was 323 g/m^3, and density was 1.3 $fish/m^2$.

Group B: Fingerlings of *Macquaria ambigua* arrived at Walkamin on 24 Nov 2000, and

Table 1. In plastic chemotherapeutic trials. As all fish had severe lesions, not all survived for the full length of the trial. At least two fish from every trial were alive at the end. None of these trials were effective in stopping division of T corlissi or in killing them'.

Chemical	Concentrations	Duration	Method
$KMnO_4$	1, 5, or 15 mg/L	1 and 20 hrs	20L bucket
Malachite green	0.1 or 1 mg/L	1 and 20 hrs	20L bucket
Methylene blue and salt	1 or 3 mg/L MB 10‰ salt	1 and 20 hrs	20L bucket
Formalin and salt	70 mg/l formalin salt (12‰)	1 and 5 hrs	20L bucket
$CuSO_4$	25 mg/l	1 hr	20L bucket
Salt	10‰	5 to 7 days	100L tank

were weaned and stocked into nursery ponds on 22 Dec 2000. These were harvested, graded and stocked into new ponds on 14 May 2001. Fish were sampled with a seine net for measurements once every two weeks during nursery. During grading fish were treated with a salt bath (12‰) salt for 60 to 90 minutes. They were then sedated, caught and transported back to new ponds in 10‰ salt water, on 15-17 May. Fish from all ponds were mixed for statistical purposes, to have a complete mix of fish from a previous experiment. Ten ponds were stocked, with maximum biomass of 244 g/m^3 and maximum density of 2 fish/m^2.

Group C: Fingerlings of *Macquaria ambigua* arrived at Walkamin on 1 Nov 2001, and were weaned, and 1266 were stocked into each of 6 ponds on 19 Dec 2001. These ponds had been treated with 20mg/L chlorine and then sun dried for 6 months. Fish were sampled once every four weeks. These ponds were treated with Platypus Probiotic (International Animal Health products Pty. Ltd., Huntingwood, Australia)[1], a program of weekly treatments administered over a four-week cycle to introduce large numbers of beneficial *Bacillus sp*. bacteria into the water. The probiotics were administered according to the manufacturer's instructions. Maximum biomass achieved was 1.1 kg/m^3, and density was 3.95 fish/m^2.

Gross pathology, microbiology and histology

From the outset and throughout all infections, gross observations and fresh scrapes and smears were taken from sick and undamaged fish at least weekly to assess progress of infections, check efficacy of therapeutic treatments, and to ensure that the same infection was present throughout the course of the epizootics. In May 2001, January and July 2002 samples (each sample n=5-10) moribund golden perch from these epizootics were submitted to Oonoonba Veterinary Laboratory (OVL), for gross, histological, parasitological and microscopical examination. In July 2002, further samples of golden perch with 3 differing types of skin lesion were submitted for histological examination to determine the aetiology of each type of lesion.

In January 2002, a sample of dead tadpoles of the native frog *Litoria microbelos* found in a pond containing several dead golden perch were also submitted for gross and histological examination, as it was suspected the amphibians may be carriers of *Tetrahymena.*

In June 2001, fish with grossly visible small red 'pinpoint' skin lesions, 1 to 2 mm diameter, were observed to monitor progression of the skin lesions over time.

Fish and tadpoles were sacrificed, organs and tissues including skin, gills, brain, heart, liver, head and caudal kidney, spleen, pancreas, eye, stomach, intestine, abdominal fat, swim bladder, bladder, skin and skeletal muscle were fixed in Bouin's fixative for 24 hours then processed routinely for histology (Bancroft and Stevens, 1990).

[1] Use of this product does not indicate or imply endorsement

Wet mount preparations, parasitology and microbiology

Movement and morphological characteristics of live protozoan parasites observed on wet mount preparations were studied for species identification at both FFAC and OVL. Air dried skin smears were either stained with Klein's dry silver impregnation method (Lom and Dykova, 1992) or preserved in Bouin's fixative, and sent to Dr. Peter O'Donoghue (University of Queensland Department of Parasitology) for species confirmation. Skin scrapes were taken from the leading edge and from deep within the centre of skin ulcers and plated onto Sabourads dextrose agar (SDA) with added chloramphenicol and gentamycin, and marine agar with added thiamine (MAT) for fungal isolation and culture. Swabs were taken from the skin ulcers, heart and caudal kidney of several fish with skin ulcers, and plated onto sheep blood agar (SBA) and marine agar with added vitamins (MAV) for bacterial isolation and culture.

Pond treatments and *in vitro* chemical trials

Chemicals recommended by Boyd (1982), Kabata (1985) and Noga (1996) were added to ponds containing affected fish for the treatment of the *Tetrahymena* infection. Four ponds were treated with 30mg/L formalin, then 8 days later with 7 mg/L potassium permanganate ($KMnO_4$), then 3 days later $KMnO_4$ at 5 mg/L. $KMnO_4$ demand was calculated according to Boyd (1982). Two ponds were treated with 20ml/L formalin, four days later 30ml/L formalin, then five days later 7 mg/L $KMnO_4$, the 3 days later 5 mg/L $KMnO_4$. Samples were taken approximately six hours and 24 hours after each treatment from each pond and wet smears from scrapes examined. Prior to chemotherapy application the water level was dropped and an aspirator placed in each pond, to increase mixing and dissolved oxygen levels. Inflow water was added to refill the pond, and the chemical administered to the inflow water to ensure mixing of the chemical in the pond water.

Several chemicals recommended by Boyd (1982), Kabata (1985) and Noga (1996) for the treatment of ectoparasites were tested on infected fish from the 2001 outbreak as small volume treatments. For each chemical tested, 5 small (<50g) fish with skin ulcers were placed in an aerated 20 l plastic bucket containing the chemical. At the end of each chemical treatment, skin scrapes were taken from the edges and at the centre of skin ulcers of each fish, wet mounts were prepared and observed for the presence and activity of *Tetrahymena sp.* under a light microscope.

In June 2002, fish in ponds affected with tetrahymenosis were administered Emtryl Soluble (400 g /kg dimetridazole soluble powder, Rhône-Poulenc/ Aventis Animal Nutrition Pty Ltd) at a dose rate of 30 mg dimetridazole/kg/fish for 10 days. Emtryl was dissolved and sprayed onto the food with gelatine. Gelatine was used to help the drug stick to the pellets and increase retention time on pellets while in the water. Feed consumption was monitored daily using feed trays. Several fish affected with tetrahymenosis were taken out of ponds and placed in 200L tanks and similarly administered with Emtryl for observational purposes to assess healing of skin ulcers and health in response to administration of the antiprotozoal. Skin scrapings were then done from the edge and centre of skin ulcers to determine whether *T. corlissi* was still present.

RESULTS

Mortality

The first mortalities occurred in April 2001, one day before fish were handled, graded and transported to new ponds. Mortality rates of affected fish in the 14 ponds in 2001 varied from 32% to 86%, with an average of 40% ± 18.7%. In the winter of 2002 mortalities was 92% in the single pond affected. In both years, mass mortalities coincided with cool water temperatures (Figures 1 and 2).

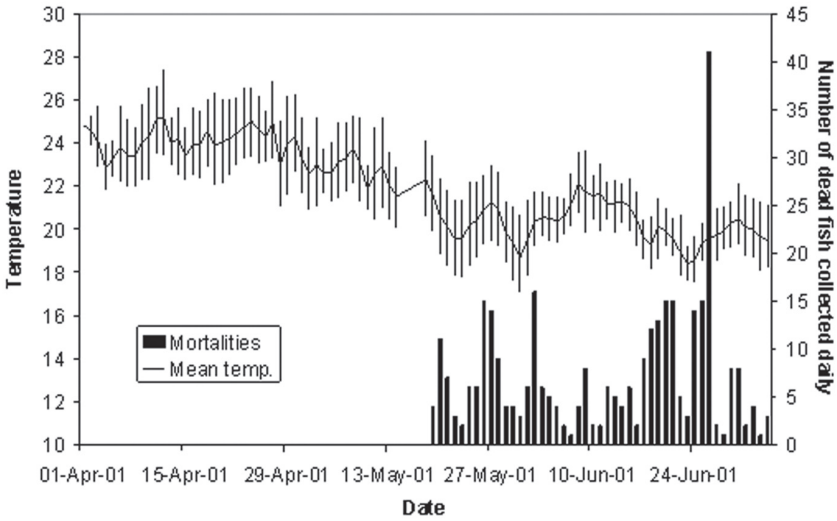

Figure 1. Mortalities caused by *Tetrahymena* in a typical pond, and temperature, in the 2001 epizootic (Group B).

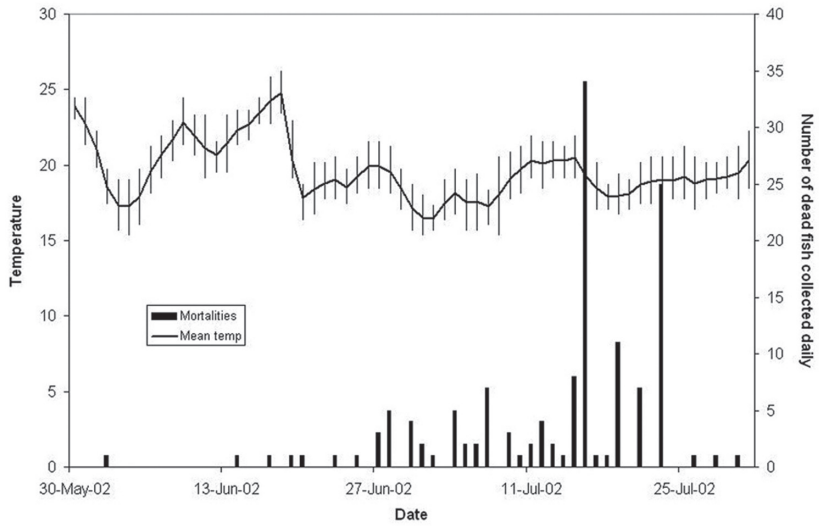

Figure 2. Mortalities in the single pond affected in 2002.

Water quality

Water quality parameters were within normal range for golden perch. pH varied from measured 7 to 9 during the entire period of the growth trials. Dissolved oxygen was maintained at above 90% saturation. Pond water temperature varied during 2001 from a minimum of 17.6°C in winter to a maximum of 26.6°C in summer (Figure 1).

Gross pathology

Fish submitted in May 2001 showed reddening of the pectoral, dorsal and anal fins, and ulceration of the skin (Figure 3) with 'saddleback' lesions under the dorsal fins typical of infection with columnaris disease caused by *Flavobacterium* sp. The gills of all infected fish were anaemic. Fish had large, round deep skin ulcers with hyperaemic margins exposing the skeletal muscle (Figure 4). The skin ulcers were located variously on the body of the fish but were most common laterally or the caudal peduncle. Several fish had white, fuzzy growths attached to the skin ulcers, resembling that of fungal infection. Gross internal examination of several infected fish with deep skin ulcers showed many had peritonitis with a red-brown exudate typical of motile aeromonad septicaemia.

Figure 3. Early lesion on side of perch, about 10 mm across. This is a shallow lesion with haemorrhage around margins.

Fish submitted in July 2002 had 4 different types of grossly visible skin lesions; including small 1-2 mm dark marks on intact skin on the caudal peduncle; deep round skin ulcers with hyperaemic margins varying in size from 0.5 to 8 cm; areas of shallow skin erosion beneath the dorsal fin with reddening of the caudal fins; and areas of skin that were pale in colour but with no erosion, rostral to the dorsal fin.

Observations made on ulcer development on fish held in tanks in 2001 with visible small 'pinpoint' red skin lesions, 1 to 2 mm diameter, showed the lesions grew rapidly

Figure 4. Lesion of freshly dead fish. Haemorrhagic margin of lesion with skeletal muscle necrosis and secondary infection with fungi and bacteria.

into skin ulcers that doubled in size daily, until the ulcer reached 5 to 7 cm diameter within 2 to 4 days, sometimes occupying 10% surface area of the fish. Small fish (30-120 g) died within 1-2 days whereas larger fish (>120 g) died within 2 to 5 days.

Wet mount preparations

Examination of wet mount preparations of skin smears from the leading edge of areas of skin ulceration in fish with "saddleback' lesions under the dorsal fins submitted in May 2001 showed numerous filamentous, gliding bacteria forming 'haystacks' typical of *Flavobacterium columnare*.

Examination of wet mount preparations from skin scrapes done at the leading edge and centre of deep skin ulcers from fish in 2001 revealed thousands of active hymenostome ciliated protozoan parasites, pyriform in shape with somatic cilia covering their entire surface. Scrapes prepared from the centre of the skin ulcers had fewer parasites, and presence of fungal hyphae. Skin scrapes from normal areas of skin were negative for ectoparasites. Examination of wet mount preparations from gill smears showed low numbers of the flagellated protozoan parasite *Ichthyobodo necator*, the ciliated protozoan parasite, *Trichodina* sp. and *Ichthyophthirius multifiliis.*

Air dried smears from ulcer margins, stained with Klein's dry silver impregnation method at OVL, were sent to Queensland University Microbiology Department for confirmation of identification by Dr. Peter O'Donoghue and identified as *Tetrahymena corlissi* Thompson, 1955 on the basis of their characteristic morphological features.

Table 2. Morphometric characterization of *Tetrahymena corlissi* (X = mean, SE = standard error, min = minimum, max = maximum, n = number of observations). Provided by Peter O'Donoghue, University of Queensland. All length measurements in µm.

Character	X	SE	min	max	n
Trophozoite - length (range)	55.4 (32-70)	3.8	32	70	10
Trophozoite - width (range)	38.0 (26-50)	2.2	26	50	10
Buccal cavity – length (µm)	8.0 (6-10)	0.4	6	10	10
Buccal cavity – width (µm)	4.0 (3-5)	0.2	3	5	10
Number of meridional kineties	28.1	0.5	25	30	10
Number of post-oral kineties	2	0	2	2	10
Macronucleus – length (µm)	11.8	0.6	8	14	10
Macronucleus – width (µm)	8.0	0.4	6	10	10
Micronucleus – length (µm)	3.0	0.2	2	4	8
Micronucleus – width (µm)	2.0	0.2	1	3	8

Microbiology

Fungi isolated from deep skin ulcers in May 2001 included *Curvularia* sp., *Fusarium* sp., *Paecilomyces* sp. and *Scopulariopsis* sp. These were considered secondary pathogens and

will not be considered further. *Aeromonas sobria* was isolated from the skin ulcers, heart and caudal kidney of fish submitted in May 2001. *Aeromonas sobria* and *A. hydrophila* were isolated from the heart and from deep skin ulcers of fish submitted in July 2002.

Histopathology

Histological examination of deep skin ulcers from fish submitted in May 2001 and July 2002 showed the skin was ulcerated to the level of the skeletal muscle, and occasionally to the abdominal cavity. Numerous colonies of gram-negative bacilli were seen among necrotic dermis and necrotic skeletal muscle. Numerous *Tetrahymena corlissi* were detected in the scale pockets, epidermis, dermis and between necrotic bundles of skeletal muscle. In some fish, *T. corlissi* were in the in the omental tissues of the peritoneal cavity, and in the meninges of the brain. Granulomas and an increased number of melanomacrophage centres were in the liver of infected fish.

Histological examination of the three different types of skin lesion from fish submitted in January 2002 revealed differing pathology. The deep skin ulcers had similar pathology as described above, except that the ulcers extended only down to the level of the stratum compactum. Colonies of Gram negative bacteria covered the ulcerated surface and microcolonies were close to the edge of the ulcers. There were dilated blood vessels in the dermis, a generalised inflammatory infiltrate of mononuclear cells in the dermis and among the skeletal muscle and necrosis of the dermis and skeletal muscle fibres. *T. corlissi* were present in the loose connective tissues of the dermis. The paler areas of skin had epidermal hyperplasia with the epidermal layer 2 to 4 cells thicker than normal. The small dark marks visible on the caudal peduncle consisted of intact layer of epidermis. *T. corlissi* were within epidermis and dermis and were associated with necrotic cells, infiltrates of mononuclear cells in the hypodermis and a few dilated blood vessels with haemorrhage. No colonies of bacteria were detected.

Pond treatments and *in vitro* chemical trials

Chemicals added to ponds during 2001 were ineffective in killing *T. corlissi.* $KMnO_4$ demand was 4 mg/L. All fish with skin lesions in ponds treated with $KMnO_4$ died the following day and fish in ponds continued to die after both $KMnO_4$ and formalin treatments had been applied. *Tetrahymena* numbers on lesions of dead and dying fish appeared to be unchanged, and they were observed actively dividing and swimming during and after the treatment periods.

In vitro trials done in 2001 on fish infected with *T. corlissi*, were all ineffective. Examination of wet mount preparations from skin scrapes done from the centre and margin of skin ulcers of affected fish showed presence of actively swimming and dividing *T. corlissi.*

Prolonged 10‰ salt baths for 7 to 10 days were effective at killing exposed *T. corlissi*. After 3 days of treatment, the ulcer hyperaemia was absent and the skeletal muscle in the centre of the skin ulcers had turned white. Shallow skin scrapes done from ulcer margins showed no evidence of *T. corlissi,* however, deep scrapes taken from the ulcer margins showed small numbers of *T. corlissi.*

In the 2002 outbreak oral treatment with Emytryl at 30mg/kg fish/day for 10 days showed cessation of mortalities within 3 days of treatment. Visual observations of fish medicated with Emytryl held in tanks showed healing of ulcers within three days.

DISCUSSION

This is the first report of *T. corlissi* causing disease in food fish. Both outbreaks of tetrahymenosis commenced during early winter when pond water temperatures dropped. The natural distribution of golden perch is 900km south of FFAC, in areas with lower temperatures. As the temperature tolerances of golden perch are 4-37°C, it is unlikely that temperature is the primary stressor. In the outbreak of 2001 handling fish may have caused skin abrasion, predisposing fish to infection with *Flavobacterium columnare*, *A. sobria* and *T. corlissi*. The fish were fed a diet for barramundi as no specific diet has been formulated for golden perch. Fish may have been immuno-compromised from low water temperatures or from a lack of particular essential nutrients. Fish may have had no effective immunity to local strains of *T. corlissi*, *Aeromonas* spp., and *F. columnare.* Jade perch (*Scortum barcoo*) kept in nearby ponds were also affected by tetrahymenosis (personal observation), whereas six endemic species of fish on site were not. The appearance of infections in winter suggests that possibly *Tetrahymena* is active in colder weather, but the absence of marked immune response in early infections in 2002, and the rapidity of death of golden perch, suggests that golden perch were naïve to the local *T. corlissi*. This suggests that, in this case, translocation of a fish beyond its native range has resulted in exposure of the fish to a virulent endemic pathogen.

In both the 2001 and 2002 outbreaks, *A. sobria* and *A. hydrophila* were identified as causing motile aeromonad septicaemia in the golden perch. Seasonal outbreaks of motile aeromonad septicaemia are often seen in stressed or immuno-compromised fish cultured in ponds (Roberts 1993). *A. sobria* produces a potent enterotoxin (Carson 1990), contributing to rapid necrosis of skin and muscle. Once motile aeromonads have invaded the integument of a compromised fish host, bacterial septicaemia can develop and result in rapid death. Lesions initiated by *T. corlissi* appeared to cause rapid sloughing of epidermis and destruction of dermal tissue. Secondary bacterial infection then ensued, and the resultant toxins, haemorrhage, osmotic stress and septicaemia resulted in death of the fish.

In this case, *T. corlissi* was differentiated from other *Tetrahymena* spp. previously described as opportunistic parasites of freshwater fish on the basis of morphological features. *T. pyriformis* is smaller in size (40 x 20 μm cf. 60 x 40 μm), possesses fewer meridional kineties (17-21 cf. 25-30) and does not possess a caudal cilium. *T. rostrata* is larger and has more meridional kineties (32-48 cf. 25-30) including up to 4 postoral kineties (cf. 2).

Only one case of tetrahymenosis in pond reared food fish has been reported, when *Tetrahymena* sp. infected Australian freshwater silver perch, *(Bidyanus bidyanus)* grown in earthen ponds, causing scale lifting, skin ulceration, muscle swelling and necrosis (Callinan and Rowland, 1994). That study did not report the haemorrhagic margins of the lesions, which were a feature of the epizootic at FFAC. Our findings were similar to these

and other studies (Ferguson *et al.*, 1987; Lom and Dyková, 1992; Imai *et al.*, 2000; Wakita *et al.*, 2002) in that *T. corlissi* invaded through the skin into the underlying skeletal muscle causing deep skin ulceration, muscle necrosis, inflammation and infection of internal organs and tissues.

We are uncertain whether the *A. sobria* or *T. corlissi* was the primary pathogen responsible for the deep skin ulcers in the 2001 outbreak, since both pathogens were isolated from the ulcers and both are capable of producing deep skin ulcers with red margins in fish (Roberts, 1993; Thune *et al.*, 1993; Imai *et al.*, 2000). In 2002 histological examination of fish with skin lesions consisting of paler than normal areas of intact skin, found *T. corlissi* in the epidermis and dermis, underneath the intact epithelium, and no bacterial colonies were discernable. *T. corlissi* was detected on areas of apparently healthy skin, and in the scale pockets and subcutaneous tissues, further suggesting that *T. corlissi* was the primary pathogen in the 2002 outbreak. *T. corlissi* is histophagous and invades the scale pockets in other species of fish (Imai *et al.*, 2000), so is likely to be the primary pathogen in these infections.

Several species of indigenous, native freshwater fishes (sleepy cod, *Oxyeleotris lineolatus*; barramundi, *Lates calcarifer*; long finned eels, *Anguilla reinhardtii*) cultured in earthen ponds at FFAC, using the same water supply, were unaffected. Species of native fish living in the settlement ponds where water drained from ponds containing golden perch affected *T. corlissi*, including rainbow fish, *Melanotaenia splendida*, sleepy cod and hardyheads, *Craterocephalus stercusmuscarum* were also unaffected. It is possible that native freshwater fish species had innate immunity to *T. corlissi.*

The *T. corlissi* infecting golden perch appeared to be resistant to all bath treatments of chemicals tested. Callinan and Rowland (year) recommended 10g/L salt for 60 mins or 25 mg/L formalin for treatment of tetrahymenosis in silver perch reared in tanks. However we found salt and formalin baths were ineffective against *T. corlissi*. Our experience indicated that only long term bath treatments were effective against the surface dwelling *Tetrahymena*, but not the deep tissue infection, indicating treatment with a systemic drug. Indeed, the only treatment effective in treating *T. corlissi* infection was orally administered systemic anti-protozoal drug Emytryl (400 mg/g dimetridazole) at 30mg/kg/fish/day. This drug was effective within 3 days of treatment as mortalities ceased in ponds, and healing of ulcers was observed in fish in tanks. Dimetridazole has been used effectively to control other motile internal protozoal infections such as *Hexamita spp*. (Hoffman and Meyer, 1974; Gratzek, 1993).

The 2002 outbreak of tetrahymenosis occurred in a pond in which probiotics were used. Although *A. sobria* was not isolated from established skin lesions, *A. hydrophila* was isolated, but only from deep skin lesions. The probiotics used in this case are used in crustacean aquaculture to reduce populations of *Vibrio* and viruses (Moriarty, 1998; Gatesoupe, 1999). The less acute nature of the 2002 outbreak may indicate that the probiotics were effective in reducing susceptibility to infection with *Aeromonas sobria* and *A. caviae*. In the previous six years, *A. sobria* has been isolated from all golden perch from ponds submitted for veterinary examination. This implies that the probiotic treatment impacted on pathogenic bacteria in the pond.

The challenge presented by *A. sobria* and *T. corlissi* is significant to golden perch culture. Both are ubiquitous, and as such present a potential threat to successful pond culture, particularly if fish are introduced and are naïve to a local strains of *Aeromonas* spp. or *Tetrahymena* spp. Our results showed that the *T. corlissi* present on golden perch was resistant to most commonly used ectoparasite chemotherapeutants. Preliminary results from the outbreak in 2002 showed that dimetridazole administered orally is effective against *T. corlissi*.

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Disease and Health Management of Farmed Exotic Catfish *Pangasius hypopthalmus* in Mymensingh District of Bangladesh

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ABSTRACT

The study was carried out to examine the status of disease and health management practices in *Pangasius hypophthalmus* in Mymensingh District of Bangladesh during the period from April 2004 to March 2005 using questionnaire interview and participatory rural appraisal tools such as focus group discussion (FGD). Financial losses of farmers due to fish disease was also determined. A total of 100 fish farmers were interviewed and six FGD sessions were conducted. The most prevalent symptoms of disease as reported by the farmers were red spot, followed by anal protrusion, tail and fin rot, pop eye, dropsy and gill rot. Other conditions like cotton wool type lesion, ulceration and white spot were also reported but with lower incidence. This study indicated that there were economic losses of approximately 3.6% of farmers' total yearly income from fish production due to ill-health. These losses varied among different farm categories. This study also highlighted health management problems of *P. hypophthalmus* farming which include lack of technical knowledge of farmers on fish health and disease, lack of assistance from government and non-government organizations, unavailability of appropriate therapeutants and lack of knowledge on their application. In conclusion, the present study provided valuable information on disease and health management in *P. hypophthalmus* in Bangladesh based on field data. In order to develop sustainable pangasius culture in both Bangladesh and within the Southeast Asian region, more research is needed for proper identification and characterization of pathogens and for the development of farmer-oriented disease control and health management packages.

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INTRODUCTION

Pangasius hypophthalmus (Sauvage) is a fast-growing fish, which has recently become a vey popular food fish and valuable aquaculture species in South-East Asia. The fish is extensively cultured by commercial fish farms in Thailand, India and Myanmar. The fish, popularly known as 'Thai pangas', was introduced to Bangladesh from Thailand during the 1990s and has since developed into a very popular species amongst fish farmers and consumers. The fish proved to be a great success in Bangladesh aquaculture and is at present the only catfish species used for commercial aquaculture in the country (Rahman *et al.,* 2005). Two types of culture systems have been practiced in Bangladesh for *P. hypophthalmus* farming: monoculture (following intensive culture strategy) and polyculture (following semi-intensive culture strategy). In the polyculture systems the production of *P. hypophthalmus* is about 10-12 tons/ha. In the case of the intensive commercial culture, production is about 25-30 tons/ha with animal protein rich diets and water exchange (BFRI, 1998). The last few year achieved rapid development on *P. hypophthalmus* farming in Mymensingh District of Bangladesh. Farmers have been converting their rice fields into *P. hypophthalmus* farms for quick profit. Presently, in Mymensingh District, there are about 1,364 pangasius farms covering an area of 774 ha producing 19,203 tons fish per year (DoF, 2003).

Although *P. hypophthalmus* as an air-breather is very able to tolerate poor water quality conditions compared to other freshwater fish species, there are some reports about diseases affecting this species. Aeromonads, notably *Aeromonas hydrophila*, are the most significant problem noted in the literature (Subagja *et al.,* 1999). Ferguson *et al.,* (2001) first described an important disease, Bacillary Necrosis, of Vietnamese *P. hypophthalmus*, which Crumlish *et al.,* (2002) later identified to be caused by *Edwardsiella ictaluri*. Currently in Bangladesh, disease is also the major problem restricting *P. hypophthalmus* farming in the country. However, due to lack of diagnostic support and appropriate therapeutants, farmers are suffering from increasing financial losses due to diseases. As a consequence, the price obtained for this species is decreasing and the livelihood of farmers is under threat. This study was carried out to examine the status of disease and health management practices of *P. hypophthalmus* in Mymensingh District of Bangladesh.

MATERIALS AND METHODS

Study area

The study was conducted in three selected *upazilas* (local administrative unit – Mymensingh District is consists of 11 *upazilas*) in Mymensingh District namely Trishal, Bhaluka and Muktagacha (Figure 1) where the highest cluster of *P. hypophthalmus* farms are located.

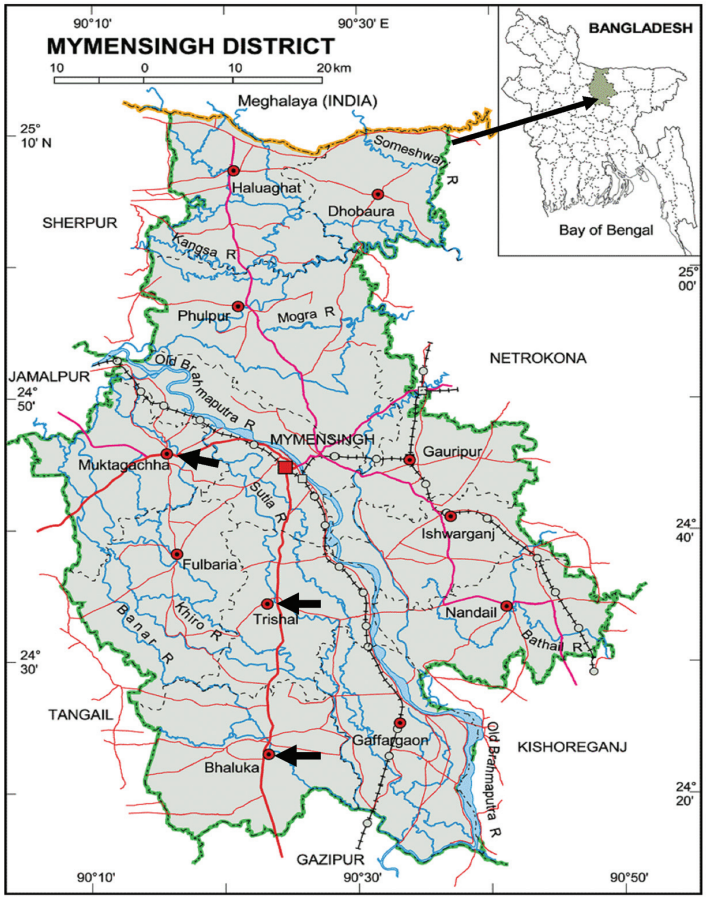

Figure 1. Map of Mymensingh District. Arrows show study areas.

Data collection

Data was collected through the questionnaire interview and participatory rural appraisal (PRA) with fish farmers. For questionnaire interview, a set of preliminary questionnaire was prepared. This was pilot-tested with a few *P. hypophthalmus* farmers of each representative location and necessary modifications were made based on their feedback. Simple random sampling method was followed for the interview. A total 100 farmers having different farm size were interviewed. The questionnaire was divided into several sections. The first section focused on general farming and farmer's information, the second section on pond preparation information, the third one covered information related to fish stocking and pond management, and the final section focused on fish health and disease problems, their economic loss, management interventions used to control disease. This section was used only when the farmers reported health problems in their ponds.

PRA tools including focus group discussion (FGD) was conducted with farmers to get an overview of particular issues concerning fish health. Six FGD sessions in different

upazilas were conducted where each group size was between 6 and 12 farmers. Cross check interviews were conducted with key informants such as District Fisheries Officer, Upazila Fisheries Officer (UFO) and NGOs working in aquaculture.

RESULTS

Farmers category

All together, data from 100 farmers were analysed. The survey split farmers into three catagories depending on their pond area. The first category comprised of small farmers having less than 0.5 ha pond area which represented about 45% of the total farmers interviewed (Table 1). The second category was medium farmers who had pond areas between 0.5 and 1.0 ha, and the third category was larger farmers having pond areas over 1.0 ha. The medium and large category farmers represented 35% and 20%, respectively.

Table 1. Summary of the respondents with different pond categories.

Pond category (ha.)	Trishal	Bhaluka	Muktagacha	Total
	n=40	n=33	n=27	n=100
<0.5	20	10	15	45
0.5-1.0	12	13	10	35
> 1.0	8	10	2	20

n:sample size

Farmer's age, family, experience and education

All the farmers interviewed were male with an average age of 37 years and an average family size of 6 (Table 2). The average years of experience with *P. hypophthalmus* farming by the farmers was 5 years. About 44% farmers had education up to high school level (under ten class) followed by Secondary School Certificate (ten class) (29%) and Higher Secondary Certificate (twelve class) (20%). Only few respondents (7%) had graduation degrees. When the farmers' were aked about the reasons for fish culture, majority of the farmers (71%) reported that they culture fish for income and 29% farmers reported that they culture fish for both income and food.

Table 2. Average age, family size and experience of the respondents.

Pond category (ha)	Average age (yr)	Average family size (no)	Average experience (yr)
<0.5	37	6	4
0.5-1.0	36	5	5
> 1.0	38	7	6
Overall average	37	6	5

Pond preparation and stocking

Of the 100 interviewed farmers, 70 respondents said that they used only lime during pond preparation, 25 respondents used lime and cow dung and only 6 farmers used lime, cow dung and some inorganic fertilizers like urea and triple super phosphate. About 62 farmers reported that they do not remove undesirable species from their ponds before stocking fingerlings of *P. hypophthalmus* and 38 farmers said that they remove undesirable species by using netting. Few farmers dried their ponds before stocking. The average pond preparation cost which include mainly the costs of application of lime, fertilizer and netting was about Tk1,910/ha (1US$ = 68 Bangladesh Taka = Tk). All the interviewed farmers practiced monoculture of *P. hypophthalmus*. The average stocking density of fish was 39,581 fingerlings/ha and average stocking cost was Tk 55,852 (US$821.35)/ha (Table 3).

Table 3. Stocking density and stocking cost.

Pond category (ha.)	Average stocking density (fingerling/ha)	Average stocking cost (Tk/ha)
<0.5	37,213 (± 7193)	52,226 (± 18170)
0.5-1.0	41,515 (± 7017)	55,555 (± 9265)
> 1.0	40,014 (± 3757)	59,774 (± 12516)
Overall average	39,581 (± 5989)	55,852 (± 13317)

* Values in parenthesis are ± standard deviation of mean, 1 US $= 68 Tk

Treatment used by the farmers before fry stocking

About 84 respondents reported that they do not use any pretreatment prior to stocking the fish in the pond and only 16 respondents reported that they dip their fish in potassium permanganate or salt solution before being released into the ponds.

Fish health problem and management

During the present survey, the areas for data collection were selected mostly on the basis of having previous disease history. When farmers were asked whether they had fish health problems in their ponds, the majority (98%) of the farmers said they had problems during previous years or the year before and only 2 respondents indicated that they did not find any disease in their ponds. The average prevalence of ill-health was about 7.2% in a production cycle. The highest prevalence was found with small-scale farmers (7.6%) followed by medium (7.2%) and large scale farmers (6.7%). Farmers reported about 3.4% mortality of their fish in a single production cycle due to ill health (Table 4). Ninety-six farmers reported that they found disease during winter season in their ponds and only four farmers reported that they found disease in rainy season.

Table 4. Prevalence of health problem and mortality of fish.

Pond category (ha.)	Disease prevalence (%)	Mortality (%)
<0.5	7.6	4.0
0.5-1.0	7.2	3.3
> 1.0	6.7	3.0
Average	7.2	3.4

Type of health problems

When farmers were asked about the kind of health problems and clinical signs in their ponds, a range of conditions was reported by the farmers according to their occurrence. The most prevalent symptom of health problem was red spot (19.1%) followed by anal protrusion (18.9%), tail and fin rot (14.3%), pop-eye (12.5%), dropsy (10.9%) and gill rot (9.0%). Other conditions like ulceration, cotton wool type lesion and white spot were also reported by the farmers but with lower incidence (Figure 2). Clinically diseased *P. hypophthalmus* with ulceration and fin rot is shown in Figure 3.

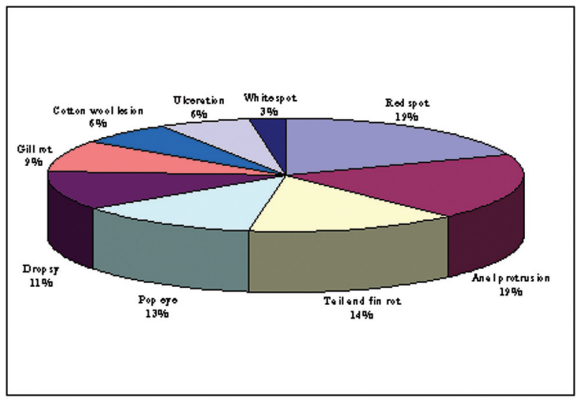

Figure 2. Type of symptom of diseases (% occurrence) of *Pangasius hypophthalmus* in Mymensingh District of Bangladesh.

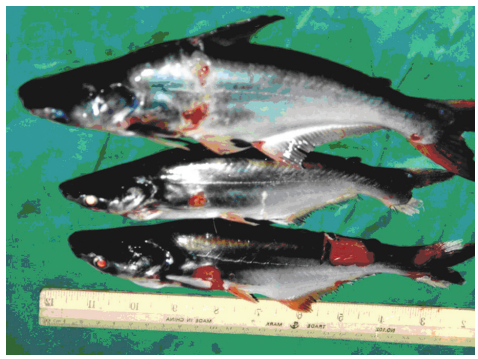

Figure 3. *Pangasius hypophthalmus* with ulceration and fin rot collected from farmer's pond.

Response of farmers to disease problems

Only 10% of farmers said that they go to the government extension officers to report diseases and for seeking advice from them while 80% farmer does not go to extension officer for advice. Generally, most of the farmers turned to other farmers for advice when disease occurred in their ponds and applied a range of treatments. About 82% farmers treated their fish after occurrence of diseases in their ponds. A diverse number of treatments were reported, many in multiple combinations. Liming was the most common treatment followed by application of salt, potassium permanganate, antibiotics, pesticides and insecticides. Of the antibiotics, oxytetracycline and tetracycline were commonly used by most of the farmers. During FGD sessions it was observed that farmers indiscriminately used chemicals for disease control without knowing their effect. It was also found that farmers were under pressure by the pharmaceutical companies to buy their products.

Disease control cost

Average disease control cost per production cycle was Tk 4,285/ha which included prevention cost (Tk 2,827/ha) and treatment cost (Tk 1,458 ha) (Table 5). Costs of preventive measures include the cost of pond drying, addition of water, use of lime before disease outbreak and removal of water turbidity; while treatment cost include the cost of chemicals used for treating after occurrence of fish diseases.

Table 5. Disease control cost (Tk/ha), of *P. hypophtalmus.*

Pond category (ha.)	Prevention cost (Tk/ha)	Treatment cost (Tk/ha)	Total (Tk/ha)
<0.5	2342 (± 786)	1220 (± 1106)	3,562 (± 1892)
0.5-1.0	2433 (± 548)	1447 (± 847)	3,880 (± 1395)
> 1.0	3705 (± 753)	1707 (± 709)	5,412 (± 1462)
Average	2827 (± 696)	1458 (± 2662)	4,285 (± 3358)

*Values in parenthesis are ± standard deviation of mean, 1 US$= 68 Tk

Fish production

Fish production varied with different farmers category. Farmers were asked about their expected production when they had no disease problems and the actual production obtained due to disease problems at the end of the production cycle. Large category farmers had the highest average expected production (Tk 656,031/ha) followed by medium (Tk 620,649/ha), and small farmers (Tk 588,126/ha). Average actual production, that the farmers received after selling fish at the end of the production cycle was also highest in large category farmers (Tk 633,636/ha) and the lowest was in small-scale farmers (Tk 565,022/ha) (Table 6).

Economic losses due to ill-health

The results of the study indicated that there are average economic losses of Tk 21,500/ ha to farmers from fish health problem. These losses varied with the size of farms. The economic loss was estimated by the differences between the expected production and actual production, here prevention and treatment cost of fish diseases was not calculated. The highest average loss as high as Tk 23,104/ha was found with small-scale farmers followed by large (Tk22,395) and medium scale farmers (Tk18,999) (Table 6 and Figure 4). The average estimated loss was 3.6% of the total yearly income from fish production to farmers.

Table 6. Fish production (Tk/ha) and economic loss (Tk/ha) in the study area.

Farm category (ha)	Expected production (Tk/ha)	Actual production (Tk/ha)	Economic loss (Tk/ha)	Percentage of actual production
<0.5	588,126	565,022 (± 126938)	23,104 (± 10435)	4.1
0.5-1.0	620,649	601,650 (± 116008)	18,999 (± 5424)	3.1
> 1.0	656,031	633,636 (± 101092)	22,395 (± 7892)	3.5
Average	621,602	600,102 (± 114679)	21,500 (± 7917)	3.6

**Values in parenthesis are ± standard deviation of mean, 1 US$= 68 Tk*

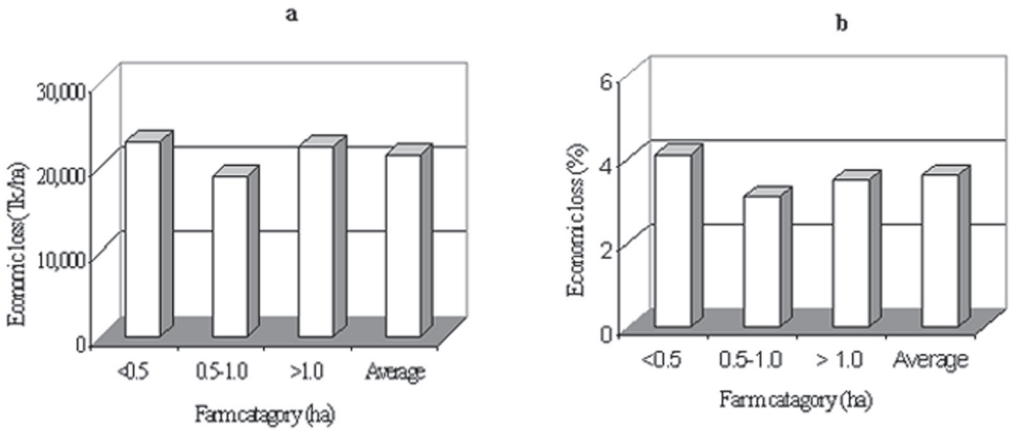

Figure: 4. Economic losses of ~~of~~ fish farmers due to ill-health of *Pangasius hypophthalmus*. (a) Economic loss (Taka/ha). (b) Economic loss percentage of actual production.

Problems in fish health management

Farmers faced several problems when they encountered particular disease in their ponds which include lack of appropriate support from government and non government organizations (49%), lack of knowledge on fish health and disease (35%), unavailability of medicine (10%) and lack of training facility about fish disease treatment (6%).

Importance of fish disease to farmers

About 46% of farmers mentioned ill-health of fish as a major problem in fish culture while 22% considered it as a moderate problem, 21% of farmers as minor and 11% of farmers mentioned that they do not think disease a problem.

DISCUSSION

Aquaculture in Bangladesh is growing rapidly with respect to both the quantity and variety of species. Aquaculture production has shown a sharp annual average growth of 28% from 0.12 million tons to 0.66 million tons during the period 1984-1985 to 1999-2000 (Mazid, 2002). Diseases of fish are one of the major constraints resulting from intensification of aquaculture and may eventually become a limiting factor to the economics of a successful and sustainable aquaculture industry. The present study identified a range of clinical signs of diseases and conditions of *P. hypophthalmus* farming in Bangladesh as was reported by the farmers according to their occurrence. The most prevalent symptom of disease was red spot, followed by anal protrusion, tail and fin rot, pop eye, dropsy, gill rot, cotton wool type lesion and ulceration. Similar conditions were also reported in the rural carp culture by several authors (Faruk *et al*., 2004a; DoF, 2002; Mazid, 2001; Amin, 2000).

Prevalence of fish disease has negative economic impact on aquaculture. A global estimate of disease losses to aquaculture by World Bank in 1997 was in the range of US$3 billion per annum (Subasinghe *et al.,* 2001). The results of this study indicated that the cost of ill-health of fish was approximately 3.6% to the income of fish farmers from fish production and this loss varied slightly according to the size of farms. Small-scale farms suffered from highest economic losses than large-scale farms. In a recent study on the economic impact of fish diseases on rural freshwater aquaculture Bangladesh, Faruk *et al.,* (2004a) reported average loss of farmers due to fish disease was 14.0% of total fish production. They conducted the survey mainly on farmers practicing carp polyculture. Similarly, Brown and Brook (2002) reported that average loss for farmers due to fish disease was 18.5% of total average yearly income from fish production. Thus, it is clear that the impact of diseases of *P. hypophthalmus* on production was less than those of carp polyculture system. Small-scale farmers have very little knowledge in fish culture and they are reluctant to use any new technologies. Consequently, their production per hectare was low and the losses were high. On the other hand, since large farmers invested more in fish culture, they tended to feed and monitor fish health more regularly.

Economic losses from diseases are likely to increase as aquaculture expands and intensifies. Assessing the impact of disease in aquaculture systems is not easy, as only acute losses are recognized and quantified. Chronic mortalities and poor growth caused by disease are generally not recognized. In order to quantify disease losses, farmers should be able to identify disease as the reason for crop loss, slow growth or poor harvest (Mohan and Bhatta, 2002). Therefore, it is important to train farmers to carry out field-level diagnosis and assess the likely impact of diseases.

In the present study, the reporting of diseases by fish farmer was found very low which is due to the lack awareness of farmers about fish disease and also the lack of reporting places or diagnostic laboratory from where farmers can get advice and other support services. As

a consequence there were severe lack of prevention, diagnosis and treatment. According to fish farmers, most of the diseases mainly occurred during the winter season. During this time when the water level of farmer's pond become very low and the water quality also become very poor that the fish immune system are suppressed due to low temperature. As a result fish become more susceptible to disease. Therefore, farmers could be suggested to take some preventive measures at the beginning of the winter season which include, application of lime and salt, disinfecting of equipment, addition of water, etc. (Faruk *et al*., 2004b).

Farmer responses to disease problems generally involved application of chemicals, with little understanding of their effectiveness, when better results might have been obtained by changes in management practices. They learned about the treatment they used from other farmers, chemical and feed sales persons, hatchery owners and from themselves. The advice received from feed and chemical sales persons may have been biased and which generally involve the sale promotion of chemicals. Advice from government extension services and NGOs was low, indication that efforts are required to increase their profile in this particular area.

In the present study, farmers were found to practice only few health managemant techniques which included application of lime during pre-stocking pond preparation, removal of undesirable species by netting, periodic removal of gases by manual dredge on the pond bottom, periodic checking of fish health condition and some times disinfection of nets and other equipment.

The study identified some problems faced by the farmers in fish health management which included lack of technical knowledge on fish health management, lack of assistance from government and non-government organizations, unavailability of appropriate therapeutants, lack of knowledge on the application of therapeutants, pressure on farmers from pharmaceutical companies and chemical sales person, indiscriminate use of chemicals, low quality chemicals and financial problem. Therefore, in order to sustain the industry in Bangladesh few recommendations could be made which include training of farmers and extension agents on simple diagnostic procedures and effective therapies, awareness creation among farmers on fish health management, legislation development on the safe use of chemotherapeutic agents for prevention and control, establishment of diagnostic centers and support service.

In conclusion, culture of *P. hypophthalmus* in freshwater pond has become very popular and economically beneficial among rural fish farmers of Bangladesh. However, diseases are among the most common and serious problems in *P. hypophthalmus* farming and is responsible for substantial source of monetary loss to farmer. There is no systematic and in-depth research on this particular area from which rural farmers could be benefitted. The present study provided some valuable information based on field data and there is a need to verify this under laboratory condition. In order to develop sustainable pangasius culture in both Bangladesh and within the southeast Asian region, more research is needed for proper identification and characterization of pathogens and development of farmer-oriented disease control and health management packages.

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In vivo Transcription Analysis of Seabream Iridovirus (RSIV) Using DNA Microarrays

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ABSTRACT

Red seabream iridovirus (RSIV) is the causative agent of an infectious disease in marine fish that is listed to be notifiable to the Office International des Epizooties. To better understand the molecular mechanisms of its pathogenesis, we explored the expression of almost all the putative RSIV open reading frames (ORFs) over the time-course of an *in vivo* infection in red seabream using DNA microarrays. Expression of about 45% of total RSIV ORFs was detected at about 5 days post-infection (d.p.i.). Almost all the ORFs (97% to 99%) were expressed at their maximum levels during the period 7-9 days post-infection (dpi). The expression levels and the number of expressed ORFs started to decrease at 10 dpi. Our results suggest that the pathogenesis of RSIV infection began at around day 5, and continued with high levels of viral multiplication until viral clearance, apparently by the host antiviral immune defenses, starting from around 10 dpi. RSIV ORFs were preferentially expressed in the spleen, which may be the primary target of RSIV. The spleen may thus be a susceptible organ for diagnosis of iridoviral disease in fish.

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INTRODUCTION

Systemic iridoviral diseases have been observed in more than 100 freshwater and marine fish species worldwide with high mortalities ranging from 30% to 100% (Hyatt *et al.*, 2000; Iwamoto *et al.*, 2002; Qin *et al.*, 2003; Tidona *et al.*, 1998). One of these infectious diseases is red seabream iridoviral disease (RSIVD) that has been recorded in at least 31 marine fish species (Kawakami and Nakajima, 2002). The causative pathogen was first isolated from diseased red seabream (*Pagrus major*) in Japan in 1992 and hence named red seabream iridovirus (RSIV) (Inouye *et al.*, 1992). Because of geographical range and occurrence in fish involved in international trade, the RSIVD is notified to be quarantined by the Office International des Epizooties (OIE).

RSIV-infected fish showed diseased symptoms from 5 days of infection, and mortality commenced at day 6 and increased up to 90% at day 9 (Oshima *et al.*, 1998). The infected fishes displayed enlarged cells in spleen, kidney, liver and gills (Inouye *et al.*, 1992). Although some rapid, sensitive diagnostic methods, and control strategies have been developed (Caipang *et al.*, 2004; Caipang *et al.*, 2003; Caipang *et al.*, 2006; Jeong *et al.*, 2004; Kurita *et al.*, 1998; Nakajima *et al.*, 1995; Oshima *et al.*, 1998; Oshima *et al.*, 1996), the molecular mechanisms of its pathogenesis are poorly understood. Recently, the whole RSIV genomic sequence of about 112 kbp has been determined (Kurita *et al.*, 2002), providing an important basis for studies on its pathogenicity at the molecular level both *in vitro* and *in vivo*. *In vitro* expression analyses of individual viral genes at various time points during the viral life cycle can provide a better understanding of the viral DNA replication and gene expression strategies, while *in vivo* genome-wide transcription analyses can provide possible clues for the pathogenesis of the virus, and provide insights into the complex host-virus interactions (DeFilippis *et al.*, 2003; Martinez-Guzman *et al*., 2003; Ye *et al.*, 2001).

The potential use of DNA microarray technology in virology has been comprehensively discussed in numerous reviews (Clewley, 2004; Cummings and Relman, 2000; DeFilippis *et al.*, 2003; Ye *et al.*, 2001). This technology is well suited for genome-wide transcription studies, and has been applied to explore gene expression patterns of viruses by both cell culture and animal model studies (see Lua *et al.*, 2005 and references therein). In a previous study (Lua *et al.*, 2005), we used DNA microarrays to monitor the *in vitro* transcription program of RSIV over the time-course of an infection. Individual RSIV ORFs were characterized at the transcriptional level and were also classified into temporal kinetic classes by their dependence on *de novo* protein synthesis and viral DNA replication. The gene expression of RSIV occurred in a temporal kinetic cascade with 3 stages, which includes Immediate-Early (IE), Early (E) and Late (L) transcripts, following a common feature of the family *Iridoviridae*. IE genes are expressed immediately after primary infection and encode transcription factors associated with *trans*-activations. E genes are normally expressed later and include enzymes associated with DNA replication. L genes are expressed after the onset of viral DNA replication and encode mainly structural proteins of viral particles. In the present study, we aimed to have a better understanding of the RSIV pathogenic mechanisms at the molecular level by monitoring the viral transcription profiles over the time-course of an *in vivo* infection in a fish model through the use of RSIV DNA microarrays.

MATERIALS AND METHODS

Virus stock

RSIV was obtained from a spleen homogenate of RSIV-infected red seabream, and propagated in Grunt fin (GF) cells (Clem *et al.*, 1961) as previously described (Lua *et al.*, 2005). The virus titer was determined using the 50% tissue culture infective dose ($TCID_{50}$) method (Reed and Muench, 1938). The viral stock was stored in 1 ml aliquots at -80°C until further use.

In vivo virus infection and time-course sampling

Red seabream juveniles were experimentally infected with 150 μl of the RSIV inoculum (5.0×10^5 $TCID_{50}$/ml) and held in tanks supplied with running seawater at 25°C. Control fishes were injected with the same volume of phosphate buffered saline. Thirty fishes were sacrificed immediately after the RSIV infection for use as reference (control) samples. These fish are referred to as 0 day post-infection (dpi) fish. Five fish were randomly selected from the experimental population on each of 2, 3, 5, 7, 9, 10, and 14 dpi for use as target (test) samples. The spleens and kidneys were removed from the collected fish and stored in RNAlater (Ambion, USA) according to the manufacturer's protocol.

Construction of RSIV DNA microarray chip

The DNA microarray chips containing almost all the putative RSIV open reading frames (ORFs) (92 ORFs) were constructed exactly as described by Lua *et al.* (2005). Briefly, specific primer sets were designed to amplify approximately 300-1500bp fragments of each ORF using viral genome as a template. All PCR products showing a single band of the appropriate size by gel electrophoresis were purified, and reconstituted in TE buffer at a final concentration of about 500 μg/ml for spotting onto the glass slides. Each ORF was spotted in duplicate at different parts of the slides to assess the consistency of hybridization and facilitate comparison during the analysis. Piscine β-actin genes from Japanese flounder, red seabream, and Japanese flounder natural embryo (HINAE) cells (Kasai and Yoshimizu, 2001) were included as internal controls to normalize the microarray data. In addition, distilled water was also used as a negative control.

Microarray hybridization experiment

Total RNA was extracted from the collected spleens with TRIzol (Invitrogen, USA) and subjected to DNase I treatment (Promega, USA) according to the manufacturer's protocols. For each time-course target sample and control sample, cDNAs were generated from 50 μg total RNA using an RSIV antisense-strand specific primer mixture. The cDNAs were first labeled with aminoallyl-dUTP using a LabelStar™Array Kit (Qiagen, USA) and purified with a QIAquick PCR Purification Kit (Qiagen, USA) following the manufacturer's recommendations. The target and control aminoallyl-cDNAs were then

coupled with Cy5- and Cy3-monofunctional dyes (Amersham Biosciences, England), respectively, and purified with MinElute™ Spin columns (Qiagen, USA) according to manufacturer's instructions.

At each indicated time point, the Cy5/Cy3-dUTP labeled cDNAs were combined and hybridized to the microarray chips for 16-18h at 42°C. The chips were rinsed several times and finally dried following the DNA microarray standard method (Bowtell and Sambrook, 2002) as modified by Lua *et al.* (2005).

Microarray statistical data analysis

The microarray chips were scanned using a GenePix 4000B array scanner and images were analyzed by GenePix Pro 4.0 array analysis software (Axon Instruments, Inc., USA). The Cy5 and Cy3 signal intensities of viral genes were normalized to the signal intensities of the spotted β-actin gene. The background signal was subtracted from the median signal intensity to obtain the absolute viral gene expression. Only genes exhibiting signal intensity at least twofold greater than the signal intensities of the reference samples collected at 0 d.p.i. were used for statistical analysis. The significance of differences between viral infected samples and reference samples was determined with a paired *t*-test on replicated spots for each gene. *P* values of less than 0.05 were considered significant.

The microarray data was also reported as the calibrated expression ratio, which was the ratio of the fluorescence intensity of a RSIV transcript in infected spleens compared to that of the β-actin transcript (Lua *et al.*, 2005; Tsai *et al.*, 2004). The expression ratio data was imported into the cluster program 3.0 in conjunction with an average linkage hierarchical clustering algorithm using Euclidian distance as the similarity metric. After clustering, the results were visualized in a tree structure by using a tree view program (Eisen *et al.*, 1998).

Reverse Transcription (RT) - PCR

RT-PCR assay was used to confirm the microarray data and to investigate a susceptible organ of RSIV infection. Several RSIV ORFs, with different expression patterns as determined by the microarray results, were selected from the three temporal kinetic classes (IE, E and L genes). Twenty μl of cDNA was synthesized from 5 μg total RNA derived from spleens and kidneys by using M-MLV Reverse Transcriptase (Invitrogen, USA) according to the manufacturer's protocols. RT-PCR was carried out in a 30 μl reaction volume containing 1 μl cDNA using Taq polymerase. The same specific primers for each RSIV ORF used in the amplification of microarray probes were also employed here. Cycling parameters consisted of an initial denaturation at 95°C for 2 min, followed by 23 and 27 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and elongation at 72°C for 1 min, and a final elongation step at 72°C for 5 min. A 23 cycle PCR was used to determine differences in expression of RSIV transcripts between spleen and kidney at the high level spread stage of the infection (7-9 dpi) while a 27 cycle PCR was performed to show differences in expression at the early stage (5 dpi) and late stage (10-14 dpi) of the infection.

RESULTS

In vivo RSIV transcription program

The microarray analysis showed that no viral transcripts were detected in spleens at 2 and 3 dpi (data not shown), but viral ORFs showed significant changes in expression from 5 d.p.i. onwards. At 5 days after infection, 44 viral ORFs were significantly expressed, accounting for 44.6% ($p<0.05$) of total RSIV ORFs. Almost all (about 97% to about 99%, $p<0.001$) of viral ORFs were significantly expressed during the period 7-9 dpi (Table 1). As shown by the cluster analysis (Figure 1), the expression levels of viral ORFs were at their maximal levels during this period, showing high levels of viral multiplication. However, the numbers and the expression levels of expressed ORFs started to decrease at 10 dpi. The expression of only 25% ($p<0.05$) of the ORFs was detected at 14 dpi (Table 1, Figure 1).

Confirmation of microarray results by RT-PCR

Six RSIV ORFs were selected for confirming the microarray results by RT-PCR (Fig. 2). These ORFs included IE transcripts 097R and 591R, E transcripts 092R and 324R, and L transcripts 291L and MCP (Major Capsid Protein). The β-actin transcript was used as an internal control. As expected, no viral band was amplified at day 0, day 2 or day 3 of the infection. From 5 d.p.i. onwards, the selected ORFs were observed with different expression levels over the time-course of the infection. Therefore, the RT-PCR results (Figure 2) confirmed the microarray data showing the same expression patterns of selected ORFs. In addition, the β-actin transcript levels, as determined by RT-PCR, were similar between samples, confirming that the β-actin gene can be used to normalize the viral gene expression results across the microarrays.

Identification of a susceptible organ of RSIV infection

Four ORFs were selected for identifying a susceptible organ of RSIV infection by RT-PCR (Figure 3). These ORFs consisted of IE transcript 097R, E transcript 407R, and L transcripts 291L and MCP. Differences in expression of these ORFs between spleens and kidneys during the spreading stage of the virus (7-9 dpi) were detected after both 23 and 27 PCR cycles (Figures 3A and 3B), while the differences at the early stage (5 dpi) and the late stage (10-14 dpi) of the infection were detected after 27 PCR cycles (Figure 3B). The expression levels of the selected ORFs were all higher in the spleen than in the kidney. These findings indicate that the spleen may be a susceptible organ of RSIV infection.

Table 1. Microarray analysis of in vivo RSIV transcription program.

No.	ORF	Putative Function	Accession No.	Signal Intensity Value[a]					Calibrated Expression Ratio[b]					Kinetic class[c]
				5 d.p.i.	7 d.p.i.	9 d.p.i.	10 d.p.i.	14 d.p.i.	5 d.p.i.	7 d.p.i.	9 d.p.i.	10 d.p.i.	14 d.p.i.	
1	ORF016L	Hypothetical protein		430	1287	728	135		0.45	2.38	1.61	0.23		L
2	ORF018R	Hypothetical protein			1177	1398	362			2.18	3.09	0.63		L
3	ORF029R	Hypothetical protein			480	209				0.89	0.46			ND
4	ORF033R	Cytosine DNA methyltransferase	AAT71861		223	157	153			0.41	0.35	0.27		E
5	ORF037R	Hypothetical protein		560	2103	1586	506		0.59	3.89	3.51	0.88		L
6	ORF042R	Hypothetical protein		1370	5346	1650	404	69	1.44	9.90	3.65	0.70	0.14	L
7	ORF049R	RING-finger-containing E3 ubiquitin ligase	AAT71876	192	2212		293	63	0.20	4.10		0.51	0.13	IE
8	ORF054R	Putative RNA guanylytransferase	AAL98788		242	116	127			0.45	0.26	0.22		E
9	ORF063R	Subunit of DNA-dependent RNA polymerase	BAA82753		505	207				0.94	0.46			E
10	ORF077R	Putative DNA-binding protein	AAT71873		746	492	258			1.38	1.09	0.45		E
11	ORF092R	Putative replication factor	AAS18131	117	3126	938	843	80	0.12	5.79	2.08	1.47	0.16	E
12	ORF097R	Hypothetical protein		471	11601	8886	5031	217	0.50	21.48	19.66	8.75	0.44	IE
13	ORF101R	Hypothetical protein			1115	1318	627	64		2.06	2.92	1.09	0.13	L
14	ORF106R	Hypothetical protein		135	1783	1384	1312	148	0.14	3.30	3.06	2.28	0.30	L
15	ORF111R	Hypothetical protein		804	13835	6500	4051	261	0.85	25.62	14.38	7.05	0.52	L
16	ORF122R	Hypothetical protein			513	608	301			0.95	1.35	0.52		L
17	ORF128R	Hypothetical protein			763	1219	291			1.41	2.70	0.51		E
18	ORF135L	Hypothetical protein		90	847	1415	802		0.09	1.57	3.13	1.39		L
19	ORF140R	Cytosine DNA methyltransferase	AAT71861		530	472	158			0.98	1.04	0.27		ND
20	ORF145R	Hypothetical protein			820	565	259			1.52	1.25	0.45		E
21	ORF151R	Hypothetical protein			387	311	169			0.72	0.69	0.29		E
22	ORF156R	Thiol oxidoreductase	AAP33193		268	232	118			0.50	0.51	0.21		E
23	ORF161L	Hypothetical protein			471	349				0.87	0.77			E
24	ORF162R	Hypothetical protein			368	269	170			0.68	0.60	0.30		E
25	ORF171R	Hypothetical protein			684	289	144			1.27	0.64	0.25		E
26	ORF179L	Hypothetical protein			223	157	180			0.41	0.35	0.31		E
27	ORF180R	Hypothetical protein		138	1171	584	187		0.15	2.17	1.29	0.33		ND
28	ORF186R	Hypothetical protein			300	283	139			0.56	0.63	0.24		L
29	ORF197L	Hypothetical protein		119	2377	1047	460	90	0.13	4.40	2.32	0.80	0.18	L
30	ORF198R	Hypothetical protein			197		108			0.36		0.19		E
31	ORF224L	RNA polymerase beta subunit	AAT71848		584	390	112			1.08	0.86	0.19		L
32	ORF226R	Hypothetical protein		263	501	767	216		0.28	0.93	1.70	0.38		L
33	ORF234L	Deoxyribonucleoside kinase	AAT71846		150	191				0.28	0.42			E
34	ORF237L	Subunit of DNA-dependent RNA polymerase	AB018418		896	626				1.66	1.39			E
35	ORF239R	Subunit of DNA-dependent RNA polymerase	BAA82753		561	427	118			1.04	0.94	0.21		E

Table 1. (continued)

No.	ORF	Putative Function	Accession No.	Signal Intensity Value[a]					Calibrated Expression Ratio[b]					Kinetic class[c]
				5 d.p.i.	7 d.p.i.	9 d.p.i.	10 d.p.i.	14 d.p.i.	5 d.p.i.	7 d.p.i.	9 d.p.i.	10 d.p.i.	14 d.p.i.	
36	ORF256R	DNA repair protein RAD2	BAA82754		1057	1174	507			1.96	2.60	0.88		L
37	ORF261R	Hypothetical protein			208	106				0.39	0.23			L
38	ORF268L	Ribonucleotide reductase small subunit	BAA82755		762	818	333			1.41	1.81	0.58		E
39	ORF291L	Laminin-type epidermal growth factor-like domain	AAT71838	2477	6859	1729	561	157	2.61	12.70	3.83	0.98	0.32	L
40	ORF317L	DNA polymerase	AB007366	118	963	405	120		0.12	1.78	0.90	0.21		E
41	ORF321R	DNA polymerase	AB007366		95	146				0.18	0.32			E
42	ORF324R	DNA polymerase	AB007366		870	707	415	143		1.61	1.56	0.72	0.29	E
43	ORF333L	Hypothetical protein		98	1396	870	513		0.10	2.59	1.92	0.89		IE
44	ORF342L	Hypothetical protein		141	1149	698	258		0.15	2.13	1.54	0.45		IE
45	ORF349L	Serine/threonine protein kinase catalytic domain	AAT71828	69	964	1909	766		0.07	1.79	4.22	1.33		L
46	ORF351R	Hypothetical protein			205	265				0.38	0.59			E
47	ORF353R	Hypothetical protein			668	766	465			1.24	1.69	0.81		IE
48	ORF373L	Hypothetical protein		222	1483	1164	367		0.23	2.75	2.58	0.64		L
49	ORF374R	Hypothetical protein		434	8571	6712	1752	54	0.46	15.87	14.85	3.05	0.11	L
50	ORF380R	Major capsid protein	BAC66968	309	1645	1464	1017	136	0.33	3.05	3.24	1.77	0.27	L
51	ORF385R	Catalytic domain of ctd-like phosphatase	AAT71821		411	459	204			0.76	1.02	0.35		E
52	ORF390R	Hypothetical protein			652	365				1.21	0.81			L
53	ORF394R	Hypothetical protein				215					0.48			L
54	ORF396R	Transmembrane amino acid transporter	AAT71816	190	1562	1084	401		0.20	2.89	2.40	0.70		IE
55	ORF401R	Hypothetical protein		140	611	780	251		0.15	1.13	1.73	0.44		L
56	ORF407R	ATPase	AB007367	396	5551	1952	671	138	0.42	10.28	4.32	1.17	0.28	E
57	ORF412L	ATPase	AAO16492	88	532	744	363	53	0.09	0.99	1.65	0.63	0.11	E
58	ORF413R	ATPase	AB007367		303	562	318			0.56	1.24	0.55		E
59	ORF420L	Hypothetical protein			854	362	113			1.58	0.80	0.20		L
60	ORF423L	RING-finger domain-containing protein	AAT71906		123	251				0.23	0.56			L
61	ORF424R	Putative ankyrin repeat protein	AAL98801	115	1673	786	213		0.12	3.10	1.74	0.37		E
62	ORF426R	Hypothetical protein	AAT71837	370	1947	983	835	421	0.39	3.61	2.17	1.45	0.85	L
63	ORF430L	Putative phosphatase		264	5175	1696	610	85	0.28	9.58	3.75	1.06	0.17	E
64	ORF458L	Hypothetical protein		299	5939	3039	952	101	0.31	11.00	6.72	1.66	0.20	L
65	ORF463R	Hypothetical protein	NP078615		707	507	120			1.31	1.12	0.21		L
66	ORF487L	Proliferating cell nuclear antigen	AAL98835		424	389	108			0.79	0.86	0.19		E
67	ORF488R	Putative tumor necrosis receptor associated-factor	AAS18067		308	277				0.57	0.61			L
68	ORF493R	D5 family NTPase			1244	642	215			2.30	1.42	0.37		L
69	ORF502R	Hypothetical protein			111	212				0.21	0.47			ND
70	ORF506R	Hypothetical protein		190	395	313			0.20	0.73	0.69			L

Table 1. (continued)

No.	ORF	Putative Function	Accession No.	Signal Intensity Value[a]					Calibrated Expression Ratio[b]					Kinetic class[c]
				5 d.p.i.	7 d.p.i.	9 d.p.i.	10 d.p.i.	14 d.p.i.	5 d.p.i.	7 d.p.i.	9 d.p.i.	10 d.p.i.	14 d.p.i.	
71	ORF515L	Hypothetical protein			92	106				0.17	0.23			ND
72	ORF522L	Hypothetical protein			171					0.32				L
73	ORF534L	Ankyrin repeat-containing protein	AAT71909	101	111	230				0.21	0.51			E
74	ORF535R	Hypothetical protein		501	1434	1844	733	122	0.11	2.66	4.08	1.27	0.25	IE
75	ORF543R	RING-finger domain-containing protein	AAT71906	388	5996	2862	1312	222	0.53	11.10	6.33	2.28	0.45	L
76	ORF550R	Hypothetical protein		95	4080	1732	749	202	0.41	7.56	3.83	1.30	0.41	L
77	ORF554R	Hypothetical protein			626	548	368	60	0.10	1.16	1.21	0.64	0.12	L
78	ORF562R	Hypothetical protein			1041	531	241			1.93	1.17	0.42		E
79	ORF569R	Hypothetical protein		103	705	470	191			1.31	1.04	0.33		E
80	ORF575R	Hypothetical protein			2386	1222	439		0.11	4.42	2.70	0.76		L
81	ORF586L	Hypothetical protein			463	385	209			0.86	0.85	0.36		E
82	ORF589L	Hypothetical protein		171	363	273				0.67	0.60			E
83	ORF591R	Hypothetical protein		85	873	674	396		0.18	1.62	1.49	0.69		IE
84	ORF596L	dsRNA-specific ribonuclease	AAT71898		1199	830	222		0.09	2.22	1.84	0.39		L
85	ORF600L	Hypothetical protein		92	283	311	128			0.52	0.69	0.22		E
86	ORF606R	Hypothetical protein			396	517	167		0.10	0.73	1.14	0.29		L
87	ORF617L	Hypothetical protein			258	363	125			0.48	0.80	0.22		E
88	ORF618R	Hypothetical protein		224	137	144				0.25	0.32			E
89	ORF628L	Hypothetical protein		277	1905	782	1027	110	0.24	3.53	1.73	1.79	0.22	E
90	ORF632L	Hypothetical protein		139	4141	1203	362	122	0.29	7.67	2.66	0.63	0.25	E
91	ORF635L	Hypothetical protein		141	2143	1201	708		0.15	3.97	2.66	1.23		IE
92	ORF641L	Putative ankyrin repeat protein	AAL98801		1538	576	149		0.15	2.85	1.27	0.26		E
				44.6*	**98.9****	**96.7****	**79.3****	**25***						

[a]Signal intensity value is the background-subtracted median value
[b]The calibrated expression ratio is the ratio of the expression of the RSIV ORF in viral-infected cells compared to the β-actin control gene
[c]Abbreviation: IE, Immediate-Early; E, Early; L, Late; ND, Not Detected (adapted by Lua *et al.* 2005); *($p<0.05$); **($p<0.001$)

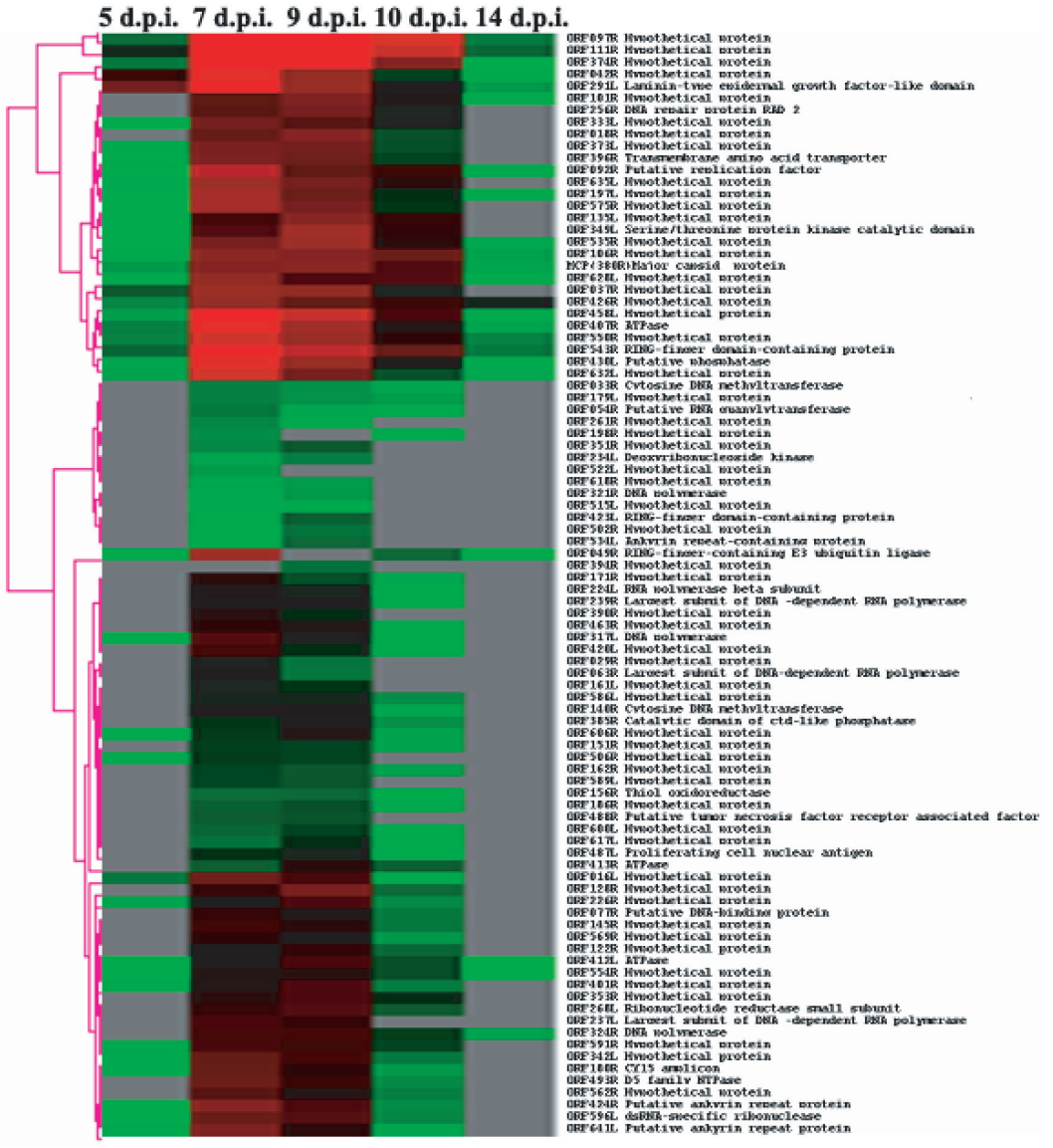

Figure 1. Hierarchical cluster analysis of *in vivo* RSIV transcription program**.** Calibrated expression ratios for each ORF were categorized by an average linkage hierarchical clustering program. Each row represents the expression profile of a single ORF, and each column indicates time points after infection. The normalized expression levels across all the time points are color-coded. Green boxes indicate expression ratios lower than the mean. Red boxes indicate expression ratios greater than the mean. Black boxes indicate an intermediate level of expression and gray boxes indicate missing or not detected. The magnitude of up-regulation from the mean is shown by differing intensities of red, with deep red showing lower expression and bright red showing the highest levels of expression.

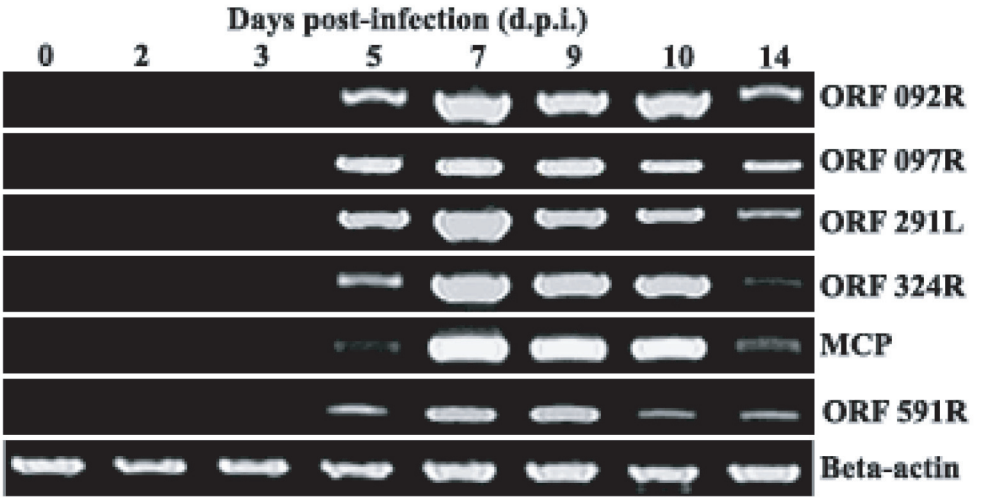

Figure 2. RT-PCR analysis of RSIV gene expression in viral-infected spleen. cDNAs were synthesized from 5 μg total RNA taken from the same samples used for the microarray experiments. One μl cDNA was used for 30 μl RT-PCR reaction with cycling conditions as follows: an initial denaturation at 95°C for 2 min, followed by 27 cycles of denaturation of 95°C for 30 sec, annealing at 55°C for 30 sec and elongation at 72°C for 1 min, and a final elongation step at 72°C for 5 min.

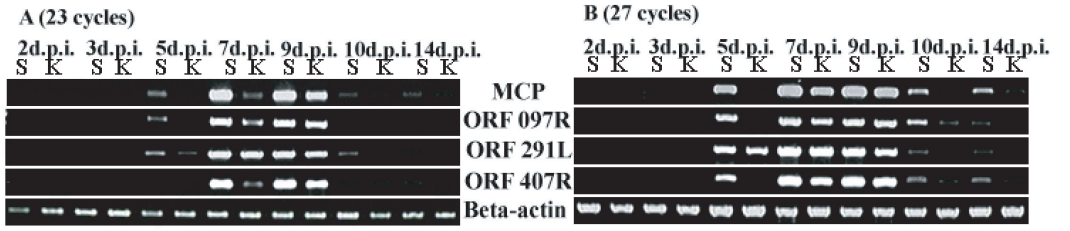

Figure 3. Difference in expression of RSIV genes between spleen (S) and kidney (K). cDNAs were synthesized from 5 μg total RNA derived from spleens and kidneys. One μl cDNA was used for 30 μl RT-PCR reaction with cycling conditions as follows: an initial denaturation at 95°C for 2 min, followed by 23 and 27 cycles of denaturation of 95°C for 30 sec, annealing at 55°C for 30 sec and elongation at 72°C for 1 min, and a final elongation step at 72°C for 5 min. A 23 cycle PCR was used to determine differences in expression of RSIV transcripts between spleen and kidney at the high level spread stage of the infection while a 27 cycle PCR was performed to show differences in expression at the early and late stages of the infection.

DISCUSSION

Outbreaks of system iridoviral diseases associated with high mortality (30-100%) have been reported in cultured freshwater and marine fish species in many parts of the world including Africa, America, Asia, and Europe (Chao *et al.*, 2004; Iwamoto *et al.*, 2002; Qin *et al.*, 2003). Among them, an iridoviral disease caused by RSIV has been considered as a serious, important disease because of its wide geographical distribution and host range. The disease is documented not only in Japan, but also in Korea, Taiwan and Indonesia (Chao *et al.*, 2002; Chou *et al.*, 1998; Do *et al.*, 2004; Inouye *et al.*, 1992; Jeong *et al.*, 2003; Mahardika *et al.*, 2004; Wang *et al.*, 2003). In Japan, the disease has been recorded in at least 31 marine fish including 3 main cultured marine fish, red seabream, Japanese flounder and yellow tail (Kawakami and Nakajima, 2002). Due to the devastating effects of this pathogen to marine aquaculture, an understanding of RSIV pathogenic mechanism, at the molecular level, is necessary and may provide possible clues for disease control and diagnosis strategies.

The concurrent development of DNA microarray technology and the complete sequencing of a number of viral genomes are providing the opportunity to speed our understanding of various aspects of both sides of the host-virus interaction at the molecular level. In fact, the DNA microarray technology has been successfully applied in virological studies in both cell culture systems and experimental animal models. In our previous study (Lua *et al.*, 2005), RSIV DNA microarrays were used for rapid analysis of the RSIV gene transcriptional profile over the time-course of an *in vitro* infection in HINAE cells and for grouping genes into temporal kinetic classes, providing a global picture of transcription and kinetics of RSIV genes during the replication cycle. In the present study, the same RSIV DNA microarray was used to characterize the viral gene expression profiles over the time-course of an *in vivo* infection in red seabream, providing a better understanding of the pathogenic mechanisms of RSIV infection at the transcription level.

The time-course experiments have allowed us to monitor the expression of each RSIV ORF through an *in vivo* infection. The timing of viral transcripts that we observed (beginning at 5 dpi and peaking at 7-9 dpi) is similar to what has been observed in previous studies (Nakajima *et al.*, 1995; Oshima *et al.*, 1998). In an immunoassay of RSIV-infected red seabream (Nakajima et al., 1995), the virus was not detected in the spleen at 1 or 3 dpi, was moderately detected at 5 dpi and was strongly detected at 7 dpi. Using a PCR assay, PCR products corresponding to a portion of the ribonucleotide reductase small subunit gene were not amplified from RSIV-infected red seabream at 1 and 2 dpi but were amplified starting at 5 dpi (Oshima *et al.*, 1998). Similar results were observed in Taiwan grouper iridovirus (TGIV) infection, a piscine iridovirus classified into the same group with RSIV, in which the viral particles were determined in some internal organs of groupers at 4-5 days after intramuscular infection (Chao *et al.*, 2002). In Singapore grouper iridovirus (SGIV) infection, viral antigens were detected in virus-infected fish blood at 3 dpi by a Western blot analysis (Qin *et al.*, 2002). Taken together, our results suggest that the pathogenic mechanism of RSIV is probably similar to that found in other piscine iridoviruses, such as TGIV and SGIV. Although viral particles were detected at slightly different times in the above studies, piscine iridoviruses seem to begin to spread at around 4-5 dpi.

Our finding that the *in vivo* expression profiles of RSIV gradually declined in both the numbers and the expression levels after 10 dpi. (Table 1, Fig. 1) indicates that the virus was being gradually cleared by host antiviral immune defenses. Similarly, Caipang *et al.* (2003) showed with real-time PCR that RSIV was cleared from both the vaccinated and unvaccinated red seabream after viral challenge, and Chao *et al.* (2004) showed with H & E staining and *in situ* hybridization that the number of basophilic enlarged cells (virus-containing cells) gradually decreased in groupers 7 days after TGIV infection. Chao *et al.* (2004) attributed the viral clearance to either an improved host defense or to depletion of susceptible cell types.

Differences in expression of selected RSIV ORFs between spleens and kidneys at high spread stage of the infection could be observed with only 23 PCR cycles (Figure 3A). Although differences in expression at the early and late stages of the infection were not detectable after 23 cycles, they were detectable after 27 cycles (Figure 3B). Overall, the expression levels of the selected ORFs were all higher in the spleen than in the kidney. Among the selected ORFs, the MCP gene was found to be expressed at significant higher levels in the spleen than in the kidney over the time-course of infection. MCP gene contains highly conserved domains and codes for the major structural component of viral particles (Schnitzler and Darai, 1993; Tidona *et al.*, 1998; Williams, 1996). The MCP gene has been used to detect and measure RSIV as well as other iridovirues (Caipang *et al.*, 2003; Tidona *et al.*, 1998). Thus, our RT-PCR results confirmed, at the transcription level, the hypothesis that the spleen is a susceptible organ for RSIV infection in particular and for iridoviral infections in fish in general. The spleen also appears to be where TGIV begins replicating (Chao *et al.*, 2004), and thus has been suggested to be used for early screening of TGIV. Our results support this conclusion.

CONCLUSION

In conclusion, the present study is the continued analysis of RSIV gene expression patterns *in vivo* to complete transcriptional profiles of RSIV both in cell culture and fish model systems. The results demonstrate that RSIV DNA microarrays can be used to study RSIV infection in a fish model at the molecular level. This study describes the first use of DNA microarrays to explore gene expression patterns of a marine fish-pathogenic virus in fish. Such studies should impart a greater understanding of pathogenesis of RSIV infection at the molecular level, contribution to the thorough knowledge of RSIV infection and further provide a possible clue for selection of a susceptible organ for detection of iridoviral infections in aquaculture.

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Immunomodulatory Activity of *Solanum trilobatum* Leaf Extracts in *Oreochromis mossambicus*

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ABSTRACT

The application of plant-derived immunostimulants in aquaculture for the prevention of diseases is a promising new development. The objective is to study the effect of crude extract, water and hexane soluble fractions of *Solanum trilobatum* leaves administered intraperitoneally on the specific immune response, non specific immunity and disease resistance in *Oreochromis mossambicus*. The specific immune response was assessed in terms of primary and secondary antibody responses to *Aeromonas hydrophila* and serum lysozyme activity was measured to study the non-specific immune mechanism. The functional immunity in terms of percentage mortality and Relative Percent Survival (RPS) was assessed by challenging the fish with live *A. hydrophila*. All the doses of three fractions except 32 mg kg^{-1} of crude extract and 800 mg kg^{-1} of water soluble fraction significantly enhanced the primary and secondary antibody responses on most of the days tested. Almost all the doses of both water and hexane soluble fractions enhanced the serum lysozyme activity. In disease resistance test, the percentage mortality was significantly reduced in the treated groups which are reflected by RPS values ranging from 45 to 70. This preliminary study indicates the potential of using *S. trilobatum* for controlling infectious diseases in aquaculture.

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INTRODUCTION

Intensive fish farming creates a stressful environment for the animal resulting in suppression of their immune response (Kajita *et al*., 1990), which leads to disease and disease outbreaks in aquaculture. There are basically three methods for controlling fish diseases: vaccination, chemotherapeutics and immunostimulants. Treatment with synthetic chemicals and antibiotics to prevent the above problem in fish has had limited success and the emergence of antibiotic resistant microbes is an important consideration for their continued use. The higher dependence of fish on the nonspecific immune mechanisms than on the specific immunity and problems associated with the development of efficacious vaccines such as cost, antigenic heterogeneity of microbial strains and viral antigenic drift have forced fish health scientists to look for effective immunostimulants from different sources including plants for prophylactic and therapeutic purposes. The use of immunostimulants enhances specific and non-specific immunity of farmed fish and offers a promising alternative to antibiotics and vaccines (Anderson, 1992).

The immunostimulants which have been tested for application to aquaculture include peptides like FK-565 (Kitao and Yoshita, 1986; Kitao *et al*., 1987), glucan (Chen and Ainsworth, 1992), extracts from a tunicate (McCumber *et al*., 1981), or an abalone (Sakai *et al*., 1991), lactoferin (Sakai, 1999), levamisole (Anderson and Jeney, 1992), chitosan (Siwicki *et al*., 1994), GH (Sakai *et al*., 1996), Vitamin C (Qin *et al*., 2000; Sahoo *et al,*. 1999), extra cellular products of *Mycobacterium* spp. and oligonucleotides (Sakai *et al*., 2001). These immunostimulants have been reported to increase various aspects of innate immunity such as the number of phagocytes, lysozyme and complement activities as well as serum Ig levels. Some immunostimulants have been shown to protect rainbow trout (Wahli *et al.,* 1998) against furunculosis and to decrease unspecific mortality in rockfish (Kim *et al*., 1999).

Solanum trilobatum Linn (Family: Solanaceae) is one of the common Indian medicinal plants and it has been used in traditional medicine for many centuries (Mohan *et al.,* 1998). This plant is commonly used to treat asthma, cough, dyspnoea, chronic febrile infections and difficult parturition. Its pivotal action is cardiac, tonic and carminative. The constitutents of this plant include, sobatum, β solamarine, solaine, solasodine, glycoalkaloid, diosogenin and tomatidine. This plant possesses a broad spectrum of antibiotic, antibacterial, and anticancer activity (Mohan and Devi, 1996). This plant is being widely used in human medicine in India but there is no report on its application in preventing fish diseases.

The objective was to study the effect of crude extract, water and hexane soluble fractions of *Solanum trilobatum* leaves administered intraperitoneally on the specific, nonspecific immune responses and the disease resistance in *Oreochromis mossambicus*.

MATERIALS AND METHODS

Fish and their maintenance

Oreochromis mossambicus (Mossambique tilapia), a common fresh and brackish water cichlid fish was used in this study. Male fish weighing 25±5 g were used. All experiments

were carried out in Fiber Reinforced Plastic tanks (vol. 150 l). They were kept at an ambient, uncontrolled temperature of 28±2ºC under natural photoperiod. Water was changed on alternate days. Fish were fed *ad libitum* with a balanced fish diet prepared in the laboratory.

PLANT EXTRACT PREPARATION

The plant *Solanum trilobatum* was procured from the market and the plant species was identified and confirmed by Dr. D. Stephen, P.G. Department of Botany, The American College, Madurai, Tamil Nadu, India. The voucher specimen (Specimen No: CFIS01) was deposited in the Herbarium of Department of Botany, Lady Doak College, Madurai. The leaves were collected and washed in sterile distilled water. They were shade-dried, powdered and stored at -20^0C until further use. The extraction was done by following the methods of Lee *et al.* (2000) and Xu *et al.* (2000).

Preparation of crude extract

Ten grams of leaf powder was exhaustively extracted with sterile distilled water and it was filtered through sterile muslin cloth and the filtrate was allowed to stand for 30 min at room temperature. The filtrate was collected and the solvent was removed using rotary vacuum evaporator (Buchi SMP, Switzerland). The residue obtained after evaporation was dissolved and the desired doses were prepared in sterile distilled water.

Preparation of water and hexane soluble fractions (WSF/HSF)

Ten grams of leaf powder were exhaustively extracted with methanol and it was filtered through sterile muslin cloth and the filtrate was allowed to stand for 30 min at room temperature. The filtrate was collected and the solvent was removed using rotary vacuum evaporator (Buchi SMP, Switzerland). The residue obtained after evaporation was mixed with sterile distilled water. The extract was taken in a separating funnel and equal volume of hexane was added and mixed carefully by intermittent releasing of the pressure inside the separating funnel. The content was allowed to stand without any disturbance until two distinct layers (lower water soluble and upper hexane soluble fraction) were seen. The fractions were collected separately. This process was repeated until the colourless hexane fraction was obtained which indicates the completion of hexane fractionation. The water and hexane soluble fractions were concentrated in rotary vacuum evaporator. The desired doses of water and hexane soluble fractions were prepared in distilled water and pure coconut oil (to dissolve the non-polar compounds), respectively and stored at -20^oC until used for experimentation.

Bleeding and serum collection

Fish were bled from common cardinal vein using 1 ml tuberculin syringe fitted with 24-gauge needle (Michael *et al*., 1994). For serum separation, 200 µl of blood was drawn and the whole bleeding procedure was completed within 1 min to minimize the stress to

fish. The blood was collected in serological tubes and the clot was stored in a refrigerator overnight. The clot was then spun down at 400g for 10 min. The serum collected was stored in sterile Eppendorf tubes at -20^{o}C until used for assays.

Bacterial agglutination assay

Preparation of heat killed whole cell vaccine

Overnight culture of A. hydrophila was subjected to 60^{o}C for 1 hr in a water bath (Karunasagar *et al.*, 1997). The culture was centrifuged at 800g for 15 min. The packed cells were washed and the required dose was prepared in phosphate buffered saline.

Primary and secondary antibody responses

After acclimation for a period of 2 weeks, fish (n=6) were intraperitoneally injected with 6.4, 32, 160 or 800 mg kg^{-1} body weight (0.2 ml/fish) of crude extract, water or hexane soluble fractions of *S. trilobatum* whereas the corresponding control fish received 0.2 ml of water or purified coconut oil (control for HSF group). Two days after the leaf extract administration, the fish were vaccinated intraperitoneally with heat killed *A. hydrophila* vaccine (10^9 cells/fish). Both extract administration and vaccination was done using a tuberculin syringe with a 24-gauge needle. To study the secondary antibody response, fish were administered with the same dose of antigen on day 59-post primary immunization. The blood was collected at the interval of seven days and the bacterial agglutination assay was performed. The serum was separated and the complement was inactivated at 47^{0}C (Sakai, 1981) in a water bath for 30 min and stored at –20^{0}C until used for assay. Serially double diluted antibacterial antisera were titrated by bacterial agglutination assay (Roberson, 1990). The highest dilution of serum giving detectable macroscopic agglutination was expressed as $\log_2$ antibody titre (to represent $\log_2$ of the inverse of titre) of the serum.

Lysozyme activity

After acclimation, fish (n=6) were intraperitoneally injected with (0.2ml/fish) 0, 4, 40 or 400 mg kg^{-1} body weight of crude extract, water or hexane soluble fraction whereas the corresponding control fish received 0.2 ml of distilled water or purified coconut oil respectively. The fish were bled 2 days prior to and 2, 4, 6, 8 and 10 days after treatment. Lysozyme activity was measured by the method of Parry *et al.* (1965) in combination with the microplate adaptation of Hutchinson and Manning (1996). In this turbidimetric assay, 0.03% lyophilized *Micrococcus lysodeikticus* in 0.05mM sodium phosphate buffer (pH 6.2) was used as substrate. Ten microlitres of fish serum was added to 250 µl of bacterial suspension in a "U" bottom microtitre plate and the reduction in absorbance at 490nm was determined after 0.5 and 4.5min incubation at 22^{o}C using a microplate reader (Biorad, USA). One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 per min.

Disease resistance

Group of 10 fish in triplicates were administered with 0.2ml of 0, 4, 40 or 400 mg kg^{-1} body weight of crude extract, water or hexane soluble fraction of *S. trilobatum* on day 1.

The control fish received 0.2 ml of water or oil. After the administration of plant extracts fish were challenged (injected) with virulent *A. hydrophila* (1 x 10^8 cells/fish) on day 7. Earlier, the challenge dose was adjusted to give 80% mortality in the untreated groups. An untreated and a phosphate buffered saline injected control groups were also maintained. Mortality was recorded for 15 days and RPS was calculated by the following formula (Ellis, 1988) below:

$$RPS = 1 - \left\{ \frac{\text{Percent mortality in treated group}}{\text{Percent mortality in control group}} \right\} \times 100$$

Statistical analysis

Data were expressed as arithmetic mean ± standard error (SE). Statistical analysis of data involved one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparison test. The levels of significance were expressed as P-value less or greater than 0.05.

RESULTS

Bacterial agglutination assay

The highest dose of 800 mg kg^{-1} of crude extract significantly enhanced the primary and secondary antibody responses on most of the days tested ($P<0.05$). The enhancement in the secondary antibody response was found to be on day 21 in all other groups (Figure 1). The highest dose of water soluble fraction (800 mg kg^{-1}) was found to be lethal to fish. The other doses have advanced the peak antibody response by a week and significantly enhanced the primary and secondary antibody responses on most of the days tested ($P<0.05$, Figure 2). All the doses of hexane soluble fraction except 800 mg kg^{-1}, advanced the peak day by 1 week ($P<0.05$), whereas 800 mg kg^{-1} treated groups showed the peak response on day 14. Further, all the concentrations enhanced the antibody response on most of the days tested ($P<0.05$). The enhanced secondary antibody response for the group administered with 160 mg kg^{-1} of hexane fraction was found to be on 7, 14 and 21 ($P<0.05$). For 800 mg kg^{-1} treated groups, it was only on 14 ($P<0.05$, Figure 3).

Lysozyme activity

The lysozyme activity was enhanced by the administration of 40 mg kg^{-1} of crude extract (Figure 4) or WSF (Figure 5) on day 4, 6 and 8 ($P<0.05$). The 400 mg kg^{-1} of crude extract or 4 mg kg^{-1} of WSF enhanced the activity on day 2 and 4($P<0.05$). It is also enhanced in the group treated with 4 mg kg^{-1} or 400 mg kg^{-1} of HSF on most of the days tested ($P<0.05$) (Figure 6).

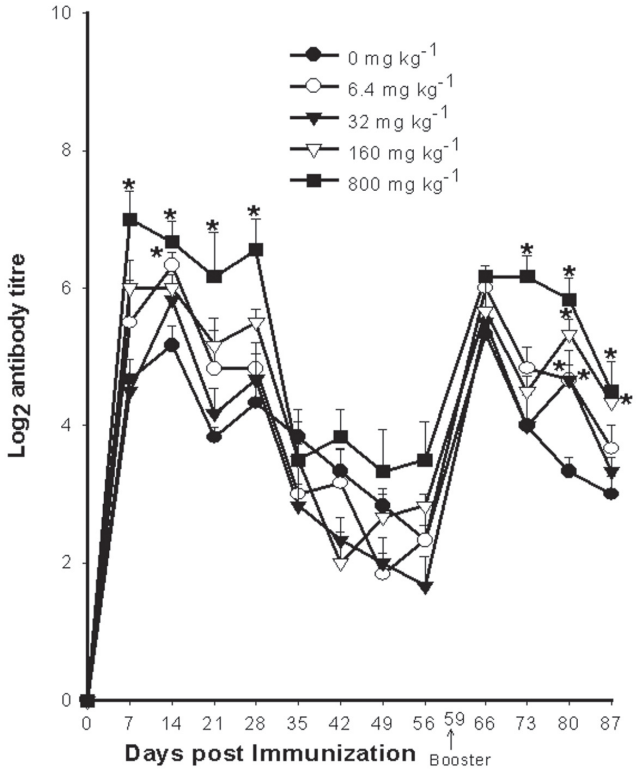


Figure 1. Effect of *S. trilobatum* crude leaf extract on the antibody responses against heat killed *A. hydrophila* in *O. mossambicus*. Each point represents the arithmetic mean value of 6 fish ± standard error (* P <0.05).

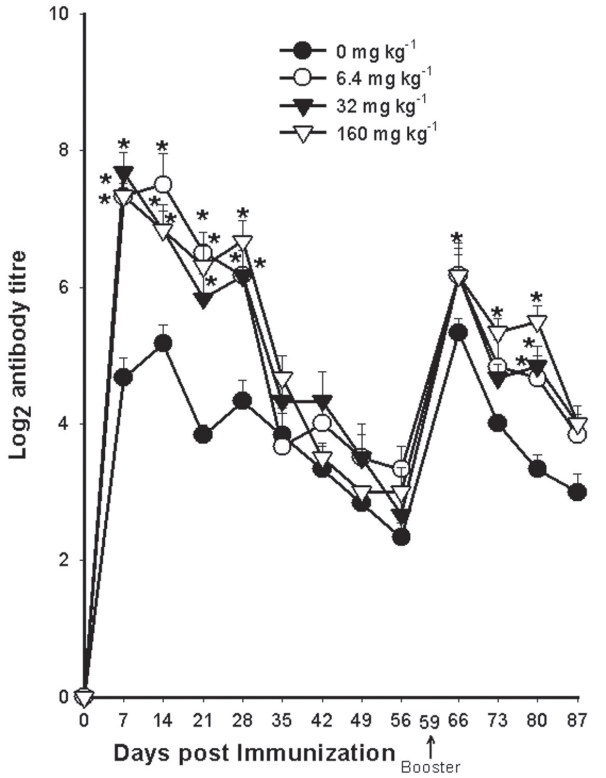


Figure 2. Effect of *S. trilobatum* water soluble fraction (WSF) on the antibody responses against heat-killed *A. hydrophila* in *O. mossambicus*. Each point represents the arithmetic mean value of 6 fish ± standard error (* P <0.05).

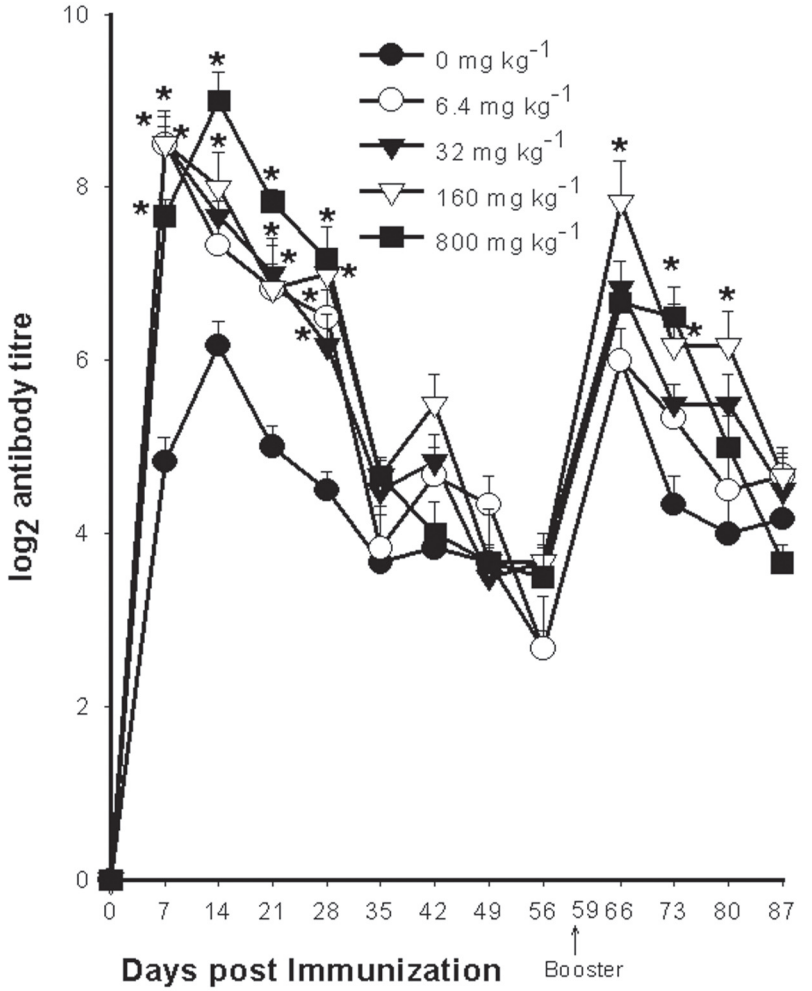


Figure 3. Effect of *S. trilobatum* hexan soluble fraction (HSF) on the antibody responses against heat killed *A. hydrophila* in *O. mossambicus*. Each point represents the arithmetic mean value of 6 fish ± standard error (* P <0.05).

Disease resistance

The percentage mortality was decreased (Table 1) in fish challenged with live *A. hydrophila* by extract administration. Among the groups administered with plant extracts, the lowest percentage mortality of 23.33% was observed in the group treated with highest dose of 400 mg kg^{-1} of WSF or 4 mg $kg^{-1}$of HSF with the RPS value of 70.84, and 65.01, respectively (Table 1).

Figure 4

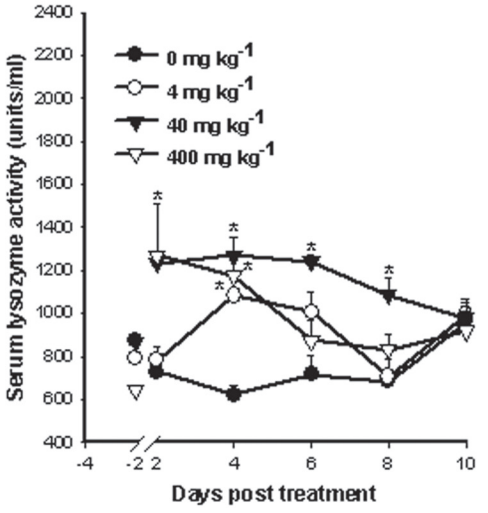
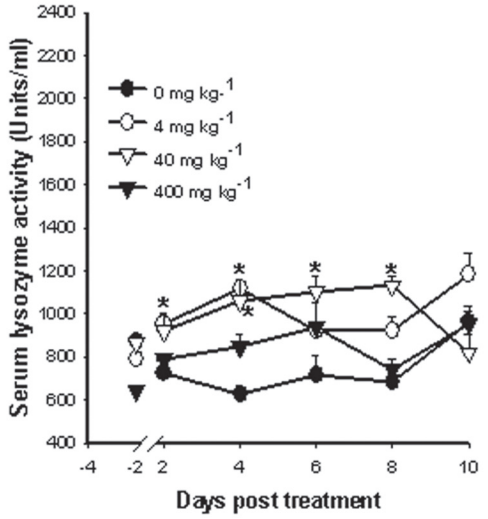


Figure 5



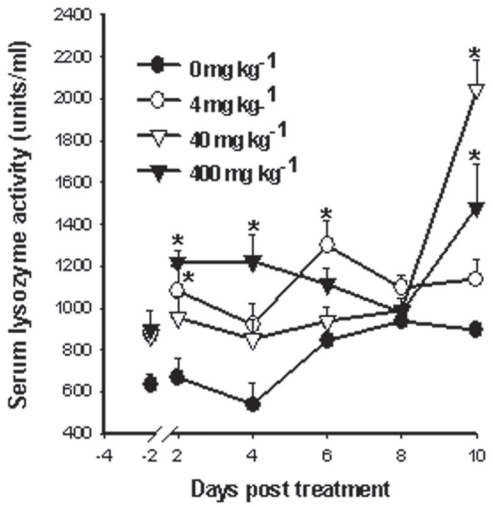


Figure 6

Figures 4-6. Effect of *S. trilobatum* crude leaf extract, water and hexane soluble fractions (WSF/HSF) on the serum lysozyme activity in *O. mossambicus*. Each point represents the arithmetic mean value of 6 fish ± standard error (* $P < 0.05$).

Table 1. Effect of *S. trilobatum* leaf extracts on the percentage mortality and Relative Percent Survival (RPS) in *O. mossambicus* challenged with live *A. hydrophila*. The percentage mortality values are mean ± SE, n=10 fish per group in triplicate; a posteriori Tukey comparison of control and treated groups shown with different alphabets representing significant difference (P<0.05).

No.	Type of sample	Percentage mortality (%)	Relative percent survival (RPS)
1	Distilled Water (Control)	80.00 ± 0.00^{a}	-
2	Crude – 4 mg kg^{-1}	40.00 ± 5.77^{bc}	50.00
3	Crude - 40 mg kg^{-1}	30.00 ± 0.00^{c}	62.50
4	Crude - 400 mg kg^{-1}	46.67 ± 3.33^{b}	41.66
5	Distilled Water (Control)	80.00 ± 0.00 ^{a}	-
6	WSF – 4 mg kg^{-1}	30.00 ± 5.77 ^{b}	62.50
7	WSF - 40 mg kg^{-1}	33.33 ± 3.33 ^{b}	58.34
8	WSF - 400 mg kg^{-1}	23.33 ± 3.33 ^{b}	70.84
9	Oil (Control)	66.67 ± 6.67 ^{a}	-
10	HSF – 4 mg kg^{-1}	23.33 ± 6.67 ^{b}	65.01
11	HSF - 40 mg kg^{-1}	30.00 ± 5.77 ^{b}	55.00
12	HSF - 400 mg kg^{-1}	30.00 ± 5.77 ^{b}	55.00

DISCUSSION

In the present study, crude extract, water and hexane soluble fractions of *S. trilobatum* leaves were found to have significant stimulatory effect on the specific, nonspecific immunity tested and the disease resistance in *O. mossambicus*. Most of the doses significantly enhanced the primary and secondary antibody responses to *A. hydrophila*. The magnitude of the antibody response is very high in the groups treated with water or hexane soluble fractions. This finding is in agreement with an earlier finding where the leaf extract of *Ocimum sanctum* produced a significant stimulatory effect on both primary and secondary responses to *A. hydrophila* (Logambal *et al.,* 2000) and SRBC (Venkatalakshmi and Michael, 2001) in the same species of fish. This treatment not only enhanced the magnitude of the response but also shortened the lag period of both primary and secondary responses with corresponding advancement of the peak day of antibody response as seen in the present study. The primary and secondary response to sheep erythrocyte was enhanced by the treatment of Azadiractin*,* a triterpenoid extracted from neem seed kernel of *Azadirachta indica* (Logambal and Michael, 2001) in *O. mossambicus*. Similarly, feeding of 0.5% root extract of *Achyranthes aspera* for 4 weeks significantly enhanced the haemagglutination titre in *Labeo rohita* (Rao *et al.,* 2004) and anti BSA antibody level in *Catla catla* (Chakrabarti and Rao, 2006).

Lysozyme is an important component in the immune system of fish. It is bactericidal by hydrolyzing β (1→4) linkages of bacterial cell wall peptidoglycans resulting in bacteriolysis. It is also known to act as opsonin and activate the complement system and phagocytes (Magnadottir, 2006). In the present study, it was observed that the lysozyme activity was substantially enhanced on treatment with crude extract, water or hexane soluble fractions of *S. trilobatum.* Similar results of elevated lysozyme activity was observed on 20, 25 and 30 days after feeding Jian carp (Jian and Wu, 2004) and large yellow croaker, *Pseudosciaena crocea* (Jian and Wu, 2003) with traditional Chinese medicine (TCM) formulated from Astragalus root (*Radix astragalin seu* heydsari) and chinese Angelica root (*Radix angelicae sinensis*) at a ratio 5:1 (w/w). *Oreochromis niloticus* fed with 0.1 and 0.5% *Astragalus radix* for 1 week (Yin *et al*., 2006) and *Labeo rohita* fed with 0.5% of *Achyranthes aspera* seed extracts for 4 weeks were shown to enhance lysozyme activity (Rao *et al*., 2006).

For testing efficacy of an immunostimulant, it is very essential to estimate the increased protection in treated fish. (Sakai *et al*., 2001). The enhancement of nonspecific immune parameters by *S. trilobatum* leaf preparation is possibly an important factor in reducing the percentage mortality and thereby protecting the fish against live *A. hydrophila* challenge. Earlier studies in this laboratory also revealed that dietary supplementation of *Ocimum sanctum* (Logambal *et al*., 2000) and *Nyctanthes arbortristis* (L. D. Devasree, Centre for Fish Immunology, Lady Doak College, unpublished data) leaves and intraperitoneal injection of water and hexane soluble fraction of *Eclipta alba* (D. Christybapita, Centre for Fish Immunology, Lady Doak College, unpublished data) leaves enhanced the disease resistance against *A. hydrophila* in *O. mossambicus.* The present finding is in agreement with the results of Abutbul *et al*. (2004) in tilapia fed with a diet containing ethyl acetate extract of *Rosmarinus officinalis* leaf powder and Rao *et al*. (2006) where the disease resistance against *A. hydrophila* was enhanced in *L. rohita* fed with 0.5% of *A. aspera*. The methanolic herbal extracts (*S. trilobatum*, *Andrographis paniculata* and *Psoralea corylifolia*) helped to increase the survival and growth and reduced the bacterial load even in the shrimp, *Penaeus monodon* post larvae (Citarasu *et al.*, 2003).

Even though all the fractions enhanced the specific and nonspecific immunity, the water and hexane soluble fractions were more protective than the crude extract was. The immunostimulatory effect of water soluble fraction might be due to alkaloids and carbohydrates. The low and high molecular weight compounds of several medicinal plants have been shown to have immunostimulating potential *in vivo* and *in vitro* (Wagner, 1990). The enhancement in nonspecific immune responses and the protection by hexane soluble fraction might be due to the presence of the compound, sobatum. Sobatum, a partially purified component of the plant *S. trilobatum* was obtained from the petroleum ether/ethyl acetate (75:25) extractable portion, which was identified as β -sitosterol by comparison with an authentic sample and proved to be an anticancer agent by *in vitro* and *in vivo* experiments (Govindan *et al*., 2004). This phytosterol seems to enhance the activity of lymphocytes and the production of cytokines in mammalian models (Bouic *et al*., 1996)**.**

CONCLUSION

The present study reveals that the administration of crude extract, water or hexane soluble fraction enhanced the specific, nonspecific immune parameters and disease resistance against *A. hydrophila* in tilapia. The water and hexane soluble fractions seem to be a better immunostimulant, which can have a promising role in aquaculture to prevent diseases and disease outbreaks. Also further investigation on the immunostimulatory and disease protective effects of this plant preparations when administered along with feed (which is the preferred route of administration in the culture situation) for disease prevention in aquaculture in warranted.

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Diversity and Distribution of External Parasites from Potentially Cultured Freshwater Fishes in Nakhonsithammarat, Southern Thailand

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ABSTRACT

Twenty-one species from 16 genera of potentially cultured freshwater fishes were examined for external parasites. Ten individuals of each fish species (mainly local species) were examined from various places in Nakhonsithammarat, southern Thailand. Five parasitic groups containing a total of 51 species were identified. They were a single ciliated protozoan, myxozoans (2 spp.), monogeneans (44 spp.), digeneans (2 spp.) and crustaceans (2 spp.). Monogenea was regarded as a major parasitic group of the fish. *Dactylogyrus* (Monogenea) had the highest number of species (12 spp.), whereas *Trichodina* sp. (Ciliophora) was the most widely distributed species, being observed on seven fish species from seven families. Most of the parasites (43 taxa or 86 %) found in this study were specific to their host species. All except red pacu (*Piaractus brachypomus*) and swamp eel (*Monopterus albus*) were infected with parasites (91 %).

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INTRODUCTION

In Thailand, many species of freshwater fish, both native and introduced species have been successfully cultured. In the past two decades some exotic species have been introduced into the Southeast Asian region including Thailand for food fish aquaculture, for example, common carp (*Cyprinus carpio*), Nile tilapia (*Oreochromis niloticus*), African shaft-toothed catfish (*Clarias gariepinus*) and red pacu (*Piaractus brachypomus*). Hence, fish culture are now extending into all parts of Thailand.

Nakhonsithammarat, a representative province of the southern region of Thailand is situated at about 7°55′ - 9° 20′N and 99° 15′- 100° 20′ E, facing both the Pacific and Indian Oceans. The province is remarkable in having three main watersheds, the Pak-panang, the Tapi and the Trang including many tributaries that support both agricultural and fishery activities. A recent survey of fish diversity showed that there are 121 fish species of inland waters in Nakhonsithammarat (Lerssutthichawal *et al.*, 2005). Although only a few species of fish are cultured, there are more species that has aquaculture potential, both feral and introduced species.

To address the increasing demand for food protein, fish culture has become a major sector of fish production. Some indigenous as well as introduced species have been induced to the culture program. Nevertheless during the culture period, it could experience many serious problems, particularly those caused by parasites. The aim of this project is to document the diversity and host distribution of the external parasites of potentially cultured fishes, which were regarded as a major cause of low production in aquaculture system.

MATERIALS AND METHODS

Fish samples

Various species of freshwater fishes were obtained from natural waters (rivers, canals, swamps) and cultured areas (ponds, net cages and ditches) including the local fish markets of Nakhonsithammarat, southern Thailand to examine their external parasites. Fish species examined were chosen based on their potential value as aquaculture (both indigenous and introduced species). Fish were identified using the keys given by Kottellat *et al.* (1993, Lerssutthichawal *et al.* 2005, Rainboth, 1996, Roberts, 1989, Smith, 1945, Vidthayanon, 2001) and Vidthayanon *et al.* (1997). A list of fish species examined and the external parasites identified is given in Table 1.

Examination for parasites

Fish were measured and killed. Skin and fin mucous were scraped and examined using compound and phase-contrast microscopy. Gills were removed and scraped into a Petri dish filled with clean water to dislodge the parasites and examined under a stereo microscope. The parasites found were transferred onto a glass slide and covered with coverslip. Parasites were fixed and preserved using the appropriate methods for each group as outlined by Tonguthai *et al.* (1999).

Identification and determination of prevalence and intensity

External parasites were identified using the following relevant works (e.g. Arthur and Lumanlan-Mayo, 1997; Bondad-Reantaso and Arthur, 1989; Chinabut and Lim, 1993; Dykova and Lom, 1988; Ergens, 1981; Gussev, 1976; Hanek and Furtado, 1973; Kabata, 1985; Kaewviyudth and Chinabut, 1999; Lerssutthichawal, 1999; Lerssutthichawal and Lim, 1997; Lom and Dykova, 1992; Lim, 1986, 1987, 1991, 1995, 1998; Lim and Furtado, 1983, 1985, 1986; Paperna, 1996; Sirikanchana, 2003). New parasitic species are indicated as sp. n. Prevalence and mean intensity for selected parasites were determined according to Margolis *et al.* (1982).

RESULTS AND DISCUSSION

Species and occurrence of fish parasites

Five parasitic groups with a total of 51 species were collected from 19 fish host species belonging to 16 genera and 14 families). They were a single species of ciliate protozoan and two species of myxozoans, 44 monogenean species, two digenean species and two crustacean species (Table 1). All hosts except *Piaractus brachypomus* (red pacu) and *Monopterus albus* (swamp eel) were infected with external parasites (91 %).

Table 1 also shows that cyprinid and bagrid fishes accommodated the highest number of parasite species, exemplified by *Barbodes gonionotus* and *Hemibagrus nemurus*, each having seven species of parasites. Except for red pacu and swamp eel, which were uninfected, the majority of fish host species (12 of 19) harbored two to three parasite species.

Diversity and specificity of freshwater fish parasites

Monogenea was the most common parasitic group encountered, with 44 species found on 18 fish species (86 %) from 13 genera and 11 families. *Dactylogyrus* had the highest number of species (12 spp.), followed by *Bychowskeylla*, *Cichlidogyrus*, *Thaparocleidus* and *Trianchoratus* with four species each (Table 2).

Table 2 indicated that there are specific interaction between the parasites, especially monogeneans and their host. *Bifurcohaptor* and *Cornudiscoides* were found only on the Bagridae (*Hemibagrus*), *Bychowskyella* and *Quadriacanthus* from the Clariidae (*Clarias*) and *Cichlidogyrus* from the Cichlidae (*Oreochromis*). However, some monogenean genera present on different fish families, indicating a low degree of host specificity, exemplified by *Trianchoratus* from Anabantidae (*Anabas*), Channidae (*Channa)* and Belontidae (*Trichogaster*) or *Thaparocleidus* from Bagridae (*Hemibagrus*) and Notopteridae (*Notopterus*). *Sundanonchus*, however, was found on Channidae (only *Channa micropeltes*) and Pristolepidae (*Pristolepis fasciatus*). The previous works reported some monogenean genera found in this study were found on another fish group. For example *Bychowskyella* could be found on Bagridae, Schilbeidae, Siluridae and Sisoridae (see Lerssutthichawal, 1999; Lim, 1998).

The absence of clinical signs of disease on feral fish and the high diversity of parasite species encountered, suggest that there are factors controlling any drastic increase in any of the co-existing species within the community thereby keeping populations of the different species within the carrying capacity of their environment. These factors help to maintain species diversity, resulting in the stability of the ecosystem concerned.

Table 1. Prevalence and mean intensity of freshwater fish parasites in Nakhornsithammarat.

Fish families	Fish species	Parasite species	Parasite group	Prevalence (%)	Mean intensity
Anabantidae	*Anabas testudineus*	*Trianchoratus gussevi*	Mo	40	13
		T. parvulus	Mo	10	1
		Trichodina sp.	Ci	40	NC
Bagridae	*Hemibagrus filamentus*	*Bifucohapter baungi*	Mo	20	2
		Cornudiscoides malayensis	Mo	20	3
		C. sundanensis	Mo	20	2
Belontidae	*Trichogaster trichopterus*	*Trianchoratus trichogasterium*	Mo	20	1
	T. pectoralis	*Gyrodactylus* sp. A	Mo	10	1
		Transversotrema partialense	D	10	2
		Trichodina sp.	Ci	60	NC
Channidae	*Channa micropeltes*	*Sundanonchus micropeltis*	Mo	50	150
	C. striata	*Trichodina* sp.	Ci	10	7
		Lamproglena chinensis	Cr	10	7
		Trianchoratus ophiocephali	Mo	40	13
Characidae	*Piaractus brachypomus*	-	-	-	-
Cichlidae	*Oreochromis niloticus (black tilapia)*	*Trichodina* sp.	Ci	10	NC
		Cichlidogyrus longicornis	Mo	50	4
		C. sclerosus	Mo	60	9
		C. tilapiae	Mo	40	3
		C. tubicirus	Mo	20	10
	O. niloticus (red tilapia)	*C. sclerosus*	Mo	70	4
		C. thurstonae	Mo	80	4
		C. tilapiae	Mo	40	12
		Trichodina sp.	Ci	20	NC
Pristolepidae	*Pristolepis fasciatus*	*Sundanonchus triradicatus*	Mo	100	17
Synbranchidae	*Monopterus albus*	-	-	-	-

Ci:Ciliophora; Cr:Crustacea; Di:Digenea; Mo :Monogenea ; My :Myxozoa ; NC :Not calculated

Table 1. continued

Clariidae	*Clarias batrachus*	*Bychowskylla tchangi*	Mo	60	10
		Quandricanthus kobiensis	Mo	60	14
	Clarias hybrid (C. macrocephalus x C. gariepinus)	*Q. bagrae*	Mo	30	1
		Gyrodactylus fernandoi	Mo	10	2
	Clarias macrocephalus	*Bychowskyella tchangi*	Mo	7	5
		G. fernandoie	Mo	7	5
		Quadricanthus sp.n. A	Mo	26	46
		Trichodina sp.	Ci	7	NC
	Clarias nieuhofi	*Bychowskyella* sp.n. A	Mo	10	1
		Bychowskyella sp.n. B	Mo	10	4
		Bychowskyella sp.n. C	Mo	10	4
Cyprinidae	*Barbodes gonionotus*	*Dactylogyrus kanchananuriensis*	Mo	70	4
		D. pseudosphyrna	Mo	60	14
		D. puntii	Mo	60	16
		D. siamensis	Mo	70	11
		D. tonguthai	Mo	70	41
		Gyrodactylus sp. B	Mo	10	NC
		Thelohanellus jiroveci	My	10	NC
	Cyprinus carpio	*Dactylogyrus minutes*	Mo	80	10
		Dactylogyrus sp.n. A	Mo	10	4
		Gyrodactylus sp. B	Mo	40	5
	Leptobarbus hoeveni	*Dactylogyrus leptobarbi*	Mo	100	25
		Dactylogyrus sp.n. B	Mo	100	19
		Dactylogyrus sp.n. C	Mo	90	4
		Dactylogyrus sp.n. D	Mo	70	5
		Dactylogyrus sp.n. E	Mo	100	18
Eleotridae	*Oxyleotris marmorata*	*Pseudodactylogyroides marmoratae*	Mo	20	1
		Pseudodactylogyroides sp.n. A	Mo	10	2
Helostomatidae	*Helostoma temincki*	*Transversotrema patialense*	Di	10	5
		Trihodina sp.	Ci	50	NC
Notoptoridae	*Notopterus notopterus*	*Lamproglena inermis*	Cr	10	1
		Malayanodiscoides bihamuli	Mo	80	1
		Thaparocleidus platamauxilli	Mo	20	1
Osphronemida	*Osphoronemus goramy*	*Henneguya shaharini*	My	40	NC
		Percianyrocephalus sp. A	Mo	20	3

Table 2. Distribution of parasites in freshwater fish species.

Parasite species	**Fish hosts**		**Parasite species**	**Fish hosts**	
	Fish species	**Fish families**		**Fish species**	**Fish families**
Protozoa			Monogenea		
Henneguya shaharini *Thelohanellus jiroveci*	*Osphronemus goramy*	Osphronomidae			
	Barbodes gonionotus	Cyprinidae	*Dactylogyrus n.* sp. B	*Leptobarbus hoeveni*	Cyprinidae
	Anabas testudineus	Anabantidae	*Dactylogyrus n.* sp. C	*L. hoeveni*	Cyprinidae
	Channa striata	Channidae	*Dactylogyrus n.* sp. D	*L. hoeveni*	Cyprinidae
	Clarias macrocephalus	Clariidae	*Dactylogyrus n.* sp. E	*L. hoeveni*	Cyprinidae
	Helostoma temincki	Helostomatidae	*Gyrodactylus fernandoi*	*Clarias hybrid (C. macrocephalus x C. gariepinus)*	Clariidae
	Oreochromis niloticus (common type)	Cichlidae		*C. macrocephalus*	Clariidae
	Osphronemus goramy	Osphronemidae	*Gyrodactylus n.* sp. A	*Trichogaster pectoralis*	Belontidae
	Trichogaster pectoralis	Belontidae	*Gyrodactylus n.*sp. B	*Barbodes gonionotus*	Cyprinidae
Monogenea				*Cyprinus carpio*	Cyprinidae
Bifurcohaptor baungi	*Hemibagrus filamentus*	Bagridae	*Malayanodiscoides bihamuli*	*Notopterus notopterus*	Notopteridae
Bychowskyella tchangi	*Clarias macrocephalus*	Clariidae	*Perciancyrocephalus n.* sp. A	*Osphronemus goramy*	Osphronomidae
	C. batrachus	Clariidae	*Pseudodactylogyroides marmoratae*	*Oxyeleotris marmorata*	Eleotridae
Bychowskyella sp.n. A	*C. nieuhofi*	Clariidae	*Pseudodactylogyroides* n.sp. A	*O. marmorata*	Eleotridae
Bychowskyella sp.n. B	*C. nieuhofi*	Clariidae	*Quadricanthus bagrae*	*Clarias hybrid (C. macrocephalus x C. gariepinus)*	Clariidae
Bychowskyella sp.n. C	*C. nieuhofi*	Clariidae	*Q. kobiensis*	*C. batrachus*	Clariidae
Cichlidogyrus longicornis	*Oreochromis niloticus (common type)*	Cichlidae	*Quadricanthus* n.sp. A	*C. macrocephalus*	Clariidae
C. sclerosus	*O. niloticus (common type)*	Cichlidae	*Sundanonchus triradicatus*	*Pristolepis fasciatus*	Pristolepidae

Table 2. continued

	O. niloticus (tub-tim)	Cichlidae	*S. micropeltis*	*Channa micropeltis*	Channidae
C. thurstonae	*O. niloticus* (common type)	Cichlidae	*Thaparocleidus platamauxilli*	*Notopterus notoperus*	Notopteridae
	O. niloticus (tub-tim)	Cichlidae	*Thaparocleidus* n.sp. A	*Hemibagrus filamentus*	Bagridae
C. tilapiae	*O.niloticus* (common type)	Cichlidae	*Thaparocleidus* n.sp. B	*H. filamentus*	Bagridae
	O. niloticus (tub-tim)	Cichlidae	*Thaparocleidus* n.sp. C	*H. filamentus*	Bagridae
C. tubicirrus	*O.niloticus* (common type)	Cichlidae	*Trianchoratus ophiocephali*	*Channa striata*	Channidae
Cornudiscoides malayensis	*Hemibagrus filamentus*	Bagridae	*Trianchoratus trichogasterium*	*Trichogaster trichopterus*	Belontidae
C. sundanensis	*H. filamentus*	Bagridae	*Trianchoratus gussevi*	*Anabas testudineus*	Anabantidae
Dactylogyrus kanchanaburiensis	*Barbodes gonionotus*	Cyprinidae	*T. parvulus*	*A. testudineus*	Anabantidae
D. leptobarbi	*Leptobarbus hoeveni*	Cyprinidae	*Digenea*		
D. minutus	*Cyprinus carpio*	Cyprinidae	*Transversotrema patialense*	*Trichogaster pectoralis*	Belontidae
D. pseudosphyrna	*B. gonionotus*	Cyprinidae		*Helostoma temincki*	Helostomatidae
Monogenea			*Digenea gen sp. metacercaria*	*Hemibagrus filamentus*	Bagridae
D. puntii	*D. puntii*	D. puntii	*Crustacea*		
D. siamensis	*D. siamensis*	D. siamensis	*Lamproglena chinensis*	*Channa striata*	Channidae
D. tonguthai	*B. gonionotus*	Cyprinidae	*Lamprolegna inermis*	*Notopterus notopterus*	Notopteridae
Dactylogyrus n. sp. A	*Cyprinus carpio*	Cyprinidae			

CONCLUSIONS

Despite of the presence of number of parasites (both cases of single species and co-existing species), the fish hosts show no clinical signs of disease. This strongly suggests that the presence of parasites on their fish hosts is a common phenomenon, and it is not necessary to remedy. Pond and water quality as well as feeding could be more concerned to keep the fish healthy.

The important issue here is to determine which of the co-existing species would become potential pathogen(s) under intensive culture. It is difficult presently to ascertain whether a specific species or a species with wide host range could become a potential pathogen.

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Growth and Resistance to *Aeromonas hydrophila* of Indian Major Carp, Rohu (*Labeo rohita*) in Cisterns Treated with Sugarcane Bagasse as Artificial Substrate

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ABSTRACT

Biofilm of *Aeromonas hydrophila* developed *in vitro* when used as an oral vaccine gave a higher antibody titre and protection in carps compared with the free cells. The present study evaluated the effect of a biofilm developed on artificial substrates in carp ponds and their resistance to *A. hydrophila* infection. Fingerlings of rohu, *Labeo rohita,* were reared for 98 days in three treatments, namely: (i) sugarcane bagasse + cattle dung (SCD), (ii) sugarcane bagasse (S) and (iii) cattle dung (CD), on an equal dry weight basis. At the end of the 98 day period the specific growth rate of rohu was significantly higher ($P<0.05$) in SCD compared with CD and S groups. However, there was no significant difference ($P<0.05$) in the average survival between the 3 treatments. Rohu reared in the biofilm promoted ponds (SCD and S) had significantly higher antibody titres and protection upon challenge against *A. hydrophila* compared with those from cisterns treated alone with cattle dung. Promotion of biofilm had dual advantage in increasing fish growth and resistance against *A. hydrophila*.

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INTRODUCTION

Heterotrophs are the essential component of the food web, as these organisms decompose organic matter and release nutrients for algae and are also consumed directly by fish and fish food organisms (Colman and Edwards, 1987; Moriarty, 1997). Furthermore, they are not dependent on light and their nitrogen uptake potential to produce protein is higher than algae (Avnimelech *et al.,* 1986, 1992). Therefore, there is great scope for promotion and exploitation of heterotrophs for increasing aquaculture production. However, natural exploitation of heterotrophs by fish and fish food organisms is difficult and not economical, because of their small size which is a limiting factor. Exploiting the potential of heterotrophs through promotion of biofilm immobilized on artificial substrates was demonstrated as a strategy for boosting aquaculture production by Shankar *et al.* (1998). The potential of microbial biofilm on artificial substrate to increase fish production was later confirmed by several investigators (Ramesh *et al.,* 1999; Umesh *et al.,* 1999; Joice, *et al.,* 2002; Mridula *et al.,* 2003). In these studies, microbial biofilm promoted on artificial substrate such as sugarcane bagasse, paddy straw and *Eichhornea* leaves could boost fish growth by 50% compared with substrate free control. Furthermore, employing artificial substrates such as bamboo increased the growth of rohu by 77% (Wahab *et al.*, 1999) and growth of mahseer, *Tor putitora,* by 42% (Keshavanath *et al.,* 2001). Higher yield of planktophagic fish have also been recorded in traditional fishing methods such as acadja fisheries of West Africa (Welcome, 1972), brush parks of Sri Lanka (Sena Nayake, 1981), artificial reefs of the Philippines (Waltermath and Schirm, 1995) and floating islands of Loktak in India (Suresh, 1999).

Growth performance of *Oreochromis mossambicus* (Huchette *et al.,* 2000), *Catla catla, Labeo rohita, Cirrhinus mrigala* and *Cyprinus carpio* (Ramesh *et. al.,* 1999; Umesh *et al.,* 1999), *Labeo calbasu* (Wahab *et al.,* 1999), mahseer (Keshavanath *et al.,* 2001) and *Labeo fimbriatus* (Mridula *et al.,* 2003) have been evaluated with different substrates. Among these, in general browsers with fringed lips such as rohu, calbasu and tilapia grew better than others and the production was 50 to 77% higher in substrate-based culture systems compared with control systems.

Several observations, as well as controlled experiments, have indicated that heterotrophic production could also act as antagonists against pathogens in aquaculture systems (Avnimelech and Ritoo, 2001). Interestingly, in our preliminary studies, significantly higher serum agglutinating antibody titres and protection against *Aeromonas hydrophila* was recorded in common carp fry grown in ponds promoted with biofilm on sugarcane bagasse compared with the control ponds (Joice *et al.,* 2002). Earlier in our studies, biofilm of *A. hydrophila* developed *in vitro* and fed to Indian Major Carps (IMCs) as an oral vaccine through their feed induced significantly higher humoral and protective responses compared with the free-cell vaccine (Azad *et al.,* 1999a, 1999b and 2000). In biofilm mode, a bacterial pathogen occurring in several layers act as a biocapsule, which can withstand foregut destruction and can reach immune responsive hindgut to give better response and protection. The bacterium *A. hydrophila* is found ubiquitously in the aquatic environment and is regarded as an opportunistic pathogen frequently associated with haemorrhagic septicemia in carps, eels, milkfish, channel catfish, tilapia and ayu (Roberts *et al.,* 1992; Kuge *et al.,* 1992; Thune *et al.,* 1993; Leung *et al.,* 1995; Angka *et al.,* 1995).

Higher serum antibody agglutination titres and protection against *A. hydrophila* was observed in common carp reared from spawn to fry in a biofilm promoted system (Joice *et al*., 2002). These findings encouraged us to initiate further detailed study on growth and resistance in rohu - a column feeder with browsing habitat considered as appropriate model for biofilm exploitation in cisterns treated with sugarcane bagasse, with or without cattle dung.

MATERIALS AND METHODS

The experiment was carried out for 98 days in nine cement cisterns (5×5×1m) each with 15 cm soil base and filled with water to 80 ± 2 cm. Among the three treatments, treatment SCD received 0.8 unit (4 kg) of sugarcane bagasse and 0.2 unit (5 kg) of cattle dung, treatment S received 1.0 unit (5 kg) of sugarcane bagasse and treatment CD received 1.0 unit (25 kg) of cattle dung on dry weight basis. Cattle dung was procured from a local farm and sugarcane bagasse from juice extraction centers. The dosage of sugarcane bagasse and cattle dung was fixed based on dry weight of cattle dung, which is usually applied in fish culture ponds at 10,000 kg wet weight/ha. Sugarcane bagasse tied in 2-3 feet long bundles were suspended in the water column from horizontal bamboo beams. Fresh cattle dung was applied in the form of slurry.

Fingerlings of rohu, *Labeo rohita* (average weight of 2.08 g), procured from the government fish farm, B.R. Project, Karnataka State, were stocked at a density of 25/cistern (10000/ ha.), one week after the addition of bagasse and cattle dung.

Water parameters

Water was analysed once every three days for a number of different parameters. Temperature and pH were recorded using a Horiba water quality analyzer (Model U-10). Dissolved oxygen, total ammonia, nitrite and nitrate were measured following standard procedures (APHA, 1995).

Microbial enumeration

Total plate count (TPC) of bacteria in water and on sugarcane bagasse was estimated weekly on nutrient agar by the spread plate method (Ramesh *et al.,* 1999; Umesh *et al*., 1999). *A. hydrophila* was enumerated weekly by spread plate technique using *Aeromonas* isolation medium consisting of *Aeromonas* selective supplement (Hi Media, Bombay). *Nitrosomonas* sp*.,* and *Nitrobacter* sp*.,* in water and on substrate were enumerated weekly by the method of Rodina (1972). For estimation of TPC of bacteria, *A. hydrophila* and nitrifying bacteria on substrate, a known weight of bagasse was taken in a test tube containing physiological saline and bacterial cells in biofilm dislodged by vortexing for 3 mins. The cells were enumerated after culture and expressed as No/g.

Fish growth and survival

At least 50% of the stocked fish were collected and their individual length and total weight recorded at 15 days interval. However, on termination, at the end of 90 days, length and weight of all the surviving fish were recorded. Fish growth data obtained was subjected to ANOVA and Duncan's multiple range tests at $P < 0.05$ (Duncan, 1955; Snedecor and Cochran, 1968).

Evaluation of immune response and protection against *A. hydrophila*

Ninety days post-rearing of the fish, the substrate was removed in SCD and S and water exchanged fully in all the 3 treatments. Fishes were reared in these tanks for further 45 days on an artificial diet at 5% body weight. During this rearing period blood was drawn from five fishes in each treatment at an interval of 15 days and antibody titre estimated by agglutination assay (Sundick and Rose, 1980), and titre expressed as $\log_2$ values based on visual observations. The bacterium *A. hydrophila* (isolate AAh 2/96) was recovered from naturally infected *Clarius batrachus* and was obtained from the Central Institute of Freshwater Aquaculture, Bhubaneswar, India and used for the agglutination assay.

After 30 days of rearing in ponds providing artificial diet, twelve fishes from each treatment were transferred to 50L fiber-reinforced plastic tubs and each fish challenged with *A. hydrophila* (AAh 2/96). The bacterial isolate (*A. hydrophila*) was grown for 24 hrs in Tryptone Soya Broth (TSB) and used for challenge experiments. Bacterial growth from nutrient agar slants were harvested into 10 ml of 0.01M sterile phosphate buffered saline (PBS), centrifuged at 4,000 rpm for 20 min and the cell pellet resuspended in 10ml of 0.01M sterile PBS. After washing a further 3 times with sterile PBS, the bacterial concentration was determined using a spectrophotometer at 575nm and the cell density adjusted to 10^8 cell/ml. The dosage was determined by LD_{50} of the bacterium by injecting different doses of *A. hydrophila*. Fish from different treatment groups were administered with 0.1 ml of the pathogen (10^8 cells/ml) suspension by intramuscular injection. Fishes were observed for a week and external signs and mortality rates were recorded. Specific mortality of fish was confirmed by the re-isolation of the pathogen from the fish. Relative percentage survival (RPS) of fish was determined according to Amend, (1981) using the following formula:

$$RPS = 1 - \frac{\text{Percentage mortality in treatment}}{\text{Percentage mortality in control}} \times 100$$

RESULTS

Growth and survival

Significant difference ($P<0.05$) in average growth of rohu between the treatments was observed (Table 1). The growth was highest in SCD (38.97 g), followed by CD (31.93 g) and S (18.46 g). The overall survival of the fish was highest in the SCD (91.33%), followed by S (85.33%) and CD (84.00%). However, there was no significant difference ($P<0.05$) in the average survival of rohu between the treatments.

Table 1. Growth, survival and specific growth rate of *Labeo rohita.*

Parameter	Treatments		
	SCD	S	CD
Av. initial weight (g)	2.08 [a]	2.08 [a]	2.08 [a]
Av. final weight (g)	38.97 [a]	18.46 [b]	31.93 [c]
SGR (%/day)	1.41 [a]	1.06 [b]	1.32 [c]
Survival (%)	91.33 [a]	85.33 [a]	84.00 [a]

* Values with the same superscript in each row are not significantly different ($P < 0.05$)
SCD – Cisterns with sugarcane bagasse and cattle dung
S - Cisterns with sugarcane bagasse alone
CD - Cisterns with cattle dung alone

Microbial count

TPC of bacteria

In Table 2, details of microbial enumeration, total plate count (TPC) of bacteria *A. hydrophila* and nitrifying bacteria are given. Following the addition of cattle dung and bagasse, TPC of bacteria in water increased rapidly reaching a peak on day 15 in all the treatments (Figure 1a). Mean TPC ($\times 10^4$/ml) was significantly higher in SCD (13.51) followed by S (11.41) and CD (10.47). TPC of bacteria on bagasse also reached a peak on day 15, following which there was a gradual decrease (Figure 1b). The mean TPC of bacteria on bagasse ($\times 10^6$/g) was significantly higher in SCD (19.14) than in S (9.85). Overall, bacterial number on bagasse per unit weight was 100 times higher than that in water.

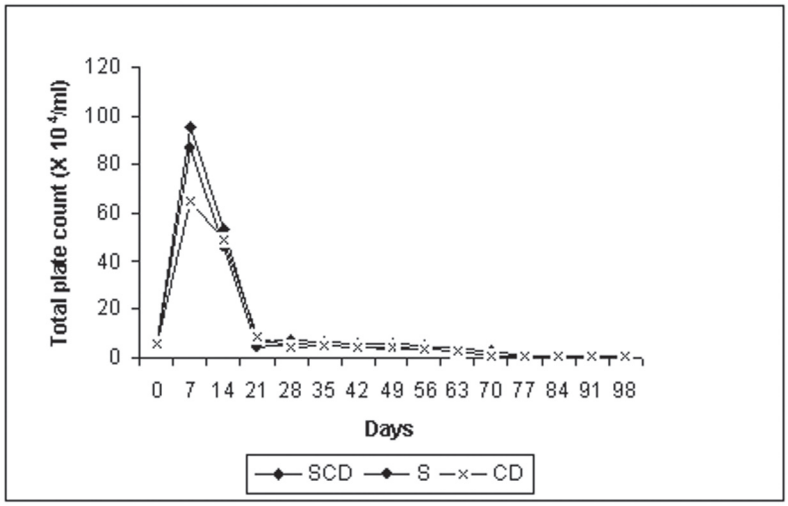
Aeromonas hydrophila count

There was a gradual increase in the *A. hydrophila* count in water after the addition of bagasse and cattle dung in S and SCD groups compared with CD. However, this was not statistically significant (Table 2). The peak was observed on day 21 in water and on bagasse in all the treatments following which there was a gradual decline. The mean *A. hydrophila* count in water ($\times 10^2$/ml) was almost similar in all the treatments with slightly higher values recorded in SCD (0.55) followed by S (0.45) and CD (0.40) (Table 2). The *A. hydrophila* count on bagasse ($\times 10^4$/g) was significantly higher in SCD (4.79) than in S (3.48).

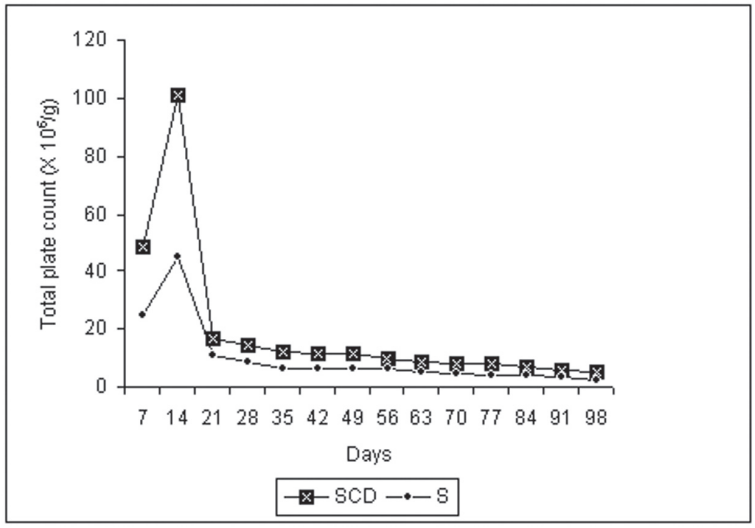
Water parameters

The mean values of water parameters are presented in Table 3. Following the addition of bagasse and cattle dung, dissolved oxygen dropped sharply and it was below 2.0 mg/l in all the treatments during the first week. However, during the subsequent weeks there was marked improvement in the dissolved oxygen. Mean dissolved oxygen (mg/l) was

(a)



(b)



SCD – Cisterns with sugarcane bagasse and cattle dung

S - Cisterns with sugarcane bagasse alone

CD - Cisterns with cattle dung alone

Figure 1. Total plate count of bacteria in (a) water and (b) on substrate in different treatments.

Table 2. Mean values of total plate count (TPC) of bacteria, *Nitrosomonas* sp., *Nitrobacter* sp. and *Aeromonas hydrophila.*

Parameter	Treatments		
	SCD	S	CD
TPC of bacteria in water ($\times 10^4 ml^{-1}$)	13.51 [a] (0.60-123.0)	11.41 [b] (0.38-59.0)	10.47 [c] (0.50-63.0)
TPC of bacteria on substrate ($\times 10^6 g^{-1}$)	19.14 [a] (4.0-127.0)	9.85 [b] (0.09-51.0)	-
Nitrosomonas sp. in water ($\times 10^2 ml^{-1}$)	1.75 [a] (0.0-5.0)	1.31 [a] (0.0-4.0)	1.80 [a] (0.0-5.0)
Nitrosomonas sp. on substrate (No.$\times 10^3$ g^{-1})	9.79 [a] (2.00-23.0)	7.95 [b] (1.0-19.0)	-
Nitrobacter sp. in water (No.$\times 10^2$ ml^{-1})	2.22 [a] (0.0-6.0)	1.82 [a] (0.0-3.0)	1.99 [a] (0.0-4.0)
Nitrobacter sp. on substrate (No.$\times 10^3$ g^{-1})	11.95 [a] (2.0-27.0)	8.17 [b] (2.0-19.0)	-
Aeromonas hydrophila in water (No.$\times 10^2$ ml^{-1})	0.55 [a] (0.10-1.54)	0.45 [a] (0.10-1.35)	0.40 [a] (0.06-1.32)
Aeromonas hydrophila on substrate (No.$\times 10^4$ g^{-1})	4.79 [a] (0.14-27.20)	3.48 [b] (0.06-21.0)	-

* Values are means of three tanks and fifteen sampling days (N = 45) for water and three tanks and fourteen sampling dates for substrates (N = 42).

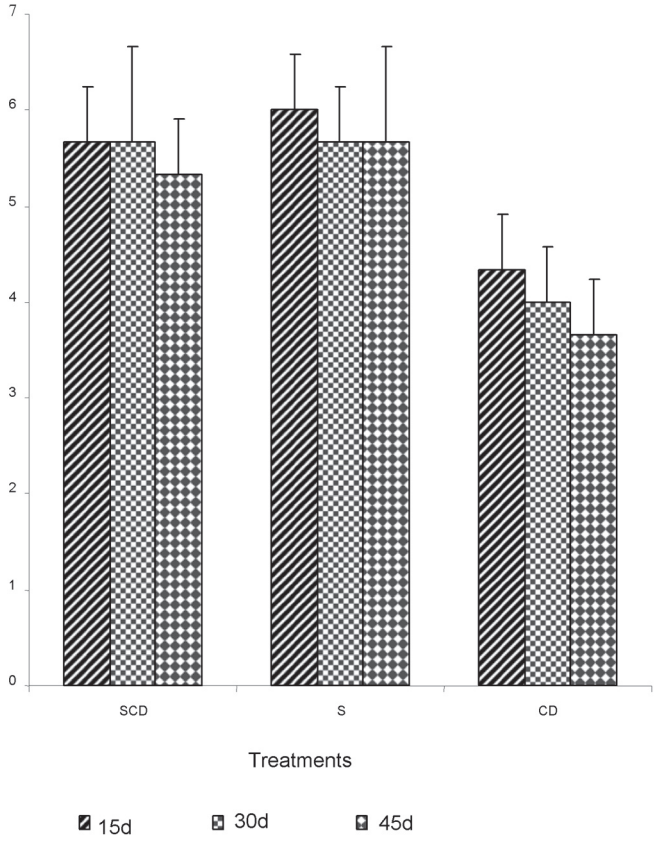
** Values in parenthesis indicate range

*** Values with the same superscript in each row are not significantly different ($P < 0.05$)

SCD – Cisterns with sugarcane bagasse and cattle dung

S - Cisterns with sugarcane bagasse alone

CD - Cisterns with cattle dung alone



SCD – Cisterns with sugarcane bagasse and cattle dung

S - Cisterns with sugarcane bagasse alone

CD - Cisterns with cattle dung alone

Figure 2. Serum agglutination titer at 15, 30 and 45 days after 90 days of rearing in biofilm promoted tanks in different treatments.

significantly higher ($p<0.05$) in SCD (8.06) than that in CD (7.90) and S (7.72). The mean total ammonia (µg at./l) was significantly lower in SCD (3.49) and S (3.99) than that in CD (5.07), while the mean nitrate-nitrogen (µg at./l) was significantly higher in SCD (2.19) compared with S (1.80) and CD (1.80).

Immune response and protection in *Labeo rohita* against *A. hydrophila*

Antibody titre and protection

Mean antibody titre ($-\log_2$) in the fish was significantly higher in SCD (5.55) and S (5.77) than that of CD (3.99). A reduction in the antibody titre was recorded 15 days after harvest in all the treatments which was more pronounced in CD (Figure 2).

Relative percentage survival (RPS)

The mortality rate was 8.33% in SCD, 0% in S and 75% in CD after challenging with *A. hydrophila,* AAh 2/96 (Table 4). Relative percentage survival was higher in S (100) than that of SCD (88.89).

Table 3. Mean values of water quality parameters.

Parameter	Treatments		
	SCD	S	CD
Water temperature (°C)	28.20 [a] (24.2-31.5)	28.00 [a] (24.0-31.3)	28.40 [a] (24.4-31.2)
pH	7.80 [a] (7.06-8.22)	7.60 [a] (7.02-8.23)	7.80 [a] (7.02-8.27)
Dissolved oxygen (mg/l)	8.06 [a] (2.28-9.13)	7.72 [a] (2.28-9.13)	7.90 [a] (1.37-9.58)
Total ammonia (µg at./l)	3.49 [a] (2.09-7.35)	3.99 [a] (2.50-6.75)	5.07 [b] (2.48-9.68)
Nitrite-nitrogen (µg at./l)	1.29 [a] (0.44-2.04)	1.36 [a] (0.60-2.16)	1.81 [b] (0.78-2.96)
Nitrate-nitrogen (µg at./l)	2.19 [a] (0.54-5.24)	1.89 [b] (0.54-4.24)	1.80 [b] (0.54-4.04)

* Values are means of three tanks and thirty-four sampling days (N = 102).
** Values in parenthesis indicate range
*** Values with the same superscript in each row are significantly different (P>0.05)

SCD – Cisterns with sugarcane bagasse and cattle dung
S - Cisterns with sugarcane bagasse alone
CD - Cisterns with cattle dung alone

Table 4. Relative percentage survival in different treatments after challenging against *A. hydrophila*.

Treatments	No. of fish challenged	No. of fish survived	Percentage survival (%)	Relative percentage survival (RPS)
SCD	12	11	91.66	88.89
S	12	12	100	100
CD	12	3	25	-

SCD – Cisterns with sugarcane bagasse and cattle dung
S - Cisterns with sugarcane bagasse alone
CD - Cisterns with cattle dung alone

DISCUSSION

Growth of rohu was highest in SCD followed by CD and S and there was significant difference in growth between them. However, there was no significant difference in survival of fish between the treatments. Better growth and survival of rohu and other IMCs with substrate supplemented with manure compared with manure or substrate alone has been reported by us and other authors (Ramesh *et al.,* 1999; Umesh *et al.,* 1999; Joice, *et al.,* 2002; Mridula *et al.,* 2003). The TPC of bacteria in the water was higher in SCD compared with S and CD treatment groups. It is interesting that CD had a lower total plate count than the group in the S treatment, which could be due to a higher density of TPC on the S substrate. Total bacterial plate counts on this substrate was 100 fold higher than those calculated for the water per unit. However, there was significant difference in TPC between SCD and S. Therefore, overall, it is total higher TPC on substrate and in water in the SCD than that in S or CD on which rohu could browse. This may be the reason for the enhanced growth and survival in these treatment groups. Rohu is a column browser (Das and Moitra, 1955; Dewan *et al.,* 1991) and is known to thrive well by browsing on substrates (NFEP, 1997). Plankton production was not enumerated because our earlier studies have demonstrated clearly that higher TPC on substrate favour higher plankton production (Ramesh *et al.,* 1999; Umesh *et al.,* 1999; Joice, *et al.,* 2002; Mridula *et al.,* 2003). Better growth and survival in SCD compared with CD or S could also be due to lower ammonia and nitrite in the former treatment. Such low level of ammonia and nitrite have been observed in substrate added ponds (Langis *et al.,* 1988; Ramesh *et al.,* 1999; Umesh *et al.,* 1999; Joice, *et al.,* 2002; Mridula *et al.,* 2003). Additional growth of nitrifying bacteria on the substrate could have acted as an *in situ* biofilter.

Antibody titre against *A. hydrophila* in rohu was highest in SCD followed by S and CD. Little difference was detected in the antibody titres between the SCD and S-treatment groups compared with the wide difference between SCD and CD or S and CD. Interestingly, growth pattern was also different being higher in SCD followed by CD and S. The *A. hydrophila* count in the water was slightly higher in the SCD group followed by S and CD. However, A. *hydrophila* count on the substrates was 100 fold higher in the SCD than in S. The data generated from this study would suggest that the higher *A. hydrophila* concentration on the substrate has favoured a higher antibody titre in the SCD group.

Biofilm of *A. hydrophila* on the substrate could have acted as *in situ* immunomodulator such as a bacterin, in rohu when exposed to the pathogen. Oral vaccination of carps with biofilm of *A. hydrophila* incorporated in feed has given better antibody titre and protection compared with free cell (Azad *et al*., 1999a and 1999b). Furthermore, it was interesting to note that the antibody titre dropped more rapidly after 15 days in CD compared with that in SCD and S. This could be due to retention of *A. hydrophila* biofilm antigen in immune response sites of the fish for a longer duration compared with that of free cell antigens in CD treated groups. Longer retention of biofilm antigen in immune responsive sites with higher antibody titre in carps has been demonstrated by antigen localization with monoclonal antibody in immunohistochemistry (Azad *et al*., 2000).

The relative percentage survival of the experimental groups was higher in those given SCD and S treatments which corresponded with a higher antibody titre in both these group. In contrast, a very poor survival was noticed in the CD fish group in which the antibody titre was low and was decreasing rapidly. This supports the hypothesis that there was an immunomodulation in the SCD and S groups leading to an enhanced protection against bacterial challenge. This also clearly shows the importance of biofilm on substrate to boost resistance against opportunistic bacterial pathogens found ubiquitously in the aqueous environment such as *A. hydrophila*. These findings support our earlier observations of higher antibody titre and RPS recorded in common carp spawn reared in substrate-based ponds compared with control (Joice *et al*., 2002). Several observations as well as controlled experiments have indicated that heterotrophic population in pond act as antagonists against pathogens (Avnimelech and Ritoo, 2001).

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Two Cases of Streptococcal Infections of Cultured Tilapia in Asia

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ABSTRACT

Tilapia (*Oreochromis niloticus*) is one of the most popular cultured species in both Thailand and Indonesia. Initial outbreak of mass mortality in cultured tilapia in Thailand was observed in floating net cages of the Mekong River in Mukudahan City, northeast Thailand in May 2001. The mortality in the cages lasted approximately two weeks and reached around 40 to 60%. Affected fish showed abdominal distention due to ascites, a watery-like substance in the intestinal cavity and abscesses on the peduncle. In tilapia ponds, lower total percentage overall mortalities were observed daily, and continuously in the ponds with running water from the irrigation channel in Lubuk Linggau City, South Sumatra in 2002 and 2003. These fish had opaque discoloured eyes with bilateral exophthalmia. The bacteria isolated from the brains and other organs of the affected tilapia from Thailand and Indonesia were identified as Gram-positive, non-motile cocci. These bacterial isolates from infected tilapia in both Thailand and Indonesia were identified as *Streptococcus agalactiae* and *S. iniae*, respectively.

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INTRODUCTION

Tilapia *Oreochromis niloticus* is one of the most popular fish species produced in freshwater aquaculture in many Asian countries. This fish species is popular due to its rapid growth and high adaptability to the environment. A serious obstacle to the sustainability and development of tilapia culture, however, is the frequent occurrences of mortality due to bacterial infection. The most commonly reported bacterial infection resulting in high mortalities was associated with the β-hemolytic streptococci, *Streptococcus iniae* (Sako, 1993; Ramesh *et al*., 1994; Sugita, 1996). *Streptococcus shiloi* was found responsible for a bacterial meningoencephalitis in tilapia in Israel (Eldar *et al*., 1994), which has become a synonym for *S. iniae* (Eldar *et al*., 1995). The Lansfield B type cocci identified as *Streptococcus agalactiae* was more known to cause mastitis of cattle, but recently this species has been found in wild mullet *Liza klunzingeri*, causing mass mortality in Kuwait in 2001 (Evans *et al*., 2002). A streptococcus that was recovered and isolated from infected tilapia was originally identified as *Streptococcus difficile* (Eldar *et al*., 1994) but this has been finally characterized as *S. agalactiae* (Evans *et al*., 2002).

Identification of *S. iniae* and *S. agalactiae* can be completed by biochemical tests using API 20 *strep* or API 32 *strep* test (BioMerieux, USA) or a serological test using the Slidex strepto kit for grouping of β-hemolytic streptococci groups (BioMerieux, USA) (Yuasa *et al*., 1999; Evans *et al*., 2002). Recently an oligonucleotide probe array has been recognized as a rapid and accurate diagnosis for bacterial infection of fish (Matsuyama *et al*., 2006). In the present study, *S. agalactiae* and *S. iniae* were both recovered from diseased tilapia farmed in two different aquaculture systems in Thailand and Indonesia, respectively.

MATERIALS AND METHODS

Mortality and clinical signs of fish in the sampling sites

In May 2001, mass mortality of cultured tilapia occurred in the net cages floating on Mekong River in Mukdahan City, Morth-east Thailand (Figure 1; Plate 1, Photo A). In a colony of floating cages including 10 units (farms), 3 units located in the middle of the floating cages were affected by the mortality. Cumulative mortalities of the units reached 40-60% during two weeks, and total losses of fish in each unit reached around 2 tons. Affected fish, around 300-500 g in body weight, showed expanded abdomen, deposit of water-like in the intestine and swelling at the peduncle with grayish pus inside (Plate 1, Photos B, C, and D). Bacterial detection and identification in Thailand were attempted in one of the units.

On the other hand, in Indonesia, mass mortalities of cultured tilapia were observed at several farms where fish were reared in concrete ponds with running water system in Lubuk Linggau, South Sumatra, Indonesia in 2002 and 2003 (Figure 1; Plate 1, Photo E). Inlet water for all farms was supplied from an irrigation channel and water temperature in the channel was at around 23^{o}C through the year. Mortality was lower than that in the Thailand case, maximum of 0.1-0.2% per day, but continuously observed throughout

the year. Affected fish characteristically showed the bilateral exophthalmia with opaque coloration (Plate 1, Photo F). Bacterial detection from affected fish in Indonesia was attempted at 3 farms.

Detection and isolation of the bacteria

Recovery of the bacteria was performed by insertion of flamed needle into the spleen, kidney, liver and grayish pus in peduncle of 5 moribund fish in Thailand and into additional brains and eye socket of 5 moribund per site in Indonesia, and streaked directly on to brain heart infusion (BHI) agar and incubated at 25°C for 2 days. The colony growth was observed after 2 days and sub-cultured onto fresh BHI agar if the culture onto fresh BHI agar if the cultures were not pure.

A small section of each of the organs in the fish in Indonesia was aseptically removed from the animals with clinical signs of disease and a tissue smear prepared on a clean microscope glass slide. This was fixed with absolute methanol for 3 min and stained with 5% Giemsa solution for 30 min at room temperature, and viewed by light microscopy at x 100 magnification under immersion oil.

Bacterial identification tests

Conventional identification of bacteria

Single colonies of pure isolated bacteria was applied on the slide glass with a drop of distilled water, left to air dry and then heat-fixed by passing through a flame. These slides were then gram-stained using Gram stain kit (Nissui, Japan). Production of oxidase was observed by using the cytocrome-oxidase paper test (Nissui, Japan). Catalase production was confirmed by bubble production in 3% hydrogen peroxide (Wako, Japan). The oxidative-fermentative (O/F) test, production of H_2S and haemolysis tests were conducted using O/F medium (Nissui, Japan), SIM medium (Nissui, Japan) and 5% sheep blood agar (Nissui, Japan), respectively. Motility of bacteria was confirmed by turbidity in SIM medium. Growth of bacteria at 45 °C was determined by incubation in BHI broth (Nissui, Japan) for 24 hr. Additional biochemical profiles of the pure isolates were identified using API 20 Strep. kit (BioMerieux, USA) examined at 25°C or 35°C. Isolates used in the tests were B44005 for tilapia from Thailand and JF0375 and JF0381 from tilapia of Indonesia. Additionally, 2 known bacterial isolates identified as NJM0101 from yellowtail *Seriola quinqueradiata* of Japan and 8-1 Br from mullet *Liza klunzingeri* of Kuwait were used as references of *Lactococcus garvieae* and *Streptococcus agalactiae*, respectively (Table 1).

Oligonucleotide probe array

Oligonucleotide probes targeting one or two specific regions for each species in the 16S rDNA were used for spotting and immobilizing on a nylon membrane (Table 2). Prepared probes included 5 kinds of species causing streptococci in fish. These probes were designed from the unique regions to each species based on the alignment of sequences described in a GenBank database. A specific probe for *S. agalactiae* and *Enterococcus* sp. was prepared. Two probes were prepared for *L. garvieae*, *S. iniae* or *S. dysgalactiae*, which were used for spotting and immobilizing on a nylon membrane. Extracted DNA of each isolate (Table

1) was amplified using universal primer set AGAGTTTGATCMTGGCTCAG (20F) and GGTTACCTTGTTACGACTT (1500R), by PCR in order to amplify the conservative region of each isolate. The PCR reaction was conducted by temperature cycling on an iCycler (Bio-Rad, USA) including an initial denaturation (94^0C for 4 mins), 30 cycles of core PCR production (30s at 94^0C, 30s at 72^0C), and a final elongation (7 mins at 72^0C). Hybridization of Digoxigenin (DIG)-labeled PCR product with each probe was conducted on the nylon membrane that was prepared according to the description by Matsuyama *et al*. (2006). Polaroid film was exposed with luminescent signals produced by alkaline phosphatase-conjugated anti-DIG antibody to observe white spots due to the luminescent exposure. *Lactococcus garvieae*, *S. iniae* and *S. dysgalactiae* were identified when two spots for each species were reacted since the result of single spot was not enough to identify these species and *S. agalactiae* and *Enterococcus* sp. were identified when one spot was reacted.

Table 1. Isolates used in this study

Isolates No.	Hosts	Organs	Sites	Countries	Date of Isolation
JF 0375	tilapia *Tilapia niloticus*	eye socket	running water pond	Indoneisa	Oct. 2003
JF 0381	tilapia *Tilapia niloticus*	brain	running water pond	Indoneisa	Oct. 2003
B 44005	tilapia *Tilapia niloticus*	kidney	Floating cage on river	Thailand	May. 2001
8-1 Br*	mullet *Liza klunzingeri*	brain	Gulf of Kuwait	Kuwait	Oct. 2001
NJM 0101	yellowtail *Seriola quinqueradiata*	kidney	Floating cage on sea	Japan	Oct. 2001

* This isolate was obtained in the reseach on streptococci of fish in the Gulf of Kuwait in cooperature with the Mariculture and Fisheries Department of the Kuwait Institute for Scientific Research of Kuwait in 2001

Table 2. Oligonucleotide probes used for DNA hybridization

Species	Probe No.	Sequence
Lactococcus garvieae	a	CGCCGCTCTTCATAAAAATAGCAGGCTATCTTTAATCATCGCTCGACTTG (110r)
Lactococcus garvieae	b	GTAGTGAAGCAATTGCTTCTTTTAAATAAGAATCATGCGATTCTCATTGT (240r)
Streptococcus iniae	c	TTCGCAACTCTTTGGATTAGTGCAAGCACCAATCCTCAGCGTTCTACTTG (110r)
Streptococcus iniae	d	ATAGTGAAGCAATTGCTCCTTTTAAATTAAGTACATGTGTACTCTAGTGT (240r)
Streptcoccus dysgalactiae	e	TTCGCAACTCCTTGGACCGGTGCAAGCACCAGTCCTCAGCGTTCTACTTG (110r)
Streptcoccus dysgalactiae	f	ATAGTGATGCAGTTGCACCTTTCAAATGAAAGACATGGGTCCTCCATTGT (240r)
Streptcoccus agalactiae	g	ACAGTGAAGCAATTGCTCCTTTTAAATAACTAACATGTGTTAATTACTCT (250r)
Enterococcuc sp.	h	AGCGACACCCGAAAGCGCCTTTCACTCTTATGCCATGCGGCATAAACTGT(240r)

RESULTS

From all internal organs of examined fish in both Thailand and Indonesia, unique small colonies, whitish in color, were isolated using the BHI agar within 24 hr at 25^0C. The number of bacterial colonies obtained from all examined organs were equally numerous in affected fish in Thailand. Whereas on the other hand, the colonies from the brain and eye-socket were much higher in number than these from the spleen, kidney and liver of

Table 3. Biochemical profiles of the isolates used in this study

Items of identification	Isolate No.			
	B44005	8-1Br (*S. agalactiae*)	JF0375, JF0381	BHR9502[3] (*S. iniae*)
Gram Stain at 25°C	+[1], coccal	+, coccal	+, coccal	+, coccal
Size	0.8-1.0μm	0.8-1.0μm	0.8-1.0μm	0.8-1.0μm
Motility at 25°C	-[2]	-	-	-
Cytochrome oxidase	-	-	-	-
Catalase	-	-	-	-
O/F test (25°C)	F	F	F	F
H_2S	-	-	-	-
Haemolysis (25°C)	βtype	βtype	βtype	βtype
Gowth at 45°C	+	+	-	-
Voges-Proskauer	+	+	-	-
Hipputate	+	+	-	-
Esculin	-	-	+	+
Pyrrolidcnylarylamidase	-	-	+	+
αgalactosidase	-	-	-	-
βglucuronidase	+	+	+	+
βgalactosidase	-	-	-	-
Alkaline phosphatase	+	+	+	+
Leucine arylamidase	+	+	+	+
Arginine dihydrolase	+	+	+	+
Acid formation from				
ribose	+	+	+	+
arabinose	-	-	-	-
mannitol	-	-	+	+
sorbitol	-	-	-	-
lactose	-	-	-	-
trehalose	+	+	+	+
inulin	-	-	-	-
raffinose	-	-	-	-
amygdalin	-	-	+	+
glycogen	-	-	+	+

1) +: positive reaction

2) -: negative reaction

3) Referring to Yuasa et al (1999)

affected fish in Indonesia. Stamp smears from all examined organs in fish of Indonesia showed existence of coccal bacteria, 0.8-1.0 μm in diameter, in each organ, but the location of bacteria in the spleen and kidney was limited inside the leucocytes (Plate 1, Photo G).

Conventional identification of bacteria

According to the fundamental bacteriological identification studies performed, bacteria isolated from both fish of Indonesia and Thailand showed the same morphology. These were all Gram-positive, coccal, 0.8 to 1.0 μm in diameter (Plate 1, Photo H). None of the bacteria were motile, and they were negative for the production of catalase and oxidase,

utilized glucose fermentative and showed β haemolysis on blood agar. The main difference between the Thai and Indonesian isolates was growth in the Thai isolate at 45^0C and none from the Indonesian isolate at this temperature.

Biochemical identification using API 20 *strep* at 25^0C and 35^0C were ended within 24 hr and the results between both temperatures were the same. The result of API 20 *strep* for isolates JF0375 and JF0381 from Indonesia were consistent with the result described for *S. iniae* (Yuasa *et al.*, 1999) recovered from infected rabbitfish in Bahrain. The API 20 *strep* results for isolate B4405 from Thailand was consistent with the result for the isolate 8-1 Br from wild mullet, which was identified as *S. agalactiae* at the Laboratory of Fish Pathology of Nippon Veterinary and Animal Science University.

Oligonucleotide probe array

In the oligonucleotide probe array, the PCR products from conservative regions of B4405 or 8-1 Br and NJM0101 were hybridized with only oligonucleotide spots for *S. agalactiae* and *L. garvieae*, respectively. The PCR products from conservative regions of JF0375 and JF0381 were hybridized with two oligonucleotide spots for *S. iniae* as well as an oligonucleotide spot for *S. dysgalactiae*, but not hybridized with another spot for *S. dysgalactiae* (Figure 2). In this case, JF0375 and JF0381 were identified as *S. iniae*.

DISCUSSION

In this study, *S. agalactiae* and *S. iniae,* were recovered and identified from affected fish at a site in Thailand and *S. iniae* recovered from the affected fish at several sites in Indonesia, respectively. *Streptococcus spp.* are recognized globally as causative agents of streptococcal infections in several kinds of seawater and freshwater fishes. However, all isolates from tilapia in Indonesia were identified as *S. iniae*; no *S. agalactiae* was isolated through the year. This may be due to the lower water temperature of Indonesian culture system favouring the recovery of *S. agalactiae*. Actually mass mortality of wild mullet *Liza klunzingeri* due to *S. agalactiae* in Kuwait occurred when temperature of seawater was higher than usual (Evans *et al*., 2002). Unfortunately water temperature at a sampling site in Thailand was not taken in this study; but our sampling was conducted in the hottest season, expectedly with higher temperature; more than 28^0C in the Mekong River at the sampling site. On the contrary, streptococci due to *S. iniae* has been reported in tilapia culture in Japan, a temperate country, and *S. agalactiae* has not been detected from any sick fish species in Japan yet.

Mortality of rabbitfish *Siganus canaliculatus* due to *S. iniae* in Bahrain were more frequently observed in May to June and September to October when water temperature is lower than that in July to August (personal data). These findings suggest that occurrences of streptococcal infection due to two species are water temperature- dependent. It may be considered that *S. agalactiae* may be a pathogen suited to more tropical regions compared with *S. iniae* which may prefer the cooler, temperate regions. In this study, it was found that the *S. agalactiae* grew at 45^0C in the medium, whereas *S. iniae* did not, thus supporting the

Figure 1. Locations of outbreaks of streptococcal infections.

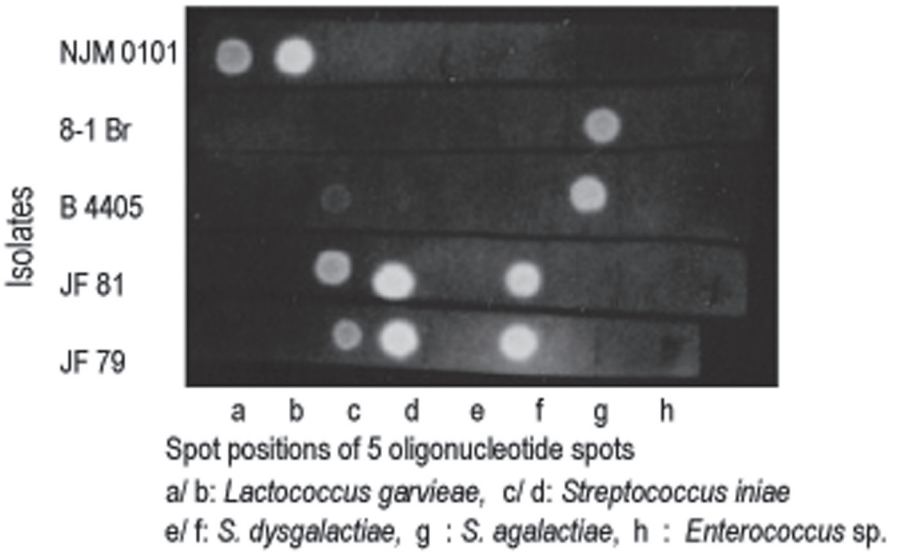

Figure 2. Result of oligonucleotide probe arrays for 5 isolates.

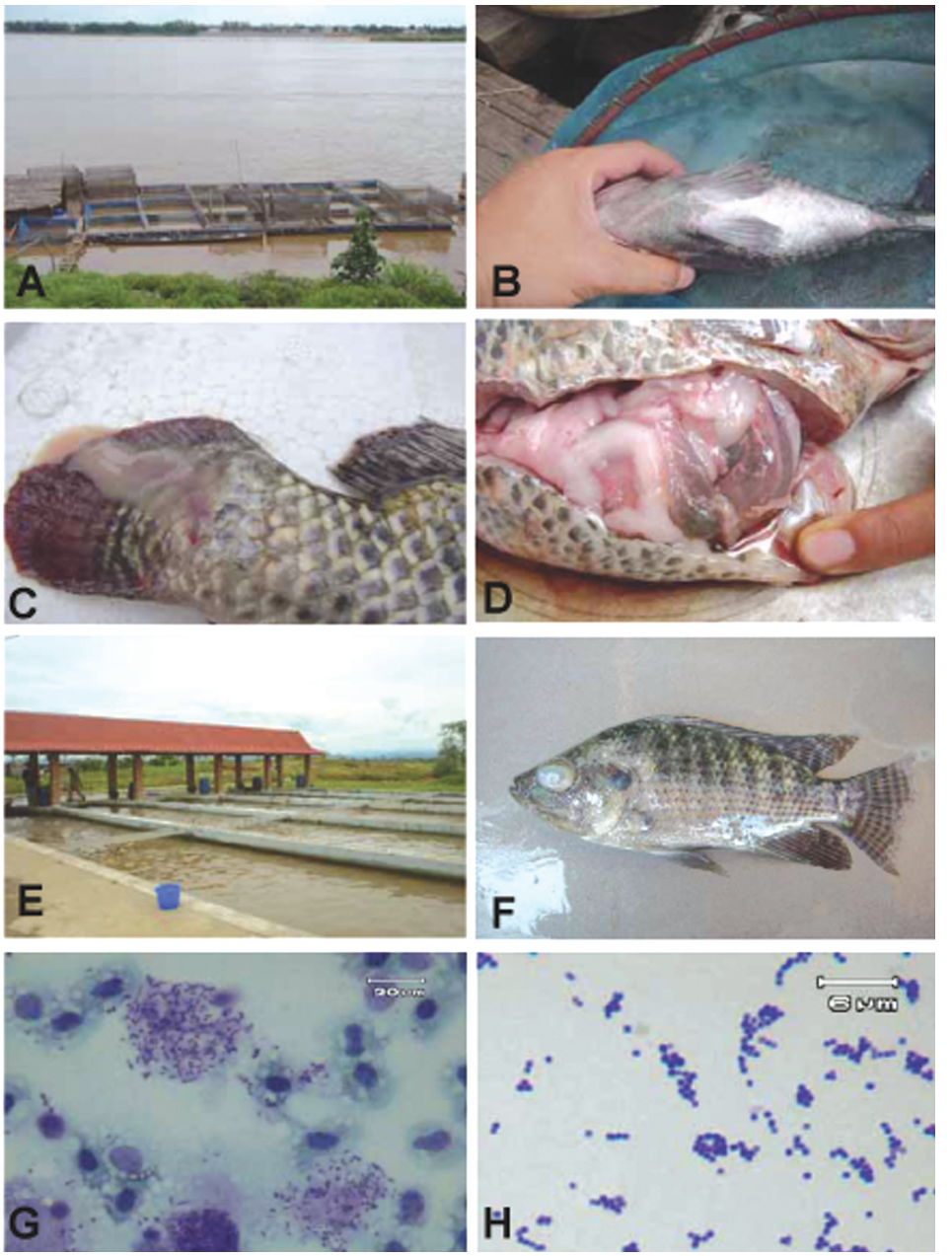

Plate 1. Photographs used in the present study. Photo A. Floating net cages for tilapia culture in Mekong River of North-East Thailand. Photo B. Expanded abdomen of affected fish observed in Thailand. Photo C. Deposit of grayish pus in peduncle of moribund fish in Thailand. Photo D. Deposit of water-like in the intestine of affected fish in Thailand. Photo E. Pond culture of tilapia with running water from irrigation channel in South Sumatra of Indonesia. Photo F. Affected fish in Indonesia, characteristically showing opaque and exophthalmia. Photo G. Stamp smear of spleen from affected fish in Indonesia. Bacteria were mainly located inside leucocytes (Giemsa stain, x1000). Photo H. Gram stain of bacteria isolated from affected fish in Indonesia, showing gram positive, chained, coccal-shaped cells (x1000).

hypothesis that *S. agalactiae* may prefer higher temperature for its growth. Furthermore, *S. agalactiae* was primarily isolated from human and bovine sources, causing neonatal meningitis infections and mastitis, respectively (Evans *et al*. 2002).

The observed clinical signs of the affected tilapia in this study were different depending on the aetiological agent recovered. Infection due to *S. agalactiae* caused high and acute mortality with systemic infection. From these animals numerous bacterial colonies were obtained from the main organs. However, it was observed that *S. iniae* caused chronic mortality with more localised pathology, mainly limited into the brain and eye socket.

Identification of *Streptococcus* species has been generally conducted based on the results of biochemical profiles or serology (Ramesh *et al.*, 1994; Yuasa *et al.*, 1999; Evans *et al.*, 2002). Advances in the use of genetic identification procedures and probes have rapidly developed for other bacterial species and the sequences of the 16S rDNA in bacteria obtained from many microbes are currently widely available in repositories such as Genebank. Matsuyama *et al*. (2006) developed rapid identification method by oligonucleotide DNA array targeting intergenic transcribed spacer regions for bacterial fish pathogens *Vibrio* and *Photobacterium* spp., which is useful for rapid and easy discrimination of *Vibrio* species. The oligonucleotide probe array in this study was conducted following the method described by Matsuyama *et al.* (2006) and were prepared based on the alignment of the 16S rDNA. The results of identification of each isolate by the oligonucleotide probe array was completely consistent with that of biochemical characterization, thus proving that both methods are reliable for identification and differentiation of *Streptococcus* spp. in fish. The oligonucleotide probe array is superior to conventional methods by its rapidity (completed within 8 hrs). Additionally, this new method is available for ethanol-fixed sample without bacterial isolation. It is anticipated that this method may become popular as a rapid and confirmatory diagnosis, due to the accuracy and convenience of use.

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Chinese Herbs (*Lonicera japonica* and *Ganoderma lucidum*) Enhance Non-Specific Immune Response of Tilapia, *Oreochromis niloticus*, and Protection Against *Aeromonas hydrophila*

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ABSTRACT

Three diet variations, in addition to control (no herbs), were used to determine the effect of Chinese herbs (*Lonicera japonica* and *Ganoderma lucidum*) on non-specific immune response of tilapia. These contained 1.0% of *Lonicera*, 1.0% of *Ganoderma* and a mixture of *Ganoderma* (0.5%) and *Lonicera* (0.5%). The diets were fed for 3 weeks. The respiratory burst activity of blood leukocytes, phagocytosis, plasma lysozyme, total protein and total immunoglobulin were monitored. Following three weeks after feeding, fish were infected with *Aeromonas hydrophila* and mortalities recorded. The results of this study showed that feeding tilapia with Ganoderma and Lonicera alone or in combination enhanced phagocytosis by blood phagocytic cells during the whole experimental period and stimulated lysozyme activity after two weeks. Respiratory burst activity of phagocytic blood cells, total protein and total immunoglobulin in plasma were not enhanced. Both herbs when used alone or in combination increased the survival of fish after challenge with *A. hydrophila.* The highest mortality was observed in control fish – 58% and fish fed with Lonicera extract – 43%, while 30% of fish died in the group fed with Ganoderma and the lowest mortality (21%) was observed when fish were fed with a combination of two herbs. Thus, it can be concluded that the herb extracts added to diets acted as immunostimulants and appeared to improve the immune status and disease resistance of fish.

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INTRODUCTION

Diseases caused by *Aeromonas hydrophila* are some of the most widespread in freshwater fish culture. Septicaemia caused by motile aeromonads is a ubiquitous problem that affects fishes found in warm, cool, and cold fresh water around the world (Plumb, 1999). The bacterium, *A. hydrophila* has been associated with disease in carp, eels, milkfish, channel catfish, tilapia and ayu and can also be an opportunist in stress-related diseases in salmonids (Miyazaki and Jo, 1985; Rahman *et al*., 1997; Plumb, 1999). Antibiotics are fequently used to control disease caused by this bacteria, but there is an increasing risk of developing antibiotic resistant strains of bacteria. Vaccines are being developed against *A. hydrophila* and atypical *A. salmonicida* but these are not yet commercially available and as *A. hydrophila* is such a heterogeneous species (multiple strains), vaccine development is extremely complex.

Our research is directed in an alternative, promising area. Herbs can act as immunostimulants, conferring early activation to the non-specific defense mechanisms of fish and elevating the specific immune response. Herbs have been used as medicine and an immune booster for humans for thousands of years in China. Recently, a growing interest has developed in using herbs in animal feeds by both researchers and feed companies. Chinese herbs contain many immunologically active components such as polysaccharides, organic acids, alkaloids, glycosides and volatile oils, which can enhance immune functions. Chinese herbs have been used as medicine to treat different fish diseases in China for many years. Herbs have also been used in the other countries for control of shrimp and fish disease, and successful results have been reported in Mexico, India, Thailand and Japan (Auro de Ocampo, 1993; Dey and Chandra, 1995; Direkbusarakom, 1996; Logambal and Michael, 2000). Recently, there has been increased interest in the immune stimulating function of some herbs in aquaculture. The non specific immune functions such as bacteriolytic activity and leukocyte function were improved by some mixtures of Chinese herbs in shrimp (*Penaeus chinensis*) and tilapia (Luo, 1997; Chansue *et al*., 2000).

Two herbs were selected for the current study: *Lonicera japonica* and *Ganoderma lucidium*. Tilapia (*Oreochromis niloticus*) were fed with the extracts of Chinese herbs either alone or in combination for three weeks to investigate the effect of these substances on the non-specific immune response of tilapia and to examine protection levels against a challenge by *A. hydrophila.*.

MATERIALS AND METHODS

Fish

Tilapia (*O. niloticus)* (52.5±3.50g) were acclimatised in a recirculation system at the Research Institute for Fisheries, Aquaculture and Irrigation (HAKI, Szarvas, Hungary). Fish were fed with a dry feed, produced in the experimental milling facility of the institute and kept in 2000 l fibreglass tanks with water temperature maintained between 22-23^{o}C.

Herbal extracts

Lonicera extract containing 25% of chlorogenic acid, and *Ganoderma* extract containing 30% of *Ganoderma* polysaccharide were commercial products obtained from Xuancheng Baicao Plants Industry and Trade Ltd. China.

Experimental design and sampling procedure

Experiments were performed in the recirculation system of HAKI, Szarvas, Hungary. Batches of three-month old tilapia with an average initial weight of 52.5±3.50 g were held in 100 l fibreglass tanks. Fish were divided onto 8 groups, each group contained 60 fish. Water temperature and pH were constant (22-23^{o}C; pH 8.5) during the experimental period, and dissolved oxygen was maintained at 80-90% of saturation. Water flow was maintained at 7 l/min.

Fish were fed *ad libitum* 6 times daily with the appropriate pelleted feed using an automatic feeder. Three diet variations, in addition to control (no herbs), were used. These contained 1.0% of Lonicera, 1.0% of *Ganoderma* and a mixture of *Ganoderma* (0.5%) and *Lonicera* (0.5%). Each group was fed in duplicate. The diets were fed for 3 weeks. Blood samples (6 fish/group) were collected from caudal vein one, two and three weeks after start of feeding. Heparin was used as an anticoagulant. Individual fish were sampled only once to avoid any influence on the assays due to multiple bleeding and handling stress on the fish.

Separation of leukocytes from the blood

Leucocytes for assay were separated from each blood sample by density-gradient centrifugation. One ml of histopaque 1.119 (Sigma) containing 100 µl of bacto hemagglutination buffer, pH 7.3 (Difco, USA) was dispensed into siliconised tubes. One ml of a mixture of 1.077 density histopaque and hemagglutination buffer and 1 ml of blood was carefully layered on the top. The sample preparations were centrifuged at 700 g for 15 min at 4°C. After centrifugation, plasma was collected and stored at -80°C for future analysis. Separated leukocytes were gently removed and dispensed into siliconised tubes, containing phenol red free Hanks Balanced Salt Solution (HBSS, Sigma). Cells were then washed twice in HBSS and adjusted to $2x10^6$ viable cells/ml.

Respiratory burst activity

Respiratory burst activity of isolated leukocytes was quantified by reduction of ferricytochrome c (Secombes, 1990). Briefly, 100 µl of leukocyte suspension and an equal volume of cytochrome C (2 mg/l in phenol red free HBSS) containing phorbol 12-myristate 13-acetate (PMA, Sigma) at 1 µg/ml were placed in triplicate in microtiter plates. To test specificity another 100 µl of leukocyte suspension and solutions of cytochrome C containing PMA and superoxide dismutase (SOD, Sigma) at 300 U/ml were prepared in triplicate in microtiter plates. Samples were then mixed and incubated at room temperature

for 15 min. Extinctions were measured at 550 nm against a cytochrome C blank in a multiscan spectrophotometer. Readings were converted to nmoles O_2^- by subtracting the O.D. of the PMA/SOD treated supernatant from that treated with PMA given alone for each fish, and converting O.D. to nmoles O_2^- by multiplying by 15.87. Final results were expressed as nmoles O_2^- produced per 10^5 blood leukocytes.

Phagocytosis assay

Phagocytosis activity of blood leukocytes was determined spectrophotometrically by the method of Seely *et al.,* (1990). This assay involves the measurement of congo red-stained yeast cells that have been phagocytosed by cells. To perform the assay, 250 µl of the leukocyte solution was mixed with 500 µl of the congo red-stained and autoclaved yeast cell suspension (providing a yeast cell: leukocyte ratio of 40:1). The mixtures were incubated at room temperature for 60 min. Following incubation, 1 ml of ice-cold HBSS was added and one ml of percoll (1.055) was injected into the bottom of each sample tube. The samples were centrifuged at 850 g for 5 min to separate leukocytes from free yeast cells. Leukocytes were harvested and washed twice in HBSS. The cells were then resuspended in 1 ml trypsin-EDTA solution (5.0 g/l trypsin and 2.0 g/l EDTA, Sigma) and incubated at 37°C overnight. Absorbance of the samples was measured at 510 nm using trypsin-EDTA as a blank.

Lysozyme assay

Plasma lysozyme activity was measured spectrophotometrically by the method of Sankaran and Gurnani (1972). The lysozyme substrate was a 0.02 % (w/v) suspension of Micrococcus lysodeikticus in phosphate buffer (0.05 M, pH 6.2). Lyophilised hen egg white lysozyme was used as a standard. A new standard curve was prepared for each assay. Standard solutions as well as samples were added to the substrate at 25°C. The results were expressed as mg/ml equivalent of hen egg white enzyme activity.

Total protein

Plasma total protein was measured by the biuret method using a commercially available kit (Reanal, Hungary)

Total immunoglobulin

Plasma total immunoglobulin was measured using the method described by Siwicki *et al*., (1994). Analysis of total immunoglobulin in plasma is based on the biuret method. However, primary separation of immunoglobulins from the plasma was achieved by precipitation with polyethylene glycol (PEG) and the resulting supernatant analysed. To perform the assay 100 µl of the plasma was combined with 100 µl 12% PEG and incubated at room temperature for 2 hr while being continuously mixed. Following incubation the mixture was centrifuge for 10 min at 400 g and total protein concentration in the supernatant was determined by the biuret method. Total immunoglobulin value for individuals was calculated from total protein value less the quantity of protein in the supernatant.

Bacterial challenge

Groups of 30 fish from each treatment were challenged by intraperitoneal injection after three weeks of feeding. *A. hydrophila* (strain B-02/12 Bacteriology Laboratory, Institute of Aquaculture University of Stirling, Scotland), isolated in Bangladesh was used as the challenge strain. The bacteria were cultured in tryptone soya broth (TSB) overnight at 28ºC, washed twice with sterile phosphate buffered saline (PBS) and then resuspended in PBS. The concentration of the suspension was determined spectrophotometrically using a pre-made standard curve relating concentration to absorbance at 610 nm. The concentration of bacterial suspension was adjusted accordingly with sterile PBS, prior to performing challenges.

A preliminary challenge using an unrelated, but similar, group of tilapia was performed to establish the LD_{50} dose of the bacterium prior to performing the challenge of fish under study. The LD_{50} was found to be 3×10^6 cells/ml and was used at this rate for the subsequent challenge. Fish were injected intraperitoneally (i.p.) with 0.1 ml of the bacterial suspension. Mortalities were observed for 10 days. Bacterial swabs were taken from the kidneys. All the surviving fish were killed using an overdose of anaesthetic.

Statistics

Results are presented as the average ($\pm$ standard error) for five fish, and were compared at each time point using one way ANOVA and Dunn's multiple range tests (Sigma Stat 3.2). Significant differences between experimental groups were expressed at a significance level of $P<0.05$.

RESULTS

The non-specific defence mechanism values for the fish fed with herb extracts are shown in Figures 1-5. Phagocytic activities of leukocytes were elevated for the whole period of the experiment and each treatment showed values higher than untreated control, the highest value was measured on week three in the group fed a combination of herbs (Figure 1). Herb supplementation in tilapia had no effect on respiratory burst activities of isolated phagocytic cells for the duration of the experiment. The production of oxidative radicals measured during 3 weeks of feeding the fish with all diets remained on the same level (Figure 2). The plasma lysozyme activities were significantly higher after two weeks in groups fed with herb extracts. However, there were no significant differences between treated groups (Figure 3). There were no significant changes in plasma protein (Figure 4) and total immunoglobulin during the whole experiment (Figure 5).

The results from the virulent pathogen challenges are shown in Figure 6. The highest mortality was observed in control fish (–58%) and fish fed with *Lonicera* extract (–43%), while 30% of fish died in the group fed with *Ganoderma* and the lowest mortality (21%) was observed when fish were fed with a combination of two herbs.

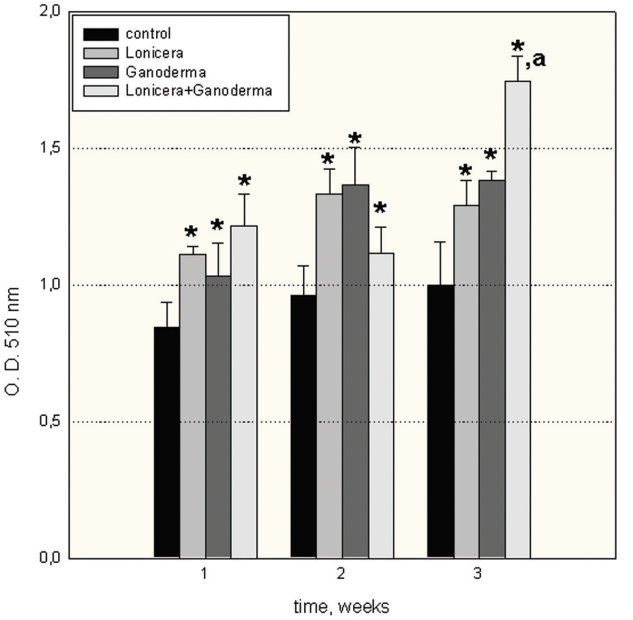

Figure 1. Phagocytic activity of isolated phagocytic cells in tilapia in control group and groups fed diets containing different kind of herbs. Data is expressed as the mean of six fish±SEM. Significance differences ($P<0.05$) from the untreated control are indicated by asterisks. Significant differences among group are indicated by letters.

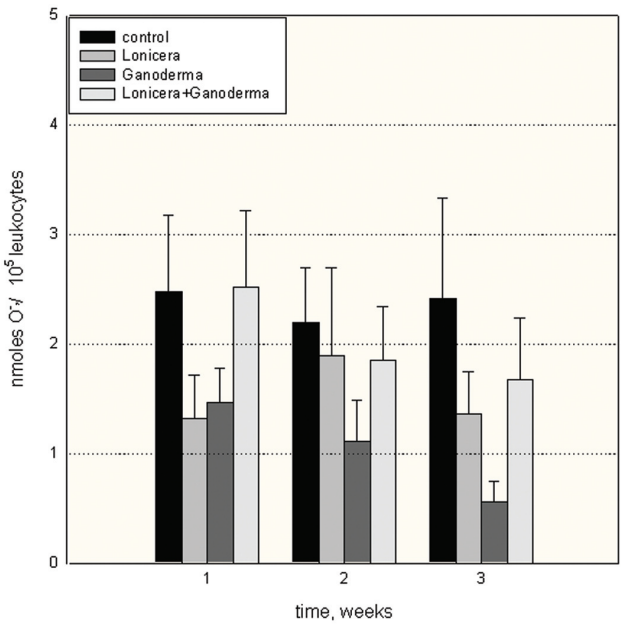

Figure 2. Changes in respiratory burst activities of phagocytic cells isolated from blood in tilapia in control group and groups fed diets containing different kind of herbs. Legends are the same as in Figure 1.

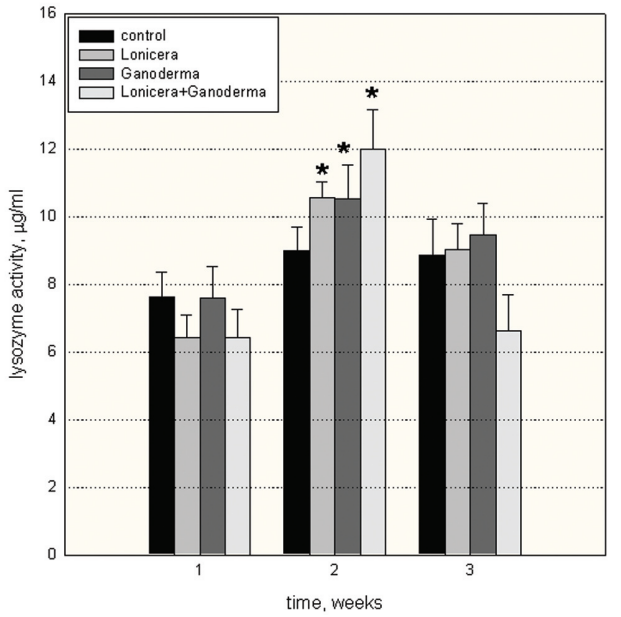

Figure 3. Changes in plasma lyzosyme activities in tilapia in control group and in groups fed diets containing different kind of herbs. Legends are the same as in Figure 1.

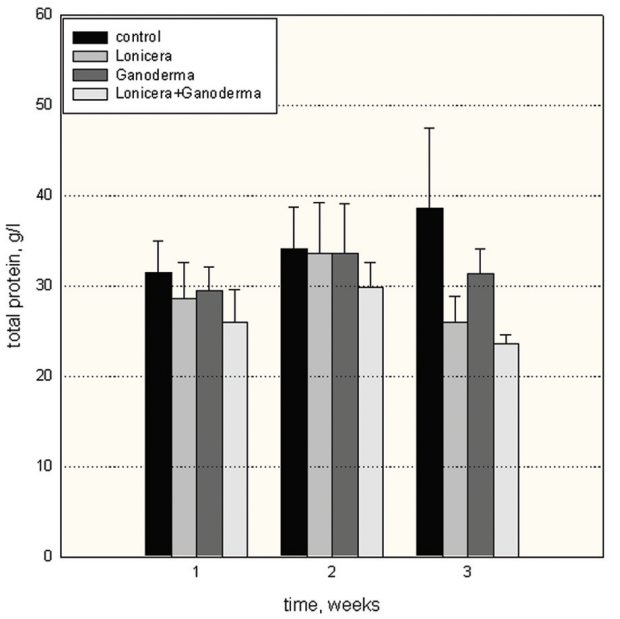

Figure 4. Changes in plasma plasma total protein values in tilapia in control group and in groups fed diets containing different kind of herbs. Legends are the same as in Figure 1.

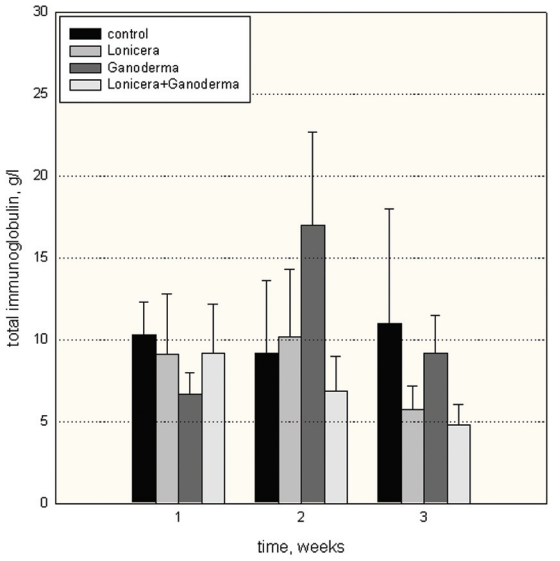

Figure 5. Changes in plasma plasma total immunoglobulin values in tilapia in control group and in groups fed diets containing different kind of herbs. Legends are the same as in Figure 1.

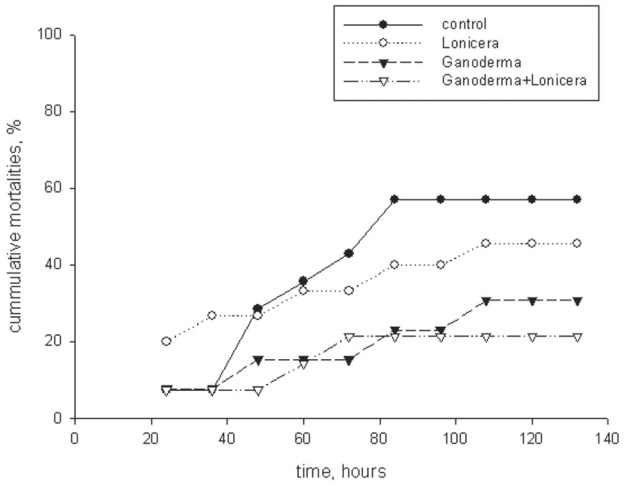

Figure 6. Cumulative mortalities (%) of tilapia in control and in groups fed diets containing different kind of herbs and over a 6 days after artificial challenging with *Aeromonas hydrophila.* Thirty fish from each group were used.

DISCUSSION

In this study, extracts of two Chinese herbs from Ganoderma **(***Ganoderma lucidium*) and from Lonicera (*Lonicera japonica*) were chosen because of their recorded ability to enhance the activity of the immune system. *Ganoderma lucidium* is a traditional Chinese medicine used for the prevention and treatment of various human diseases in China and other Asian countries (Lin, 2001). It has been shown that an aqueous extract from *G. lucidum* will promote phagocytosis by macrophages in mice immunosuppressed by cyclophosphamide (Tang, 2000; Wang *et al.*, 2003), it will stimulate proliferation of lymphocytes induced by concanavalin A or lipopolysaccharide and influence gene expression of cytokines (Wang *et al.,* 1997). The second herb, *L. japonica* has been known as an anti-inflammatory agent and used widely for upper respiratory tract infections, diabetes mellitus and rheumatoid arthritis (Lee *et al.,* 1998). It has been reported that Lonicera significantly increased blood neutrophil activity and promoted phagocytosis by the neutrophils in bovine at the correct concentration (Hu *et. al*., 1992).

The results from this study showed that both *Ganoderma* and *Lonicera* were able to modulate some parameters of the innate immune system of tilapia. The non-specific defence mechanisms of fishes include neutrophil activation, production of peroxidase and oxidative radicals, together with initiation of other inflammatory factors (Ellis, 1977; Ainsworth *et al.,* 1991). In this study fish fed with herb extracts showed an elevated phagocytosis over the whole period of the experiment. Fishes treated with immunostimulants usually show enhanced phagocytosis. Several studies have reported that oral administration of yeast products (MacroGard; Vitastim; *Saccharomyces cerevisiae*), (Aisworth *et al.,* 1994; Siwicki *et al.,* 1994; Jeney *et al.,* 1997), chitin (Sakai *et al.,* 1992), plant extracts, such as ginger increased the phagocytic capability of the cells in rainbow trout (Dügenci *et al.,* 2003) and extracts of four Chinese herbs (*Rheum officinale, Andrographis paniculata, Isatis indigotica, Lonicera japonica*) increased phagocytosis of white blood cells of crucian carp (Chen *et al.*, 2003).

Total lysozyme is a measurable humoral component of the non-specific defence mechanism, and while reports on modulation of the lysozyme activity in fishes are rare, increased values have been recorded by various authors after activation of the immune system with immunomodulants (Engstadt and Robertsen*.,* 1993; Siwicki *et al.,* 1994; Thompson *et al.,* 1995) and by feeding four different Chinese herbs (*Rheum officinale, Andrographis paniculata, Isatis indigotica Lonicera japonica*) (Chen *et al.,* 2003). In our previous study it was shown, that Astragalus enhanced lysozyme activities in tilapia during the whole period of the experiment when fed low (0.1%) and medium (0.5%) doses of herbs, while no effect was found when fish were fed with Scutellaria (Yin *et al.,* 2006).

In this study we used the reduction of ferricytochrome *c* to determine extracellular superoxide anion. Jeney *et al.,* (1997) showed that extracellular activity was very high in fish fed with dietary glucan. Rainbow trout fed with ginger (*Zingiber officinale*) extract had significantly higher extracellular activity of phagocytic cells in blood (Dügenci *et al.,* 2003). However, in this study we could not detect differences in respiratory burst activity in fish nor in our previous studies, when the tilapia were fed with Astragalus extract. Respiratory burst activity of phagocytic cells was not elevated and in the case of

fish fed with Scutellaria there was significant inhibition of extracellular superoxide anion production (Yin *et al.,* 2006). It was shown that in trout fed with nettle and mistletoe extracts the production of extracellular superoxide anion was of a similar level to that in the control fish (Dügenci *et al.,* 2003).

Total protein level and total immunoglobulin was not affected by feeding tilapia with herbs. Immunostimulants usually do not affect total protein levels, but fish with nutritional deficiencies may show lower plasma total protein levels (Siwicki *et al.,* 1994). On the other hand a decrease of total protein level in Russian sturgeon plasma was found after treatment of fish with glucan, chitosan and finnstim by immersion (Kolman *et al.,* 1998). Immunostimulants may raise the total immunoglobulin levels. Sturgeon treated with epin by immersion showed a higher concentration of total immunoglobulin (Kolman, 2001), whereas when chitosan, glucan and finnstim were administered by immersion the level of total immunoglobulin was decreased (Kolman *et al.,* 1998).

After challenge with *A. hydrophila* survival of fish fed with herb extracts was improved when compared with the control group. The survival was further enhanced in the group fed the Ganoderma extract supplement and when both herbs were used together. It is possible that this is the result of enhancement of some components of non-specific immune system of the fish by Ganoderma and a combination of Ganoderma and Lonicera. There is strong evidence that feeding glucans can modify the activity of the innate immune system of fish and increase the disease resistance in several fish species (Anderson, 1992; Sakai, 1999; Raa, 2000). Feeding carp with chitosan (1%) and levamisole (250 mg/kg of diet) also increased survival of fish following challenge with *A. hydrophila* (Gopalakannan and Arul, 2006).

The results of this study showed that feeding tilapia with *Ganoderma* and *Lonicera* alone or in combination enhanced phagocytosis by blood phagocytic cells during the whole experimental period and stimulated lysozyme activity after two weeks, but not respiratory burst activity of phagocytic blood cells, total protein or total immunoglobulin in plasma. Both herbs when used alone or in combination increased the survival of fish after challenge with *A. hydrophila.* Thus, it can be concluded that the herb extracts added to diets act as immunostimulants and appear to improve the immune status and disease resistance of fish.

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Effects of Waterborne Chloramphenicol and Oxytetracyclene Exposure on Haematological Parameters and Phagocytic Activity in the Blood of Koi Carp, *Cyprinus carpio*

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ABSTRACT

Chloramphenicol and oxytetracycline are used in ornamental fish culture to treat bacterial infections. The present study was aimed at evaluating the effects of different concentrations of chloramphenicol (2-10 mg L^{-1} for 10 days) and oxytetracycline (20 mg L^{-1} for 3 and 10 days, 100 mg L^{-1} for 3 days) on hematological parameters and phagocytic activity in the blood of koi carp, *Cyprinus carpio*. Results showed that treatment of fish with 10 mg L^{-1} chloramphenicol for 10 days depressed haematocrit, erythrocyte counts and mean corpuscular volume leading to anaemia. It also induced leucocytosis coupled with neutrophilia, thrombocytosis and lymphocytosis. The phagocytic index of the fish was also enhanced significantly with respect to the controls. The treatment of fish with 100 mg L^{-1} oxytetracycline for 3 days induced leucopenia coupled with neutropenia. Phagocytic index of these fish was significantly depressed compared to the controls. Results revealed that precautions should be taken when high concentrations of chloramphenicol or oxytetracycline are used in koi carp culture especially for long term treatments.

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INTRODUCTION

Chloramphenicol and oxytetracycline are two antibiotics used in aquaculture to treat bacterial infections in fish. These antibiotics are suitable as injections, orally medicated food and as immersions (Schaperclaus *et al.,* 1991). Immersion concentrations of chloramphenicol commonly practiced in finfish culture include 2 mg L^{-1} (Subasinghe, 1992) and 5 to 10 mg L^{-1} (Supriyadi and Rukyani, 1992). An immersion concentration of oxytetracycline recommended for finfish culture varies from 10 mg L^{-1} to 100 mg L^{-1} (Noga, 2000).

The use of chloramphenicol is restricted in food animals (Noga, 2000). It causes idiosyncratic aplastic anemia in humans. The CONH group of chloramphenicol is suspected for the bone marrow suppression in the process of haemopoiesis in mammals (Brander *et al.,* 1991). Interperitonial injections of chloramphenicol caused suppression of erythroblast numbers in the peripheral blood of European eel (*Anguilla anguilla*). In these fish, the heterophils and thrombocytes dropped but monocytes, basophils and lymphocytes elevated in the peripheral blood whereas phagocytosis of monocytes were stimulated three days after chloramphenicol injection (Kreutzmann, 1977). Rijkers *et al*. (1980) reported that oxytetracycline injections (180 mg kg^{-1}) for three days caused alteration of several haematological parameters in koi carp and suppressed the immune response in fish.

The present study was aimed at evaluating the effects of prolonged exposure by therapeutic immersion to different concentrations of chloramphenicol and oxytetracycline on the primary and secondary erythrocyte indices, total and differential leukocyte counts and phagocytic activity in the circulating blood of koi carp, *Cyprinus carpio*.

MATERIALS AND METHODS

Test fish

Apparently healthy koi carp of 30-70 g in body weight and 12-15 cm in total length were obtained from a commercial ornamental fish farm. The fish were acclimated to laboratory conditions in glass aquaria filled with aged, aerated tap water under the natural photoperiod for 14 days. During the acclimation period, the fish were fed once daily with commercially prepared fish feed (Prima, Sri Lanka) *ad libitum*.

Chloramphenicol and Oxytetracycline treatment Chloraphenicol manufactured by State Pharmaceuticals Company in Sri Lanka and Oxytetracycline (TETRAN-VET®) from Unjha Formulations Limited, India were used in this study. The treatment concentrations of chloramphenicol (2, 5 and 10 mg L^{-1}) and oxytetracycline (20 and 100 mg L^{-1}) were prepared by diluting appropriate amounts of antibiotics in separate glass aquaria containing aged tap water.

For the chloramphenicol exposure, three sets of glass aquaria each filled with 45 L of specific concentrations of chloramphenicol (2, 5 and 10 mg L^{-1}) in aged tap water were used. Four fish were introduced to each aquarium and the treated waters were continuously aerated using air pumps. Fish maintained in another set of glass aquaria each filled with 45

L of aged, aerated tap water at comparable densities served as controls. Control fish and fish exposed to chloramphenicol were fed once daily with commercially prepared fish feed at 1% of body weight. The treated water and water in the control aquaria were renewed every three days. After continuous exposure to chloramphenicol for 10 days, the exposed fish and control fish were sacrificed (n = 8) for blood sampling.

The same treatment procedure was followed for exposure of fish to 20 mg L^{-1} and 100 mg L^{-1} oxytetracycline for 3 days. In addition, a sample of acclimated fish were maintained in another set of glass aquaria each filled with 45 L of 20 mg L^{-1} oxytetracycline for 10 days. The fish maintained in aged tap water at comparable densities served as controls. Fish were fed daily at 1% of body weight and water was continuously aerated. The aquaria were covered with black coloured paper to minimize the photoinactivation of oxytetracycline. After the specified oxytetracycline exposure, fish exposed to oxytetracycline and control fish (n = 8) were sacrificed for blood sampling.

Physico-chemical parameters in aquaria water

During the exposure period, pH, temperature and dissolved oxygen concentration (DO) were measured daily using water quality monitors (HI 8314 membrane pH meter HANNA® instruments, TOA®, WQC-22A water quality checker).

Haematological parameters

Fish were sacrificed by pithing and blood samples were taken by severing the caudal vein of the fish. Before drawing the blood, pipettes were washed with heparin solution (Heparain Leo®, Leo Pharmaceuticals, Denmark) to delay blood coagulation. Haematocrit and leukocrit values in the blood samples were determined after centrifuging the blood in heparinized microcapillary tubes at 5000 rpm for 5 minutes using a haematocrit centrifuge. The microhaematocrit reader was used to measure haematocrit values whereas the heights of the packed leucocytes were measured using a calibrated micro-eyepiece under the light microscope for determination of leukocrit values.

Erythrocyte count and leukocyte count in the blood samples were determined using Shaw's solutions as a diluting fluid (Hesser, 1960). Haemoglobin concentration in the blood was determined using cyanohaemoglobin method using Sigma® test kits (Sigma, MO, USA). Using the haematocrit, erythrocyte count and hemoglobin data, the secondary erythrocyte indices namely Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC) were calculated as described by Houston (1990).

For the determination of abundance of different leukocytes, blood smears of each fish were stained with Wright's and Giemsa as described by Chinabut *et al.* (1991). Neutrophils, monocytes, lymphocytes and thrombocytes in the blood smears were identified as described by Hibiya (1982) and Chinabut *et al.* (1991). Abundance of each cell type was calculated using the total leukocyte count and percentages of each cell types in the blood smears. At least two blood smears were prepared from each fish.

Phagocytic activity

Phagocytic activity of neutrophils and monocytes in blood was determined as described by Anderson and Siwicki (1995) using *Staphylococcus aureus* (Sigma, MO, USA) with slight modifications. A sample (0.1 mL) of blood was placed in a microtiter plate well, 0.1 mL of *S. aureus* 1×10^7 cells suspended in phosphate buffered saline at pH 7.2 was added and then mixed well. The bacteria–blood solution was incubated for 25 minutes at room temperature. Five µL of this solution was taken on to a clean glass slide and a smear was prepared. The smear was air dried, fixed with ethanol (95%) for 5 min, air dried and stained with Giemsa for 10 min. Duplicate smears were made from each fish. A total of 100 neutrophils and monocytes from each smear were observed under the light microscope and the number of phagocytizing cells and the number of bacteria engulfed by the phagocyte were counted. Phagocytic capacity and phagocytic index were calculated as follows: Phagocytic capacity equals the number of bacteria engulfed cells divided by the total number of neutrophils and monocytes (phagocytes) examined. Phagocytic index is expressed as the total number of bacteria engulfed by the phagocytes, divided by the total number of phagocytes containing engulfed bacteria.

Statistical analysis

The data are presented as mean ± standard deviation of the mean for each group. For each antibiotic exposure, erythrocytic and leucocytic parameters and phagocytic activities in the blood of control fish and antibiotic exposed fish were compared using one-way analysis of variance (ANOVA). Where differences were significant, Tukey's test was used in multiple comparisons of the means. The accepted level of significance was $p < 0.05$ (Zar, 1999).

RESULTS

Temperature (27-29 ^{0}C), pH (6.5 – 7.0) and DO (5 - 6 mg L^{-1}) in the aquaria water were within favorable limits for fish during the study period. No mortality of Koi carp occurred during chloramphenicol or oxytetracycline exposure. However the fish exposed to 100 mg L^{-1} oxytetracycline for 3 days displayed anorexia.

Effect of chloramphenicol or oxytetracycline exposure on erythrocytic indices

Erythrocyte indices in the blood of control fish and fish exposed to chloramphenicol are presented in Figure 1. The haematocrit values of fish exposed to 10 mg L^{-1} chloramphenicol for 10 days (mean value 32.6%) were significantly lower than that of the control (mean value 44.3%) and the fish exposed to 5 mg L^{-1} chloramphenicol (mean value 43.8%). The erythrocyte counts and MCV in the blood of fish exposed to 10 mg L^{-1} chloramphenicol (mean values 1.84 x 10^6 cells mm^{-3}, and 1.59 x 10^{-4} fl respectively) was significantly lower than that of the controls (mean values 2.21 x 10^6 cells mm^{-3}, and 2.28 x 10^{-4} fl respectively), and the fish exposed to lower concentrations of chloramphenicol (mean values 2.12-2.27 x 10^6 cells mm^{-3}, and 2.10-2.12 x 10^{-4} fl respectively). Haemoglobin concentration and MCH value in the blood of fish exposed to 2 mg L^{-1} chloramphenicol (mean values 13.8

g dL^{-1} and 6.81 x 10^{-5} pg respectively) were lower than that of the control group (mean values 18.48 g dL^{-1} and 8.94 x 10^{-5} pg respectively). However, MCHC value in fish exposed to chloramphenicol was not significantly different from that of controls. None of the oxytetracycline treatments used in this study had any significant effect on primary and secondary erythrocytic indices of the fish (results not shown).

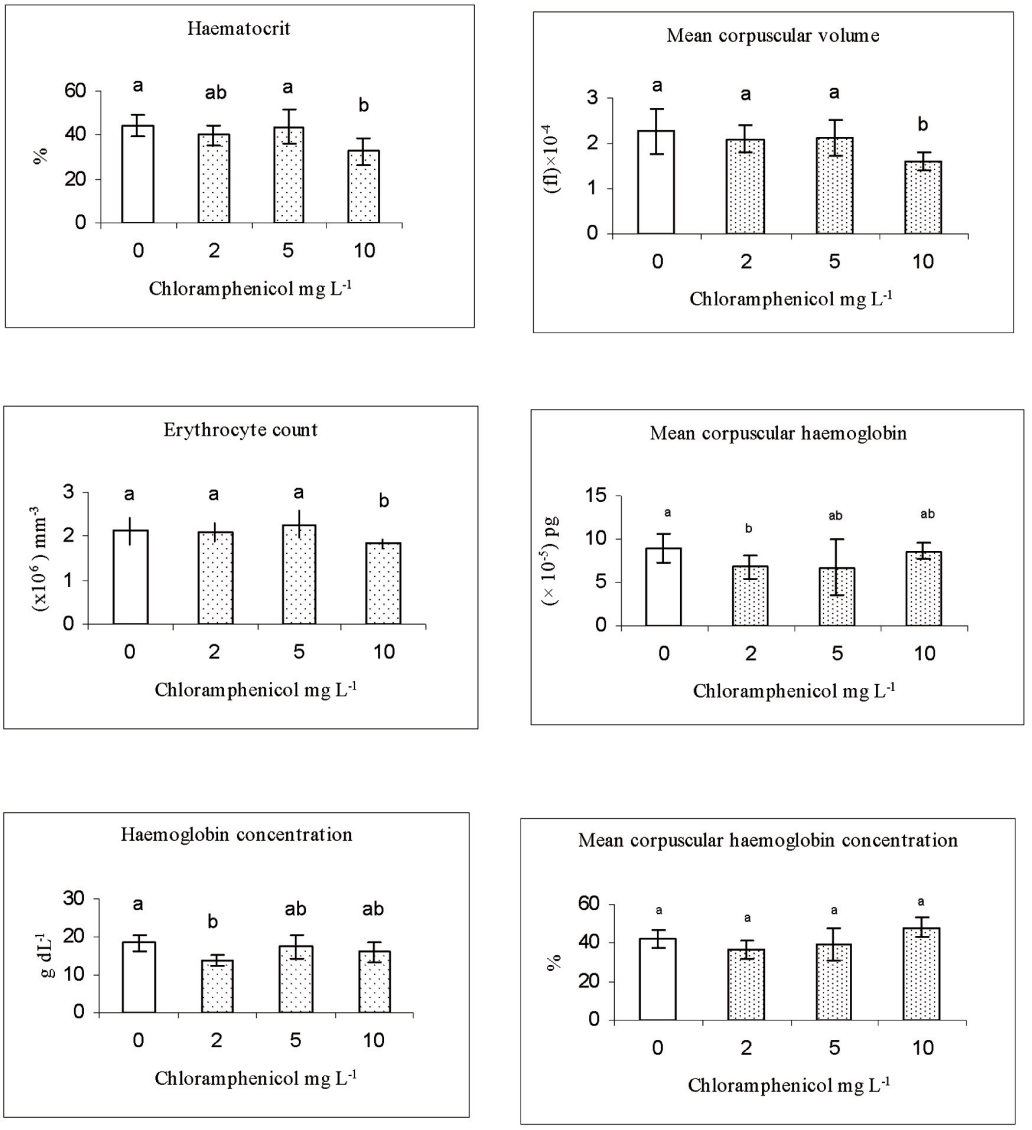

Figure 1. Effect of exposure of koi carp (*Cyprinus carpio*) to different concentrations of chloramphenicol for 10 days on erythrocytic indices in the blood. Results are presented as mean and standard desviation, n = 8 per group. For each parameter, bars indicated with different letters are significantly different from each other (ANOVA, Tukey's test, $P<0.05$)

Effect of chloramphenicol or oxytetracycline exposure on leukocytes in the blood

Leukocrit levels and total leukocyte counts in the blood of control koi carp and fish exposed to chloramphenicol or oxytetracycline are presented in Figure 2. The leukocrit in the blood of fish exposed to 5 mg L^{-1} (mean value 2.27%) or 10 mg L^{-1} (mean value 2.50%) chloramphenicol was significantly higher than that of the control group (mean value 1.45%) and fish exposed to 2 mg L^{-1} (mean value 1.1%) chloramphenicol. Total leukocyte count of fish exposed to 10 mg L^{-1} chloramphenicol (mean value 1.9 $x10^4$ mm^{-3}) was significantly higher than that of the control group (mean value 1.1 $x10^4$ mm^{-3}) and the fish exposed to 2 mg L^{-1} chloramphenicol (mean value 0.95 $x10^4$ mm^{-3}). The leukocrit and total leukocyte count of fish exposed to 100 mg L^{-1} oxytetracycline for 3 days (mean values 0.64% and 0.91 $x10^4$ mm^{-3} respectively) was reduced in comparison to that of the control fish mean values 0.88% and 1.47 $x10^4$ mm^{-3} respectively) and the fish exposed to other concentrations of oxytetracycline (mean values 1.01 -1.04% and 1.5-1.55 $x10^4$ mm^{-3}, respectively).

Absolute counts of neutrophils, monocytes, lymphocytes and thrombocytes in the blood of chloramphenicol-exposed fish and respective controls are presented in Figure 3. The absolute neutrophil counts and thrombocyte counts in the blood of fish exposed to 5 (mean values 3.9 $x10^3$ mm^{-3} and 5.19 $x10^2$ mm^{-3} respectively) or 10 mg L^{-1} (mean values 4.3 $x10^3$ mm^{-3} and 5.52 $x10^2$ mm^{-3} respectively) chloramphenicol were significantly higher than that of fish exposed to 2 mg L^{-1} chloramphenicol (mean values 2.07 x 10^3 mm^{-3} and 1.24 x 10^2 mm^{-3} respectively) and controls (mean values 3.32 x 10^3 mm^{-3} and 2.1 x 10^2 mm^{-3} respectively). There was no significant difference in absolute monocyte counts in the blood among experimental and control groups. The absolute counts of lymphocytes in fish exposed to 10 mg L^{-1} chloramphenicol (mean value 12.5 x 10^3 mm^{-3}) were significantly higher than that of fish exposed to 2 mg L^{-1} chloramphenicol (mean value 6.67 x 10^3 mm^{-3}) and controls (mean value 7.55 x 10^3 mm^{-3}).

Absolute counts of neutrophils, monocytes, lymphocytes and thrombocytes in the blood of oxytetracycline- exposed fish and controls are presented in Figure 4. Absolute neutrophil count in the blood of fish exposed to 100 mg L^{-1} oxytetracycline (mean value 0.89 x 10^3 mm^{-3}) for 3 days was significantly lower than that of the control fish (mean value 2.84 x 10^3 mm^{-3}) and the fish exposed to 20 mg L^{-1} oxytetracycline for 3 days (mean value 3.13 x 10^3 mm^{-3}). There was no significant difference in the absolute monocyte, lymphocyte and thrombocyte counts in the blood of oxytetracycline-exposed groups compared to the control group.

Effect of chloramphenicol or oxytetracycline exposure on phagocytic capacity and phagocytic index in the blood

The effect of chloramphenicol exposure on phagocytic capacity and phagocytic index in the blood of koi carp are presented in Table 1. The phagocytic index of fish exposed to 10 mg L^{-1} chloramphenicol (mean value 3.9) was significantly higher than that of the control fish (mean value 3.1), and fish exposed to 2 and 5 mg L^{-1} chloramphenicol (mean values 2.7-2.8). However the phagocytic capacity in the blood of fish exposed to chloramphenicol

Table 1. Effect of exposure of koi carp (*Cyprinus carpio*) to different concentrations of chloramphenicol for 10 days on phagocytic capacity and phagocytic index in the blood.

Fish	Phagocytic capacity	Phagocytic index
Controls	97.1 ± 1.8^a	3.1 ± 0.2^a
Chloramphenicol–exposed		
2 mg L^{-1}	94.5 ± 1.9^a	2.8 ± 0.2^a
5 mg L^{-1}	94.7 ± 2.5^a	2.7 ± 0.3^a
10 mg L^{-1}	95.2 ± 2.8^a	3.9 ± 0.3^b

Results are presented as mean and standard deviation, n = 6-8 per group. In each column, means indicated with different superscripts are significantly different from each other (ANOVA, Tukey's test, $P < 0.05$)

was not significantly different from the controls. With respect to the phagocytic capacities in the fish exposed to oxytetracycline, there were no significant differences among experimental and control groups (Table 2). However, the phagocytic index of fish exposed to 100 mg L^{-1} oxytetracycline for 3 days (mean value 3.5) was significantly lower than that of the other groups of fish (mean values 4.1-4.4).

DISCUSSION

Chloramphenicol and oxytetracycline are antibiotics used to control bacterial infections in aquaculture (Subasinghe, 1992; Supriyadi and Rukyani, 1992; Tonguthai and Chanratchakool, 1992). Even though they are used as chemotherapeutic agents, these antibiotics could adversely affect the health status of the fish. Chloramphenicol is a highly penetrable antibiotic (Brander *et al*., 1991). In the present study, chloramphenicol exposure (10 mg L^{-1}) for 10 days had a significantly negative effect on haematocrit, erythrocyte counts and MCV in the circulating blood of koi carp. This could lead to anaemia. However haemoglobin content, MHC and MCHC levels in the blood were not significantly affected by 10 mg L^{-1} chloramphenicol treatment. The reduced MCV in fish exposed to 10 mg L^{-1} chloramphenicol indicate the presence of immature erythrocytes in the circulating blood. Rijkers *et al*., (1980) found that high doses of oxytetracycline injections (180 mg kg^{-1}) could inhibit the erythropoiesis of common carp *(Cyprinus carpio)*. The present study found that exposure of koi carp to oxytetracycline had no significant effects on erythrocyte indices in the blood.

Kreutzmann (1977) found the percentage of heterophiles (neutrophil) and thrombocytes percentage were dropped, monocytes and lymphocytes percentages were increased in kidney and peripheral blood of European eel (*Anguilla anguilla*) after chloramphenicol injections (repeated injections 2 mg / 100 g dose rate at 1st, 3rd and 5th day). In the present study, total leukocyte counts in the blood were significantly increased in koi carp exposed

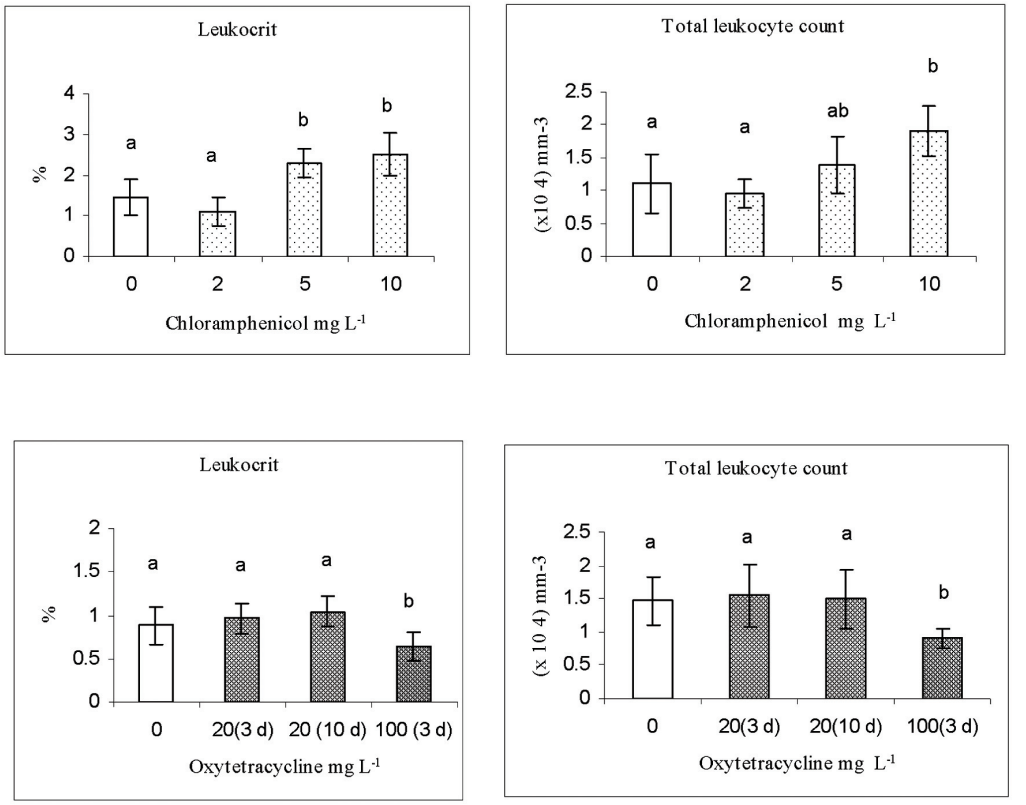

Figure 2. Effect of exposure of koi carp (*Cyprinus carpio*) to different concentrations of chloramphenicol for 10 days or oxytetracycline for 3 and 10 days on leukocrit and total leucocyte counts in the blood. Results are presented as mean and standard deviation, n = 8 per group. For each parameter, bars indicated with different letters are significantly different from each other (ANOVA, Tukey's test, $P<0.05$)

to 10 mg L^{-1} chloramphenicol for 10 days. Absolute number of neutrophils, lymphocytes and thrombocytes in the blood were increased in fish exposed to 10 mg L^{-1} chloramphenicol coupled with leucocytosis. In addition absolute number of neutrophils, and thrombocytes in the blood were increased in fish exposed to 5 mg L^{-1} chloramphenicol. Results indicate that exposure to high concentrations of chloramphenicol seem to increase the rate of release of leukocytes to the circulating blood especially neutrophils. The thrombocytes also coupled with this process. But the absolute monocyte count in the blood of fish exposed to chloramphenicol was not affected.

Kreutzmann (1977) reported that no significant reduction of granulocytes occurred after oxytetracycline injection (repeated injections 2 mg / 100 g dose rate at 1st, 3rd and 5th day) in European eel (*Anguilla anguilla*). The present study found that exposure of koi carp to high concentration of oxytetracycline (100 mg L^{-1} for 3 days) could reduce

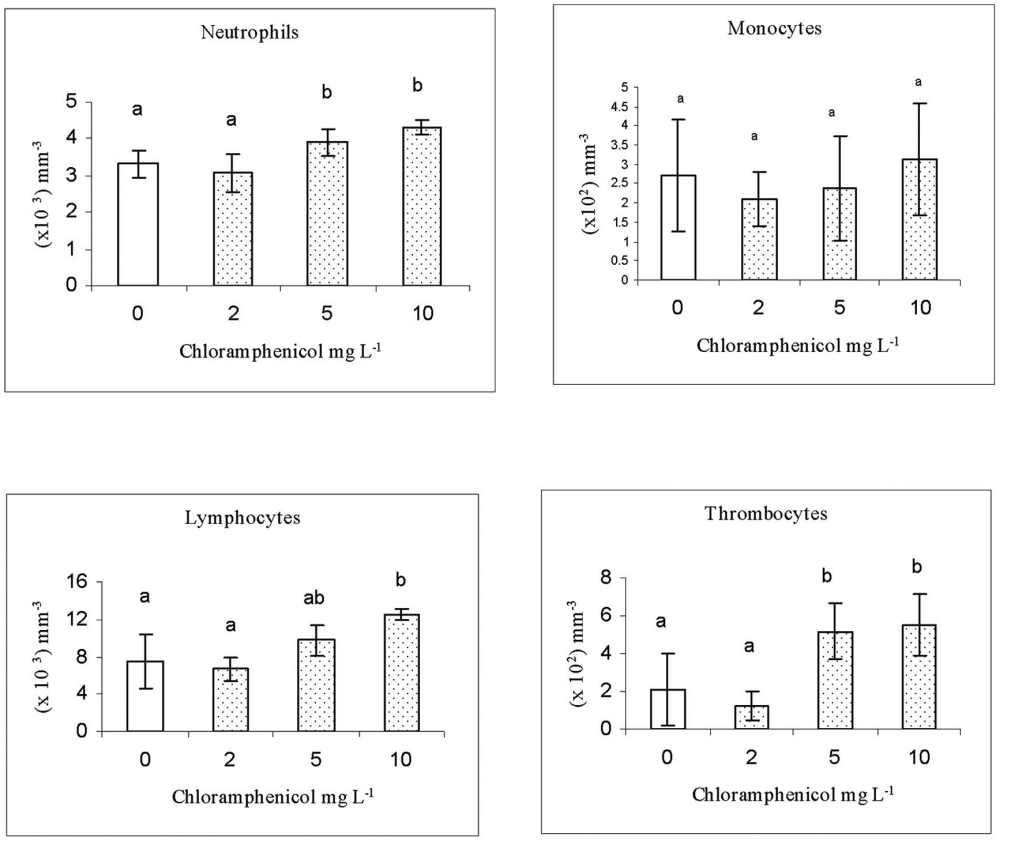

Figure 3. Effect of exposure of koi carp (*Cyprinus carpio*) to different concentrations of chloramphenicol for 10 days on absolute counts of neutrophils, monocytes, lymphocytes and thrombocytes in the blood. Results are presented as mean and standard deviation, n = 8 per group. For each parameter, bars indicated with different letters are significantly different from each other (ANOVA, Tukey's test, $P<0.05$)

the leukocyte counts and absolute number of neutrophil counts in the blood leading to leucopenia coupled with neutropenia. It indicates that high doses of oxytetracycline could induce suppression of leukocytes in the blood stream of this fish. Neutrophils released to the blood stream of the fish may be reduced by high doses of oxytetracycline. However oxytetracycline exposure doesn't seem to affect the release of thrombocytes to the blood stream of koi carp as the absolute thrombocyte count was not changed in fish exposed to oxytetracycline immersions. Rijkers *et al*., (1980) also reported that parental administration of oxytetracycline (180 mg kg^{-1}) did not affect the thrombocyte counts in common carp (*Cyprinus carpio)*.

Kreutzmann (1977) found stimulated phagocytic activity of monocytes in peripheral blood and spleen after chloramphenicol injections (repeated injections 2 mg/100 g dose rate at 1st, 3rd and 5th day) in European eel (*Anguilla anguilla*). In the present study, the phagocytic

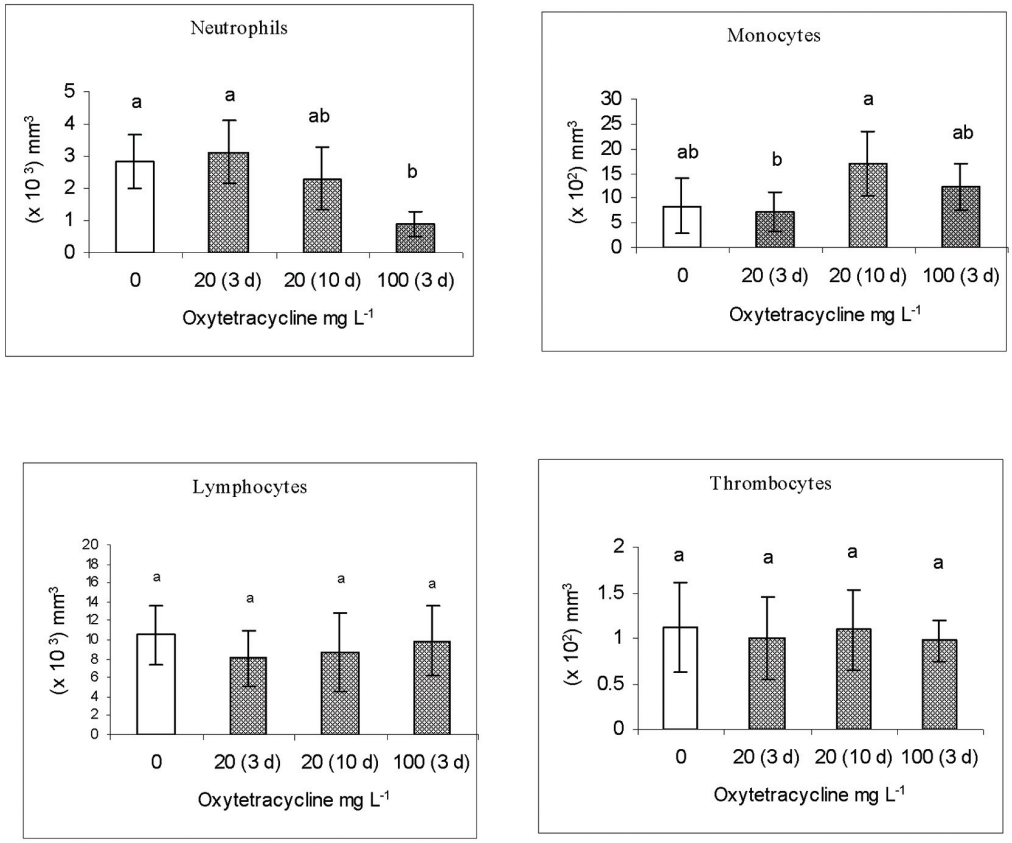

Figure 4. Effect of exposure of koi carp (*Cyprinus carpio*) to different concentrations of oxytetracycline for 3 or 10 days on absolute counts of neutrophils, monocytes, lymphocytes and thrombocytes in the blood. Results are presented as mean and standard deviation, n = 8 per group. For each parameter, bars indicated with different letters are significantly different from each other (ANOVA, Tukey's test, P<0.05)

Table 2. Effect of exposure of koi carp (*Cyprinus carpio*) to different concentrations of oxytetracycline on phagocytic capacity and phagocytic index in the blood.

Fish	Phagocytic capacity	Phagocytic index
Controls	93.1 ± 4.5[a]	4.1 ± 0.5[a]
Oxytetracycline-exposed		
20 mg L^{-1} for 3 days	94.7 ± 4.5[a]	4.4 ± 0.6[a]
20 mg L^{-1} for 10 days	97.8 ± 2.7[a]	4.2 ± 0.4[a]
100 mg L^{-1} for 3 days	94.4 ± 2.6[a]	3.5 ± 0.2[b]

Results are presented as mean and standard deviation, n = 6 per group. In each column, means indicated with different superscripts are significantly different from each other (ANOVA, Tukey's test, P < 0.05)

index in the blood of koi carp exposed to 10 mg L^{-1} chloramphenicol was significantly increased in comparison to the controls. The elevated phagocytic index may be linked with the elevated neutrophil count in the blood. The leukocytes, especially absolute neutrophil counts were high in fish exposed to 10 mg L^{-1} chloramphenicol. Evelyn (2002) reported that neutrophils could enhance the phagocytic activity of monocytes. It may also be a result of the acquisition of myeloperoxidase activity from the neutrophils. The increase in cellular elements of the immune system in fish may have contributed to the stimulated phagocytosis observed in these fish.

Tafalla *et al.* (1999) described the non-specific functions of the head kidney macrophages (phagocytosis and respiratory burst) were not suppressed in fish when oxytetracycline was administered *in vivo* (medicated feed, 200 mg oxytetracycline kg $^{-1}$ day $^{-1}$ and 20 mg L^{-1} for 2 hours bath treatment) to turbot (*Scophthalmus maximus*). In the present study, the phagocytic index was reduced but phagocytic capacity was not affected in koi carps exposed to 100 mg L^{-1} oxytetracycline for 3 days. Phagocytic index indicates the total number of bacteria engulfed by the phagocytes, in relation to the total number of phagocytes containing engulfed bacteria. Phagocytic capacity is expressed as the number of bacteria engulfed cells in relation to the total number of examined phagocytes which include both neutrophils and monocytes. Reduction in phagocytic index in fish exposed to the highest concentration of oxytetracyclin may have resulted due to oxytetracycline induced suppression of total neutrophils in the blood stream. Neutrophils could enhance the phagocytic activity of monocytes (Evelyn, 2002). Reduction of neutrophils in the blood of koi carp exposed to 100 mg L^{-1} oxytetracycline may have suppressed their positive effect on the phagocytic activity of monocytes which resulted in thereduced phagocytic index of these fish compared to the controls.

CONCLUSIONS

The present study evaluated the effects of different concentrations of chloramphenicol and oxytetracycline given for different time periods on haematological parameters and phagocytic activity in the blood of koi carp. The results showed that low concentrations of chloramphenicol (2 mg L^{-1} or 5 mg L^{-1} for 10 days) or oxytetracycline (20 mg L^{-1} for 3 days or 10 days) had no or mild effects on the parameters studied. However, treatment of fish with 10 mg L^{-1} chloramphenicol for 10 days depressed haematocrit, erythrocyte counts and mean corpuscular volume leading to anaemia. It also induced leucocytosis coupled with neutrophilia, thrombocytosis and lymphocytosis. The phagocytic index of the fish was also enhanced significantly with respect to the controls. The treatment of fish with 100 mg L^{-1} oxytetracycline induced leucopenia coupled with neutropenia. Phagocytic index of these fish was significantly depressed compared to the controls. Results revealed that precautions should be taken when high concentrations of chloramphenicol or oxytetracycline are used in koi carp culture especially for long term treatments.

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Nocardial Infections in Fish: An Emerging Problem in Both Freshwater and Marine Aquaculture Systems in Asia

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ABSTRACT

Rising numbers of infections with *Nocardia seriolae* have caused increasing damage to the yellowtail and amberjack industry in Japan. However, nocardial infections in fish are not limited to Japan. Over the last few years, routine disease investigations in cultured fish in the Southeast Asian region revealed that nocardiosis is omnipresent in countries such as Malaysia, Taiwan, Indonesia and China. The disease was observed in marine species such as pompano, four-finger threadfin, big-eye trevally, snapper and grouper, but also in the freshwater fish tilapia. Various levels of mortality could be found in association with the disease. Typical clinical signs included nodules in gills, spleen, kidney and liver, with or without multiple skin ulcers and nodules. Isolation of the pathogen was difficult unless samples were taken from fresh lesions and cultured on nutrient-rich media. Although clear clinical signs were present in all cases and impression prints showed massive presence of typical *Nocardia*-like cells, only in 55% of cases could *Nocardia* be recovered by culture method. Impression prints represented a fast and reliable method to demonstrate the presence of *Nocardia* sp. Histopathologically, the observed nodules were typical granulomas. A selected set of biochemical tests allowed us to identify the causative agent as *N. seriolae* and allowed differentiation from other pathogens. Specific PCR primers were designed and used to confirm the identity up to species level. Experimental infection has demonstrated the role of *N. seriolae* as a primary pathogen in fish.

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INTRODUCTION

Nocardiosis caused by *Nocardia* sp. in fish was first described by Rucker (1949) as *Streptomyces salmonicida* infecting sockeye salmon (*Oncorhynchus nerka*). Later, the bacterium was transferred to the genus *Nocardia* as *Nocardia salmonicida,* based on the presence of meso-diaminopimelic acid, arabinose and galactose in whole organism hydrolysates and subsequently confirmed by an almost complete sequence of 16S rDNA analysis (Pridham *et al.*, 1969; Isik *et al.*, 1999). Since then, four species of *Nocardia* [*N. asteroides*, *N. seriolae* (formerly called *N. kampachi*), *N. salmonicida* and *N. crassostreae*] have been isolated from diseased aquatic animals (Valdez *et al.*, 1963; Kusuda *et al.,* 1974; Kudo *et al.*, 1988; Chen, 1992; Friedman *et al.*, 1998; Brandsen *et al.*, 2000; Chen *et al.*, 2000; Wang *et al.*, 2005). *N. seriolae* has by far been the most frequently isolated *Nocardia* sp. from fish and in recent years its damage to the fish industry has been increasing. In particular, the yellowtail (*Seriolae quinqueridiata*) and amberjack (*S. dumerelli*) industries in Japan have suffered from *N. seriolae* infections. The economic losses are significantly high as outbreaks tend to occur several months after stocking when fish are between 300 to 1,000 g (Kariya *et al*., 1968; Kubota *et al*., 1968; Kusuda *et al*., 1974; Kudo *et al*., 1988). However, not only Japan appears to suffer from Nocardiosis as *N. seriolae* has been found to affect Japanese sea bass (*Lateolabrax japonicus*) and yellow croaker (*Larimichthys crocea*) in Taiwan and China, reportedly causing more than 15% mortality in each species (Chen *et al.*, 2000; Wang *et al.*, 2005).

Nocardiosis is a systemic bacterial disease caused by a Gram-positive, partially acid-fast, aerobic, filamentous bacterium. Typical disease signs include nodules in gills, spleen, kidney and liver with or without multiple skin ulcers/nodules. Histopathologically, the observed lesions are typical granulomas (Egusa, 1992).

From 2002 to 2006, we have investigated the causes of mortality associated with typical clinical disease signs indicative of nocardial infections in Indonesia, China, Singapore and Malaysia. This isolation, identification and prevalence of nocardial infections in South East Asia will be discussed to illustrate the role of *N. seriolae* as a primary pathogen of fish.

MATERIALS AND METHODS

Sampling

In this study, the samples were taken at fish farms in China (4 sites), Singapore (1 site), Indonesia (1 site) and Malaysia (3 sites). The sample sites in China included Guandong and Hainan provinces. In Indonesia, samples were taken at fish farms in Sumatra and in Malaysia, samples were taken at fish farms in the states of Selangor and Johor. Sampling covered the period from February 2002 to June 2006.

Samples were only taken when abnormal behaviour and mortality were observed and no apprently normal fish were sampled. Lesions and clinical signs were recorded, as well as the weight and length of each fish sampled. Fish were sampled on-site, at the farm and affected areas such as abscesses, internal organs such as spleen, kidney, liver or

brain displaying granulomatous lesions were plated on selective (Ogawa agar) and non-selective media (tryptone soy agar, Oxoid) supplemented with 1.5% NaCl (w/v), brain heart infusion agar (Becton Dickinson) supplemented with 1.5% NaCl (w/v), blood agar (Biomedia laboratories) and Eugon agar (Becton Dickinson). Affected organs (including skin ulcers/lesions) were plated using disposable loops and the agar plates were incubated at 26°C until growth was observed. Thereafter, the colonies were purified by sub-culturing on Eugon agar and incubating at 26 °C for 3-5 days. From affected organs, organ imprints were made by sampling the organ and imprinting the cut-through section onto a clean microscope glass slide. Presumptive diagnosis was achieved based on the characterisation of clinical signs, Gram-staining of the impression prints and, where appropriate histology. Samples for histology were fixed in 10% (v/v) formalin, embedded in paraffin wax and sectioned at 5 µm. The sections were stained by haematoxylin and eosin (H&E). Some sections were stained with Gram stain, Ziehl-Neelsen (ZN) and modified ZN (Fite-Faraco (FF) (Faraco, 1938; Fite *et al*., 1947).

Phenotypic/biochemical characterisation

The purified bacterial cultures recovered were Gram-stained (MERCK commercial staining kit, according to manufacturer instructions) and selected cultures identified as representative strains were examined for acid fastness using a modified ZN stain.

For biochemical characterisation, bacterial colonies were collected after 5 days of incubation. The isolates were tested for oxidase by placing some growth (using a platinum loop) on filter paper impregnated with oxidase reagent (Biomerieux). The catalase test was performed by placing some growth on a glass slide and flooding it with 3% H_2O_2. Urease production was tested by stabbing urease agar slants (urea: 20 g/L, NaCl: 5 g/L, KH_2PO_4: 2 g/L, peptone: 1 g/L, glucose: 1 g/L, phenol red: 0.01 g/L, agar 15 g/L) with some collected growth, followed by streaking the slanted surface of the agar. Results were read after 5 days incubation at 26°C. Colonies were suspended into sterile 0.5% NaCl (w/v) and adjusted to turbidity equivalent of 5-6 McFarland standard Nitrate reduction was tested by inoculating three drops of the above suspension in nitrate broth (Difco) supplemented with 1.5% NaCl (w/v). Results were read after 3 and 5 days of incubation at 26°C. McConkey agar (Oxoid) was inoculated with the bacterial suspension and incubated for 5 days at 26°C. The same suspension was used to inoculate API ZYM (Biomerieux) which was read after 5 days incubation at 26°C according to the manufacturer's instructions.

Confirmation of identity by PCR

A specific PCR test was used to confirm identity of the strains. DNA extraction was performed using QIAamp DNA mini kit (QIAGEN) according to the manufacturer's recommendation. The partial sequence (~1.2 kb) of the *N. seriolae* 16S ribosomal RNA gene was determined and gene-specific primers (N5F1 and N5R1) were designed. Primer sequences were tested for specificity using Primer Designer 4 software.

The forward and reverse primers used were N5F1: 5'-TGA GCC TGA ACT GCA TGG TTC-3' and N5R1: 5'-ACG GTA TCG CAG CCC TCT GTA-3'. Two PCR mixes were used namely puReTaq Ready-To-Go PCR beads (Amersham Biosciences) or a cocktail

consisting of 0.2µm of forward and reverse primers each, DNA template 1.0µl, 2.5µl of 1X PCR buffer minus $MgCl_2$, 1.5mM of $MgCl_2$, 0.16mM dNTP, 1U Taq DNA Polymerase in a total reaction volume of 25µl. Amplification was carried out using Hybaid Thermal Cycler with the following cycling parameters: 95ºC (2 mins) x 1 cycle, followed by 30 cycles at 95 ºC for 30 sec; 58ºC for 1 min and 72ºC for 1 min and finally 72ºC for 5 mins. The expected specific amplified product was 1069 bp. PCR products were analysed on 1.2% (w/v) agarose gel (BST Techlab) in TAE buffer. The gels were run at 120V for 75 min, stained with ethidium bromide and photographed using the SynGene Bio Imaging System. The specificity of the primers was tested on isolates, type strains *N. seriolae* CCUG 46828T and *N. asteroides* CCUG 10073T, and unrelated species, as shown in Table 1.

Table 1. Strains used for specificity testing of PCR primers.

Name of strain	Isolation	Country	Year
N. seriolae-YT-Japan	Yellowtail	Japan	2003
N. seriolae-SB-Taiwan	Japanese Sea bass	Taiwan	2003
N. asteroids-SB-Taiwan	Japanese Sea bass	Taiwan	2003
*N. seriolae-TF-Malaysia-**5***	Threadfin	Malaysia	2002
*N. seriolae-PF-Malaysia-**2***	Pomfret	Malaysia	2002
N. seriolae (CCUG 46828T)	Yellowtail	Japan	1988
N. asteroides (CCUG 10073T)	See J. Gen microbial 20 (1), 129-35, 1959		
Vibrio (Listonella) anguillarum	Yellowtail	Japan	2000
Lactococcus garvieae	Rainbow trout	Belgium	2003
Photobacterium damsela subspecies *piscicida*	Yellowtail	Japan	2001
Tenacibaculum maritimum	Asian sea bass	Singapore	2003

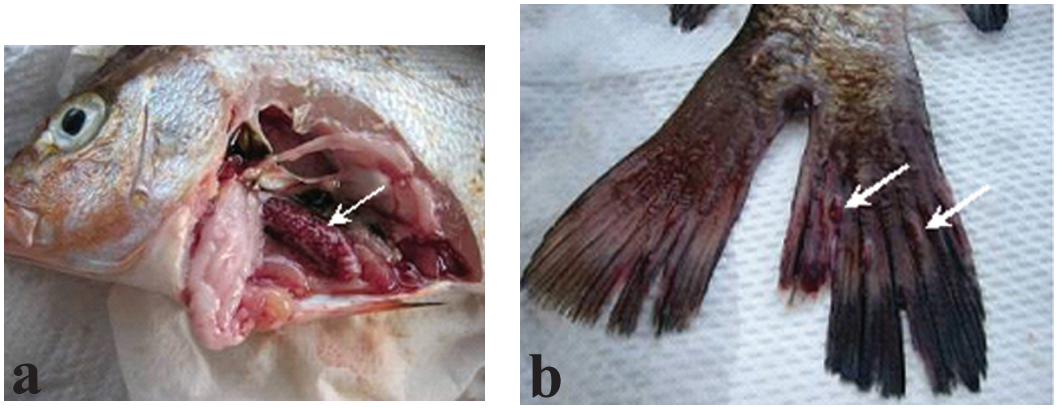

Figure 1. Red snapper (weight: 150 g) showing typical clinical signs of nocardial infections. a: ascites, splenomegaly, macroscopic nodules in spleen (arrow). b: typical nodules (arrow) in tail region.

Experimental infection

Experimental infection was performed on yellowtail (*S. quinqueradiata*) with average weight of 93 g. A *N. seriolae* strain from Japan was cultured in Eugon broth (100ml) for approx. 60 hr and bacterial suspensions of 1.2 x 10^6 CFU/ml, 1.5 x 10^7 CFU/ml and 8.5 x 10^7 CFU/ml were prepared in sterile 1.5% NaCl (w/v). Ten fish per group were injected intraperitoneally with 0.1 ml of the challenge suspension after sedation using AQUI-S (AQUI-S New Zealand Ltd.). Fish were held in a 50-L tank in full strength seawater (approx. 30 ppt) at 28 °C and observed for a period of 19 days.

RESULTS

Clinical signs

Common gross clinical signs included lethargy, multiple skin ulcerations and appearance of red spots with or without an ulcerative mouth. Other common clinical signs observed grossly, were brownish or haemorrhagic gills, abscess inside the operculum, greyish or haemorrhagic liver with white nodules often associated with a brittle texture, fibromatosis in the abdominal cavity, spleen necrosis associated with the presence of macroscopic white nodules, ascites, hemorrhagic brain and swollen kidney often associated with the presence of white nodules.

On-farm mortality associated with nocardial infections was difficult to estimate. Overall, nocardial infections seemed to induce low level chronic mortality in most of the sampled farms. Nonetheless, mortality was considered as economically important as the disease usually occurred several months after stocking, when fish were big. However, in some species, such as Pompano, cumulative mortalities of up to 30% were seen in farms in China causing very significant financial loss.

Bacterial recovery results

In Malaysia, mainly pompano (*Trachinotus blochii*) was affected. However, nocardial infections were also found in mangrove snapper (*Lutjanus argentimaculatus*), four-finger threadfin (*Eleutheronema tetradactylum),* tiger grouper *(Epinephelus fuscoguttatus)* and big-eye trevally (*Caranx sexfasciatus*). Samples from China and Singapore showed that these same fish species were affected there also (see Table 2). In Indonesia, nocardial infections were found in tilapia. *Nocardia* was typically isolated from larger fish (> 100 g, up to 600 g). The causative agent was often isolated from the skin, brain and spleen but could also be isolated from the liver and gills. For all the fish mentioned in Table 2, clinical signs were indicative of nocardial infection; however, in 20 out of 45 cases, no bacteria were isolated through culture method. Those 20 cases were presumptive infections based on clinical signs and impression prints (see Figure 2) where typical *Nocardia*-like cells were seen indicating that a *Nocardia* infection was present.

When clear clinical signs were observed in association with the presence of the bacteria on organ imprints, there was only a 55% chance that the organism was isolated from the diseased fish. In all cases where *Nocardia* was isolated, the bacteria could be seen in organ imprints but likewise the cases where no positive isolation was obtained. Table 2 shows

Table 2. Confirmed and presumptive diagnosis of nocardial infections found in China, Malaysia, Indonesia and Singapore.

Country of isolation		Common fish name	Latin name	Isolation organ	Fish weight/ size	Isolation date (d/m/y)
	1	Pompano	*Trachinotus blochii*	B S	20 cm	2/12/2002
	2	Pompano	*Trachinotus blochii*	B SK	19 cm	"
	3	Threadfin	*Eleutheronema tetradactylum*	SK	15 g	12/12/2002
	4	Pompano	*Trachinotus blochii*	B	120 g	"
	5	Threadfin	*Eleutheronema tetradactylum*	B	208 g	"
	6	Pompano	*Trachinotus blochii*	L[1]	133 g	"
	7	Pompano	*Trachinotus blochii*	B[1]	15 g	"
	8	Pompano	*Trachinotus blochii*	S[1]	32 g	"
	9	Wild fish	-	B SK[1]	25 g	"
Malaysia	10	Pompano	*Trachinotus blochii*	S	> 200 g	23/01/2003
	11	Mangrove snapper	*Lutjanus argentimaculatus*	S[1]K	150 g	28/07/2003
	12	Grouper	*Epinephelus fuscoguttatus*	-[1]	8 g	"
	13	Pompano	*Trachinotus blochii*	B	150 g	"
	14	Trevally	*Caranx sexfasciatus*	SK	204 g	22/10/2004
	15	Pompano	*Trachinotus blochii*	S L[1]	150 g	28/07/2003
	16	Pompano	*Trachinotus blochii*	SK	100 g	13/01/2005
	17	Red snapper	*Lutjanus erythopterus*	L	50 g	07/10/2005
	18	Red snapper	*Lutjanus erythopterus*	K	25 g	"
	19	Red snapper	*Lutjanus erythopterus*	S	180 g	"
	20	Red snapper	*Lutjanus erythopterus*	B L K S[1]	100 g	"
	21	Pompano	*Trachinotus blochii*	SK[1]	60 g	16/08/2003
	22	Snapper	*Lutjanus sp.*	B	400 g	17/08/2003
	23	Green grouper	*Epinephelus coicoides*	SK[1]	11 g	"
	24	Green grouper	*Epinephelus coicoides*	SK[1]	30 g	"
	25	Green grouper	*Epinephelus coicoides*	SK[1]	40 g	"
	26	Pompano	*Trachinotus blochii*	SK	220 g	6/11/2003
	27	Pompano	*Trachinotus blochii*	G	175 g	"
	28	Pompano	*Trachinotus blochii*	SK	200 g	"
	29	Pompano	*Trachinotus blochii*	SK	220 g	7/11/2003
China	30	Pompano	*Trachinotus blochii*	S	70 g	27/12/2005
	31	Red snapper	*Lutjanus eryhtropterus*	B L	75 g	1/9/2005
	32	Red snapper	*Lutjanus eryhtropterus*	L[1]	50 g	"
	33	Pompano	*Trachinotus blochii*	K[1]	500 g	14/11/2005
	34	Pompano	*Trachinotus blochii*	SK	350 g	15/11/2005
	35	Pompano	*Trachinotus blochii*	L K[1]	350 g	"
	36	Pompano	*Trachinotus blochii*	K	320 g	"
	37	Pompano	*Trachinotus blochii*	L[1]	350 g	"
	38	Pompano	*Trachinotus blochii*	S[1]	-	10/4/2006
	39	Pompano	*Trachinotus blochii*	S L[1]	-	"
	40	Red snapper	*Lutjanus eryhtropterus*	S	46.3 g	20/01/2005
Singapore	41	Pompano	*Trachinotus blochii*	SK	12 cm	4/10/2005
	42	Tilapia	*Oreochromis sp.*	SK	600 g	9/6/2005
Indonesia	43	Tilapia	"	S	450 g	"
	44	Tilapia	"	S L[1]	700 g	"
	45	"	"	SK	200 g	15/6/2006

[1] Indicates presumptive diagnosis based on the dominant presence of typical Gram-positive filamentous branching bacteria seen in impression prints in association with typical clinical signs of nocardiosis. B: brain, S: spleen, L: liver, K: kidney, SK: skin ": same as above

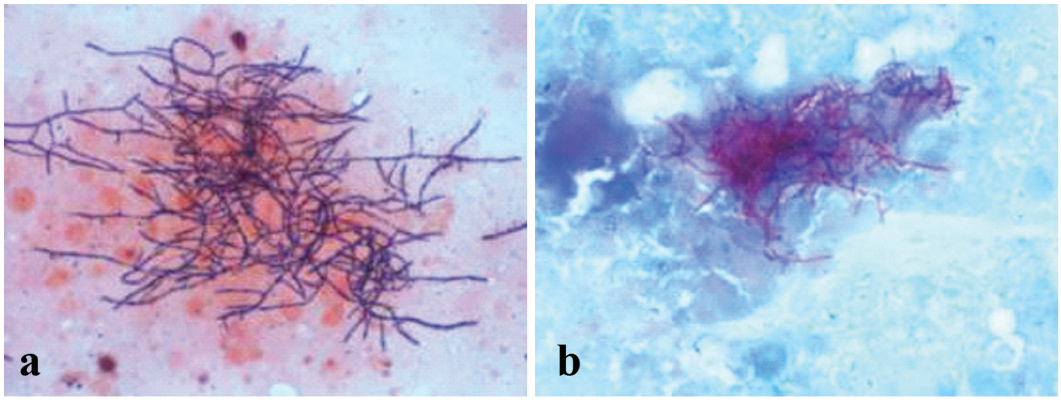

Figure 2. **a:** Gram-stain of impression print from infected fish brain showing typical branching Gram-positive hyphae indicative of nocardial infections (oil immersion X1000). **b:** Ziehl-Neelsen stain of impression print from infected fish brain showing typical acid-fast branching hyphae (oil immersion X1000).

the isolation results over the sampling period showing confirmed and presumptive cases of nocardial infections. When *Nocardia* was isolated, it was always found in association with clinical signs of disease. From the original isolation plates, typical *Nocardia*-like growth was observed after 5-7 days of incubation. Optimum recovery was obtained with Eugon agar. After purification, colonies appeared matt to velvety and dry in appearance with a granular surface, irregularly shaped edges and were light brown in colour.

Histology was performed on several fish showing typical clinical signs of infection. Organs such as brain, liver, stomach, kidney, gills, spleen and skin were sampled. In the liver, mild to moderate diffuse fatty degeneration of the hepatocytes was seen in association with granulomata. Observation of Gram stained tissue sections showed these granulomata to have proliferating Gram-variable rod-shaped bacteria in the centre of the granulomata. The exocrine acinar cells of the pancreatic islets were devoid of zymogen granules. In the brain, large granulomata located at the base of the cerebellum were present; these consisted of necrotic cells, inflammatory cells and, in Gram stain tissue sections there was much proliferation of Gram-variable or Gram-negative mostly thin filamentous, segmented bacteria with a strong infiltration of inflammatory cells and macrophages. Similar granulomata were observed in other foci in the brain tissue. In the head and posterior kidney, parenchyma was often replaced by large, old granulomata or coagulated necrotic areas adjacent to the capsula unilateral. The granulomata were composed of necrotic cellular debris in the centre surrounded by a thick wall of epitheloid flattened cell layers in which proliferating, Gram-variable filamentous bacteria were observed. Remaining parenchyma showed mild necrosis and congestion in association with a large presence of either single or grouped melano-macrophages. The kidney seemed to be more or less affected depending on the case. Usually, the posterior kidney interstitial tissue and tubuli showed only minimal degenerative lesions. When gill lesions were seen, lamellar epithelium showed severe necrosis with the presence of proliferating coccoid rod- or filamentous-shaped bacteria. The capillaries of the primary lamellae showed thrombi composed of necrotic cells. If lesions in the operculum were seen, severe degeneration of the pseudobranchial epithelium

was noted and severe inflammation (consisting of necrosis, haemorrhage and leucocyte infiltration) were found in the surrounding tissue caused by a large number of proliferating Gram-positive filamentous bacteria. In addition, large granulomata composed of Gram-positive rod-shaped bacteria were found. In the stomach, multi-focal mild necrosis of the epithelium was more pronounced in the crypts. In the spleen, most of the pulpa was usually replaced by large old?? granulomata composed of necrotic cellular debris in the centre surrounded by epitheloid flattened cell layers. Younger granulomata were also found and showed the presence of proliferating thin filamentous Gram-negative bacteria. A mild to moderate diffuse presence of single or grouped melano-macrophages was noted and Gram stain revealed the presence of Gram-negative rod-shaped thin and filamentous bacteria in some of the granulomata. In the skin, the dermis showed focally severe inflammatory reactions around major granulomatous, i.e., necrotic foci that spread into the hypodermis. In Gram stain, the necrotic foci were composed of Gram-variable thin filamentous bacteria (see Figure 3).

Phenotypic/biochemical characterisation

All strains showed typical Gram-positive to Gram-variable (often with a bead-like appearance) branching cells after purification on Eugon agar. Some strains were used for acid-fast staining and gave partially acid-fast results (Figure 2).

The results of phenotypical and biochemical profiles of the isolated bacterial strains as well as *N. seriolae* CCUG 46828^T and *N. asteroides* CCUG 10073^T type strains are given in Table 3.

Overall, results indicated that the field isolates were closer to the *N. seriolae* type strain than *N. asteroides* (as determined by growth on McConkey agar, urease production, nitrate reduction, α-chymo trypsin negative, and α-galactosidase and β-galactosidase positive). Results amongst the strains were consistent; however, variable results were obtained when strains were tested for valanine arylamidase (19/25 strains positive), cystine arylamidase (20/25 strains positive), trypsin (18/25 strains negative) and α-galactosidase (22/25 strains positive). When the strains were analysed through cluster analysis (unweighted pair group average, percent disagreement, Statistica data analysis software system, version 6 - 2004, Stat Soft Inc.), the analysed strains formed a tight cluster (linkage distance < 0.2, Figure 4) and showed a clear difference with *N. asteroides* type strain (linkage distance > 0.3).

Confirmation of identity by PCR

The N5F1 and N5R1 primers specifically amplified only *N. seriolae* and clearly distinguished it from the closely-related species *N. asteriodes*. The amplification was specific to *N. seriolae* and did not cross react with the other unrelated Gram-negative and Gram-positive bacteria tested (*Listonella anguillarum*, *Lactococcus garvieae* and *Tenacibaculum maritimum*; Figure 5). The primers also proved to be effective to confirm *N. seriolae* infection in clinically-infected fish. All amplifications yielded the expected specific amplification product of 1,069 bp indicated by arrow heads in Figure 5.

Table 3. Results of phenotypic/biochemical characterisation of *Nocardia* strains.

	A	B	1	2	3	4	5	10	13	14	16	17	18	19	22	26	28	27	29	30	31	34	36	40	41	42	43
Gram	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
McConkey growth	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease production	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 26°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
API ZYM																											
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alk. Phos.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Esterase (C4)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Esterase Lip. (C8)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lipase (C4)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Leuc. Aryl.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Val. Aryl.	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	-	-	+	+	-	+	+
Cyst. Aryl.	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	-	+	+	+	-	+	+
Tryp.	+	-	+	-	+	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-
α-chymo trypsin	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acid Phos..	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NAPHT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α–Gal.	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
β-Gal.	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
β-Glucoron.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-GLucosidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
β-GLucosidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NAC.β-Glucos.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-mannosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-fucosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

A : N. asteriodes-type CCUG 10073T B : N. seriolae-type CCUG 46828T

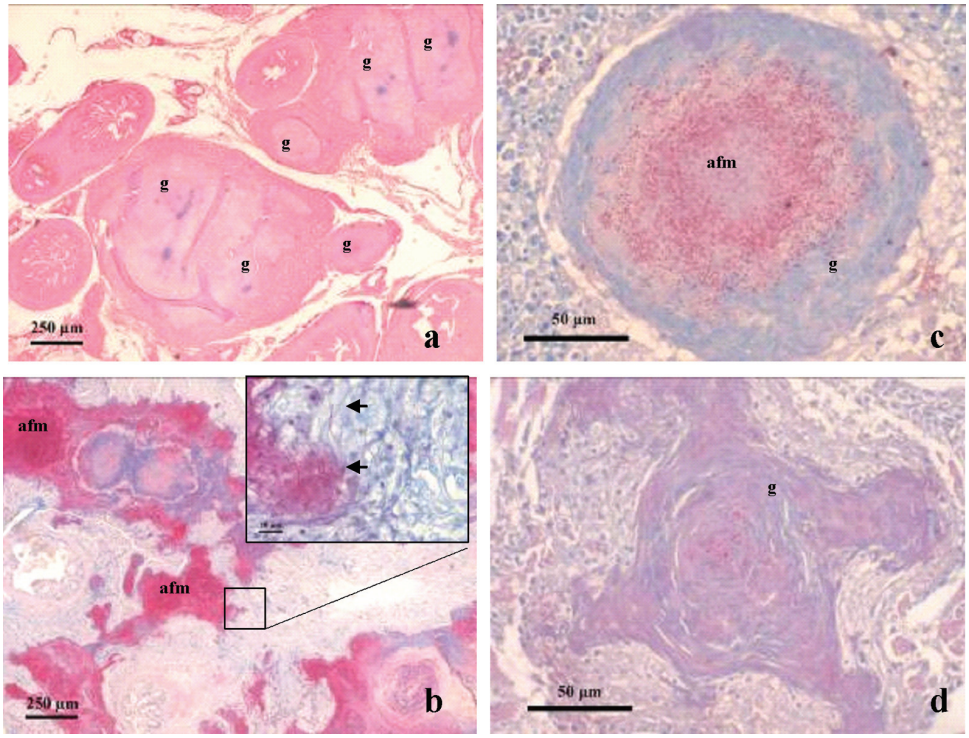

Figure 3. Examples of histological findings in internal organs of a pompano infected with *N. seriolae*. Picture a: H&E stain of granulomata (g) in peritoneal cavity - note severe granulomata with necrotic centre (X40). Picture b: Modified Ziehl Neelsen stain (Fite-Faraco; FF) of severe granulomatous lesions in peritoneum - note massive presence of acid-fast material (afm) at centre and surrounding granulomata (X400). Insert: details of acid-fast typical *Nocardia*-branched cells at edges of granulomata (arrow). Picture c: granulomata in kidney with FF stain - note clear acid-fast material at centre (X400). Picture d: details of a typical granuloma found in the heart (FF, X400).

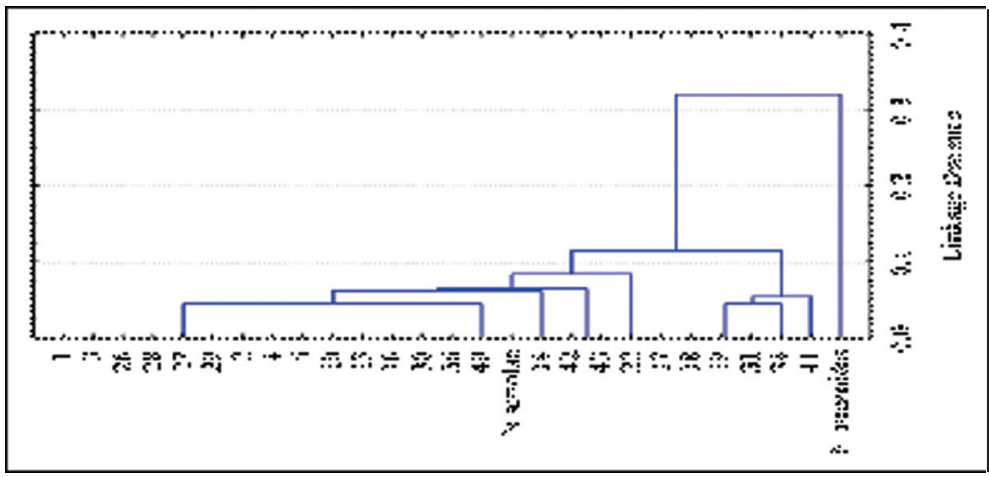

Figure 4. Linkage distance of field isolates and type strains *N. seriolae* and *N. asteroides* (Unweighted pair group average, percent disagreement).

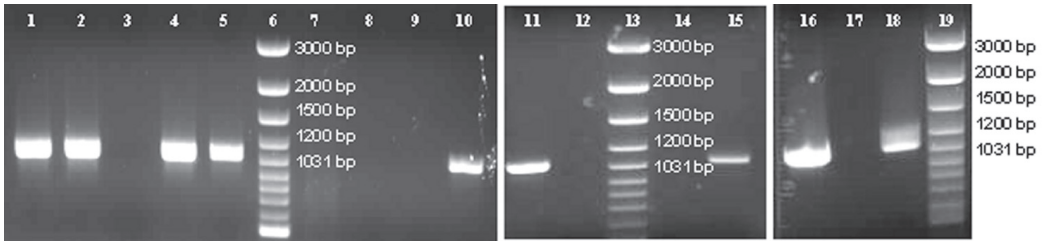

Figure 5. 1.2% agarose gel electrophoresis of PCR products of purified strains from field isolates identified as *N. seriolae* through biochemical/phenotypical identification, other closely-related bacteria and unrelated bacteria. Lanes: 1, *N. seriolae*-YT-Japan; 2, *N. seriolae*-SB-Taiwan; 3, *N. asteroides*-SB-Taiwan; 4, *N. seriolae*-TF-Malaysia-**5;** 5, *N. seriolae*-PF-Malaysia-**2**; 6, DNA ladder; 7, *L. anguillarum*; 8, *L. garvieae*; 9, *T. maritimum*; 10, *N. seriolae*-YT-Japan; 11, *N. seriolae* CCUG 46828T type strain; 12, *N. asteroides* CCUG 10073T type strain; 13, DNA ladder; 14, *N. asteroides*-SB-Taiwan; 15, *N. seriolae*-YT-Japan; 16, *N. seriolae*-infected tissue from clinically-infected fish; 17, *N. asteroides*-SB-Taiwan; 18, *N. seriolae*-YT-Japan; 19, DNA ladder.

Experimental bacterial infection

Experimental infection was performed on yellowtail (*S. quinqueradiata*) originating from Japan. Fourteen days after challenge, all fish showed severe granulomata in the internal organs and some fish developed typical skin and/or mouth ulcers and nodules.

The injection of *N. seriolae* at a concentration of 1.2 x 10^6 CFU/ml, 1.5 x 10^7 CFU/ml and 8.5 x 10^7 CFU/ml resulted in a clear dose response (Figure 6). The injection of 8.5 x 10^7 CFU/ml resulted in the highest mortality, reaching 90% at Day 19. With this dose, mortality started on Day 4 and continued until Day 15. With a six times lower dose (1.5 x 10^7 CFU/ml), mortality started one day later and peak mortality was seen at Day 8. The end mortality was 80%. With a dose of only 1.2 x 10^6 CFU/ml, mortality started only at

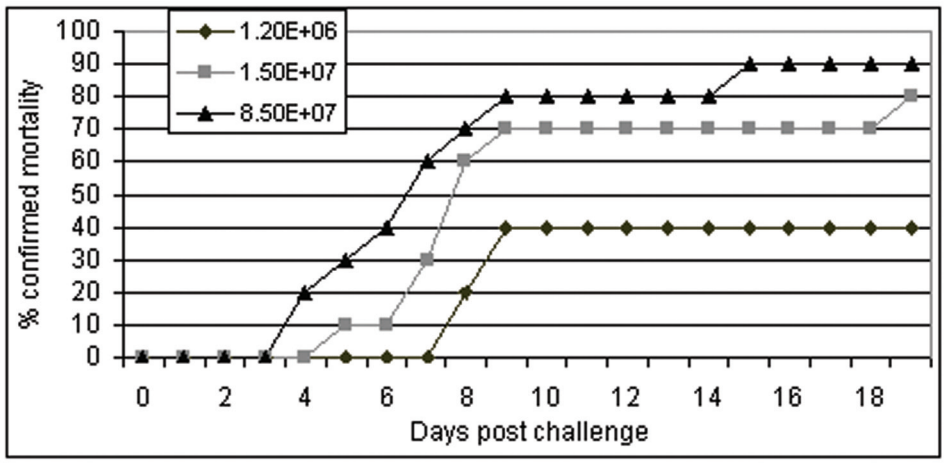

Figure 6. Cumulative percent mortality in 93-g yellowtail (*S. quinqueradiata*) after intraperitoneal injection with different concentrations of *N. seriolae*.

Day 8 and an end mortality of 40% was obtained (see Figure 6). However, upon dissection, an additional 50% of the fish showed the presence of severe granulomas in the internal organs, resulting in 90% of severely affected fish even in the lowest dose group.

DISCUSSION

From 2002 to 2006, cases associated with the typical clinical disease signs indicative of nocardial infections have been observed in countries such as Indonesia, China, Singapore and Malaysia. The causative agent of those infections was indeed found to be *N. seriolae*.

The clinical signs observed amongst the affected fish were typical of nocardial infection, nocardial infection having already been described in fish species in various countries in the Asian region. Chen *et al*. (2000) described multiple yellowish white nodules in the gills, heart, spleen and kidney in pond cultured Japanese sea bass (*Lateolabrax japonicus*) in Taiwan. Similar clinical signs were seen in yellow croaker (*Larimichthys crocea*) in China (Wang *et al.*, 2005) and have often been described in yellowtail (*S. quinqueradiata*) and amberjack (*S. dumrelii*) in Japan (Karyia *et al.*, 1968; Kusuda *et al.,* 1974; Kumamoto *et al*., 1985; Kudo *et al.*, 1988; Shuzo, 1992). From our observations in the field, a clear indication of the presence of the disease was seen based on impression prints, where typical branching, beaded, filamentous Gram-positive bacteria were present in high numbers in association with typical clinical signs. However, isolation results suggest that, even when an infection is present in the population, there is only a 50% chance that the bacteria will be isolated. Although positive differentiation with similar pathogens (like *Mycobacterium* sp.) can only be made by isolation and identification of the causative agent, impression prints of affected organs were a useful tool for the establishment of a presumptive diagnosis. Morphologically, *Nocardia* appear filamentous, branched and beaded and are 5-50 µm long, while mycobacteria are usually shorter (1-3 µm) (Wolke *et al.*, 1987). In addition, *Nocardia* will only give a positive acid-fast staining when FF acid-fast stain is used (Woo *et al.*, 1999), whereas mycobacteria would readily give a positive acid-fast reaction with the (unmodified) ZN stain. Histology can also be used for the establishment of a presumptive diagnosis and is best interpreted when counter stained with Gram or FF stain.

Identification tests indicated that all the isolates were close to *N. seriolae* and phenotypically and biochemically distinct from *N. asteriodes*. Reaction profiles of our field isolates were similar to those of *N. seriolae* described by Goodfellow (1971), Kusuda *et al.* (1974) and Holt *et al.* (1994). Confirmation of identification was done by PCR. Specific primers N5F1 and N5R1 were used to specifically differentiate *N. seriolae* from *N. asteroides* and from other Gram-positive and Gram-negative bacteria, kidney cells and virus. As the isolation of *N. seriolae* is unreliable by culture method, PCR can be a good alternative for confirmation of the presence of the disease. Laurent *et al.* (1999) described a rapid method to identify clinically-relevant *Nocardia* sp. to genus level by 16S rRNA gene PCR. Kono *et al.* (2002) have described the use of a specific PCR method to identify *N. seriolae*. Recently, a loop-mediated isothermal identification technique has been described for the identification of *N. seriolae* based on 16S-23S rRNA internal transcribed spacer region and was found to be more sensitive than PCR (Itano *et al.*, 2006).

The economical importance of the disease was difficult to estimate as only low level chronic mortality was frequently observed. However, it was clear from our isolation data that mainly bigger fish (> 100 g) were affected and, therefore, the economical impact on the farm can be very significant, even at the lower levels of mortality. In addition, it seemed that species such as silver pomfret (*P. argenteus*) and pompano (*T. blochii*) were more susceptible to nocardial infections and could suffer up to 30% mortality during a single outbreak. Furthermore, it was shown that experimental infection was able to induce a significant level of mortality within a relatively short observation period of 19 days, indicating the role of *N. seriolae* as a primary pathogen. There are few, if any, effective treatments against the disease and antibiotic treatment is often ineffective in controlling the disease. Indeed, the abundant use of some drugs has been implicated in the increasing occurrence of antibiotic resistance amongst *N. seriolae* isolates (Itano *et al.*, 2002).

Our findings suggest that it is very likely that nocardial infections due to *N. seriolae* are largely underestimated in South East Asia due to the difficulty of isolation. The significance and prevention of *Nocardia seriolae* in economically-sustainable aquaculture in this region must be given serious attention.

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Multiple Streptococcal Species Infection in Cage-cultured Red Tilapia But Showing Similar Clinical Signs

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ABSTRACT

From year 2000, periodic mortality outbreaks of cage-cultured red tilapia in the reservoirs of Tasik Kenyir, Terengganu and Tasik Pergau, Kelantan were recorded. These incidents were associated with the onset of the dry season from March until June. Bacterial isolation of the sampled organs especially the eyes, brain and kidney conducted during the sampling study from September 2002 until December 2003, revealed small pinpoint or minute transparent colonies on Blood Agar. The colonies were characteristically presumptive of *Streptococcal* spp., Gram positive cocci, confirmed by API 20 STREP. *Streptococcus agalactiae* made up 70% of the total streptococcal species identified. The remaining 30% were other species of Streptococcus that included *Leuconostoc* spp. and *S. constellatus.* Although multiple streptococcal species infection was identified, clinical signs and gross morphological changes were similar. Typical signs observed included swimming in isolation prior to erratic swimming behavior and not feeding. Almost all of the sick tilapia indicated eye abnormalities such as corneal opacity or cloudiness; unilateral or bilateral exopthalmia or sunken eyes. Popeye was hemorrhagic at times and inflammation of the ventral region was more obvious in bigger tilapias. In Tasik Kenyir, streptococcal infection was more abundant in 150-250g compared with Tasik Pergau where the affected tilapias were between 300-450g.

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INTRODUCTION

Recent years have shown an increase in tilapia production in Malaysia (Table 1; Ann. Fisheries Stats., 2004). This appeared to be more pronounced after the introduction of red tilapia (*Oreochromis niloticus* hybrid) to Malaysia in the mid-1980s (Figure 1;http://agrolink.moa.my).

Table 1. Production of two main freshwater fish species culture in Malaysia from 1990-2004, including wholesale values.

Year	Species	Total of fish (ton)	Wholesale (RM)
1990	Red Tilapia	314.14	1,523,800
	Cat fish	133.33	1,110,000
1995	Red Tilapia	4827.03	26,646,350
	Cat fish	1752.26	8,195,940
2000	Red Tilapia	15636.02	76,782,370
	Cat fish	12115.68	48,001,910
2004	Red Tilapia	21458.88	67,990,380
	Catfish	20848.92	53,825,360

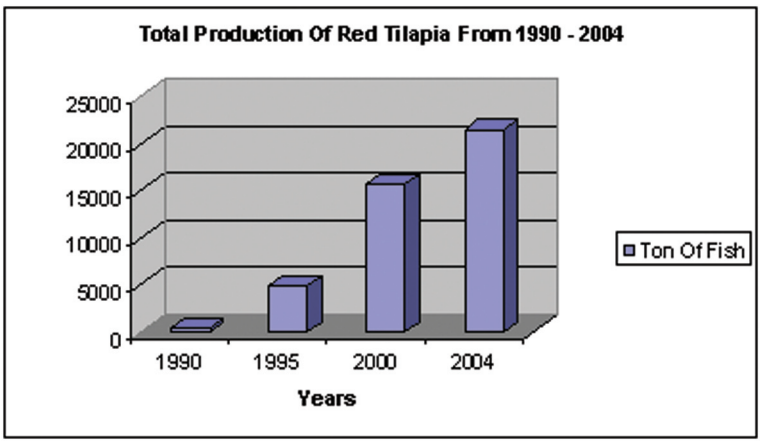

Figure 1. Total red tilapia production recorded from 1990-2004. (Source http://agrolink.mao.my).

The red tilapia is mostly cultured in ponds but now there is an increasing number of floating net-cages located in natural or man-made inland water bodies (Annual Fish. Stats. 2000). These include reservoirs or ex-mining pools. Cage-cultured red tilapia is also being produced along the river banks in Sungai Pahang, Temerloh, Pahang and Sungai Terengganu, Terengganu (Siti-Zahrah *et al.,* 2004. With the rapid growth in the Malaysian red tilapia industry, intensification of culture systems has resulted. It is anticipated that with this intensification there may be an increase in the incidence and severity of microbial

pathogens. Even though tilapia has been otherwise reported to be hardy (Roberts and Sommerville, 1982), tilapia continue to succumb to bacterial pathogens after a period of culture in cages.

The increased susceptibility was reported in 1997 at Temerloh, Pahang in cage-cultured fish at Sg. Pahang (Siti-Zahrah *et al.,* 2004). The case study conducted revealed that a high mortality rate was closely related to increased water temperature of 30-32^{0}C during the dry season. Laboratory diagnosis showed only the presence of *Aeromonas hydrophila* from kidney of affected animals, thus it was suggested that the mortality was possibly due to `heat–stress related syndrome'. This syndrome affected tilapia of more than 300g causing erratic swimming behavior and/or eye abnormalities such as exophthalmia, (Figure 2).

Figure 2. Bilateral exophthalmia in affected tilapia

The clinical signs observed for the previous disease outbreaks were again later observed in red tilapia reared in floating cages at Tasik Kenyir, Terengganu and Tasik Pergau, Kelantan of east coast, Peninsular Malaysia. The high tilapia mortality or outbreak was initially reported in June 2000, which was subsequently reported later every year during the dry season.

Thus, a study was initiated to examine the etiology of the disease affecting red tilapia causing very high mortality to the farmers. It was also observed that the outbreak was highly prevalent during the months of March to June which was associated with the coming of the dry season (Siti-Zahrah *et. al.,* 2004).

MATERIALS AND METHODS

Study sites

Two sites where floating cage-culture operated were selected. Both sites were in the east coast of Peninsular Malaysia, *i.e.* Tasik Kenyir in Terengganu and Tasik Pergau in Kelantan. Four operators were involved in the study, three of which were from Tasik Kenyir, Terengganu.

Tilapia sampling

Healthy and sick tilapia were randomly sampled from each operator, the number and size of fish sampled was dependent on the number of cages belonging to each operator. However, 25-30 fish samples were taken from Risda Sdn Bhd., who had the highest number of cages (>200) while the smallest number, about 10-12 fish were from NTL Sdn. Bhd. who operated about 20 cages. Tilapia samples taken monthly were observed and recorded for any clinical signs of disease and morphological changes exhibited.

Water sampling

Samples of water were taken monthly from a few sampling points selected from both inside and outside the cages. Water quality parameters (e.g. temperature, pH, DO, nitrite, ammonia and nitrate) were taken using YSI probe (Model 556), recorded, and analyzed to see any relationship with the prevalence of the outbreak.

Bacterial diagnosis

From the clinical signs observed in the tilapia, bacterial isolation was conducted by taking samples from the eye, brain and kidney, of affected fish using Blood agar (BA Oxoid) or Brain Heart Infusion (BHI Oxoid) agar. Secondary subculture was done to obtain pure colonies prior to Gram staining and presumptive biochemical tests including API 20 STREP (Biomereux).

RESULTS AND DISCUSSION

Water quality parameters

Periodic outbreaks of streptococcal infection with sudden high mortality of tilapia, were observed during the onset of dry season which lasted from March until June. This was associated with periodical changes and fluctuations in water quality parameters. The parameters (Table 2) were mainly temperature (30.5 to 32^0C), dissolved oxygen (3.2 to 10.2ppm), pH (6.3 to 8.7), nitrite (0 to 0.02ppm), iron (0.0 to 1.13ppm) and ammonia levels (0.0 to 0.55ppm) as recorded in Tasik Kenyir. The water temperature was well above 30^0C throughout the affected months in Tasik Kenyir Terengganu, almost similar to Tasik Pergau in Kelantan. The persistent high temperature with associated fluctuating water parameters throughout the dry season as above was reported by Siti-Zahrah *et al,* (2004), causing high mortality rate in cage-cultured tilapia.

Table 2. Mean water quality parameters recorded in cultured cages at Tasik Kenyir, Terengganu, and the associated percentage mortality and clinical symptoms observed in the affected tilapia.

Water quality parameters	Temp. (^{0}C)	DO (ppm)	pH	Nitrite (ppm)	Ammonia (ppm)	Fe^{2+} (ppm)
	30-32	3.2-10.2	6.3- 8.7	0- 0.02	0.0 - 0.55	(0.0-1.13
% mortality	60-70% in most of the reported cases					
Clinical symptoms	Isolation of tilapia at either corners of the floating cage prior to erratic swimming behavior, eye abnormalities either bilateral or unilateral exophthalmia, corneal opacity or cloudiness, hemorrhagic or sunken eyes and in some cases, blindness, inflammations along the ventral region from lower mandible to anus, and sometime pustule-like swellings at basal of dorsal fins.					

Bacterial diagnosis

A total number of more than 1000 tilapias were sampled during the study from both the reservoirs. Almost all the primary isolates from the sampled organs (eye, brain and kidney) showed uniquely uniform pinpoint or minute transparent colonies in the Blood or BHI agar. Some of the pure colonies showed distinct hemolytic properties (β- hemolytic) in the Blood Agar, while some showed otherwise. The colonies were minute (0.5-1.0mm), transparent, round and convex entire and some were occasionally very tiny pinpoint colonies (< 1mm) that can hardly be seen by the naked eye, after 24-48 hrs. They were Gram positive cocci (sometime ovoid) in chains of either single or in pairs. The catalase test was negative, and the biochemical profile of the isolates confirmed them to be *Streptococcus* spp. (data not presented).

From the samples of tilapia examined, the average rate of bacterial infection was 39%. Where 29% of the isolates recovered from the affected fish were identified as *Streptococcus* spp. However, 70% of these were identified as *Streptococcus agalactiae* from the affected fish compared with any other *Streptococcus* spp. (*Leuconostoc sp., S. constellatus* and *Gemella hydrolysans)*.

The results from this study indicated that the affected tilapia displaying clinical signs of disease were mainly infected with *Streptococcus sp.*, the most common and dominant identified in this study was *S. agalactiae*. A similar finding was recently reported in Thailand and Indonesia (Yuasa *et al.,* 2005). These authors did not indicate periodic outbreaks which can be associated with dry seasons and fluctuating water quality changes (temperature, DO, etc. as reported by Siti-Zahrah *et al.* (2004). This was also true as reported by Bunch and Bejerano (1997) which indicated that certain water quality parameters affected tilapia susceptibility to streptococcal infection.

Furthermore in Kuwait Bay, which is a semi-enclosed embayment of the Arabian Gulf, *S. agalactiae,* caused massive kill to wild mullet and cultured sea bream (Gilbert *et al.,* 2002). Kitao (1993) also indicated that salmon, mullet, golden shiner, pinfish, sea trout and sturgeon were susceptible to streptococcal infection. *Streptococcus sp.* has also been isolated from a variety of ornamental fish including rainbow sharks, red-tailed black sharks, rosey barbs, danios, some cichlids including Venustus (*Nimbochromis ("Haplochromis*") *venustus*) and *Pelvicachromis sp*. and several species of tetras (Yanong and Francis-Floyd, 2002) demonstrating the global threat of *Streptococcus* spp. to both farmed and ornamental fish species.

The same typical clinical signs of streptococcal infections were observed in this study as reported by other authors (Evans *et. al.,* 2002,). Most striking or pronounced signs were isolation of tilapia at either corners of the floating cage, prior to erratic swimming behavior, with eye abnormalities either bilateral or unilateral exophthalmia, corneal opacity or cloudiness, hemorrhagic or sunken eyes and in some cases, blindness. Gross clinical signs of disease observed during this study were numerous and similar to previously reported Streptococcus infections in tilapia (Figure 3). All of these clinical signs support the morphological and behavioural changes observed in this study associated with streptococcus infections in tilapia.

A difference was found in the physical appearance of the fish depending on the size of the animals affected. As in this study the medium-sized tilapia, had a sunken body appearance, which was not observed in the larger fish. This appeared to be an additional clinical sign.

The study also showed that clear clinical signs associated with streptococcal infection prevailed more in tilapia of 150-250g in weight in Tasik Kenyir, whilst in Tasik Pergau, infected tilapia were of 300-450g in weight. The size or weight difference in affected tilapia at the respective sites of study was not fully understood but this may be due to the progression of the infection or route and duration of exposure to the pathogen.

The results of the study showed that, the high incidence of mortality in tilapia at both of the respective sites were due to a streptococcal infection. The dominant aetiological agent was identified as *S. agalactiae*, which has been reported to cause mortalities in other farmed tilapia species. The periodic disease outbreaks reported were also associated with the onset of the dry season. This resulted in increased and fluctuating water quality parameters, particularly water temperature which was 30-32^{0}C, during the dry season. Ferguson *et. al.* (1994), reported that populations of zebra danios and white cloud mountain minnows when exposed to high concentrations of *Streptococcus* spp. in the water experienced 100% mortality within 2-4 days of exposure. It is thus, important that streptococcal infections be quickly identified and managed to prevent major losses, as already experienced by operators in both reservoirs.

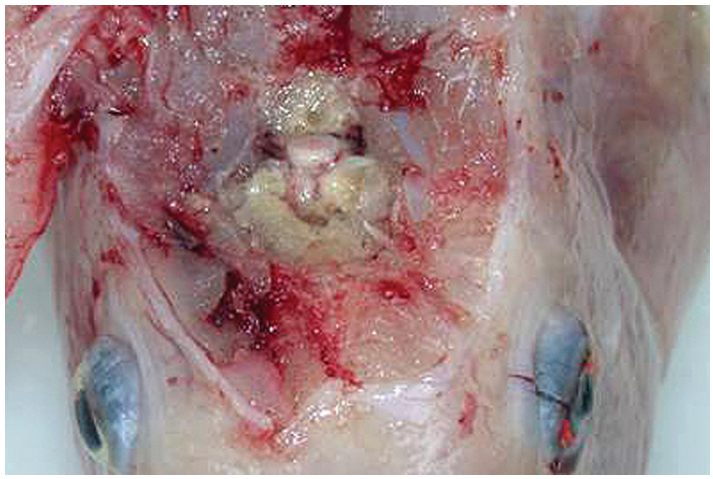

Figure 3. Haemorrhagic brain tissue observed grossly in affected tilapia

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Immune Expression Analysis and Recombinant Protein Production of a Fish Granulocyte Colony-Stimulating Factor (CSF3)

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ABSTRACT

Granulocyte colony-stimulating factor (CSF3) is a cytokine involved in the differentiation and maturation of neutrophils and its precursor cells in mammals. CSF3 orthologues has recently been identified in Japanese flounder (*po*CSF3-2), fugu (*tr*CSF3-1 and -2) and green-potted pufferfish (*tn*CSF3-1 and -2). *po*CSF3 was shown to be involved in Japanese flounder immunity, being basally well expressed in tissues and immediately up-regulated following immunostimulation. Here, we report that the CSF3 genes of fugu and green-potted pufferfish, which are in duplicates, also are constitutively expressed in several tissues but at varying levels. *po*CSF3 was further shown to be down-regulated by polyinosinic:polycytidylic acid (poly I:C), a known interferon inducer, in brain and kidney; up-regulated in PBLs (but only at 6 hr post-induction); and clearly induced in spleen. These results provide further evidence that teleost fish CSF3 is involved in the immune system. We likewise succeeded in producing a 26-kDa recombinant *po*CSF3 protein in a Japanese flounder cell line (Hirame Natural Embryo or HINAE) using a mammalian expression vector pCDNA4 HisMax C. This recombinant CSF3 fusion protein and the technique used are seen to be useful tools to further study teleost fish CSF3 function in relation to immunity.

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INTRODUCTION

The innate immune system of fish consists of nonspecific cellular responses that include monocytes/macrophages, granulocytes (neutrophils and eosinophils) and nonspecific cytotoxic cells (NCCs) (Iwama and Nakanishi, 1996). An important cytokine that is involved in the proliferation, survival, differentiation and maturation of neutrophils in mammals, granulocyte colony-stimulating factor (CSF3), has been recently and simultaneously identified in chicken and in 3 teleost fish species; Japanese flounder, *Paralichthys olivaceus* (*po*CSF3), fugu, *Takifugu rubripes* (*tr*CSF3-1 and -2), and pufferfish, *Tetraodon nigroviridis* (*tn*CSF3-1 and -2) (Santos *et al*., 2006). Chicken, *Gallus gallus* (*gg*CSF3) was shown to be the chicken Myelomonocytic Growth Factor (cMGF). On the other hand, the teleost fish CSF3 orthologues were confirmed and shown to be in duplicate or paralogous. In addition, teleost fish CSF3s have been shown to be more rapidly evolving than mammalian CSF3s, which appear to be undergoing purifying selection.

CSF3 in mammals has been reported to be produced by different kinds of cells including monocytes/macrophages and lymphocytes (Sallerfors, 1994), fibroblasts (Kaushansky *et al.*, 1988), endothelial cells (Zsebo *et al*., 1988), astrocytes (Aloisi *et al*., 1992), bone marrow stromal cells (Fibbe *et al.*, 1988a), T-lymphocytes (Ichinose *et al*., 1990) and polymorphonuclear granulocytes (Lindemann *et al.*, 1989). The increase in production of CSF3 is very sharp and abrupt (having a half life of 4-10 h in circulation and 1-2 days in tissues) in response to endotoxins or secondary mediators such as tumor necrosis factor (TNF), interleukin-1 and interferon-γ (IFN-γ), suggesting that it is a vital regulator of granulocyte production during inflammation and immune responses (Demetri and Griffin, 1991). CSF3 could also be induced by PHA and PMA (Oster *et al*., 1989a), Interleukin 3 (Oster *et al.*, 1989b), Interleukin 4 (Wieser *et al*., 1989), granulocyte-macrophage colony-stimulating factor (CSF2) (Sallerfors and Olofsson, 1991; Oster *et al*., 1989b) and macrophage colony-stimulating factor (CSF1) (Ishizaka *et al*., 1986). In teleost fish however, only the constitutive and mitogen responses of Japanese flounder *po*CSF3-2. *po*CSF3-2 has been shown to be highly expressed in major immune organs including kidney, peripheral blood leukocytes, gills and spleen and moderately in the brain. It is expressed strongly in kidney and peripheral blood leukocytes (PBLs) stimulated by lipopolysaccharides (LPS) and a combination of Concanavalin A (ConA) and Phorbol Myristate Acetate (PMA), indicating that like mammalian CSF3, it is intimately involved in the immediate immune response in Japanese flounder.

CSF3 is one of the successful cytokine therapeutics in humans and domestic mammals, which include interferons and hematopoietic growth factors (colony stimulating factors). Recombinant CSF3 protein is commercially available under the generic name filgrastim (brand name: Neupogen) or pegfilgrastim (brand name: Neulasta). These therapeutics are used for supporting cancer patients receiving chemotherapy or bone marrow transplants, patients under peripheral-blood-progenitor-cell collection and therapy, and patients with neutropenia (Vilcek and Feldmann, 2004). In lower vertebrates, particularly in teleost fish however, recombinant CSF3 has not been produced and studied for its function and potential therapeutic application.

In this study, we analyzed the constitutive expression of the CSF3 duplicate genes in fugu (*tr*CSF3-1 and *tr*CSF3-2) and pufferfish (*tn*CSF3-1 and *tn*CSF3-2). We also tested the *in*

vitro regulation of Japanese flounder *po*CSF3 in primary cultures of brain, kidney, peripheral blood leukocytes and spleen cells in response to polyinosinic:polycytidylic acid (poly I:C), a known interferon inducer. Lastly, we attempted to produce a recombinant *po*CSF3 protein in a Japanese flounder cell line (Hirame Natural Embryo or HINAE), in order to have enough material to confirm its activity, determine its structure or investigate its potential use as a stimulant to boost the fish immune system.

MATERIALS AND METHODS

1. Cell culture

HINAE cells were grown in Leibovitz's L-15 medium (Gibco-BRL, Grand Island, NY) supplemented with 15% fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS) and 100 IU ml-1 penicillin G and 100 µg ml-1 streptomycin (Gibco-BRL, Grand Island, NY).

2. RT-PCR Analysis

2.1 Constitutive expression

Fugu and pufferfish samples were dissected for the following tissues: brain, eyes, gills, heart, intestine, kidney, liver, muscle, ovary, skin, spleen, and stomach. Total RNA was extracted using Trizol reagent (Invitrogen, USA). cDNA was synthesized in the following manner: 10 µg of total RNA, 1 µl of oligo (dT) (500 µg/ml), and 1 µl of dNTP mix were mixed together, heated to 65^{o}C for 5 min then quick-chilled on ice; after which, 4 µl of 5x First-Strand buffer, 2 µl of DTT (0.1M), 0.25 µl of RNase inhibitor (TOYOBO, Japan) (40 U/µl) and MMLV RTase (Invitrogen, USA) (200 U/µl) was added, incubated at 37^{o}C for 50 min, heated to 70^{o}C for 15 min then cooled to 4^{o}C.

cDNAs were PCR amplified using fugu and pufferfish CSF3 specific primers (Table 1). PCR conditions were: initial denaturation at 95^{o}C for 5 min, 30 cycles (95^{o}C - 30s, 55^{o}C - 30s, 72^{o}C - 1 min), and final elongation at 72^{o}C for 5 min. β-actin was used as a control. PCR amplicons (5 µl) were visualized on a 1% agarose gel stained with ethidium bromide and photographed with a densitometer (Atto).

2.2 Immunostimulated expression

Japanese flounder brain, head kidney and spleen were dissected out, minced and filtered through a nylon mesh to a medium containing RPMI 1640 (Nissui, Japan), 1% streptomycin/penicillin (Gibco, USA) and 0.2% heparin (Sigma, USA). Primary PBL culture was prepared through density gradient centrifugation using Percoll solution (Pharmacia, USA). Centrifugation was done at 400x g at 4^{o}C for 3 times with each PBS washing. The cells were cultured in RPMI 1640.

Head kidney and PBL primary cultures were treated with 0.5 mg/ml of poly I:C. The cells were then sampled at 1, 3 and 6 hrs post-induction. cDNAs were synthesized as above and PCR amplified using designed primers *po*CSF3-F2 and *po*CSF3-R (Table 1) and the following conditions: initial denaturation at 95^{o}C for 5 min, 28 cycles (95^{o}C - 30s, 55^{o}C - 30s, 72^{o}C - 1 min), and final elongation at 72^{o}C for 5 min. β-actin was used as a positive control. Amplicons were visualized as above.

Table 1. Primers used for the RT-PCR analysis in this study.

Fish species	Primer name	Primer sequence (5' → 3')
Fugu (*Takifugu rubripes*)	trCSF3-1AF	TGAACATCCTGATTGTCCTCG
	trCSF3-1AR	ATCTGTCATCTGGTTCCTCGT
	trCSF3-2AF	ACAGACATGACCGACCTGACA
	trCSF3-2AR	TCCTGTAGGTGCTGATGGCT
Green-spotted pufferfiish (*Tetraodon nigroviridis*)	tnCSF3-1AF	CATGCACATCCTCATTGTCCT
	tnCSF3-1AR	TTATCTGCTTTGGTCCAGGCT
	tnCSF3-2AF	ACGGACAGACATGATCCACCT
	tnCSF3-2AR	TGCTGCTCCTGTAGCTGTTGA
Japanese flounder (*Paralichthys olivaceus*)	poCSF3-F2	ATGGACTCTGAGACAGTTGT
	poCSF3-R	CGGTAACTGCTTAGCGTGCA
	poCSF3-FProt	TTTGGATCCATGGACTCTGAGACAGTTGT
	poCSF3-RProt	TTTGATTCTTAGCGTGCACCTGCAGCTCGGC

3. Recombinant protein production

3.1 Plasmid construct

A DNA fragment containing the full *po*CSF3 ORF (containing 633 bp and a predicted mass of 21 kDa) and an added *Bam*H1 and an *Eco*R1 site was generated by PCR using designed primers *po*CSF3-FProt and *po*CSF3-RProt (Table 1). The mammalian expression vector pCDNA4/HisMax C and the PCR fragment were cut using *Bam*H1 and *Eco*R1 restriction enzymes, recovered using EASYTRAP ver. 2 (Takara, Japan) and ligated together using ligation high (TOYOBO, Japan) (Figure 1.).

3.2 Transfection and detection

HINAE cells seeded onto 35-mm tissue culture plates were transiently transfected with the pCDNA-HisMax-CSF3 construct using Lipofectamine 2000 (Invitrogen, USA) following the manufacturer's protocol. Two (2) each of 6 plates were incubated for 1, 2 and 3 days with pCDNA4-CSF3 and 2 plates were mock infected with blank pCDNA-HisMax C.

Transfected cells were harvested accordingly using a rubber scraper and then pelleted by centrifugation at 1,500 x g for 5 min. The 6 plates were separated into 2 sets, one for RT-PCR and the other for western blot. For RT-PCR analysis, the above method was used. For the recombinant protein, harvested cells were resuspended in 20 µl Phosphate Buffered Saline (PBS). This was then homogenized using a cell lysis buffer (50 mM Tris-HCl, pH 7.8; 150 mM NaCl; 1% Nonidet P-40). Samples were diluted with equal amounts of SDS sample buffer (125 nM Tris HCl, pH6.8; 4% SDS; 10% glycerol; 10% 2-mercaptoethanol; 0.004% bromphenol blue) and boiled for 10 min. The samples were resolved in 15% SDS PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was then incubated with AntiXpress antibody (Invitrogen, USA) at a 1:10,000 dilution. Recombinant CSf3 protein was then detected by using anti-mouse IgG conjugated with alkaline phosphatase (Promega, USA) following the manufacturer's protocol.

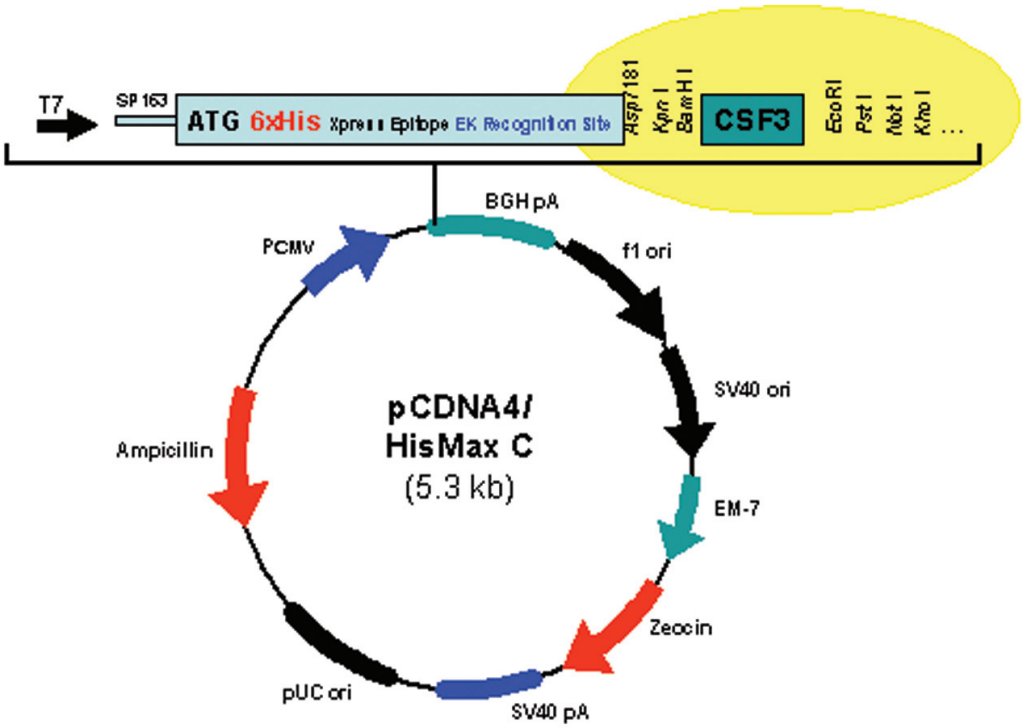

Figure 1. Mammalian expression vector pCDNA4-CSF3 construct map.

RESULTS

RT-PCR analysis revealed that both of the fugu CSF3 genes were expressed in tissues, albeit at varying degree; *tr*CSF3-1 in gills, kidney, ovary and skin while *tr*CSF3-2 in skin only. On the other hand, only one of the green spotted pufferfish CSF3 genes (*tn*CSF3-2) showed expression in gills, skin and ovary (Figure 2).

*po*CSF3-2 in response to poly I:C was also observed in primary cultures of brain, kidney, PBLs and spleen of Japanese flounder. *po*CSF3-2 expression was not detectable in the brain, only faintly observed in kidney at 1 and 3 hrs, highly expressed in PBLs at the 6th hr incubation and was inducible in spleen at 1 hr post-treatment (Figure 3).

Three clones of the pCDNA4-CSF3 construct (C6, C18, and C27) were confirmed to be in frame by sequencing (Figure 4). These same clones were transfected to HINAE cells and were able to produce the recombinant mRNA transcript of about 700 bp in size as shown by RT-PCR analysis (Figure 5A). pCDNA4-CSF3 construct C6 was selected and transfected again to HINAE cells for transient protein expression. Western blot analysis detected a 26-kDa protein (Figure 5B) and showed that its amount increased at the 3rd day of incubation. We tried to produce recombinant CSF3 with bacterial expression systems including pQE, pET32, pET28 and pNCMO2 for *Bacillus brevis* but with negative results (data not shown).

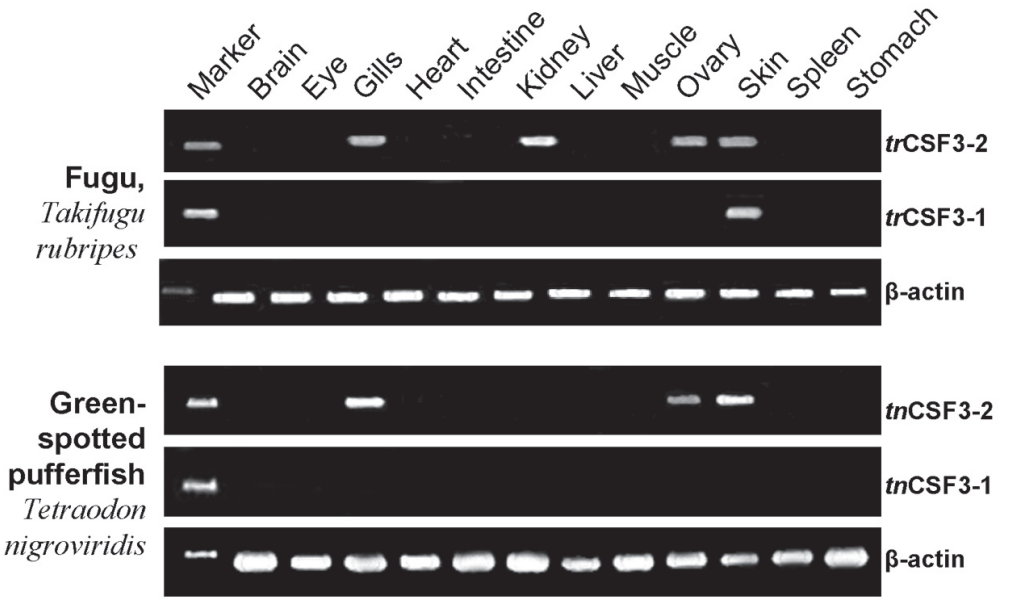

Figure 2. Expression of CSF3 genes in fugu (trCSF3-1 and -2) and green-spotted pufferfish (tnCSF3-1 and -2) in tissues.

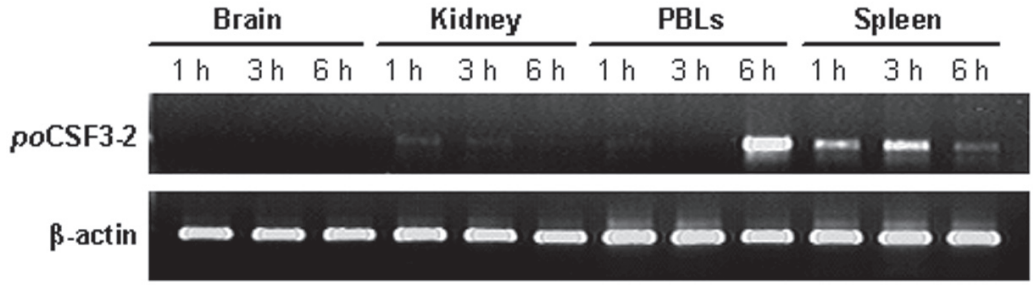

Figure 3. Expression of Japanese flounder poCSF3 gene in brain, kidney, PBLs and spleen in vitro following poly I:C treatment at 1, 3 and 6 hrs-post stimulation.

DISCUSSION

At the transcriptome level, it appears that the fugu *tr*CSF3s and green spotted pufferfish *tn*CSF3-2 are likely to be functional while the *tn*CSF3-1 is not and might have become a pseudogene. *tn*CSF3-1 was previously reported to apparently lack the conserved Cys (C) residue, a missing probable receptor-binding-glutamic-acid residue (E_{19}), a rather long hydrophobic N-terminal region, an undetected conserved domain (Santos *et al.*, 2006). If this is the case, the pufferfish paralogs probably followed a nonfunctionalization event, the classical theory of gene duplicate evolution, which predicts that one of the duplicate genes becomes fixated as a null allele incapable of transcription, translation or biological functions (Ohno, 1970). On the other hand, the fugu CSF3 paralogs were

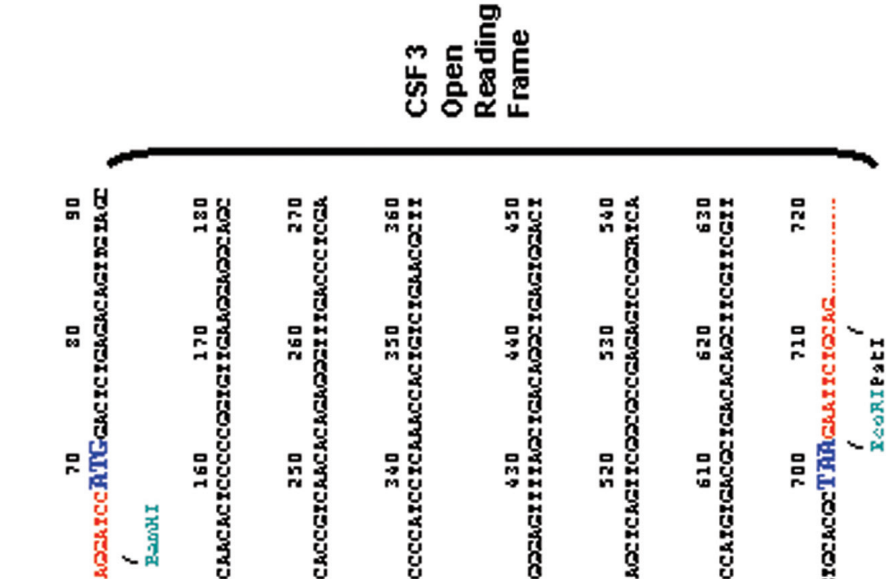

Figure 4. Confirmation of pCDNA4-CSF3 insert construct by sequencing.

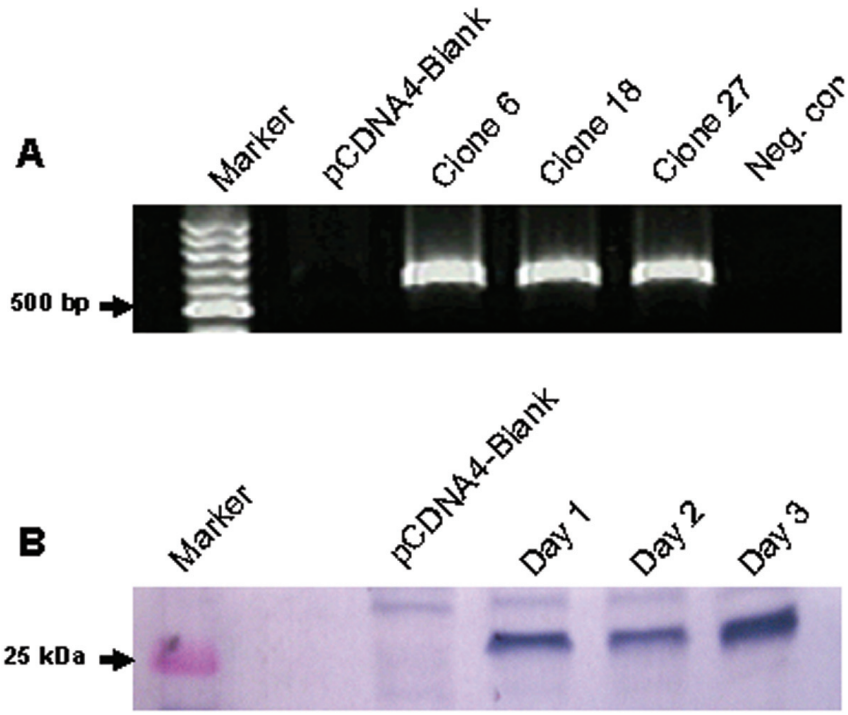

Figure 5. Recombinant Japanese flounder poCSF3-2 protein production using the mammalian expression vector pCDNA HisMax expressed in HINAE cells.
A) poCSF3-2 mRNA transient expression by 3 separate pCDNA4-CSF3 clones (C6, C18, C27) as shown through RT-PCR;

B) recombinant poCSF3-2 protein expression (Clone 6) at 1, 2 and 3 days-post transfection as detected by western blot using AntiExpress antibody.

expressed, although in different tissues. This suggests that these fugu genes undergone subfunctionalization, also called the duplication-degeneration-complementation (DDC) pathway (Force *et al.*, 1999). In the DDC pathway, the functions of both paralogs are complementary and have been preserved. The DDC pathway is not uncommon in fish. For example, evidence supporting the DDC theory has been observed in the *engrailed* genes of zebrafish. Another round of PCR (nested PCR) using some of the tissues for both fish yielded fragments of all four of the CSF3 orthologues (data not shown), indicating that all 4 mRNA transcripts are expressed and suggesting that pufferfish *tn*CSF3s is also undergoing positive selection. The functional evolution of the duplicate CSF3 genes in two species of the same fish family (fugu and green spotted pufferfish), which are thought to have diverged between 18 and 30 million years (Myr) ago (Hedges, 2002) needs to be clarified in future work.

*po*CSF3-2 production was observed in PBLs and spleen following poly I:C stimulation. In mammals, poly I:C has been reported to induce expression of CSF3 in endothelial and fibroblast cells (Fibbe *et al.*, 1988b and 1989), and in uterine epithelial cells (Schaefer *et al*., 2005). In fish, poly I:C has been shown to stimulate immune-related genes such as

viperin in mandarin fish (Sun and Nie, 2004), and the inducible isoform of nitric oxide synthase (iNOS) in small spotted cat shark (Reddick *et al*., 2006). Interleukin 6 (IL6) of fugu, which belongs to the same protein family as CSF3, and which is called Pfam IL6/CSF/MGF because of their conserved protein domains, was also significantly upregulated in the spleen when fish were injected with poly I:C (Bird *et al*., 2005). It is, however, unclear why CSF3 was not detected in brain and kidney, and in PBLs (after 1 hr and 3 hrs of incubation). In fugu, IL6 may have been increased in the kidney by poly I:C injection but the increase was not statistically significant. Nevertheless, this study provides indirect evidence that fish CSF3 is regulated by interferon or interferon-induced genes.

We were successful in constructing a mammalian expression vector producing the recombinant CSF3 protein of Japanese flounder in a fish cell line *in vitro.* The implication of such is that the recombinant protein produced is fully functional. Why bacterial expression systems failed to produce recombinant CSF3 is unclear. The immune-related activity of Japanese flounder CSF3 against possible pathogens, together with an understanding of its regulatory regions, suggests that the recombinant CSF3 protein is a good candidate for enhancing immune responses against disease. For example in mice that have been vaccinated with HIV-1 *env* and *gag/pol*, co- delivery of recombinant mice CSF3 with macrophage colony-stimulating factor (MCSF) resulted in a moderate effect on serum antibody responses and T-helper cell proliferation, upregulated INF-γ production in antigen-stimulated splenocytes and increased the serum IgG_2/IgG_1 antibody isotype ratio (Kim *et al*., 1999).

CONCLUSIONS

Granulocyte colony-stimulating factor has recently been identified in teleost fish. Howerve, little is known about where it is expressed or how it is regulated. Our results show that duplicate CSF3 genes in fugu and pufferfish are differentially expressed, and that Japanese flounder *po*CSF3-2, aside from its up-regulation by LPS and conA-PMA stimulation as previously shown, is also involved in interferon-related mechanisms and pathways. Lastly, we successfully produced a recombinant Japanese flounder *po*CSF3-2 protein and this would allow for further studies of *po*CSF3-2 structure and function, and possible use in disease management.

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Diseases of Molluscs

Parasitic Fauna of Rock Oyster (*Saccostrea forskali*) Cultured in Thailand

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ABSTRACT

Rock oysters (*Saccostrea forskali)* were sampled assuming 2% disease prevalence from two sites at the upper part of the Gulf of Thailand every four month for one year. *Marteilia* sp*., Perkinsus* sp. and trematodes in the family Plagiochiidae were found in the oyster samples. Histopathological observations revealed that *Marteilia* sp. occurred in the digestive gland, metacercaria of digenean were found in gonads and a few sporocysts containing rediae were located in the gills. *Perkinsus* sp. was histologically observed only in oysters over one year of age that were sampled from one location in November. Digenean was detected at both sites year round but histopathological observations suggested that it was not pathogenic to the host. Infection of oysters by these parasites had no apparent effect on the production of oysters at these sites. The prevalence of these parasites was low and further investigations should be undertaken to clarify their specific taxonomic identity.

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INTRODUCTION

Culture of rock oyster (*Saccostrea forskali*) in Thailand is distributed along the eastern coast of the Gulf of Thailand as well as in some parts of the southern coastal areas. Most of the oyster farms are small-scale using natural spat collection. The most popular method of oyster culture in Thailand is the hanging method, with oyster spats attached to a roll of cement disc (one inch diameter) that are joined together by a rope. Oysters are harvested at 10-12 months of age depending on size. Yields from production areas average 10 tonnes per hectare. Thailand exports oysters to many countries, including Australia, Canada and Japan. A total of 302,636 kilograms worth US$875,187 were exported in 2004.

In 1999, a *Marteilia*-like species was found in the rock oyster (*Saccostrea forskali)* sampled from the east coast of Thailand (Taveekijakarn *et al.*, 2002). *Marteilia refringens* is the causative agent of marteiliosis, one of the notifiable mollusk diseases in the aquatic animal pathogen list of the Office International des Epizooties (OIE). Little information on this oyster disease is available in Thailand and the Southeast Asian region.

Two *Marteilia* spp. are known, *M. refringens* from flat oysters (*Ostrea edulis*) in Europe, and *M. sydneyi* from rock oysters (*Saccostrea glomerata*) in Australia (Bondad-Reantaso *et al*., 2001; Berth *et al*., 2004). The *Marteilia* sp. found in the rock oyster in Thailand has not yet been identified up to a species level. Further study is required not only for classification purposes but also for epidemiological data.

Other parasitic diseases have been reported from other parts of the world where they cause significant damage to the industry (Bower *et al*., 1994; Carnegie and Cochennec-Laureau, 2004). Those include *Mikrocytos mackini* infection in Pacific oysters (*Crassostrea gigas*) cultured in western Canada and the west coast of the United States, infections with *Bonamia ostreae* and *B. exitiosa*, respectively reported in flat oysters cultured in Europe and New Zealand, *Perkinsus marinus* and *Haplosporidium nelsoni* in the Eastern oyster (*Crassostrea virginica*) populations along the mid-Atlantic region of the United States. However, less information on diseases of oysters is available in Thailand. The aim of this present work was to conduct a preliminary investigation of parasitic diseases of oysters cultured in Thailand. This study will provide a better understanding of the parasitic diseases situation of cultured oysters in Thailand, and also suggest disease prevention and control measures.

MATERIALS AND METHODS

Rock oysters (*Saccostrea forskali)* were collected in order to ensure detection of pathogens occurring at 2% prevalence (150 oysters per site). They were collected from two farms in Chonburi Province: Bangplasoy (site 1) and Bangsai (site 2, estuary of the Bangplakong River) in March, July and November 2004. In addition, 150 of over-one year-old oysters (18 months) were collected for comparison with the younger stocks (10 months). Water temperature and pH at the surface level of culture sites were also recorded. Clinical signs and macroscopic parasites of the oysters were observed. The samples infected with macroscopic parasites were freshly chopped to isolate the parasite for a further identification. For the histological study, the samples were fixed in 10% buffered formalin

for 24 hrs and then embedded in paraffin blocks. The samples were cut at a 5-μm thickness and mounted on glass slides. The sections were stained with haematoxylin and eosin (H&E) and observed under a light microscope.

RESULTS

In March and November, two kinds of parasite were found: a *Marteilia* sp.-like and a digenean trematode (Table 1). The light microscope observation revealed *Marteilia* sp. in the digestive gland. Young and mature sporulation stages of *Marteilia* sp., with refringent strongly eosinophilic inclusion bodies, occurred in the epithelium of the digestive diverticulae (Figure 1). Typically, each sporangiosorus had 2 to 6, or possibly more sporonts which contained two spores (Figure 2). Haemocytic infiltration was also found surrounding the infected area. Extra sporogonic proliferation was observed in the gill of some of the infected oysters.

The digenean trematodes were found at the both sites throughout the year (Table 1). In average, 1 to 6 digenean worms were commonly found in one oyster. Metacercaria were usually found in the connective tissue of the gonad, and a few sporocysts containing rediae were observed in gill tissues (Figures 3 and 4). Inflammatory response was not observed in association with the infection. The acetabulum of the digenean was bigger than the oral sucker and lay over the midline of the body (Figure 5). They were identified as Plagiorchiidae following Yamaguti (1958). Some samples showed focal haemocytic infiltration with no pathogenic organism.

The third sampling conducted in November was infected with *Marteilia* sp., a digenean and also a *Perkinsus* sp. in the older oyster samples. In these infected samples, focal necrosis was observed with haemocytic infiltration in the ovary. The variously-sized trophozoites were scattered in the necrotic area and among the connective tissue between degenerated tubules of digestive glands (Figure 6). Some trophozoites were also observed in the tissue as a typical signet ring cell structures, although many of them were present as balls of schizonts (Figure 7).

Table 1. Parasitic infection in rock oyster (*Saccostrea forskali),* sampled from Chonburi province, Thailand at different times of the year.

Month (2004)	Site	pH	Salinity (ppt)	Temp (°C)	Marteilia (%)	Perkinsus (%)	Digenean trematode (%)	
							Histo[1]	Macro[2]
March	1	7.6	29	30	0	0	3.2	10.1
	2	7.6	27	30	1.2	0	0	11.0
July	1	7.7	20	29	0	0	4.0	10.0
	2	8.0	20	28.5	0	0	11.3	6.0
November	1	8.1	26	29	4.3	0	19.6	15.0
	1 (>1 yr)	8.2	24	29	3	2	8	50
	2	8.3	28	29	0	0	4	12

[1] Histological observations
[2] Macroscopic observations

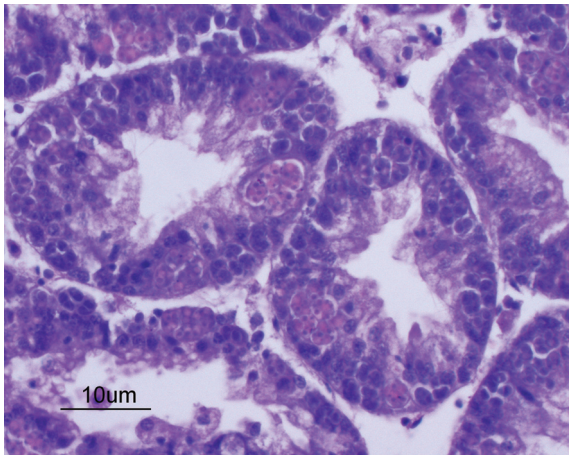

Figure 1. Severe infection of *Marteilia* sp. in digestive glands showed young and mature sporont within sporangiosorus.

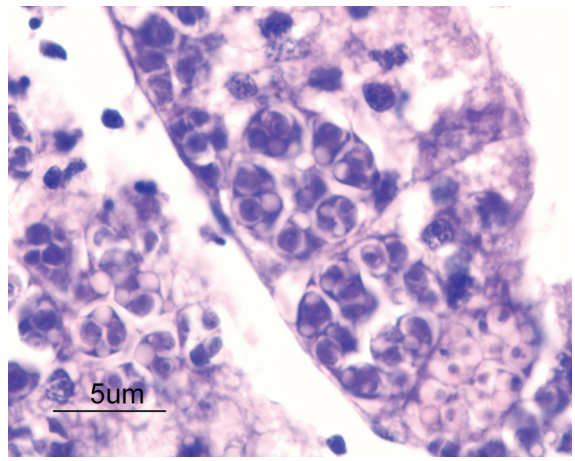

Figure 2. Sporangiosorus of *Marteilia* sp. showed 6 sporonts with 2 spores inside.

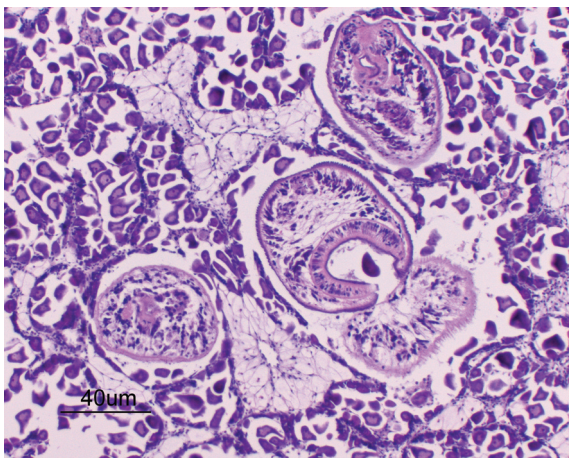

Figure 3. Digenean metacercaria was found in gonad.

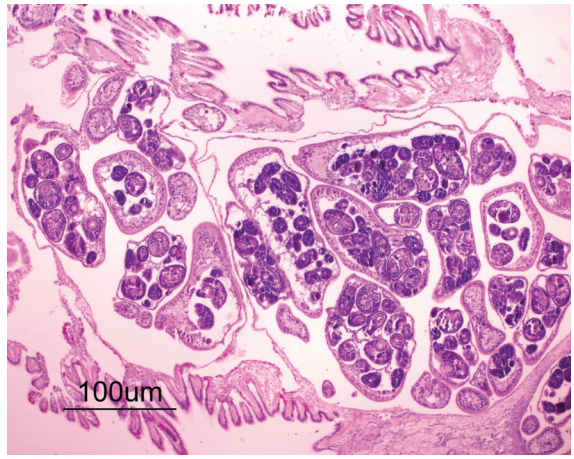

Figure 4. Sporocysts containing rediae within the gills.

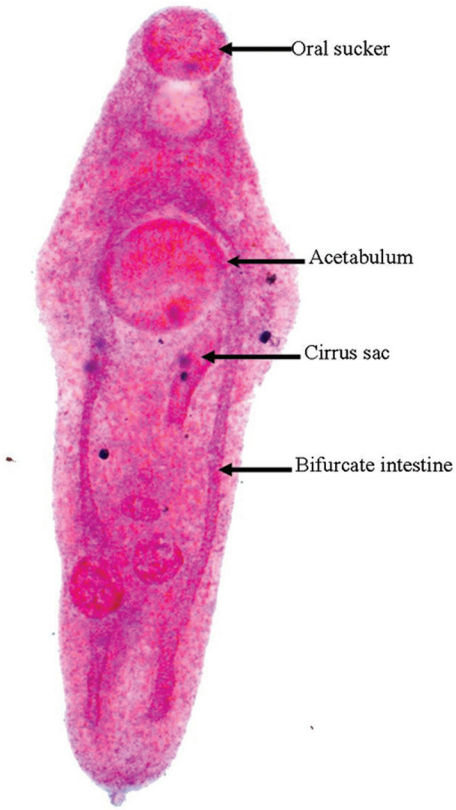

Figure 5. Metacercaria showed cirrus sac and long bifurcate intestine.

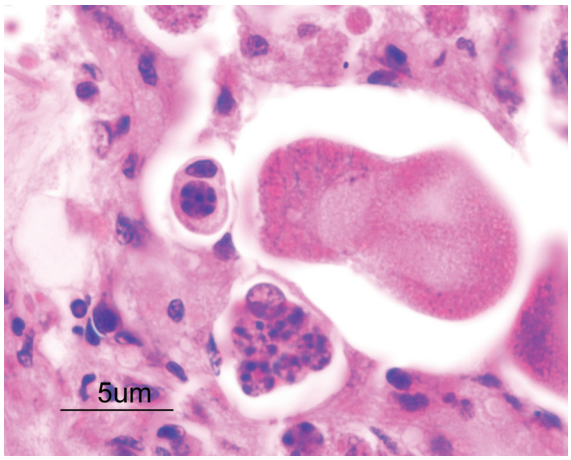

Figure 6. Tomonts of the *Perkinsus* sp. in the gonad with necrotic lesion.

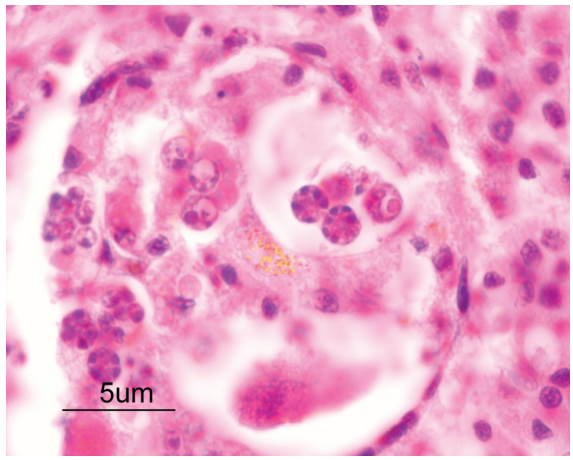

Figure 7. Trophozoites and tomonts of *Perkinsus* sp. in the connective tissue between the digestive gland tubules.

DISCUSSION AND CONCLUSION

Rock oyster sample were collected three times per year during the dry (March), rainy (July), and cool (November) season at two locations. Three kinds of parasites (*Marteilia, Perkinsus* and digenetic trematodes of the family Plagiochiidae) were observed.

Digenetic trematodes were detected in all samples. *Marteilia* infection was found only during the dry and cool seasons at 2% prevalence. *Perkinsus* infection was also observed in cool season but was only detected in the older oysters (more than one year old). In general oysters farmers keep the stocks for only 8-10 months (approximately from September to July). Only when the oysters have slow growth, farmer would keep them longer than 10 months.

Some environmental parameters such as pH and temperature were similar among the three seasons, but salinity dropped to 20 ppt during the rainy season. Interestingly, there was no *Marteilia* infection in the samples collected from neither site 1 nor 2 during the

rainy season. Salinity is believed to play an important role in the *Marteilia* infection. It is premature to make such a conclusion in our study. This proposal was supported by the report of Wolf (1979), who studied *M. sydneyi* infections in the Australian oyster, *Saccostrea commercialis*. In contrast, Berthe *et al*. (1998) suggested that temperature was a main parameter governing the life cycle and sporulation of *M. refringens*; and its high infection in European flat oyster (*Ostrea edulis*) was correlated with high water temperature (>17ºC). However, those considerations were conducted under very different environmental conditions. Importantly in our study, there was no mass mortality associated with in the samples infected with *Marteilia* sp. in Thailand unlike other outbreaks in Europe and Australia where high mortalities occurred (Alderman, 1979; Robert *et al*., 1991; Wolf, 1972; Adlard and Ernst, 1995). This might have been due to the environmental habitat, which was much different from those countries or it might have been simply due to the virulence of the pathogen. The species found in this study appears to be more similar to *M. sydneyi* than *M. refringens* because they have two spores in each sporont.

The digenean trematode was commonly found in most samples collected at different times of the year regardless of sampling sites. They could be only identified to the family Plagiorchiidae, which is closely similar to those of family Gymnophalloidae, a frequently reported group in most bivalves (Bower *et al*., 1994). The digenean found in this study showed cirrus sac and long bifurcate intestine, which are the main distinct character of the family Plagiorchiidae. Moreover, Plagiorchiidae is normally found only in the gonad, whereas the Gymnophalloidae can be found in other organs such as mantle and adductor muscle (James, 1964; Soon-Hyung and Chai, 2001). The digenean trematodes from these two families have a marine bird as a final host. This may be an important consideration for further management of this parasitic infection. Culture methods may be adapted to limit the capacity for the parasites to complete their life-cycle.

Perkinsus sp. infections are usually recognized as significant. Two species, *Perkinsus marinus* and *P. olseni,* are notifiable to the OIE. This latter species has been reported from Thailand although it was from various species, not oysters. It would be important to continue monitoring this parasite in order to better forecast potential impact on the development of oyster farming.

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New Approaches to Effective Mollusc Health Management

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ABSTRACT

Despite past and current efforts to prevent the spread of infectious diseases in molluscs, new outbreaks continue to be recorded; and, in endemic zones, diseases of molluscs continue to be a major constraint to the industry. Traditionally, efforts have focused on movements of live molluscs as a main underlying cause for transfers of diseases. The central paradigm of mollusc health management, from this point of view, has certainly been built on *Bonamia ostreae* and the history of its introduction into Europe from California. However, further consideration for other major diseases in other mollusc species shows how poorly this paradigm applies. In addition, new routes of disease transfer are becoming more and more of a concern, such as ballast waters and hull attachment. Furthermore, climate change is increasingly recognised as a driving force in pattern changes of the distribution of diseases. All of this is happening in the context of an increasingly active global mollusc aquaculture. It is therefore important to develop new approaches to better address mollusc health management issues. The main shift in paradigm is probably to consider not only exotic diseases but new diseases, defined as emerging, re-emerging and exotic. This paper reviews the lessons to be learnt from the past and illustrates some avenues for improvement of mollusc health management through early warning systems, diagnosis in multiplexed assays, and multi-layered information system at the ecosystem level.

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INTRODUCTION

Global aquaculture production has considerably increased over the past few decades, with mollusc aquaculture alone having doubled its production over the same period (FAO, 2006). This sector represents about a quarter of the global aquaculture production in volume. In some parts of the world, the annual growth rates of the industry may record highs of 30% or more, for species such as abalone or pearl oysters, for example. The global mollusc production is based on more than 42 different species. These species provide a source of income or a substantial contribution to livelihood in many coastal communities from developing as well as developed countries.

The increase of mollusc aquaculture production is underlined by improved hatching capacity and farming technology, increased transfers and introductions of live molluscs, species diversification and farming intensification, as well as international market development. Concomitant to this, risks of spreading pathogens and diseases around the world have considerably increased. Despite past and current efforts to prevent the spread of infectious diseases of molluscs, new outbreaks continue to be recorded; and, in endemic zones, diseases of molluscs continue to be a major constraint to the industry.

For example, losses due to *Marteilia refringens* and *Bonamia ostreae* in the European flat oyster, *Ostrea edulis*, were estimated at US$31 million in France between 1980 and 1983; the diseases led to a 90% reduction of the production and a direct loss of 20% of direct employments in this sector of industry (Meuriot and Grizel, 1984; Grizel and Héral, 1991). Both parasites continue nowadays to impede the flat oyster industry in Europe. Similarly, *Haplosporidium nelsoni* and *Perkinsus marinus* have severely impacted the American oyster, *Crassostrea virginica*, on the East Coast of the USA. *Haplosporidium nelsoni* (MSX) is believed to be responsible for 95% mortality of the native oysters in Delaware Bay between 1957 and 1959 (Ford and Haskin, 1987; Burreson and Ford, 2004). Difficulty in managing the diseases caused by the two parasites in Chesapeake Bay has led to the consideration of introducing of a non-native species, the Suminoe oyster, *Crassostrea ariakensis*, in replacement of the native one (Calvo *et al*., 2000). In Europe, finding a resistant non-native flat oyster species was less successful; and trials to acclimatise exotic species of flat oysters have shown their susceptibility to *B. ostreae* (Grizel *et al*., 1983; Le Borgne and Le Pennec, 1983; Bougrier *et al*., 1986; Bucke and Hepper, 1987; Pascual *et al*., 1991).

Traditionally, our efforts have focused on movements of live molluscs as a main underlying cause for transfers of diseases. *Perkinsus marinus*, *Marteilia refringens*, *Bonamia ostreae* and the diseases they cause are among those listed by the World Organisation for Animal Health, the OIE (OIE, 2006). Indeed, *Haplosporidium nelsoni* has also been listed by the OIE and, only very recently, was removed from the list; mainly based on consideration for restricted geographical distribution of the susceptible species. The OIE aquatic standards aim at reducing the risks arising from the introduction, establishment or spread of significant pathogens to protect aquatic animals, and aquatic resources, while supporting the international movements and trade of live aquatic animals and products.

However, new disease incursions continue to be recorded.

To illustrate this, *Bonamia ostreae* was first detected in British Colombia, Canada, in 2004, from farmed European flat oysters, *Ostrea edulis* (Marty *et al*., 2006). This haplosporidian parasite is known to occur on both coasts of the United States and it causes significant mortality in Europe. Archived samples of oysters obtained from the index farm between 1999 and 2004 were used to track *B. ostreae* back to 2003. All of the 3 infected farms had been stocked with *O. edulis* spat from the State of Washington, USA, where *B. ostreae* is endemic. On the East coast of North America, *Haplosporidium nelsoni* has been detected in Nova Scotia, Canada, in 2001, thus broadening its geographical distribution (Stephenson *et al*., 2003). It is not clearly understood whether the disease has long been present but undetected (because of non conducive environmental conditions), or it has only recently been introduced. Following detection of MSX, plasmodia of *Haplosporidium costale* (SSO), but no spores, were also detected in low prevalence and intensity in oysters from several locations in the Southern Gulf of St. Lawrence.

An old military adage has it that no plan survives contact with the enemy. The modern discipline studying mollusc diseases is still young and dates back to the mid-50s (Sparks, 2005). Its development was driven by most of the diseases listed above both in North America and Europe; Dermo disease in the early days followed by other major epidemics, MSX disease, marteiliosis, and bonamiosis. Here, I review the lessons to be learnt from the past and propose some avenues to be explored for improved mollusc health management.

CHALLENGING THE PARADIGM OF MOLLUSC HEALTH MANAGEMENT

The central paradigm of mollusc health management has certainly been built on *Bonamia ostreae* and the history of its introduction into Europe. *Bonamia ostreae* was initially described in 1979 after catastrophic mortality outbreaks in European flat oysters, *Ostrea edulis*, cultured in France (Comps *et al*., 1980; Pichot *et al*., 1980). Although French investigators coined *B. ostreae*, the parasite had apparently been previously observed in California (Katkansky *et al*., 1969; Elston *et al*., 1986). The case is very well documented that demonstrates introduction of the parasite into Europe, France and Spain, with infected oyster spat originating from California (Grizel, 1997; Cigarria and Elston, 1997). The disease has then rapidly spread through Europe along the Atlantic coast, from Spain to Great Britain and Denmark, and to the Mediterranean waters. The evidence is that translocations of infected stocks have been the main cause of transfer of bonamiosis.

How does this paradigm apply to other diseases?

In the case of *Haplosporidium nelsoni*, the scenario is slightly different. I already referred to the impact of this parasite on the American oyster. The parasite also occurs in the Pacific oyster, *Crassostrea gigas*, in California, Oregon and Washington State, on the west coast of the USA, as well as in France, Japan and Korea (Friedman *et al*., 1991; Friedman 1996; Renault *et al*., 2000; Kamaishi and Yoshinaga, 2002). In fact, it is believed that the parasite has long occurred in Asia (Kern, 1976; Burreson *et al*., 2000). It is now recognised that

contact of infected *Crassostrea gigas* with the native *C. virginica* caused the spread of this parasite to what proved to be a highly susceptible host (Burreson *et al*., 2000). Although *C. gigas* is certainly the most traded species, internationally, *C. virginica* remains the only known species susceptible to *H. nelsoni*. Bearing this in mind, one could say the *Bonamia* paradigm poorly applies in this instance.

Indeed, when looking more closely to diseases emergence, those scenarios used are quite varied. The Portuguese oyster, *Crassostrea angulata*, has nearly been extinguished after the outbreak of gill disease caused by an iridovirus. The disease is believed to have been introduced into Europe with first stocks of the sister species *C. gigas* in the late 60s. The two species are now believed to be con-specific with the Japanese oysters being resistant to the virus (Grizel and Héral, 1991; Renault and Novoas, 2004). In another type of scenario, after the introduction of the Japanese carpet clam, *Ruditapes philippinarum*, in Europe, this species has demonstrated a higher susceptibility to infection with *Vibrio tapetis* (Brown Ring Disease) compared to the native carpet clam species, *R. decussatus* (Paillard *et al*., 2004). In turn, *R. decussatus* appears more susceptible to *Perkinsus olseni*.

In those cases, problems have arisen from unexpected responses of an introduced species regarding endemic pathogens or the high susceptibility of a native species challenged by healthy carriers. These few examples show that we have made a simple/single storyboard out of a range of scenarios for emergence. Furthermore, our capacity to predict any of those scenarios is extremely limited because of the limited knowledge we have and the immense number of species we deal with.

In addition, new scenarios become more and more obviously significant. Ballast water and hull transfer should be cited here. Although one must admit that there is a real lack of scientific demonstration, examples are given as for *Haplosporidium nelsoni* in Nova Scotia, *Bonamia* sp. in North Carolina, *Marteilia* sp. in Florida, *Marteilioides chungmuensis* in Darwin harbour (Stephenson *et al*., 2003; Burreson *et al*., 2004; Hine, 1996). This increasing concern is also emphasised by the number of reports of alien/invasive species in aquatic systems (Carver *et al*., 2003; Bourque *et al*., 2006). Climate change is also recognised as an important factor of the change in distribution of pathogens and diseases. Change in environmental conditions is believed to be responsible for the northward expansion of *Perkinsus marinus*. Also, sea level rise could drastically alter the coastal environment, such as salinity regime in places like Chesapeake Bay, and this, as a result, may favour local diseases or emergence of new pathogens.

These considerations show that the routes of disease spreading are far more numerous that initially expected in the context of the *Bonamia* paradigm. This, in a certain extent, may explain that despite our efforts on international trade and exports certification, diseases apparently keep spreading. Obviously, by no means should we reduce our efforts towards safe trade. However, there is a need to shift paradigm to better address the issue of mollusc health management.

In order to make that shift, I propose here to consider not only exotic diseases, as we have done so far, but new diseases. New diseases are emerging, re-emerging and exotic diseases (Bower and McGladdery, 2003). Considering this is probably not as benign as it may seem

in a first attempt. Indeed, by bringing the concept new diseases under the scope of mollusc health management, we probably set the scene for new approaches.

Bearing this in mind, I foresee avenues for health management improvement by use of sentinels and development of early warning systems in surveillance, diagnosis of pathogen signatures along with host health indicators in multiplexed assays, and multi-layered information system for real-time health management at the ecosystem level.

WHAT NEW STRATEGIES?

A strategy combines specific objectives and related methodological approaches. In the previous section of this paper, I have described how there is a need to re-visit our objectives in terms of health management, and consider the new diseases as the core contention. For this to be turned into facts, it becomes obvious that emphasis is now put on surveillance of target populations. The key point, in that context, will be to enable detection of any significant change in the population health.

In the specific case of molluscs, it seems like we have very few, if any, early warning systems. During a survey performed on various mollusc species in the south Pacific, certain groups clearly appeared as very susceptible to infection with *Perkinsus* sp. (Hine, 1996). For example, members of the Arcidae (*Arca*, *Barbatia*), Malleidae (*Malleus*), Isognomonidae (*Isognomon*), Chamidae (*Chama*) and Tridacnidae (*Tridacna*) apparently tolerate relatively high prevalence and intensity rates of *Perkinsus* sp. and are likely to provide good indicators of *Perkinsus* presence in the environment. This could be used in a sentinel system to monitor the parasite burden in a specific ecosystem and prevent its impact on economically significant species. Namely, the high value the pearl oyster industry would benefit from fast tracking *Perkinsus* occurrence in a management plan.

Following the same stream of thoughts, surveillance in harbours that are involved in international (long distance) traffic or have strong connectivity with populations at risk would make a solid basis of early warning systems. With this approach, rather than trying to check some potential sources of pathogens, efforts are directed to the most probable points of entry. By doing this, index cases would most likely be very close to the source itself. This also means possible sets of actions in an attempt to mitigate potential impact or restore ecosystem integrity, when and where applicable.

The use of sentinels, or canaries, could demand to go as far as developing highly susceptible lines of animals. This may appear as a real paradox for geneticists when considering efforts to improve disease resistance is the usual mantra (Gosling, 2003). Molluscs usually show high genetic variation which is regarded as good for enhancement of desirable traits by selective breeding. While this approach has been applied towards ramping up disease resistance, so far (Ford and Haskin, 1987; Naciri-Graven *et al.*, 1998; Culloty *et al.*, 2001; Bezemer *et al.*, 2006), one could imagine such programs to provide highly susceptible lines to be deployed as sentinels. Use of caged sterile triploids for example would appropriately minimise other related risks.

Before reaching such levels of sophistication, I should stress here that basic programs for passive surveillance involving field stakeholders would greatly improve our capacity in preparedness and emergency response.

A second fold of innovative approach comes with our capacity in diagnosis. Diseases and health cannot be reduced to the simple question of presence/absence of pathogens. Although one would easily agree with this assertion, one would also admit that it does not make the reality of our diagnostic activity. In most of the cases, diagnosis is based on the presence of pathogens; this is probably the most critical drawback imposed by molecular techniques when poorly understood and mis-used.

In an era of "–omics", genomics and proteomics and sister disciplines, we could expect to overcome bug hunting and compensate the nearly absence of clinical manifestations and symptoms, by identification of key molecules (RNAs or proteins) expressing disease condition. Those markers would serve as sign-posts of host as well as pathogen physiology and state of activation. While it may be difficult to develop this for the about 65 mollusc species or so currently used in aquaculture, we may think of clusters of key molecules for the four main groups of species (oysters, clams, pectens and mussels) to be developed. Such molecules would signal disorder in the absence of specific pathogen, or in presence of usually benign infection. Such signals would assist decision making, even in the absence of specific knowledge (which is a frequent situation as illustrated by examples above).

New technologies currently enable detection of pathogens and monitoring host responses in a single assay. The muliplex fluid array system or micro-array technologies are among the most recent innovations (Adams and Thompson, 2006). The Luminex System theoretically offers simultaneous quantitative analysis of up to 100 different bio-molecules from a single drop of sample in an integrated, 96-well formatted, system. These methods are in their early stage of development but the increasing number of published articles seems to show a real trend in different fields of application (Giavedoni, 2005; Diaz and Fell, 2005).

The third component of the triad for new approaches is the use of information systems at ecosystem level as a way to predict ecosystem response to changes. Such a response may be disease emergence or re-emergence; pathogen introduction may be the change itself.

As it appears, mathematical models fail to predict host population response or provide predictions of limited accuracy. Because emergence and re-emergence are originating in local and endemic situation changes, it is important to be able to see those changes and be able to anticipate on their potential consequences. It may be interesting to think about development of a multi-layered information system providing the tools for such changes to be monitored and recorded. Roughly, layers would consist in information on environmental conditions, host populations, health status, pathogen populations, etc. Although generic systems may be developed, their use in local situation on temporal series is essential to ensure the efficiency of the systems. Such multi-layered information system would provide trends sensors that could reveal themselves as useful in now-casting, rather than fore-casting, and therefore valuable assistants in decision making process.

CONCLUSION

In conclusion, the "fortress attitude" has shown to be limited in its success to prevent spread of infectious diseases that affect molluscs. In addition to responsible movements, it sounds realistically promising to explore new approaches and put efforts to the development of efficient early warning systems, accurate health indicators and ecosystem trend sensors. Furthermore, while traditional regulation frameworks are seen by the industry as a strong impediment to its operations, the proposed approaches are likely to find a positive echo in the private sector, thus enabling real partnership towards application of shared responsibilities in mollusc health management.

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Diseases of Crustaceans

Shrimp Disease Control: Past, Present and Future

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ABSTRACT

In the period from 1970-1990, the worldwide shrimp industry was dependent on post larvae (PL) from wild sources. Grow-out systems were relatively simple, water exchange rates were high and few disease agents were known. Diagnostic capability was low and antibiotics and chemical use was common. Explosive increase in global shrimp cultivation was accompanied by viral pandemics starting around 1987 and these quickly revealed the need for changes. The need transformed the shrimp farming industry. Beginning in the early 1990's, dependence on wild PL rapidly declined and use of domesticated stocks rapidly dominated. Many significant diseases were described and diagnostic tests developed. Biosecurity measures practiced in intensive, controlled farming systems became more common, and antibiotic use decreased and became more responsible. Molecular biology is leading to a better understanding of shrimp and pathogen biology and the interaction between the two. The combined result of all of these developments has been a continuing increase in production of cultivated shrimp. In the future, it is expected that the world shrimp industry will have ready access to a variety of domesticated, genetically improved shrimp stocks free of all significant pathogens. Both laboratory and pond-side diagnostic methods/kits will be available for the most significant shrimp diseases and test standardization will be improved. Biosecurity methods will also improve

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and will be applied in all systems. Intensive and super-intensive culture systems will become more competitive with more extensive, traditional methods. Overall production efficiency will be facilitated by a better understanding of shrimp, shrimp pathogens and microbial ecology, and by the use of novel and environmentally friendly antiviral and antibacterial agents.

INTRODUCTION

According to FAO statistics (Figure 1), world production of cultivated shrimp has increased steadily since the early 1980's. It is also known that the world shrimp fishery is not growing, while the demand for shrimp is increasing steadily. Only aquaculture can meet this increasing demand. Thailand presents a good example (Figure 2). Since a peak in 1982, captured shrimp production has declined slowly while aquaculture production has steadily grown.

Despite the explosive growth in world production of cultivated shrimp, there have also been staggering, periodic losses due to disease. A global shrimp survey by the Global Aquaculture Alliance (GAA) in 2001 revealed a rough overall loss to disease of approximately 22% in a single year. Given a total production of 700,000 metric tons in 2001 valued at roughly US$8 per kg, this translated into an estimate of about US$1 billion loss in a single year. This was probably a conservative estimate, since farms with very bad results may not have responded to the survey. Thus, a conservative estimate for the total loss to disease over the past 15 years may be in the order of US$15 billion. This illustrates the importance of disease control to the industry.

With respect to disease agents, the GAA survey revealed that 60% of losses were attributed to viruses and about 20% to bacteria (Figure 3). Thus, the majority of our effort on disease control (80%) should clearly be focused on viral and bacterial pathogens. Indeed, that has been the case as the following review of disease control work will exemplify. The control effort has emphasized prevention, and this has required the development of good diagnostic tools, trained personnel and a better understanding of the hosts and their pathogens.

THE PAST (~1970-1990)

The good early years (~1970-1986)

The first shrimp cultivation systems were extensive and used post-larvae (PL) from tidal flow or hand collection, usually from nearby geographical areas. Stocking densities were low, disease problems relatively few and production relatively low. In the early 1980's, explosive, large-scale shrimp production was made possible by development of the eyestalk ablation technique to stimulate maturation of captured female broodstock. At that point, the industry shifted to dependence on hatchery produced PL, and production volume expanded rapidly. At the same time, stocking density gradually increased, especially in Asia.

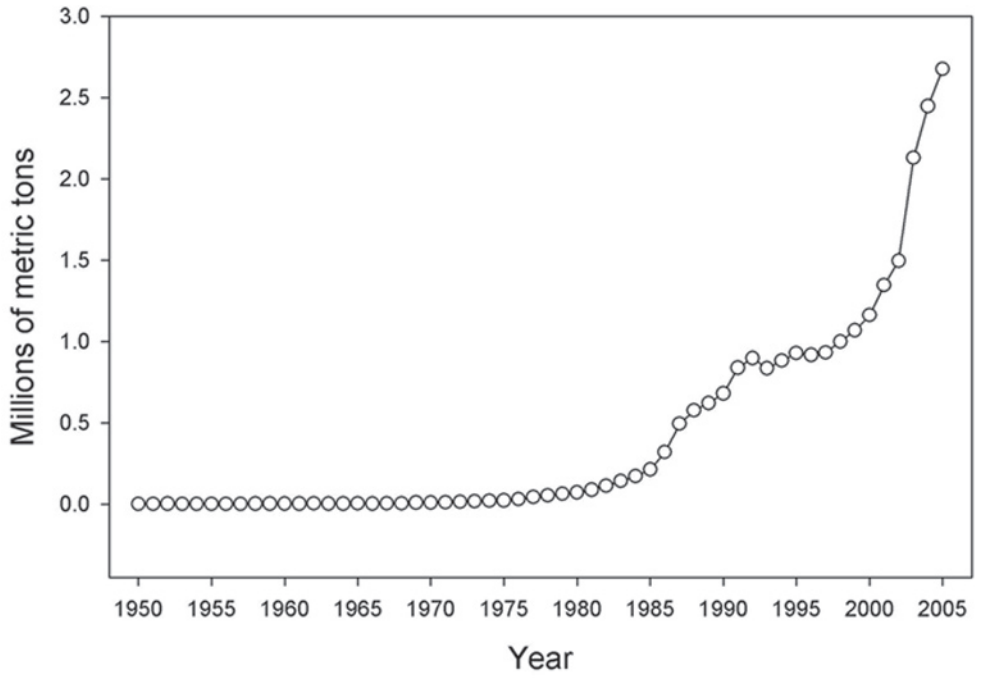

Figure 1. Global production of shrimp from aquaculture 1950 to 2005. Source FAO.

Figure 2. Comparison of captured and cultivated shrimp production in Thailand from 1985 to 2005. Source: http://www.biotec.or.th/shrinfo/.

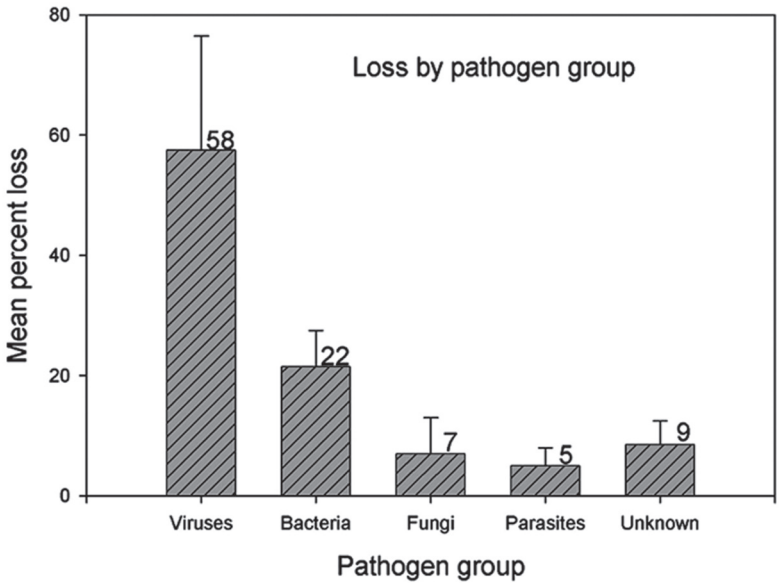

Figure 3. Relative economic loss to disease caused by various pathogen groups in the 2001 world shrimp industry survey by the Global Aquaculture Alliance.

In these early days, there were few disease control measures. Shrimp farmers used high rates of unfiltered water exchange and a wide range of chemicals and antibiotics, especially in the hatchery phase of production. There were few disease specialists available to help shrimp farmers, and diagnostic capabilities in most regions were limited. This was a vulnerable position as the industry grew exponentially with trends towards increasing farm densities in suitable farming areas and increasing rearing (stocking) intensity in individual ponds. Little was known of shrimp defense mechanisms, especially for viral pathogens. This eventually led to severe disease epidemics (epizootics) for which the industry was more or less unprepared.

The first serious disease outbreaks (~1987-1992)

The first widely reported shrimp disease epidemic was for monodon baculovirus (MBV) in Taiwan in the mid-1980's (Lin, 1989; Liao *et al.*, 1992). This was followed by epidemics caused by infectious hypodermal and hematopoietic necrosis virus (IHHNV) in the Americas (Lightner *et al.*, 1983; Lightner, 1996b), yellow head virus (YHV) in Thailand (Chantanachookin *et al.*, 1993; Flegel, 1997) and Taura syndrome virus (TSV) in the Americas (Hasson *et al.*, 1995; Brock *et al.*, 1995; Brock *et al.*, 1997).

THE WHITE SPOT VIRUS ERA (~1993-2002)

While the shrimp industry was still struggling with MBV, IHHNV, YHV and TSV outbreaks, it was hit by an even bigger disaster with the arrival of white spot syndrome virus (WSSV). After its first appearance in China in 1992, it spread rapidly around Asia (Nakano *et al.*, 1994; Flegel, 1997; Flegel and Alday-Sanz, 1998) and eventually to the Americas, first in 1996, but later with devastating losses from 1999 onwards. Total losses for this virus alone have been estimated to be in the range of US$1 billion per year since the middle of the 1990's.

LESSONS LEARNED IN THE PAST

Serious viral disease outbreaks revealed that the shrimp industry had to be better prepared with more knowledge about shrimp and their pathogens so that disease prevention methods could be improved. This need shifted attention to biosecurity, that is, possible methods of cultivating shrimp in restricted systems designed to prevent the entry of potential pathogens. The industry also realized that a good number of disease outbreaks originated from careless transboundary movement of contaminated but grossly normal aquaculture stocks.

More than any other problem, the WSSV pandemic served as a "wake up" call that shocked the industry into concerted actions. The catastrophic losses had serious impacts on whole national economies in Asia and the Americas. They resulted in increased support for research on shrimp diseases (including epidemiology) and in increased farmer awareness of the need for biosecurity.

Research on shrimp defenses and shrimp pathogens increased sharply. Many diagnostic techniques were developed, particularly PCR and RT-PCR. Training programs were carried out (e.g. SEAFDEC and the University of Arizona) and shrimp domestication and breeding programs were started with *P. vannamei* and *P. stylirostris*. In addition, hatchery and farm biosecurity measures were improved.

HIGHLIGHTS FROM THE PRESENT (~2003-2005)

Current industry overview

The shrimp industry has been transformed since 2003 by the widespread use of specific pathogen free (SPF) stocks of domesticated and genetically improved *Penaeus vannamei*, also called the American whiteleg shrimp or *Litopenaeus vannamei*. It has now replaced *P. monodon* as the main cultivated species worldwide. World production of cultivated shrimp reached an all-time high of approximately 1 million metric tons in 2004, and it continues to rise. At the time of writing, the main problem for farmers did not appear to be loss due to disease and resulting low production, but to competition in the global market and resulting low farm-gate prices. This trend is driving competition based on production efficiency, so limiting loss to disease remains an important issue.

Reaping major benefits from the 1990's

As a result of research and training in the 1990's, there are many benefits for shrimp farmers in the new millennium. For example, industry dependence on wild stocks of PL and brooders is rapidly declining. Domesticated, genetically selected stocks are now available for *P. vannamei* and *P. stylirostris*. They are also being developed for *P. chinensis* (Hennig *et al.*, 2005; Pantoja *et al.*, 2005) and *P. monodon*. In addition, many important diseases have been described and commercial diagnostic tests are available for most. A good example is the shrimp viruses (Figure 4), several of which have been listed by OIE. Increased availability of tests, test facilities and trained personnel means that the capacity for diagnosis has greatly improved. Even more important, the response time for characterization and for development of detection methods for new pathogens is more rapid and the time to development and distribution commercial test kits has been greatly reduced. Finally, biosecurity measures are widely applied and shrimp farmers are highly aware of their importance. A major benefit has been that antibiotic and chemical use has decreased and become more responsible.

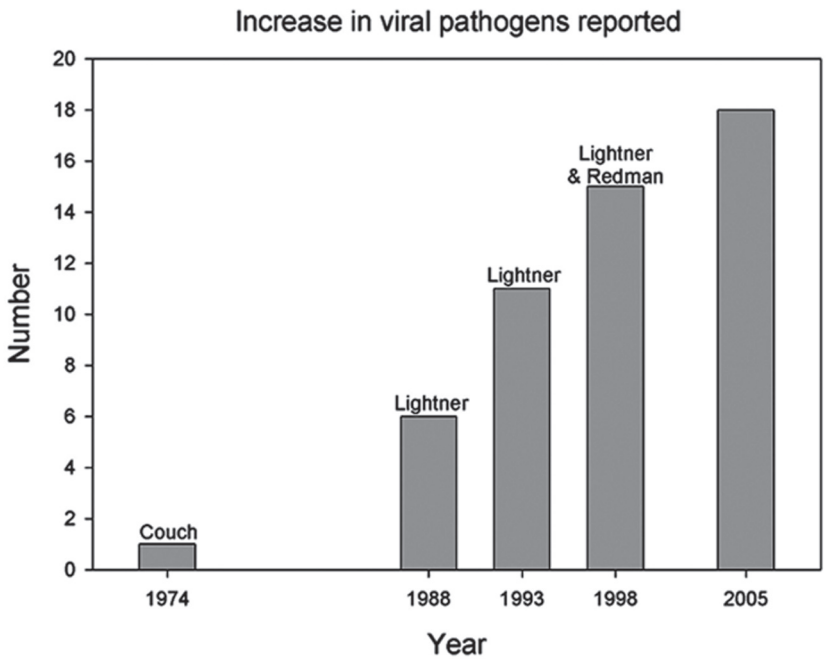

Figure 4. Increase in the number of shrimp viruses described over the period 1974 to 2005.

A number of diagnostic guides and manuals have played a fundamental role in improving diagnostic capability and are now used as standard laboratory references. These include, for example, the following: (i) Manual of diagnostic tests for aquatic animals (OIE, 2003, 2006), (ii) Disease control in fish and shrimp aquaculture in Southeast Asia (Inui and Cruz-Lacierda, 2002), (iii) Asia diagnostic guide to aquatic animal diseases (Bondad-Reantaso

et al., 2001), (iv) Diagnosis of shrimp diseases with emphasis on the black tiger prawn *Penaeus monodon (*Alday de Graindorge and Flegel, 1999), (v) A handbook of pathology and diagnostic procedures for diseases of penaeid shrimp (Lightner, 1996a), (vi) A guide to common problems and diseases of cultured *Penaeus vannamei* (Brock and Main, 1994), (vii) A handbook of normal shrimp histology (Bell and Lightner, 1988).

Several useful resources are also available on epidemiology and for guidance on safe movement of aquaculture stocks such as the following: (i) Asia regional technical guidelines on health management for the responsible movement of live aquatic animals (FAO/NACA, 2000), (ii) Manual of procedures for the implementation of the Asia regional technical guidelines of health management for the responsible movement of live aquatic animals (FAO/NACA, 2001), (iii) Survey toolbox for aquatic animal diseases (Cameron, 2002), and (iv) Diseases in Asian Aquaculture (DAA) series (Volumes 1 to 5). In addition to these books, a substantial amount of related information is available on the internet via gateways such as <www.fao.org>, <www.enaca.org> and <www.was.org>. The trend towards e-learning exemplified by the Southeast Asian Fisheries Development Center (SEAFDEC) program is an especially promising training model that can lead to even wider availability of trained personnel.

Despite current progress in the shrimp industry, a number of outstanding needs still remain to be satisfied. These include the need for more widespread use and standardization of diagnostic tests; wider application and improvement in biosecurity; better control over transboundary movement of live crustaceans for culture; investigation of the efficacy of probiotics in full-scale field trials; full understanding of the host-pathogen interaction in shrimp; more work on epidemiology and on molecular ecological studies of the microbial dynamics in shrimp ponds and tanks.

SOME PROMISING NEW DIRECTIONS

Exciting new directions are opening up in shrimp research. These should lead to new, innovative products and methodologies that will help control shrimp diseases and make shrimp aquaculture more stable and more efficient.

New diagnostic methods

An important aspect of any disease control program is the easy and convenient availability of rapid and reliable pathogen detection methods together with the ability to interpret results and apply them in a proper manner in health management programs. PCR and RT-PCR methods have been very important in helping to control the spread of major shrimp disease agents, but they have the disadvantage of requiring sophisticated equipment and highly trained personnel. Recently, lateral flow chromatographic immunodiagnostic strips similar to common drug-store pregnancy tests have begun to appear for some shrimp diseases. Using these, unskilled farm personnel can easily diagnose shrimp disease outbreaks at the pond side. The strips are relatively cheap and give an answer within 10 minutes (www.

enbiotec.co.jp; www.shrimpbiotec.com) . Other methods comparable to PCR and RT-PCR are now available or being developed for single and dual to multiple viral detection, but they too currently require advanced equipment and personnel.

Probiotics

Many shrimp farmers add preparations of living bacterial cells called "probiotics" to their cultivation ponds prompted by advertising and sales personnel with assurances that these preparations will improve water quality or prevent diseases. Sometimes the sales campaign is supported by positive results from properly controlled laboratory tests, but very few, if any, by properly controlled field tests on commercial farms. The only properly controlled field tests on probiotics revealed no significant effect on measured water quality parameters (Boyd and Gross 1998), but few proper studies on the disease control have been done (Rengpipat *et al.*, 1998; Rengpipat *et al*., 2003). If large-scale trials on commercial farms give positive evidence of efficacy with cost benefit analysis, there would be a good reason to include probiotics as part of integrated health management schemes. If not, discontinued use would reduce production costs and thus improve competitiveness.

Immunostimulant

This topic is often confused with probiotics. In fact, it is a separate issue since immunostimulant efficacy is not dependent upon the presence of living cells. They may be crude preparations such as whole, dead microbial cells (e.g. yeasts or bacteria), semi-purified products from plants and microbes or pure chemicals (Raa, 1996). A large number of these products are on the market and some have been tested and shown to be effective in proper laboratory trials but few have been tested for efficacy in full-scale field trials where laboratory successes may not be realized (Sritunyalucksana *et al.*, 1999). As with probiotics, large-scale trials on commercial farms are needed to provide positive evidence of efficacy with cost benefit analysis, if they are to be rationally included in integrated health management schemes.

Quorum sensing control of bacterial virulence

One of the problems for those who argue in favor of probiotics, especially for water quality improvement, is the small number (in the order of a few hundred or thousand per ml) of probiotic cells usually added to a shrimp pond when compared to the total number of resident bacteria (in the order of 1 to 10's of millions per milliliter). How could cells accounting for only 0.01% of the whole bacterial population have a controlling effect on water quality? For the issue of disease, the situation may not be a simple matter of percentage of population. It has been known for many years that cross-talk takes place amongst microbes via minute quantities of natural chemical messengers. These messengers can sometimes prevent a bacterial pathogen from causing disease without actually killing it. The process is called quorum sensing (Hardman *et al.*, 1998) and it is an intense area of research in many fields including medicine and environmental science but little has yet been done in aquaculture (Flegel, 2002; Defoirdt *et al.*, 2004). Controlling disease by quorum sensing would be advantageous because the chemical messengers are mostly

common and innocuous natural substances that do not kill target cells. Thus, there is no pressure for selection of non-sensitive cells, as occurs with the use of antibiotics. There have been some promising recent results for control of virulence in *Vibrio* (Misciattelli *et al.*, 1998, Manefield *et al.*, 2000; Dunlap, 2002). Perhaps some of the current probiotics actually are efficacious because they work by quorum sensing. However, more research is needed to investigate this possibility, to isolate the most effective microbes and to determine whether the can control bacterial pathogens in a cost effective manner in commercial farm settings.

Phage therapy

Another exciting area of research focuses on the use of natural bacterial viruses (called bacteriophages) to control bacterial populations. This is not a new science. The practice has reached a high level of sophistication in Russia where a common therapy for human gastrointestinal diseases such as salmanellosis is to simply drink a solution containing a mixture of appropriate bacteriophages (e.g.,www.phageinternational.com) . The technology is only just being adopted in Western countries (Sulakvilidze *et al.*, 2001) and has not yet been applied widely in aquaculture (Karunasagar *et al.*, 2005). It may be particularly useful for application in shrimp hatcheries where larval death from bacterial infections can sometimes be high (Lavilla-Pitogo *et al.*, 1990).

Shrimp-virus interactions

Although we have some knowledge of how shrimp interact with bacterial and fungal pathogens (Soderhall *et al*., 1994; Soderhall and Thornqvist, 1997; Thornqvist and Soderhall, 1997; Soderhall, 1999; Bachere *et al*., 2000b; Sritunyalucksana and Soderhall, 2000; Young Lee and Soderhall, 2002; Stet and Arts, 2005), we still know very little about how they interact with viral pathogens (Flegel, 2001). Work in this area is just beginning, but it is developing rapidly and many interesting discoveries have been made that may lead to the development of new disease control mechanisms.

There is much recent work on shrimp-viral interaction at the molecular and genetic level. Many new shrimp genes have been discovered and we hope that some of these will lead to new products for disease control. One group in Thailand has a particular interest in the process called programmed cell death (PCD) that is also called apoptosis (Kerr *et al.*, 1972). This is a common program for cell suicide in all multicellular organisms and we have some evidence that viruses may sometimes trigger this program to cause shrimp death (Sahtout *et al.*, 2001; Khanobdee *et al.*, 2002; Wongprasert *et al*., 2003). If we can find the key to the trigger, perhaps we can find novel ways of blocking it to prevent shrimp death until harvest. A few genes in the complex pathway have been discovered in shrimp (Phongdara *et al.*, 2006; Bangrak *et al*., 2002; Bangrak *et al*., 2004; Tonganunt *et al.*, 2005). The viral pathogens can also carry genes involved in controlling this host-cell process (Wang *et al.*, 2004).

We know that low temperature triggers white spot virus disease (WSD) and that hyperthermia or hypothermia can prevent it in several WSSV-infected shrimp species (Vidal *et al.*, 2001; Guan *et al.*, 2003; Jiravanichpaisal *et al.*, 2004). Although the mechanism for hyperthermic

protection has been proposed to be an increased level of apoptosis (Granja *et al*., 2003), the proposal is controversial (Wu and Muroga, 2004). It has also been suggested that osmotic stress can trigger WSSV disease outbreaks (Liu *et al.*, 2006; Yu *et al.*, 2003) and this may be true for other viruses as well. For example, it has been found that survival of viral-infected shrimp improved 13.53% ($P<0.005$) upon feeding the osmolyte betaine (Owens *et al*., unpublished). The relative risk of not responding to osmotic shock was 0.23 for shrimp fed betaine and since this was less than 1, it indicated that feeding betaine was beneficial. It is known that cultured prawns have 43% lower levels of betaine than wild prawns. Does this mean that cultured prawns are often betaine deficient? Obviously, the role for osmolytes in the diet should be further explored. Together, these examples clearly illustrate how environmental changes may trigger viral disease outbreaks. Again, understanding the process may lead to novel control measures

Another intriguing phenomenon recently reported was protection against WSD by persistent IHHNV infections in *P. stylirostris* (Tang *et al.*, 2003a). No such protection was obtained with acutely infected *P. vannamei*. It is important to realize that infection was not prevented but that disease severity was reduced. A similar phenomenon has been reported for insect cells (Burivong, 2003), suggesting that it may be a general mechanism in arthropods. This raises the question as to whether such protection is a general benefit of persistent infections, and is the reason why dual to multiple viral infections are common in shrimp (Manivannan *et al.*, 2002; Chayaburakul *et al.*, 2004; Flegel *et al*., 2004) and other arthropods (Chen *et al*., 2004).

Results from the work on insect cells (Burivong *et al.*, 2004) and whole insects (Roekring, 2004; Roekring *et al.*, 2006) revealed that defective interfering particles (DIP) of viruses may be an important product of persistent viral infections and may play some role in the lack of disease expression. Thus, it was suggested that persistent infections may serve as a kind of "memory" to somehow reduce the severity of disease (*i.e*. result in disease tolerance) possibly by preventing viral triggered apoptosis, as earlier hypothesized (Flegel and Pasharawipas, 1998). In any case, the work has shown that research on insect models can complement that done on shrimp.

Finally, there are some curious results on viral protein expression in shrimp that survive viral challenges. Palaemonid shrimp tolerant to YHV produce the viral capsid protein (p20) and one envelope protein (gp64) but not the other (gp116) (Longyant *et al.*, 2005). Similar results are sometimes seen with *P. monodon* that survive YHV-challenge (T.W. Flegel, unpublished). This is particularly intriguing because gp116 and gp64 are products of a single messenger RNA that produces a polyprotein that is later cleaved into gp116 and gp64 before viral envelope assembly (Jitrapakdee *et al.*, 2003). Since gp116 precedes gp64 in the mRNA and in the polyprotein, it is curious that gp64 can be produced in the absence of gp116.

In summary, this and other research to understand the molecular mechanisms behind shrimp tolerance to viral infections (Luo *et al.*, 2003; Xu *et al*., 2003) is showing promise but is still in early stages. It is hoped that results from continued work with shrimp and from related studies in insects will lead to a better understanding of host-viral interaction and to the development of novel methods of disease control.

Shrimp vaccines

In the year 2000, Dr. Muroga's research group in Japan reported that Kuruma shrimp survivors (*Penaeus japonicus*) in a pond two months after a WSSV outbreak could not be killed by injection of WSSV. They found a factor(s) in shrimp hemolymph that could prevent naïve shrimp from dying upon injection of WSSV (Venegas *et al.*, 2000). They called this a quasi-immune response. It is important to understand that the WSSV outbreak survivors and the protected naïve shrimp were mostly infected with WSSV after challenge, so they were not protected from infection, but protected from disease. We still do not understand the basis of this "quasi-immune" response, but the report about it led many scientists to examine the possibility of protecting shrimp from viral pathogens by using so called "vaccines". Some examples follow on the testing of new reagents with shrimp. Perhaps the most valuable thing that will come out of these tests will be a better understanding of shrimp-viral interaction. That knowledge may lead to even better methods of viral disease control.

It is unfortunate that some scientists and commercial companies refer to shrimp viral protective reagents as "vaccines". This gives listeners the false impression that the reagents can stop shrimp from getting infected by a process resembling the one that occurs in vaccinated people and other vertebrate animals. The vertebrate process involves antibodies, and we know that antibodies do not occur in shrimp. In addition, "vaccinated" shrimp generally do get infected, they just don't get sick as a result. To distinguish the difference, it has been recommended that such shrimp reagents be called "tolerines" (Flegel and Pasharawipas, 1998) rather than vaccines. The term tolerine clearly indicates that tolerance to infection rather than prevention of infection will be the result of its use.

Two general types of tolerines have been studied in shrimp. The first type was developed in Thailand in the mid-1990s and is still marketed by Charoen Pokphand Co. Ltd. (CP) under the brand name SEMBVAC. However, their developmental work for the reagent was not published. SEMBVAC and other similar products consist of inactivated whole particles of WSSV (Bright Singh *et al.*, 2005). After ingesting these products, the shrimp acquire some tolerance to WSSV, they suffer less from disease after being infected and this tolerance is very long lasting (Bright Singh *et al.*, 2005). However, field practice has proven that the protection is not absolute and can be overridden by environmental effects (unpublished field results from the use of SEMBVAC in Thailand).

The other types of tolerine consist of individual or mixed protein subunits of viral particles that are administered either by injection or by mixing with shrimp feed (Namikoshi *et al.*, 2004; Witteveldt *et al*., 2004a; Witteveldt *et al*., 2004b; Li *et al.*, 2005). In contrast to feeding inactivated whole viral particles, the shrimp apparently do not become infected when challenged with the source virus and they must be boosted at 10 to 15 day intervals to remain protected. As far as we know, no commercial product is yet available. The main factors in determining marketability will probably be cost and safety issues. With respect to novel methods of reducing production cost, some Chinese scientists (Xu *et al*., unpublished) used baculovirus-infected silkworms to express WSSV proteins and then protected shrimp by mixing the ground-up silkworms with shrimp feed.

RNA interference

RNA interference is another advanced technology that has recently been used in the laboratory to protect shrimp from viral diseases (Robalino *et al.*, 2004; Robalino *et al.*, 2005; Tirasophon *et al*., 2005). The process consists of making small fragments of double-stranded RNA with sequences that match those of viral genes. When these are injected into shrimp or exposed to shrimp cells in culture, disease protection results. It remains to be seen whether the protection is long-lived and whether the shrimp remain infected after viral challenge. Although this concept is very interesting, issues of cost, safety and public acceptance of a genetic engineering technique remain unanswered.

Antiviral and antbacterial substances in shrimp

Since the publication of ground-breaking work on the presence of antimicrobial peptides (penaeidins) in shrimp (Destoumieux *et al.*, 1997, 1999), there has been a growing interest in the field (Bachère *et al.*, 2000a, 2003, 2004; Destoumieux *et al*., 2000a, 2000b; Destoumieux-Garzon *et al.*, 2001; Munoz *et al.*, 2003; Chen *et al.*, 2005; Gueguen *et al.*, 2006; Gross *et al.*, 2001; Kang *et al.*, 2004; Supungul *et al.*, 2004). Apart from potential production for general use as anti-bacterial, -fungal and –viral agents in human and veterinary medicine, production of these substances is induced in shrimp after they become infected with microbes. Understanding the nature of these molecules, their specific antimicrobial activities and specific ways of induction could lead to new methods of disease control. Since these substances are natural shrimp compounds with homologues from invertebrates to humans they would be much better therapeutics for shrimp aquaculture than antibiotics. This is because they are already present in shrimp and their use would not lead to the development of bacterial resistance to antibiotics that are important in human medicine.

In addition to antimicrobial substances, many other kinds of proteins are also produced in response to viral pathogens. For example, over 60 proteins are up-regulated in the hepatopancreas and hemocytes of WSSV-resistant shrimp when compared to normal shrimp (He *et al.*, 2004; Pan *et al.*, 2000, 2005). These include things such as C-type lectin and an interferon-like protein (He *et al*., 2004). These results raise questions as to whether and how these substances are involved in crustacean anti-viral activity. If so, is their production a common, generic response to all viruses or does it vary specifically with each virus? Answers to such questions may lead to development of novel disease prevention methods.

Molecular epidemiology

Recent molecular work on several shrimp viruses has shown that a variety of genetic types often exist for each, either within their endemic region or elsewhere. The most extensive work in this area has been done for WSSV, YHV, TSV and IHHNV. Less has been done with other viruses such as BP and HPV and little or none with the other 10 or so remaining viruses currently known. The work is important because the types sometimes differ in pathogenicity. A good example is yellow head virus (YHV). We now know that there are 6 or more types of YHV in Asia (Walker *et al*., 2001; Peter Walker, pers. comm.) but that

only one type (Type 1) is really dangerous. In Thailand, 3 native YHV types and possibly 1 type imported from Australia are known (Soowannayan *et al*., 2003; T. Flegel, pers. comm.). Fortunately, there is a good RT-PCR diagnostic kit (Farming Intelligene, Taipei) that is useful for distinguishing amongst these 3 types. People screening broodstock and larvae can identify stocks infected with the dangerous type. Similarly, TSV is known to exist in a variety of types (Nielsen *et al*., 2005; Tang and Lightner, 2005), some of which are more virulent than others (Erickson *et al*., 2002; Erickson *et al.*, 2005; Tang and Lightner, 2005).

Although work with WSSV has shown that portions of its genome targeted by common detection systems vary insufficiently to interfere with detection (Lo *et al*., 1999; Kiatpathomchai *et al*. 2005), it has also been found that other portions contain highly variable repeat regions that can be used for molecular epidemiological work (van Hulten *et al.*, 2000, van Hulten *et al.* 2001, Wongteerasupaya *et al*., 2003). When comparisons were made in Vietnam (Hoa *et al.*, 2005) there was some indication that a particular repeat-type predominated in outbreak ponds. Similarly, variants of IHHNV in *Penaeus monodon* from various geographical regions appear to differ in virulence for *Penaeus vannamei* (Tang *et al.*, 2003b). It is not yet known whether variation found in other viruses such as BP and HPV is associated with differences in virulence.

More work is needed in this field so that shrimp farmers can be provided with tools to assess whether the viral and other pathogen types they may find in their ponds are dangerous or not. In addition, development of generic analytical tools would also allow shrimp breeders to verify that their specific pathogen free stocks were free of a wide range of potential variants.

Shrimp breeding and selection

Work with both shrimp (Moss *et al*., 2005) and their insect relatives (Roekring *et al.*, 2006; Roekring, 2004) has shown that it is possible to select for populations that do not suffer disease when infected with dangerous viral pathogens. This has been done successfully for Taura syndrome virus (TSV) in *Penaeus vannamei* and for infectious hypodermal and hematopoietic necrosis virus in *Penaeus stylirostris*. It may not be that these shrimp generally resist infection but that they simply do not become diseased after infection. Thus, it is best to say that they are tolerant to the viruses rather than resistant. Unfortunately, selection of tolerance for one virus does not seem to result in tolerance for another (Moss *et al.*, 2005). Indeed, tolerance may be specific to the strain of virus used in the selection process. For example, a new type of TSV was found to be highly virulent for a family of shrimp selected for specific tolerance to a different type of TSV (Erickson *et al.*, 2005). If we can understand the basic mechanisms of viral tolerance, we would be able to design more effective selection programs to develop disease tolerant shrimp.

There is wide interest in the shrimp research community to set up an international shrimp genome project. If this could be achieved, it would be the best way to efficiently gather shrimp genome sequences into a central, public database and assemble genetic maps. As with other economically important species, availability of complete genome sequences and genetic maps will help breeders to accelerate the process of identifying and selecting for economically desirable traits.

THE FUTURE (BEYOND 2005)

Within the next 10 years, we believe that the world shrimp industry will be overwhelmingly dominated by cultivation of domesticated lines of shrimp that are free of most, if not all, of the significant shrimp diseases. Most of the stocks used will also be improved by genetic selection for growth rate and other desirable traits like disease tolerance. We already know from experience with *P. vannamei* that such stocks are highly successful when reared with good biosecurity and good management of feed and the pond environment. The latter can be achieved by following Good Aquaculture Practices recommended by the Global Aquaculture Alliance. This combination of SPF stocks and proper management greatly reduces the risk of disease outbreaks and essentially eliminates the need for chemotherapy.

We expect that an international shrimp genome project will become a reality and that it will succeed in producing one or more genetic maps and complete shrimp genome sequences. These will constitute a valuable resource for research on all aspects of shrimp biology, including health.

Both in the laboratory and on the farm, diagnostic methods/kits will be available for the most significant shrimp diseases and many of these will be multiplex tests that can be used at the pond-side by farmers themselves. This will be complemented by easier access to information via e-learning, e-monitoring and e-assistance. Many of the tolerines, probiotics, immunostimulants, quorum sensing modulators and other disease prevention methods or tools now under development may be introduced into the industry, and biosecurity methods will be widely applied. Thus, it is expected that biosecure, controlled-environment, intensive and super-intensive culture systems will become more common and will compete well with traditional pond methods.

Increasing sophistication in the industry will essentially mimic that which has occurred with other domesticated animals such as chickens, pigs, salmon and trout. The net result will probably be a consolidation of the industry and a fall in farm-gate profit margins. Overall, production efficiency will become increasingly important, and disease control will continue to be a major factor in maintaining high efficiency. Our vision for DAA-VII in 2008 is the widespread cultivation of healthy and healthful, domesticated shrimp in biosecure ponds with no significant negative impact on the environment. We expect that new pathogens will continue to be discovered, especially if living shrimp and other crustaceans are translocated without sufficient precautions. On the other hand, the impact of newly-emerging pathogens will be counteracted by the rapid response time for their characterization and for development of diagnostic tools and by the use of domesticated SPF stocks in biosecure rearing ponds.

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Detection by PCR and Comparison of Sequences of VP28 Gene of White Spot Syndrome Virus Affecting *Penaeus monodon* in India

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ABSTRACT

White spot syndrome virus (WSSV) infection has resulted in severe production and economic losses in shrimp culture globally. Specific PCR primers were designed for the amplification of VP28 gene encoding for an envelope protein in WSSV. VP28 gene of SDDL 18/04-Indian isolate of WSSV was amplified by PCR and the PCR product was sequenced and submitted to GenBank (AY873785). The nucleotide sequence (AY873785) was compared with the VP28 gene sequences in the GenBank database (NCBI). Eleven sequences showed significant alignments with 100% homology which include the sequences from Korea (AY324881), Japan (AY249443), US south Carolina (AY249442), Indonesia (AY249441), China (AY249440, AF332093), Vietnam (AJ551447), Taiwan (AF 440570, AF272979) and The Netherlands (AF1739993, AF369029). However variations were observed in the sequences of five isolates, three from China (AY 249434, AY502435, AY682926) with a homology of 99%, one from Korea (AF380842) with a homology of 99% and one from India (AY 422228) with a homology of 98%.

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INTRODUCTION

White spot syndrome virus (WSSV) belonging to the family nimaviridae is an enveloped, non-occluded, rod-shaped DNA virus infecting penaeid shrimps and other crustaceans (Lightner, 1996). White spot syndrome has been reported in many shrimp growing countries of the world. Since WSSV has a wide geographic distribution and host range, studies on the comparison of morphology, virulence, genomic composition and protein composition among WSSV isolates have been carried out by various researchers (Wongteerasupaya *et al.,* 1996, Kasornchandra *et al*.,1998, Nadala Jr. *et al.,* 1998, Park *et al.,*1998, Lo *et al.,*1999, Wang *et al.*, 1999). VP28 envelope protein of WSSV is reported to play a key role in the systemic infection in shrimp (Van Hulten *et al.,* 2001). The present study was undertaken with an aim to know if there exists any variation in the nucleotide sequences of the VP28 gene of WSSV from different geographical regions.

MATERIALS AND METHODS

Sample collection

WSSV infected *Penaeus monodon* juveniles (6-7 g) collected from a shrimp farm located near Chennai, Tamilnadu, India were used for the study. The sample was fixed in 70% ethyl alcohol and used for polymerase chain reaction (PCR).

PCR Primer designing

The nucleotide sequences of VP28 gene encoding for the envelope protein in WSSV were collected from the GenBank database. Sequence alignments using BLAST A programme showed very few variations among the sequences of VP28 gene submitted from various countries and there is no variation at 5' and 3' end sequences. Based on the 5' and 3' end sequences of VP28 gene, the upper and lower specific PCR primers were designed so as to enable the amplification of complete VP28 gene.

PCR

DNA was extracted from the WSSV infected shrimp sample following the method described by Lo *et al*., (1996). Gills, pleopods or cephalothorax were separated and homogenized individually in NTE buffer. The homogenate was centrifuged at 3000 x g (4^0C) and the supernatant (200µl) was transferred to centrifuge tubes with 600 µl digestion buffer (100 mM Nacl, 10 mM Tris Hcl, pH 8.0, 50 mM EDTA, pH 8.0, 0.5% sodium dodecyl sulfate, 0.1 mg ml^{-1} proteinase K). After a 2 hr incubation at 65^0C the digest was deproteinised by successive phenol / chloroform / isoamyl alcohol extractions, the DNA were ethanol precipitated and dried. The dried DNA pellets were resuspended in 50µl of TE buffer (Tris-Hcl 100 mM, pH 8.0, 10mM EDTA, pH 8.0). The reaction mixture contained 2µl of template DNA, 1µM of each primer, 200µM of deoxynucleotide trisphosphate and 1.25U of Taq DNA polymerase in PCR buffer (Bangalore Genei Pvt Ltd). The PCR protocol comprised of initial denaturation for 3 min at 95^0C followed by 28 cycles of 30 sec at

95 ^{0}C; 30 sec at 58 ^{0}C; 30 sec at 72 ^{0}C with a final extension of 5 min at 72^{0}C. The PCR products were analysed by standard gel electrophoresis with 100 bp DNA marker using 1% agarose gels stained with ethidium bromide, visualized and documented in a gel documentation unit (Vilber Lourmet, France)

Sequencing of PCR products

The amplified PCR product of VP28 gene of WSSV (SDDL 18/04- Indian isolate) was purified using a silica membrane based column purification kit (Life technologies, USA). The purified PCR product was sequenced with an AB 13100 automated sequencer by a commercial company (Bangalore Genei Pvt., Ltd.).

Sequence submission, comparison and alignment

The sequence information of VP28 gene of SDDL 18/04-Indian isolate was submitted to GenBank (NCBI, USA) and the Accession number is AY873785. The sequence information was compared with the sixteen other sequences in the GenBank database submitted from India and elsewhere using BLAST A and FAST N programmes (NCBI, USA). The multiple nucleotide sequence alignment were carried out using CLUSTAL W programme (MEGA software).

RESULTS

PCR amplification of VP28 gene encoding for an envelope protein in WSSV resulted in a 615 bp product as shown in Figure 1. The details of VP28 gene sequences used for comparison, their GenBank accession numbers and their homology with SDDL

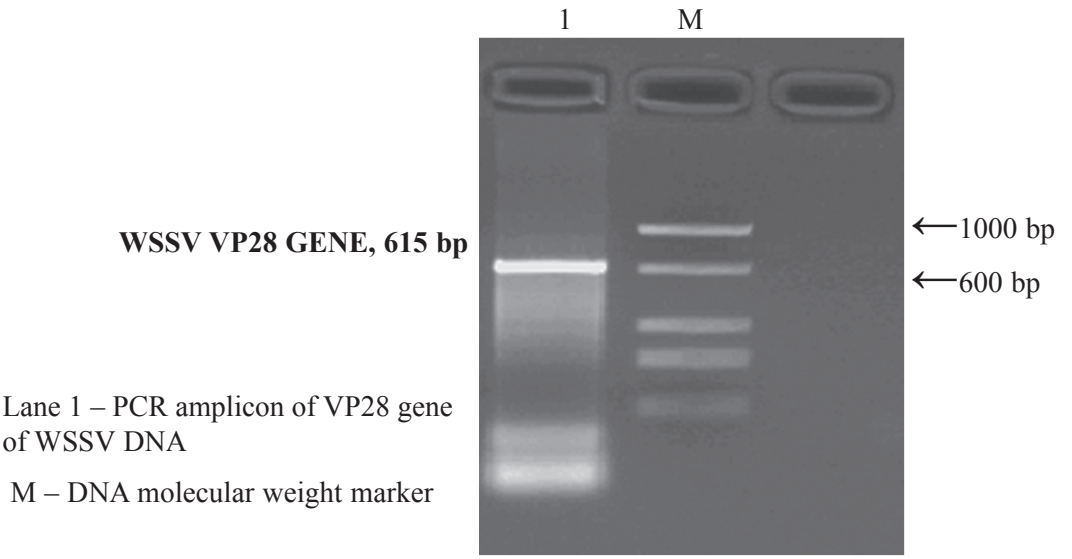

Figure 1. PCR amplification of VP28 gene of WSSV isolate from India.

18/04- Indian isolate are presented in Table.1. Sequence comparison and alignment result using CLUSTAL W programme (MEGA software) is presented in Figure 2. VP28 gene sequences showing variation on comparison with the sequence of SDDL 18/04- Indian isolate are presented in Figure 3.

Table 1. VP28 gene sequences used for comparison, their GenBank Accession numbers and their homology with SDDL 18/04- Indian isolate.

COUNTRY OF ORIGIN	GENBANK ACCESSION NO.	HOMOLOGY %
1. CHINA	AY249440	100
2. CHINA	AY249434	99
3. CHINA	AY502435	99
4. CHINA	AF332093	100
5. CHINA	AY682926	99
6. INDIA	AY422228	98
7. INDONESIA	AY249441	100
8. JAPAN	AY249443	100
9. KOREA	AF 380842	99
10. KOREA	AY324881	100
11. TAIWAN	AF272979	100
12. TAIWAN	AF440570	100
13. THE NETHERLANDS (WSSVTH-1 isolate of Thailand)	AF173993	100
14. THE NETHERLANDS (WSSVTH-1 isolate of Thailand)	AF369029	100
15. USA	AY249442	100
16. VIETNAM	AJ551447	100

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AY873785:61   gctgtatttattgtgatttttaggtatcacaacactgtgaccaagaccatcgaaacccac 120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
AF249434:85   gctgtatttattgtgatttttaggtatcacaacactgtgaccaagaccatcgaaacccgc 144

AY873785:61   gctgtatttattgtgatttttaggtatcacaacactgtgaccaagaccatcgaaacccac 120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
AF502435:61   gctgtatttattgtgatttttaggtatcacaacactgtgaccaagaccatcgaaacccgc 120

AY873785:181  ggatcaggctacttcaagatgactgatgtgtcctttgacagcgacaccttgggcaaaatc 240
               |||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
AY682926:181  ggatcaggctacttcaagatgactgatgtgtcctttgacagcgacaccttgggtaaaatc 240

AY873785:421  ccaaagattaacccatcaaaggcctttgtcggtagctccaacacctcctccttcaccccc 480
               ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
AF380842:421  ccaaagattaacccatcaaaggcttttgtcggtagctccaacacctcctccttcaccccc 480

AY873785:481  gtctctattgatgaggatgaagttggcacctttgtgtgtggtaccacctttggcgcacca 540
               ||  | ||||||||||||||||||||||||||||||||||||||||||||||||||||
AY422228:481  gtttttattgatgaggatgaagttggcacctttgtgtgtggtaccacctttggcgcacca 540
```

Figure 3. VP28 gene sequences showing variation on comparison with the VP28 sequence of SDDL 18/04- Indian isolate (AY873785).

DISCUSSION

Five major virion proteins have been reported to be present in WSSV. VP26, VP24 and VP15 are present in the nucleocapsid and VP28 and VP19 are located in the envelope. VP28 gene, which encodes for an envelope protein in WSSV, plays a key role in systemic infection in shrimp with WSSV (Van Hulten *et al.,* 2001). Studies on WSSV gene structure and their functions would help to evolve novel diagnostic techniques and new strategies for the control of the virus infection. As PCR is the widely accepted diagnostic method for screening of shrimp for viruses, specific primers were designed to amplify the VP28 gene of WSSV to study the nucleotide sequence information and variations in the VP28 gene of WSSV from different countries. Genetic variation in viruses has significant implication for diagnosis and epidemiology (Morse, 1994). Mutation in the nucleotide sequence can prevent binding of PCR primers to target sequences (Kwok *et al.,* 1990). This leads to false negative PCR results and non-specific PCR products, restricting the use of PCR based diagnostic kits to some strains of the viruses. Although WSSV has a wide geographic distribution, Lo *et al.,* (1999) showed the similarity of WSSV isolates from different geographical regions by amplifying ten different DNA fragments of the entire WSSV genome using ten different PCR primer sets. However, the significance of the amplified region has not been studied. Similarly, PCR amplification of a portion of WSSV gene from China, Japan, Indonesia, Thailand, Malaysia and India has revealed their close relatedness (Kasornchandra *et al.,* 1998). Nucleotide sequence comparison of VP28 gene of Korean isolate with Taiwanese, Thai and Chinese isolates suggested that they have originated from the same ancestor (Moon, 2003). The results of this study, which compared the VP28 gene sequence of an Indian isolate with sixteen other isolates

```
WSSV-SDDL18/04India ATG GAT CTT TCT TTC ACT CTT TCG GTC GTG TCG GCC ATC CTC GCC
WSSV-India          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-China          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-USA            ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

WSSV-SDDL18/04      ATC ACT GCT GTG ATT GCT GTA TTT ATT GTG ATT TTT AGG TAT CAC
WSSV-India          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-China          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-USA            ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

WSSV-SDDL18/04      AAC ACT GTG ACC AAG ACC ATC GAA ACC CAC ACA GAC AAT ATC GAG
WSSV-India          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-China          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-USA            ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

WSSV-SDDL18/04      ACA AAC ATG GAT GAA AAC CTC CGC ATT CCT GTG ACT GCT GAG GTT
WSSV-India          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-China          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-USA            ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

WSSV-SDDL18/04      GGA TCA GGC TAC TTC AAG ATG ACT GAT GTG TCC TTT GAC AGC GAC
WSSV-India          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-China          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-USA            ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

WSSV-SDDL18/04      ACC TTG GGC AAA ATC AAG ATC CGC AAT GGA AAG TCT GAT GCA CAG
WSSV-India          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-China          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-USA            ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

WSSV-SDDL18/04      ATG AAG GAA GAA GAT GCG GAT CTT GTC ATC ACT CCC GTG GAG GGC
WSSV-India          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-China          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea          ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-USA            ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
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Figure 2. Nucleotide sequence alignment results of VP28 gene of WSSV isolates from different countries.

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WSSV-SDDL18/04      CGA GCA CTC GAA GTG ACT GTG GGG CAG AAT CTC ACC TTT GAG GGA
WSSV-India           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-China           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-USA             ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

WSSV-SDDL18/04      ACA TTC AAG GTG TGG AAC AAC ACA TCA AGA AAG ATC AAC ATC ACT
WSSV-India           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-China           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-USA             ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

WSSV-SDDL18/04      GGT ATG CAG ATG GTG CCA AAG ATT AAC CCA TCA AAG GCC TTT GTC
WSSV-India           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-China           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea           ... ... ... ... ... ... ... ... ... ... ... ... ... ..T ... ...
WSSV-USA             ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

WSSV-SDDL18/04      GGT AGC TCC AAC ACC TCC TCC TTC ACC CCC GTC TCT ATT GAT GAG
WSSV-India           ... ... ... ... ... ... ... ... ... ... ..T .T. ... ... ...
WSSV-China           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-USA             ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

WSSV-SDDL18/04      GAT GAA GTT GGC ACC TTT GTG TGT GGT ACC ACC TTT GGC GCA CCA
WSSV-India           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-China           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-USA             ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

WSSV-SDDL18/04      ATT GCA GCT ACC GCC GGT GGA AAT CTT TTC GAC ATG TAC GTG CAC
WSSV-India           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-China           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Japan           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Korea           ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-USA             ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

WSSV-SDDL18/04       GTC ACC TAC TCT GGC ACT GAG ACC GAG TAA
WSSV-India           ... ... ... ... ... ... ... ... ... ...
WSSV-China           ... ... ... ... ... ... ... ... ... ...
WSSV-Japan           ... ... ... ... ... ... ... ... ... ...
WSSV-Korea           ... ... ... ... ... ... ... ... ... ...
WSSV-USA             ... ... ... ... ... ... ... ... ... ...
WSSV-Vietnam         ... ... ... ... ... ... ... ... ... ...
WSSV-TheNetherlands  ... ... ... ... ... ... ... ... ... ...
```

Figure 2. (continued)

from different geographical regions also showed that there is not very high variation as the homology ranged from 98%-100%. Unlike earlier studies, the present study has compared the nucleotide sequence of VP 28 gene, which plays significant role in causing WSSV infection, with all the available VP 28 sequences in the Genbank database. It is interesting to note that among the VP28 sequences which showed variation in nucleotide sequence comparison, an Indian isolate (AY 422228) showed lesser homology (98%) compared to the other isolates from distant geographical locations viz., three isolates (AY 249434, AY 502435, AY 682926) from China and one isolate from Korea (AF 380842) with a homology of 99%.

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Occurrence of Multiple Viruses in *Penaeus monodon* Shrimp Ponds and Their Effect on Shrimp Production

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ABSTRACT

The prevalence of multiple viruses (HPV, MBV and WSSV) in *Penaeus monodon* shrimp ponds along the west coast of Karnataka, India was studied by nested PCR. WSSV was found in all the 22 ponds studied (7 in non nested PCR and 15 in nested PCR). MBV and HPV were mostly found in dual infections with WSSV (19 ponds showed the presence of both MBV and WSSV and 9 ponds were positive for HPV and WSSV) or in triple infections (7 ponds were found positive for all the 3 viruses). Of these 22 ponds, 15 ponds that showed dual or triple infections by nested PCR resulted in successful crops with a total production ranging from 1.1 to 1.9 t/ha. However, mortality that resulted in emergency harvests occurred in 7 ponds where WSSV was found positive by non-nested PCR and other viruses by nested PCR. This result indicates that *P. monodon* can tolerate a low level of infection by all 3 of these viruses if environmental conditions are optimal.

Umesha, K.R., Chakraborty, A., Venugopal, Nagarajappa, M., Karunasagar, I. and Karunasagar, I.. 2008. Occurrence of multiple viruses in *Penaeus monodon* shrimp ponds and their effects on shrimp production, pp. 389-398. *In* Bondad-Reantaso, M.G., Mohan, C.V., Crumlish, M. and Subasinghe, R.P. (eds.). Diseases in Asian Aquaculture VI. Fish Health Section, Asian Fisheries Society, Manila, Philippines. 505 pp.

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INTRODUCTION

The shrimp culture industry saw important growth during the 1980s that was mainly due to technological breakthroughs and high profitability. However, viral diseases have recently become an important limiting factor for the shrimp aquaculture industry throughout the world (Leung and Tran, 2000). To date, over 22 different viruses are known to infect shrimp, and several of them have been associated with mass mortalities in cultured shrimp (Hsu *et al.,* 2000).

Hepatopancreatic parvovirus (HPV) infects several penaeid shrimp species and was first reported in *Penaeus merguiensis* and *P. indicus* (Chang and Loh, 1984) and in *P. chinensis* (Lightner and Redman, 1985). Cultured as well as wild captured penaeid shrimp have been reported to act as hosts for HPV. For example, Manjanaik *et al.* (2005) reported the prevalence of HPV in wild penaeid shrimp in India. HPV-infected shrimp do not show specific gross signs of disease and those that do show signs of disease frequently tend to be infected by other pathogens that may mask the actual effect of HPV (Flegel *et al.,* 1992, 1999).

White spot syndrome virus (WSSV) continues to be the most serious cause of shrimp disease faced by shrimp growing countries. It affects most of the commercially important species of penaeid shrimp (Lightner, 1996), and many cultured and captured crustaceans have also been found to harbour it (Hossain *et al.,* 2001a; Chakraborty *et al.,* 2002). The presence of both WSSV and MBV in *P. monodon* postlarvae in India has been reported (Otta *et al.,* 2003). The presence of triple virus infections (WSSV, MBV and HPV) in postlarvae that were showing mass mortality has been reported by Manivannan *et al*. (2002).

Monodon baculovirus (MBV) was the first virus reported for *P. monodon* and the second virus reported for penaeid shrimp (Lighter and Redman, 1981). It has been implicated in mass mortalities in shrimp that are cultured at high densities (Fulks and Main, 1992). *Penaeus monodon* larvae showing 90% mortality due to MBV infection has been reported (Ramasamy *et al.,* 1995).

Penaeus monodon postlarvae showing dual infection of MBV and WSSV (Otta *et al.,* 2003) and triple infection of WSSV, MBV and HPV (Umesha *et al.,* 2003) has been reported in India. In this communication we report the occurrence of multiple viruses in culture ponds that achieved crop success.

MATERIALS AND METHODS

Sample collection

Samples of shrimp (*P. monodon*) were collected fortnightly from 22 ponds, each of approximately 1 ha area situated in Udupi and Kundapur along the southwest coast of Karnataka, India. Each sample comprised 4-6 shrimps. Soon after collection, the samples were brought to the laboratory on ice for immediate processing.

Extraction of DNA

From each sample of shrimp, gills, stomach, hepatopancreas and cuticle were removed, pooled and total DNA was extracted following the method described by Otta *et al.* (2003). DNA extracted from gills, stomach and cuticle were used for the detection of WSSV and the DNA extracted from hepatopancreas was used for the detection of both MBV and HPV.

PCR analysis

Two sets of primers were used for the detection of HPV. The PCR protocol and primer set described by Phromjai *et al.* (2002) was expected to yield a product of 441 bp (Figure 1). For nested PCR, primers internal to the 441 bp were designed in our laboratory to bind to nucleotides 156-176 and 398-420 in the GenBank sequence AF456476 and yield a 265 bp amplicon (Figure 1). The cycling conditions for the nested reaction consisted of an initial 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, with a final extension at 72°C for 5 min. The protocol and primer set described by Pantoja and Lightner (2000) that amplifies a 592 bp product was also employed.

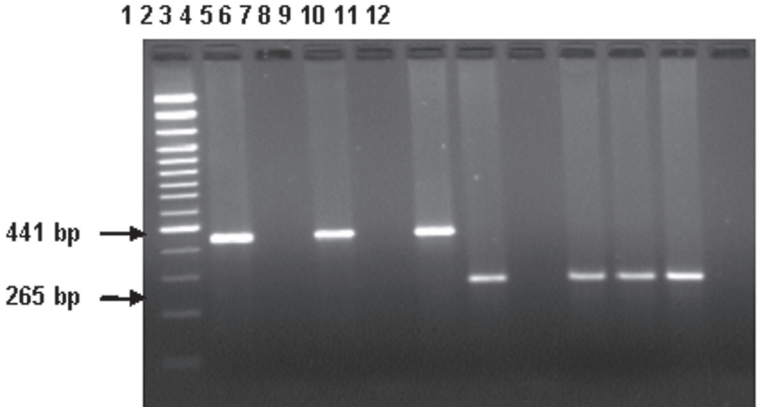

Figure.1. *Penaeus monodon*. Sample electrophoresis gel for detection of hepatopancreatic parvovirus (HPV) –specific PCR amplifications in adult *P. monodon* by 1-step. Lanes: (2 - 6) and nested (7 - 12) PCR. Lane 1: 100 bp DNA ladder Plus (Gene RulerTM genetix); Lane 2: 1-step PCR positive control; Lane 3: Negative control; Lanes 4 and 6: HPV detected by 1-step PCR; Lane 5: HPV 'negative' by 1-step PCR; Lane 7: Nested PCR positive control; Lane 8: Negative control; Lanes 9 to 11: HPV detected by nested PCR; Lane 12: HPV not detected by nested PCR

The nested primers and protocol described by Hossain *et al.* (2001a) were used for the detection of WSSV (486 bp and 310 bp respectively) (Fig. 2). For the detection of MBV, the PCR protocol and primers described by Belcher and Young (1998), yielding products of 533 and 361 bp were used (Figure 3).

PCR reactions were carried out in a 30 µl reaction mixture that consisted of 1X PCR reaction buffer, 10 pmol each primer, 200 µM dNTPs, 0.9 units of Taq DNA polymerase (Bangalore Genei, Bangalore), 2 µl of template DNA and sterile distilled water to adjust

the volume to 30 µl. All PCR reactions were carried out in an MJ Research thermocycler. The amplified products were analysed on 2% agarose gel containing ethidium bromide at a concentration of 0.5 µg/ ml and observed using a transilluminator (Gel doc system, Hero Lab).

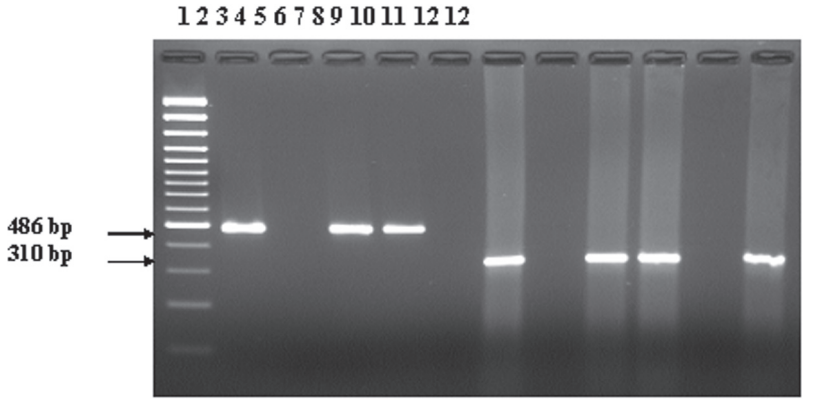

Figure 2. *Penaeus monodon.* Sample electrophoresis gel for detection of white spot syndrome virus (WSSV) –specific PCR amplifications in adult *Penaeus monodon* by 1-step (Lanes 2 to 6) and nested (Lanes 7 to 12) PCR. Lane 1: 100 bp DNA ladder Plus (Gene RulerTM genetix); Lane 2: 1-step PCR positive control; Lane 3: Negative control; Lanes 4 and 5: WSSV detected by 1-step PCR; Lane 6: WSSV 'negative' by 1-step PCR; Lane 7: Nested PCR positive control; Lane 8: Negative control; Lanes 9, 10 and 12: WSSV detected by nested PCR; Lane 11: WSSV not detected by nested PCR

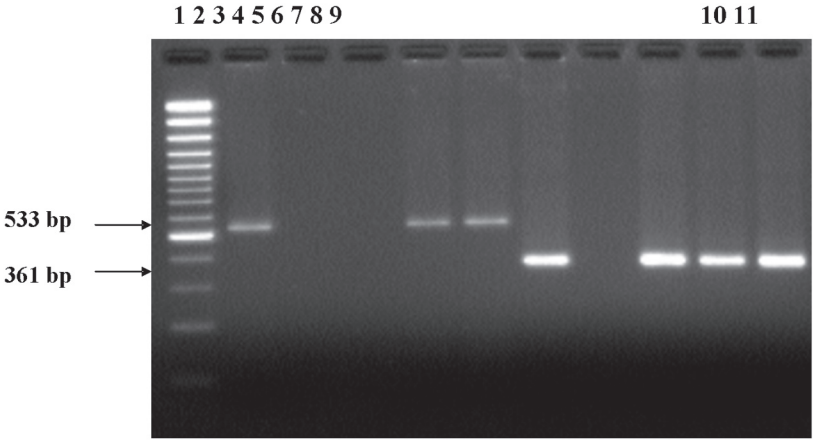

Figure 3. *Penaeus monodon.* Sample electrophoresis gel for detection of Monodon baculovirus (MBV) –specific PCR amplifications in adult *P. monodon* by 1-step (Lanes 2 to 6) and nested (Lanes 7 to 11) PCR. Lane 1: 100 bp DNA ladder Plus (Gene RulerTM genetix); Lane 2: 1-step PCR positive control; Lane 3: Negative control; Lane 4: MBV 'negative' by 1-step PCR; Lanes 5 and 6: MBV detected by 1-step PCR; Lane 7: Nested PCR positive control; Lane 8: Negative control; Lanes 9 to 11: MBV detected by nested PCR.

RESULTS AND DISCUSSION

Samples from one pond were found positive for HPV by the primers described by Phromjai *et al.* (2002) yielding a 441 bp product. Eight additional ponds were found positive for HPV by the nested PCR yielding a 265 bp product, bringing the total positive ponds to 9 (Table 1). It is interesting to note that, HPV alone was not found in any of the ponds studied, but was found in dual infections with WSSV (9/22) or in triple infections with WSSV and MBV (7/22) (Table 1). Shrimp samples from all the ponds were also analyzed for the presence of HPV by using the primers described by Pantoja and Lightner (2000), but none of the ponds were found positive. This result indicates that the strain of HPV present in India may be similar to HPV-mon reported from Thailand but different from HPV-chin. The DNA sequence of HPV in *P. monodon* from Thailand (HPV-mon) differs from that of HPV-chin in *P. chinensis* from Korea (HPV-chin) by approximately 30% (Phromjai *et al.,* 2002).

It has been reported that, HPV infection has the most significant impact in growout ponds (Flegel *et al.,* 1999; Lightner, 1996). However, in the present study, no significant difference in production ($p>0.05$) was observed between ponds with and without HPV infection.

WSSV is the most serious cause of shrimp disease in most countries where shrimp are cultivated. In the present study all the ponds studied (22/22) (Table 1) were found positive for this virus (7 ponds by non-nested PCR and 15 by nested PCR). In the initial stage of culture (2-5 weeks), WSSV alone was detected by nested PCR in 15 ponds. However, 19/22 ponds eventually showed dual infections with MBV and 9/22 showed dual infections with HPV (Table 1). Although all the ponds were positive for WSSV infection, 15 ponds were positive only by nested PCR and these ponds went through to successful harvests at between 15-17 weeks after stocking with a total production ranging from 1.1 to 1.9 t/ha (Table 1). This is normal production for India where stocking levels (6-18 postlarvae per square meter) are relatively low when compared to countries were more intensive culture (i.e., 50 or more PL per square meter) is practiced. In the 7 ponds (P15, P16, P18-P22) that were positive for WSSV by single step PCR and for other viruses by nested PCR between 5-10 weeks, mass mortality occurred and led to emergency harvests that gave production yields ranging from 0.4 – 1.4 t / ha. These results indicate that shrimp (*P. monodon*) can tolerate a low level of WSSV, WSSV/MBV, WSSV/HPV and WSSV/MBV/HPV infection. Long term presence of WSSV in shrimp culture ponds has been reported previously (Tsai *et al.,* 1999). However, stress induced by environmental factors such as pH, salinity, temperature (Hossain *et al.,* 2001b), poor pond management (Flegel *et al.,* 1995a, b) and high stocking density may promote conversion to the disease state.

Out of 22 ponds studied, MBV alone was found in only one pond (P4) in the initial stage of culture. However, 19 ponds showed dual infections with WSSV and 7 showed triple infections (Table 1). This result indicates a high prevalence of MBV in culture ponds in India. It has been reported that MBV is relatively well tolerated by *P. monodon* if other conditions are optimal (Fegan *et al.,* 1991). During the period of study, none of the shrimp from any of the ponds showed mortality that could be ascribed to MBV. We also found that MBV and HPV occurred mostly in dual or multiple viral infections, while WSSV was more frequently found alone.

Table 1. Detection of multiple viral infections in *Penaeus monodon* culture ponds by nested polymerase chain reaction.

Pond No.	Number of shrimp positive for various viruses by PCR								Pond parameters		
	WSSV	MBV	WSSV/ MBV	WSSV/ HPV	Triple	Any WSSV	Any MBV	Any HPV	Stocking density	Week of harvest	Total production
P1	3	0	4	0	0	7	4	0	7-8	16	1.4
P2	2	0	4	2	2	6	4	2	7	16	1.4
P3	3	0	3	2	2	6	3	2	9	16	1.8
P4	2	1	4	0	0	6	5	0	7	17	1.5
P5	1	0	5	3	3	6	5	3	8	17	1.5
P6	7	0	0	0	0	7	0	0	8	16	1.6
P7	4	0	3	0	0	7	3	0	9	17	1.9
P8	1	0	7	0	0	7	7	0	8	16	1.5
P9	1	0	2	0	0	3	2	0	11	15	1.4
P10	3	0	4	0	0	7	4	0	6	15	1.3
P11	2	0	3	0	0	5	3	0	8-10	15	1.6
P12	3	0	0	2	0	5	0	2	6	15	1.3
P13	0	0	3	0	0	3	3	0	7	15	1.3
P14	4	0	2	0	0	6	2	0	8-9	15	1.5
P15	0	0	4	5	4	5	4	5	16	12	1.4
P16	1	0	3	0	0	4	3	0	14	10	1.3
P17	1	0	3	2	2	4	3	2	6-7	15	1.1
P18	0	0	2	3	2	3	2	3	9	10	1.2
P19	0	0	5	1	1	5	5	1	16	7	0.8
P20	0	0	0	3	0	3	0	3	14-15	8	0.8
P21	0	0	3	0	0	3	3	0	10-11	6	0.4
P22	0	0	3	0	0	3	3	0	18	6	0.5

CONCLUSION

From this study, it can be concluded that cultured populations of P. *monodon* may serve as hosts for many viruses. While WSSV was found in single infections, MBV and HPV were mostly found as components in dual or multiple viral infections. Though *Penaeus monodon* can tolerate low-level single (WSSV), dual (WSSV/MBV, WSSV/HPV) or triple (WSSV/MBV/HPV) viral infections, it is necessary to screen the larvae for all these viruses before stocking in grow out ponds. This, together with stocking at low density can help to avoid disease outbreaks when combined with proper management practices.

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Modulation of Bio-defense Genes in WSSV-Infected *Penaeus monodon*

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ABSTRACT

Penaeus monodon is a host species to white spot syndrome virus (WSSV), the causative pathogen of a serious disease that has impacted the shrimp farming industry all over the world. The present study investigates some of *P. monodon's* cellular defense mechanisms against WSSV in a temporal analysis of 17 genes that are related to the invertebrate immune response. The results suggested that several defense mechanisms were induced upon WSSV infection, including the prophenoloxidase activation system and the JAK/STAT (Janus kinase/Signal Transducer and Activator of Transcription) signal transduction pathway. Conversely, the clotting system and the expression of anti-microbial peptide were down-regulated by WSSV infection.

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INTRODUCTION

The past two decades have shown that the shrimp aquaculture industry is vulnerable to disease, and one of the most economically significant viral pathogens is white spot syndrome virus (WSSV). Outbreaks of WSSV have caused global losses that are estimated at billions of dollars annually (Rosenberry, 2005). Shrimps lack an adaptive immune system of the type found in vertebrates, and even though there is some recent evidence of adaptive or "memory" immunity (Witteveldt *et al.*, 2004a; 2004b), shrimps mostly rely on their innate immune system to defeat infecting pathogens quickly and effectively. The innate immune system in shrimp involves several responses, including hemolymph coagulation, anti-microbial action, free radical formation, phagocytosis, cellular recognition, encapsulation and melanization (Cerenius and Soderhall, 2004; Soderhall and Cerenius, 1998). In addition, certain cellular responses such as apoptosis and heat shock response are also a significant part of the organism's defense against infection. However, these responses damage the host cell as well as the invading pathogen, and regulation of the defense responses therefore needs to be quite sophisticated. For example, in crayfish, the melanization responses are regulated by the prophenoloxidase (proPO) activation system, which is a cascade of serine proteinases that might be further regulated by the Mitogen-Activated Protein Kinase (MAPK) signaling pathway (Mavrouli *et al.*, 2005). In *Drosophila*, the genes encoding anti-microbial peptides are induced in response to microbial infection through the activation of two different but related signaling pathways, Imd and Toll (Engstrom, 1999; Imler and Hoffmann, 2000). In addition to the Imd and Toll pathways, the transcriptional profile of DCV-infected *Drosophila* suggests that the JAK/STAT (Janus kinase/Signal Transducer and Activator of Transcription) pathway might also respond to virus infection (Dostert *et al.*, 2005).

To investigate which immune response pathways might be used by *P. monodon* upon WSSV infection, in this preliminary study, we use RT-PCR to conduct a temporal analysis of 17 genes that are known to be related to immune response in invertebrates. Our results suggest that WSSV infection induces several defense mechanisms, including the proPO activation system and the JAK/STAT signal transduction pathway. Conversely, the clotting system and the expression of anti-microbial peptide are both down-regulated by WSSV infection.

MATERIAL AND METHODS

Virus

The virus used in this study, WSSV Taiwan isolate, originated from a batch of WSSV-infected *Penaeus monodon* shrimp collected in Taiwan in 1994. To prepare the inocula, the epidermis from *P. monodon* with a pathologically confirmed WSSV infection was homogenized (0.1g/ml in 0.9% NaCl) and then centrifuged at 1000Xg for 10 min. The supernatant was diluted to 1:100 with 0.9% NaCl and filtered through a 0.45 um filter.

Shrimp

Adult *P. monodon* (30 to 40 g) were collected from a culture pond at the Biotechnology Division of the Fisheries Research Institute in southern Taiwan. The shrimp used for the challenge test were checked with a commercial diagnostic kit (IQ2000^{TM}, IntelliGene) and confirmed to be WSSV-free. Prior to the experiment, shrimp were kept in a 70 L tank maintained at 25 to 28°C. Experimental shrimp were infected with WSSV by injection using a method described previously (Tsai *et al*., 1999). At various times over the course of the next 24 hrs, two or three specimens were selected at random and their pleopods were immediately frozen and stored in liquid nitrogen until used.

RNA isolation

For each time point, the frozen pleopods from WSSV-infected *P. monodon* were pooled and homogenized in 6 ml Trizol reagent (Life Technologies) and then subjected to ethanol precipitation. The pooled total RNA samples for each time point were then stored in 75% ethanol at -20°C.

Sequences of shrimp bio-defense genes

The sequences of 16 *P. monodon* bio-defense genes were obtained from the nucleotide Expression Sequence Tag (EST) database hosted by NCBI. In addition, the partial sequence of *pmstat* was obtained through RT-PCR amplification with the VNM-STAT-F/R primer set (5'-GAGTCAGTGATGGATGAGAAA-3'/5'-GTCGGAGAAACGGAGCAAGAA-3'), which was designed from a *P. vannamei* EST clone that had a 5' sequence that was similar to STAT. As Table 1 shows, the genes studied here can be categorized into two main groups. The first group includes genes that are involved in innate immunity (Table 1a), and the second group includes genes involved in other putative defense mechanisms (Table 1b).

Temporal analysis of bio-defense genes in *P. monodon* by RT-PCR

Each pooled total RNA sample in 75% ethanol was centrifuged at 14000Xg for 30 min at room temperature. The resulting pellets were re-suspended in DEPC-water and quantified by spectrophotometry at a wavelength of 260 nm. For each time point, an aliquot of 10 ug RNA was treated with 200U of RNase-free DNase I at 37°C for 30 min to remove any residual DNA and then extracted with phenol-chloroform. The DNase–treated total RNA was denatured by heating at 85°C for 10 min in 10 ul DEPC-water containing 100pmol oligo dT primer. The first-strand cDNA was synthesized by the addition of 4 ul SuperscriptII 5X buffer, 1 ul 100 mM DTT, 1 ul 10 mM dNTP, 10 ul RNAsin (Promega), and 100 U SuperscriptII reverse transcriptase (Life Technologies). DEPC-water was added to make a final volume 20 ul. The reverse transcription proceeded at 37°C for 1 hour, followed by heating at 95°C for 5 min to stop the reaction. One tenth of the products of the cDNA were taken to perform RT-PCR in a 50 ul reaction mixture containing 10 mM Tris-Cl, PH 8.8, 1.5 mM $MgCl_2$, 150 mM KCl, 0.1% Triton X-100, 0.2 mM dNTP, 100 pmol of the respective primer sets for each of the tested genes (see Table 1) and 2 U Dynazyme

Table 1. Analyzed bio-defense genes in *Penaeus monodon*.

Involved response	Genes	GenBank accession no.	Primer set	Annealing temp.
a. Genes involved in innate immunity				
Anti-microbial action	anti-microbial peptide	BI018071	5'- TGAGAAGAGCTTTCCTAGTTTAG-3' 5'-TACAAGTTTCTGTTATATTCTCTTG-3'	51
	anti-microbial peptide	BI018074	5'- CAGAAAGGAGTAGTTCATTCCC-3' 5'-TCGTCCGGGTGGTGGTCTAAG-3'	52
	penaeidin-2	BI018089	5'-TGCAGAGGCCGAAACTCCTTG -3' 5'-ACATACATCCCACATGCACTTTC -3'	53
	penaeidin 3-1	BI018080	5'-TCTCACCTGACACTCACCTGC-3' 5'-ACTACAACGAAAGTCAAACAACAC-3'	52
	lysozyme	BI018081	5'-TGGTTGGGCTTCTGGCGTTTC-3' 5'-TGAGATGACATGTATCTGACGTG-3'	53
proPO activation system	hemocyte kazal-type proteinase inhibitor	BI018078	5'-CCGATCATGATTGTATCGGCTAC-3' 5'-TTCCATTTCCTGTCACAAGGTCC-3'	53
	prophenoloxidase	BI018090	5'- TGTGAGGATATATTTGGCTCCGAAG-3' 5'-ATCCCGCAGGAAGGACACACCG -3'	53
	protein C	BI018099	5'-ACAGGTAACCAGATCGCAGGAC-3' 5'-TTGTGGAAATTGGGGTGAATGATG-3'	53
	clip-domain serine protease	BI018087	5'-GACTGCACCTCAACAGGAAGGAC -3' 5'-AATCTGTACCTTCTGCAGCACCTG -3'	54
Clotting reaction	transglutaminase	BI018082	5'-TTTCGGAGGCTGGCAGGTCAT -3' 5'-TAAATACGTATGCAGAATACTGTTC-3'	52
Signaling pathway	protein kinase C inhibitor	BI018088	5'-TGGTGCGCAACATGAGTGACG -3' 5'-ACTGGTGATATGACAAATATGCAG -3'	52
	STAT	this study	5'-GTT CAG TGT GGG AGG TGG AG-3' 5'-AGA AAC GGA GCA AGA AAG TG-3'	52
b. Genes involved in other putative defense mechanisms				
Heat shock response	heat shock protein 10	BI018100	5'-CGGTTCTGCTGCGTTAATCTC-3' 5'-ATGTGATGATTACATACATACATAG-3'	51
	heat shock protein 90	BI018096	5'-ACGAAAGAATAAATCCTTATAACAG -3' 5'-ACACCCGACCATGGCTACATG -3'	51
	heat shock protein 70	BI018094	5'-ACGTGTTACAAGTAGCTCTTAGG -3' 5'-ATACAGTGTTGTTGGGGTTCATC -3'	55
Oxidative stress	peroxidase	BI018092	5'-ACGGAGATCATGGAGTCGTCCCAC -3' 5'-TGCGCCGCCTTCACTTCCTTCATC-3'	55
Others	thymosin beta a	BI018086	5'-TGCGAGACTCACACATTACAATC -3' 5'-TGAGAAGGTCAGCAGGCACTC-3'	53

II DNA polymerase (Finnzyme, Finland). PCR was conducted as follows: 94°C for 2 min, 40 cycles of 94°C for 1 min, annealing at the temperature specified in Table 1 for 1 min, 72°C for 1 min, followed by an elongation at 72°C for 15 min. A beta actin transcript was amplified with the actin-F1/ actin-R1 primer set (5'-GAYGAYATGGAGAAGATCTGG-3' /5'-CCRGGGTACATGGTGGTRCC-3') and used as a reference gene. A WSSV genomic DNA-specific primer set IC-F2/ IC-R3 (5'-CAGACTATTAATGTACAAGTGCG-3'/ 5'-GAATGATTGTTGCTGGTTAGAACC-3') derived from an intergenic region of the WSSV genome was used to confirm that the RNA was not contaminated by any genomic DNA (data not shown).

RESULTS

Transcriptional analysis of bio-defense genes in *P. monodon* after WSSV infection

RT-PCR expression patterns of the pooled pleopod RNA (Figure 1a) suggested that eleven genes were down-regulated after WSSV infection. The proteins encoded by these genes include: five anti-microbial peptides; one protein that is involved in the proPO system; the key enzyme for the clotting reaction; a protein that is involved in anti-oxidative stress; a protein that regulates the Protein Kinase C (PKC) pathway; a protein that is involved in cell shaping and cell migration; and a member of the small heat shock protein family. Among the genes that are up-regulated upon WSSV infection (Figure 1b), two are involved in the proPO system and the third is involved in the JAK/ STAT pathway. The expression levels of two heat shock proteins and another protein involved in the proPO system remained unchanged after WSSV infection (Figure 1c). These data have not been quantified or normalized, but for each time point, the expression level of a beta-actin housekeeping gene was included for comparison (Figure 1d).

DISCUSSION

proPO activation system

One of the most important defense mechanisms, melanization, is mediated by the proPO activation system, and the results (Figure 1) suggest that this system might be activated after WSSV infection. The expression level of both prophenoloxidase and protein C, a non-proteolytic proteinase that can interact with prophenoloxidase activating precursor to enhance the activity of the proPO system (Yu *et al*., 2003), increased significantly from 4 to 6 hrs post-infection. Meanwhile, at about 6 hpi, the expression levels of a kazal family proteinase inhibitor began to decrease. Although these changes suggest that the proPO system may be activated, it should be pointed out that, to date, no direct evidence has been presented to indicate that the kazal-type proteinase inhibitors actually regulate the proPO activation system. It has been shown, however, that inhibitors from the kazal family can inhibit more than one serine proteinase with multiple domains (Kanost, 1999; Kato *et al*., 1987; Reisinger *et al*., 1987). The kazal-type proteinase inhibitor therefore remains a good candidate for controlling proteolytic activity in the proPO activation system.

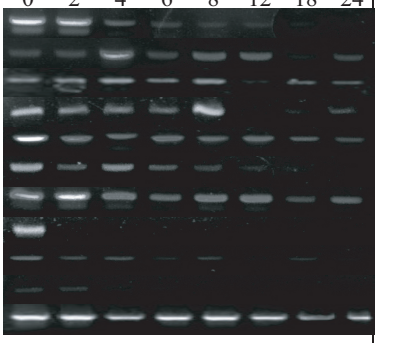
Figure 1. Time-course RT-PCR transcription levels of 17 P. monodon bio-defense genes at 0 to 24 hrs after WSSV infection. Genes are grouped according to their expression patterns. Each time point shows the RT-PCR results for pooled RNA extracted from the pleopods of 2 to 3 experimental infected shrimp.

a. Down-regulated gene expression

Involved response	Genes
Anti-microbial action	anti-microbial peptide
	anti-microbial peptide
	penaeidin2
	penaeidin3-1
	lysozyme
ProPO activation system?	hemocyte kazal-type proteinase inhibitor
Clotting reaction	transglutaminase
Oxidative stress	peroxidase
Signaling pathway	protein kinase C inhibitor
Heat shock reponse	heat shock protein10
Other responses	thymosin beta a

Hpi (hours post WSSV infection)

0 2 4 6 8 12 18 24

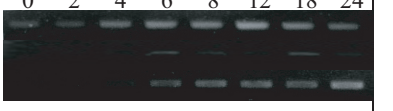


b. Up-regulated gene expression

ProPO activation system	prophenoloxidase
	protein C
Signaling pathway	STAT

Hpi

0 2 4 6 8 12 18 24

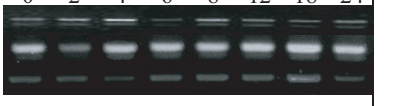


c.Unchanged expression levels

Heat shock reponse	heat shock protein 90
	heat shock protein 70
ProPO activation system	clip-domain serine protease

Hpi

0 2 4 6 8 12 18 24



d. Reference gene

Housekeeping	β - actin

Hpi

0 2 4 6 8 12 18 24

We note too that the proPO activation process produces an abundance of the free radicals that are used to destroy the pathogens (Nappi and Ottaviani, 2000). It may therefore be significant that the expression pattern of peroxidase, an anti-oxidative reagent that protects the cell from damage by free radicals, is down-regulated (Fig. 1a). This may be because the shrimp host is temporarily reducing the expression level of peroxidase to maximize the cytotoxic effect.

JAK/STAT signalling pathway

In vertebrate animals, several cellular responses are mediated by the JAK/STAT signaling pathway, including cell growth, cell differentiation and immune response (Levy and Darnell, 2002). Generally, the activation of STAT requires tyrosine phosphorylation through JAK. This allows the cytoplasmic STAT proteins to form dimers, which then enter the nucleus and bind to the recognition sequences in their respective promoters. However, in addition to JAK, a recent study suggests that inhibition of PKC activity leads to impairment of the tyrosine phosphorylation of STAT that is required for IFN-alpha mediated HCV RNA replicon clearance (Fimia *et al.*, 2004) . Hence, the decreased expression levels of PKC inhibitor (Figure1a) might act to further increase STAT activity by enhancing the activity of PKC.

In insect, the role of STAT appears to be similar. For example, bacterial infection of mosquito induced the expression of STAT, and the activated STAT successively translocated into the nucleus (Barillas-Mury *et al*., 1999) . A recent study in *Drosophila* also suggested that the JAK/STAT signaling pathway is required for the host's antiviral response (Agaisse and Perrimon, 2004; Dostert *et al.*, 2005) . In the present study, *pmstat* was transcriptionally up-regulated after WSSV infection, suggesting that the JAK/STAT signaling pathway might be part of the shrimp antiviral mechanism.

Anti-microbial action

Figure 1a shows that the anti-microbial peptides, which function as detergents to destroy the lipid membrane of pathogens, were down-regulated after WSSV infection, suggesting that anti-microbial actions were weakened by WSSV infection. Although there is no direct evidence, down-regulation of the anti-microbial peptides was presumably mediated by pathways similar to the Toll and Imd pathways in *Drosophila* (Engstrom, 1999; Imler and Hoffmann, 2000).

Clotting reaction

The clotting reaction of hemolymph is another important defense mechanism in invertebrates and it benefits the host by immobilizing the invading pathogens. In shrimp, this system is simple and mainly relies on the enzyme transglutaminase (Huang *et al*., 2004). The down-regulation of the expression of transglutaminase (Fig. 1a) therefore suggests that WSSV weakens the immunity of shrimp by impairing the clotting reaction. This assumption is consistent with the fact that the hemolymph does not coagulate in seriously WSSV- infected shrimps.

Unchanged expression levels

Even when the expression level of a gene is not affected by WSSV infection, the response mediated by that gene may still be important. For example, heat shock protein 90 (Fig. 1c) can be required by both virus and host (Garry *et al*., 1983; Hu *et al.*, 2004; Srivastava, 2002). A constant expression level might therefore simply mean that these genes are already being expressed at levels that are not detrimental to either the virus or the host.

CONCLUSION

In conclusion, although this is only a preliminary study, the transcriptional analysis results suggest that at least four defense mechanisms are modulated by WSSV infection. The proPO activation system and the JAK/STAT signaling pathway are induced after WSSV infection, while anti-microbial action and the hemolymph coagulation mechanism are repressed.

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Cluster Level Adoption of Better Management Practices in Shrimp (*Penaeus monodon*) Farming: An Experience from Andra Pradesh, India

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ABSTRACT

Disease is a major constraint to aquaculture production and profitability around the globe. Since the early 1990s, white spot disease caused by white spot syndrome virus has been causing unprecedented economic and social hardships to small-scale shrimp farmers in India. Since 2001, the MPEDA-NACA technical assistance programme on "shrimp disease and coastal management" with the additional support from Australian Centre for International Agriculture Research is working towards developing and disseminating better management practices (BMPs) and promoting farmer self help groups to collectively implement the BMPs at the cluster level. In 2005, the extension programme was conducted in modified extensive shrimp farms in 6 villages of West Godavari district. In total, 492 farmers participated with 642 ponds spread over 275 ha of water area. Farmers were organized under self-help groups called 'Aquaclubs' at village level and a set of BMPs were collectively implemented. The average crop outcomes in study ponds in terms of production, average body size, survival rate and crop duration were 1,366 kg/ha, 26.2 g, 72% and 116 days, respectively; while in non-study ponds were 764 kg/ha, 24.7 g, 46% and 98 days, respectively. Emergency harvests indicative of disease outbreaks was 2% in study ponds compared to 65% in surrounding non-study ponds and they were significantly different ($p=0.00$). In study ponds, the crop outcomes significantly ($p<0.05$) improved compared to those of the previous year (no extension support). And emergency

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harvest rate indicative of disease outbreaks significantly decreased (p=0.00) from 58% during 2004 to 2% during 2005. However in non-study ponds the emergency harvest rate marginally reduced from 68% during 2004 to 65% during 2005 (p =0.03). These results indicate that BMPs when implemented collectively by organizing the farmers in the form of Aquaclubs can significantly improve the crop outcomes and significantly reduce the impact of diseases.

INTRODUCTION

Shrimp farming is an important economic activity in India. Disease is a major threat to the aquaculture production and profitability (Subasinghe, 2005). White spot disease (WSD) caused by white spot syndrome virus (WSSV) is a major problem in shrimp (*P. monodon*) farming since early 1990s and small-scale farmers across the Asian region including India have severely suffered due to WSD epizootic (Karunasagar *et al.*, 1997; Mohan *et al.*, 1998; Shankar and Mohan, 1998). Recently some studies have identified the risk factors and developed risk management practices or so called better management practices (BMPs) to reduce the risk of disease outbreaks in shrimp farms (Leung and Tran, 1999; Corsin *et al.*, 2001; MPEDA/NACA, 2001; Turnbull *et. al.*, 2003).

In India, the Marine Products Export Development Authority (MPEDA), Ministry of Commerce and Industry, with technical assistance from the Network of Aquaculture Centres in Asia-Pacific (NACA), Bangkok, Thailand has started a project called 'Shrimp Disease and Coastal Management' during 2000 with the objectives of identifying risk factors for disease outbreaks and developing and disseminating practical measures for containing and preventing shrimp disease outbreaks on farms in the major shrimp farming state of Andhra Pradesh.

Risk factors for shrimp diseases were identified during 2001 by conducting a longitudinal epidemiology study in selected sites of Andhra Pradesh. Suitable BMPs were developed to reduce these risks at farm level. These BMPs were first pilot-tested in 5 private farms of Andhra Pradesh during 2002. Although disease occurrence could not be completely eliminated, the production obtained and the percentage of profit making ponds were encouraging (MPEDA/NACA, 2002; Padiyar *et al.*, 2003). In 2003, the cluster farm management concept was introduced and 58 farmers of Mogaltur village in West Godavari district participated with their 108 ponds by forming an Aquaclub (farmer self- help group consisting 20-30 neighboring shrimp farmers in given farm cluster). This resulted in 2.4 times better production in demonstration ponds than the surrounding non-demonstration ponds. But the prevalence of emergency harvests was not significantly different ($p>0.05$) between demonstration (82%) and non-demonstration ponds (89%) (MPEDA/NACA, 2003; Padiyar *et al.*, 2004). In 2004, extension programme was spread to extensive farming areas (in which average stocking density was about 2 shrimp/m^2 and farms with average shrimp production was about 250 kg/ha/crop) of seven villages of West Godavari district and resulted in improved crop outcomes (MPEDA/NACA, 2004).

During 2005, the extension programme was extended to modified extensive farming system (in which average stocking density was about 6 shrimp/m^2 and average shrimp

production was about 750 kg/ha/crop) in six villages in this district. In this paper, we present the crop outcomes and experiences in implementing BMPs at cluster level in six villages of West Godavari district.

MATERIALS AND METHODS

Selection of study villages

Six villages, namely Badawa, Chinamamidipalli, Dharbharevu, Kotha Navarasapuram, Y.V. Lanka and Y.V. Lanka Society, were selected from Narasapuram sub-district of West Godavari district (Figures 1 and 2). All these villages were directly dependent on Godavari estuarine water source. Salinity of water in all six villages ranged between 20-35 ppt. These villages were situated near to the earlier demonstration villages of MPEDA/NACA project during 2002-2004. Motivated by the crop results and technical services of MPEDA/NACA study team in other villages, farmers of these six villages requested for extension support in their village during 2005 which was granted. In these villages, most of the shrimp farmers were small holders with farm size of less than 2 ha/farmer and practicing modified extensive farming system.

Farmer and pond selection

Willingness of farmers to join in the village cluster level Aquaclubs (farmer self-help group) and to co-operate with the study team primarily influenced the selection. Also willingness of farmers to implement the BMPs was considered during the selection. BMPs were given as choice to the farmers and it was not absolute necessary for farmers to implement all of them if they were to be considered in the study. However, BMP implementations were recorded from each and every study pond during the cropping cycle.

Recommended BMPs

Better management practices identified and field tested by MPEDA/NACA project in West Godavari district since 2001 were used (MPEDA/NACA, 2001). Also some relevant BMPs were used from existing information (Chanratchakool *et al.*, 1998). These are given below:

(a) sludge removal and disposal away from the pond site;
- ploughing on wet soil if the sludge has not been removed completely;
- water filtration using twin bag filters of 300 μ mesh size;
- water depth of at least 80cm at shallowest part of pond;
- water conditioning for 10-15 days before stocking;

(b) seed selection and stocking:
- uniform size and colored PL, actively swimming against the water current;
- nested PCR negative PL for WSSV (using batches of 59 PL pooled together. If test turns negative it means that the prevalence of WSSV infected PL is less than 5% in that population at 95% confidence);

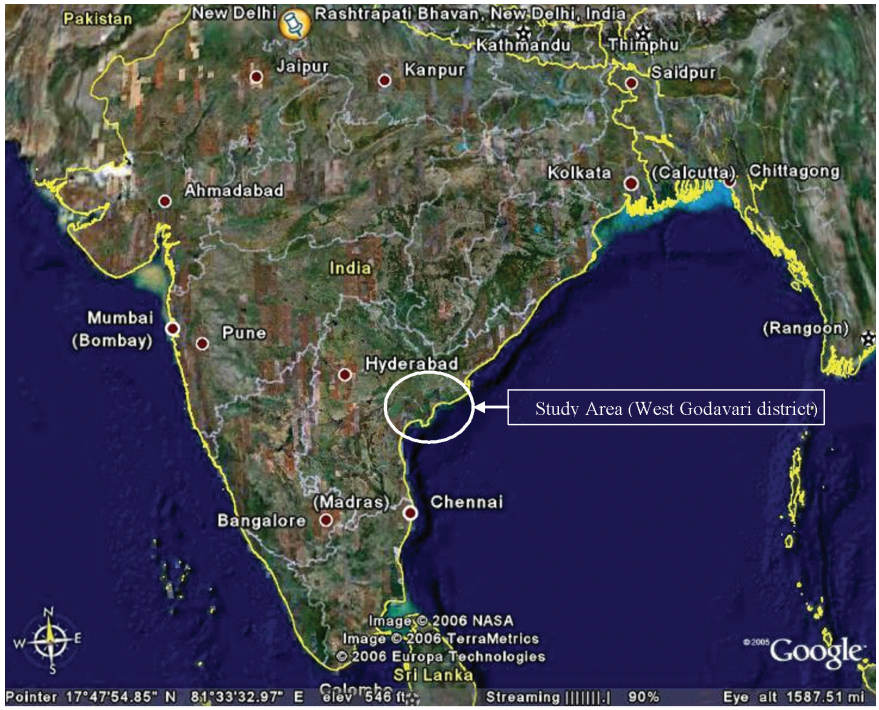

Figure 1. Map of India showing the study area.

Figure 2. Map showing the study villages (encircled) in West Godavari district.

- weak PL elimination before stocking using formalin (100 ppm) stress for 15-20 minutes in continuously aerated water;
- on-farm nursery rearing of PL for 15-20 days;
- stocking during 1st week of February to 2nd week of March;
- seed transportation time of less than 6 hrs from hatchery to pond site;
- stocking into green water and avoiding transparent water during stocking;

(c) post stocking management:

- use of water reservoirs, and 10-15 days aging before use on grow-out ponds;
- regular usage of agriculture lime, especially after water exchange and rain;
- no use of any banned chemicals;
- using of feed check trays to ensure feeding based on shrimp demand;
- feeding across the pond using boat/floating device to avoid local waste accumulation;
- regular removal of benthic algae;
- water exchanges only during critical periods;
- weekly checking of pond bottom mud for blackish organic waste accumulation and bad smell;
- regular shrimp health checks, and weekly health and growth monitoring using a cast net;
- removal and safe disposal of sick or dead shrimp;
- emergency harvesting after proper decision making;
- no draining or abandoning of disease affected stocks.

Extension method

The study team, consisting of one senior technical assistant and two junior technical assistants, stayed in one of the study villages starting two months prior to the start until end of the cropping season. The study team socialized with the villagers by keeping in touch with the farmers, villagers and village leaders on a daily basis. Through village level meetings, farmers were introduced to cluster farm management concept, formation of Aquaclubs and importance of adoption of BMPs. Key farmers from other villages involved in the MPEDA/NACA project (2002-2004) were invited to these new villages to share their experiences in the formation and management of Aquaclubs and implementation of BMPs. Depending on the willingness and availability of farmers, field visits were arranged for farmers from these six villages to other villages under the project for first-hand information exchange among the farmers. Aquaclubs prioritized the BMPs according to their local needs. Individual farmers were given freedom to implement the BMPs under the general guidance of Aquaclub leaders. Contract hatchery seed procurement system (Padiyar, 2005) was introduced in which meetings were arranged between farmers and hatchery operators for supply of good quality (BMPs) of shrimp seeds. The study team supported farmers in adopting the recommended BMPs and also to follow them through personal supervision of the farm activities on a daily basis.

Crop details in study ponds

Farm data from study ponds were collected using recording sheets and daily pond management books. The data included the following information: pond preparation (drying, sludge removal, ploughing, soil treatment, water intake), seed quality and source (activity of PL, body colour, body length in mm, deformities, diagnosis of WSSV by PCR test), stocking details (date, time, stocking density, acclimation), water management (water exchange practices, aeration, use of fertilizers, lime and other treatment chemicals, water quality parameters including water colour, salinity measured by refractometer, water pH measured by universal pH indicator, ammonia, hydrogen sulfide and total alkalinity measured by field test kits (Merck Inc.)), feed management (daily feed quantity, feed brand, feed size), health management (behaviour of shrimp, clinical signs, growth observation by weighing, use of treatment chemicals, number of dead and moribund shrimps on daily basis), harvest details (yield, average body weight, crop duration, estimated survival rate, market price) and crop expenditure details. However in this paper only the stocking densities and harvest details were considered.

Previous year crop details

The previous year crop (2004) details during same season (summer season of January - July) from the study ponds were gathered by interviewing the study farmers using a questionnaire. During 2004, there was no extension support for farmers in these six villages.

Crop details in non-study ponds

Information on farming practices and crop outcomes from non-study ponds in the above six villages and adjacent villages during demonstration period (2005) and previous year (2004) was collected using questionnaire. Each non-study ponds in the above six villages were targeted for information collection. In the adjacent villages, ponds and farmers were selected on convenience basis where in all the available farmers in those villages during the fixed 30 days of interview period were considered for interview. This kind of convenience sampling was preferred due to non-availability of the sampling frame (list of all farmers) from those of neighboring villages. Farmer interviews were conducted after completion of the cropping cycle and all the interviews were conducted in one month period.

Data analysis

The collected data were digitized and analyzed using a statistical software Epi InfoTM (downloaded from (www.cdc.gov/EpiInfo/). Mean values of two group of samples were compared using analysis of variance test (using EpiTable package in the above software). P-value lower than 0.05 is considered as significant.

RESULTS AND DISCUSSION

In the survey conducted to gather information on crop outcomes from non-study surrounding ponds, crop information from 294 ponds was gathered.

The average stocking density in study ponds was 67,850 shrimp seed/ha during 2004 and 69,900 shrimp seed/ha during 2005. In non-study ponds it was about 50,250 shrimp seed/ha during 2004 and 55,300 shrimp seed/ha during 2005. In both study and non-study ponds, stocking densities over the years did not significantly change (p=0.38 and 0.01, respectively). However, stocking density in study ponds was significantly higher (p =0.00) than that of non-study ponds during both years.

Table 1 shows the average values of crop outcomes in the study and non-study ponds in terms of production (kg/ha), average body size (g), survival rate (%), crop duration (days) and emergency harvest rate (%) indicative of disease outbreaks during 2005. They were 1,366 kg/ha, 26.2 g, 72%, 116 days and 2%, respectively in study ponds and 764 kg/ha, 24.7 g, 46%, 98 days and 65%, respectively in non-study ponds. These outcomes were significantly better study ponds than non-study ponds (p=0.00; 0.002; 0.00; 0.00; 0.00 respectively). Compared to non-study ponds, in the study ponds, average production was increased by 1.79 times, average body size was bigger by 1.5 g, survival rate was 26% higher, crop duration was 18 days longer and emergency harvest rate was lower by 5.2 times.

Table 1. Average value of crop outcomes during demonstration period (2005) and previous year (2004) (no extension year)

Crop Outcome	Study Ponds		Non-study ponds	
	2004	**2005**	**2004**	**2005**
Production (kg/ha)	1078	1366	694	764
Mean Body weight (g)	24.4	26.2	28	24.7
DOC (d)	98	116	99	98
Survival rate (%)	67	72	41	46
Emergency harvest rate (%)	58.1	2	68	65
Stocking Density	6.79	6.99	5.025	5.53

Table 2 shows the average values of crop outcomes in the study and non-study ponds in terms of production (kg/ha), average body size (g), survival rate (%), crop duration (days) and emergency harvest rate (%) indicative of disease outbreaks during 2004 (no extension period). They were 1,078 kg/ha, 24.4 g, 67%, 98 days and 58%, respectively in study ponds and 694 kg/ha, 28 g, 41%, 99 days and 68%, respectively in non-study ponds. All these average crop outcomes in the study ponds except crop duration and emergency harvest

rate were significantly better in the study ponds than in the non-study ponds (p=0.00). Compared to the non-study ponds, the study ponds had 1.55 times more production, 3.6 g smaller-sized shrimps and 26% higher survival rate.

In the study ponds, although there was no significant change in stocking density over the years during 2004-2005 (p=0.387), all the outcomes showed significant improvement ($p < 0.05$). Average production was increased by 27%, average body size increased by 1.8 g, survival rate increased by 5.8%, crop duration increased by 18 days and emergency harvest rate reduced by 56.1%. In contrast, interestingly in the non-study ponds, there was no significant change ($p > 0.05$) in average crop outcomes except that the average body size and emergency harvest rate significantly (p=0.00002; 0.033) reduced by 3.3 g and by 3% during 2005 compared to 2004. However during 2005, emergency harvest rate was 65% in the non-study ponds, which was significantly higher by 63% compared to that of the study ponds in the same year (p=0.00). This shows that over the years, in the study ponds, emergency harvests were significantly and convincingly reduced and so as average crop outcomes. But in the non-study ponds, there was no significant but marginal improvement in crop outcomes which might indicate that indirect influence of study team and extension activities in the study villages on surrounding non-study ponds of same villages and neighbouring villages.

CONCLUSIONS

Extension activities to disseminate the information on BMPs and adoption of these BMPs by farmers showed significant improvement in the success of farmers by resulting in better crop outcomes and reduced emergency harvests, which is indicative of a disease outbreak. Cluster farm management approach by promotion of farmer 'Aquaclub' proved that unity and farming discipline among shrimp farmers can effectively contain the diseases at village cluster level. It is important that the study team socializes with the farmers to effectively disseminate the BMP information and motivate the farmers to quickly adopt them. Therefore it is essential to organize active extension programme to promote BMPs and cluster management approach for shrimp farming.

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Codes of Practice and Better Management: A Solution for Shrimp Health Management?

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ABSTRACT

Following the development of the FAO Code of Conduct for Responsible Fisheries, international principles for the sustainable production of shrimp farming were prepared to address shrimp health, food safety and the environmental and socio-economical sustainability of the sector. Better Management Practices (BMP) to translate the principles into practice were also developed, disseminated and implemented in several Asian countries including India, Vietnam, Thailand and others. This manuscript describes how approaches towards sustainable shrimp production can effectively contribute to shrimp health management. It also highlights the need for broad stakeholder involvement and for providing the right incentives to producers such as reduced costs, improved production, access to information and diagnostic services and higher market prices for BMP products.

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INTRODUCTION

Over the last decade, shrimp farming has been one of the most rapidly growing sectors within aquaculture. From a production of less than 900 thousand tonnes in 1994, in 2004 global production from shrimp farming reached 2.5 million tonnes, with a farm gate value of US$9.7 billion. This production was largely dominated by Asian countries which in 2004 made up 88% of the total production with 2.2 million tonnes and a value of US$8.3 billion. Although impressive, these aggregated figures do not fully represent the extent of the growth experienced by some of the leading shrimp farming countries. Production in China showed a notable 5.5-fold increase over the 5 year period between 1999 and 2004, going from 171 thousand tonnes to 936 thousand tonnes. A similar 5-fold increase was experienced by Vietnam, where production increased from 55 thousand tonnes in 1999 to 276 thousand tonnes in 2004 (FAO, 2006).

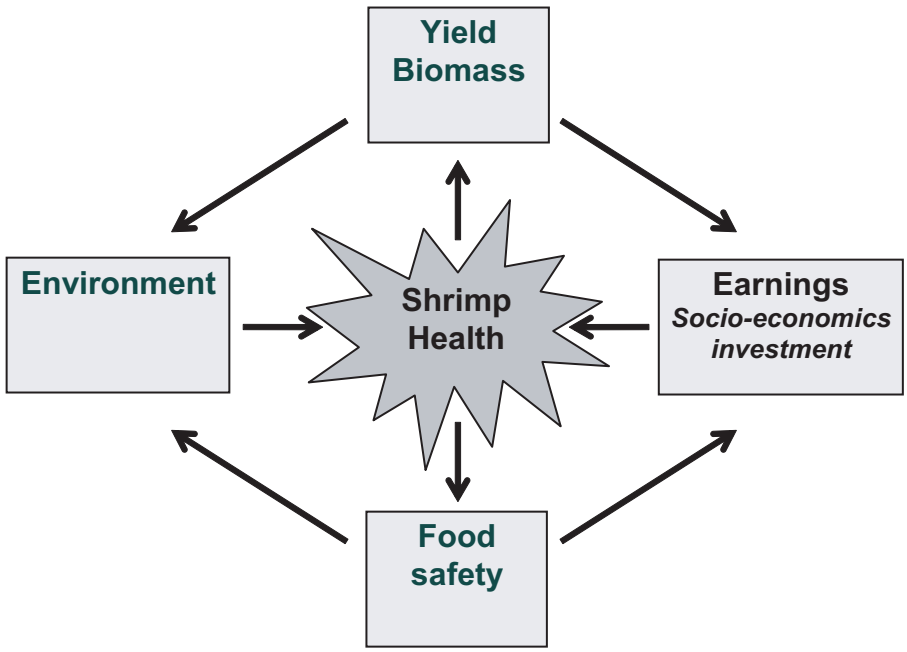

Figure 1. Diagram explaining the relation between shrimp health and other aspects of sustainability.

With this rapidly increasing production, several issues and challenges over the sustainability of the sector began to emerge (Figure 1). Outbreaks of various shrimp diseases (*i.e.* White Spot Disease, Yellow head Disease, and Taura Syndrome) have caused devastating economic damage to the sector worldwide. Increased shrimp health problems have led to the use of sometimes banned chemicals and drugs, which posed threats to the health of consumers and led to several restrictions being put in place by importing countries (Ababouch *et al.*, 2006). Major challenges included also environmental degradation and social conflicts related to the use and pollution of natural resources, sometimes leading to claims of mangrove deforestation, salination of agricultural land and eutrophication of

sensitive aquatic habitats. The sustainability of the sector was also challenged by the price in the international market, which has continued to decline, especially in the USA, in spite of the increased challenges faced by producers (Leites, 2006).

In response to these concerns, efforts were initiated to develop a more sustainable approach to shrimp farming and to the fisheries sector in general. The Food and Agriculture Organization of the United Nations (FAO) played a key role in this process. In 1995, FAO issued the Code of Conduct for Responsible Fisheries (CCRF), which set principles for the responsible development of the fisheries sector, including aquaculture. In 1999, FAO joined NACA, the World Bank, WWF and other partners to form a Consortium Programme targeted at exploring more sustainable approaches specifically for the shrimp farming sector.

This paper examines how approaches towards sustainable shrimp farming can assist shrimp health management, in particular using case studies from India, Vietnam, Thailand and Iran.

SHRIMP HEALTH MANAGEMENT AND SUSTAINABLE SHRIMP FARMING

It is increasingly being recognized that shrimp health management is strongly linked with other aspects of shrimp farming sustainability and that all those aspects should be addressed to achieve effective shrimp health management.

It is obvious that shrimp health affects yields and therefore earnings. In addition to the well known links between environment and shrimp health, the occurrence or threat of experiencing shrimp health problems, often leads farmers to use chemicals and drugs which may have been banned by importing countries. The price of rejecting batches containing such substances will also reflect on the earnings generated throughout the supply chain. As a consequence of reduced profits, farmer will have less financial resources to invest on reducing their risk of experiencing shrimp health problems, therefore increasing further the risk of diseases.

Although shrimp health is generally a major driver of the process towards sustainability, at least for producers, for the above reasons this manuscript will discuss broadly about sustainability of the shrimp farming sector, rather than only shrimp health management by itself. Sustainability of the sector is a shared responsibility and as such, will be addressed in this paper by considering the role to be played by the many stakeholders involved, and not merely by looking at shrimp farming practices. For ease of explanation, the process of achieving sustainable shrimp farming will be presented step-wise, following the diagram reported in (Figure 2).

Gather technical knowledge

There is a great deal of knowledge now available on how to successfully farm shrimp, while improving the environmental and socio-economical sustainability of farm operations and the safety of the harvested products. This knowledge should be used to address 2

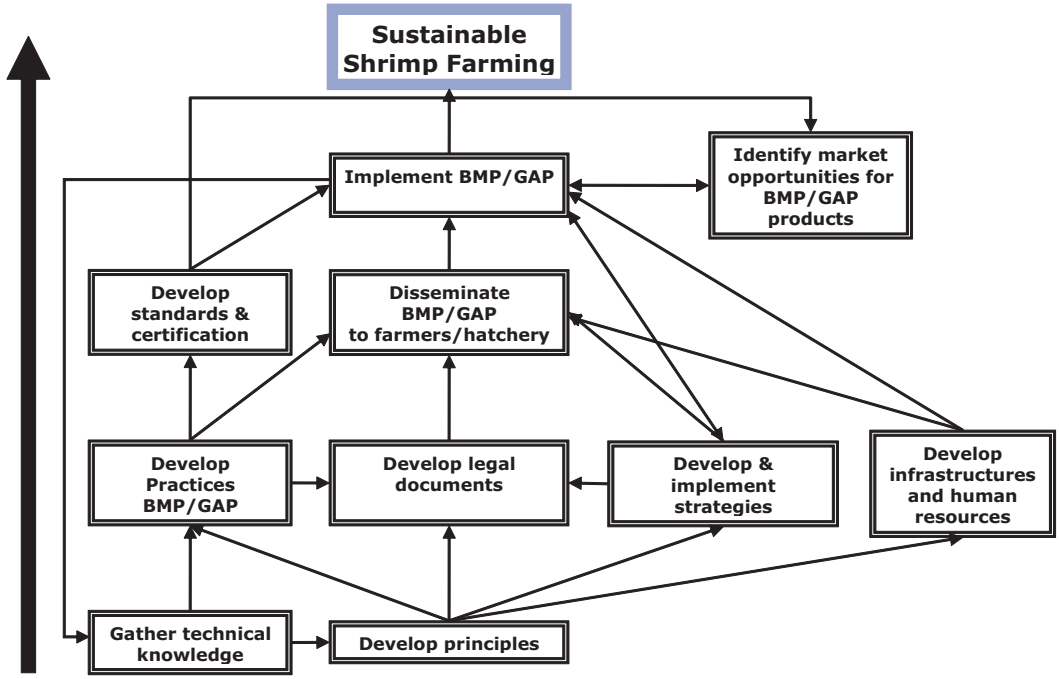

Figure 2. Diagram showing indicative steps to achieve sustainable shrimp farming, as defined by improved shrimp health management, environmental protection, food safety and socio-economical sustainability.

issues. The first concerns the development of general principles for shrimp farming sustainability, *i.e.* the identification of what concepts should be adopted to make shrimp farming sustainable. The second issue is related to the "translation" of those principles into practice, which is conducted by using technical knowledge to develop system-specific farming practices that address the sustainability of the farming operations.

The development of principles for responsible shrimp farming (see next section) was based upon several consultations which involved a broad range of stakeholders and lasted for about 7 years.

Concerning the development of practices for the implementation of the principles, this process can be assisted by the several extension documents now available. A reference manual concerning technical aspects of shrimp farming is "Health Management in Shrimp Ponds" (Chanratchakool *et al.*, 1998), a document widely used in the Asian region and around the world and which has been translated in at least 5 Asian languages. Several extension documents can also be freely downloaded from the NACA website (http://www.enaca.org/modules/wfdownloads/). The applicability of these manuals to a specific farming system however, is not straightforward and practices recommended in these documents generally require revision and amendment to fit local contexts. Knowledge on how to improve practices for the sustainability of the sector can be obtained through discussions between international and national experts and in consultation with farming communities (NACA/FSPS/MOFI, 2005). Following the development and implementation

of practices specific to a farming system, it has also proven extremely useful to review the applicability and effectiveness of these practices and to revise them if necessary. In this process, researchers often play a key role by advising and contributing to the development of Monitoring and Evaluation systems.

Develop principles

Principles, often grouped into Codes of Conduct (CoC) or Codes of Practice (CoP) if related to specific commodities, represent the "philosophical basis" for the responsible production of a product. In 1999 a Consortium Program was established with the aim of developing principles for the responsible production of shrimp, following the guidelines of the FAO CCRF and the experiences of the organizations involved. The Consortium was formed by a wide range of partners among which FAO, NACA, the World Bank and WWF were among the major, and UNEP joining it in more recent years. Benefiting from the knowledge available, from 35 complementary case studies conducted in more than 20 shrimp farming countries, and from several stakeholder consultations involving governments, private sector and non-governmental organizations, the Consortium Program developed the "International Principles for Responsible Shrimp Farming: incorporating 8 principles (FAO/NACA/UNEP/WB/WWF, 2006). In February 2006, the principles were endorsed by the 17 countries represented at the NACA Governing Council and in September 2006 they received global endorsement by the Committee on Fisheries, Sub-Committee on Aquaculture, making them the only internationally recognized set of principles for the responsible production of shrimp.

Develop Better Management Practices/Good Aquaculture Practices

The development of practices for the effective implementation of the principles is a key step towards the sustainability of the shrimp farming sector. Several names have been used to describe these practices for sustainable shrimp farming, among others Better Management Practices (BMPs) and Good Aquaculture Practices (GAP) are among the most used. Defining differences between BMP, GAP and others is beyond the scope of this review and for simplicity the most used term "BMP" will be used to define any set of practices that address broadly the sustainability of the sector.

BMP are generally specific to the system and as such they have to focus on what a farming community can effectively achieve in a cost effective way. BMP should be simple, practical and applicable with minimum effort virtually by every farmer willing to do so. In doing so, they should address especially the needs of small-scale producers, not only because of their importance in the overall supply of shrimp worldwide but also to ensure that these more vulnerable players are not excluded from these efforts towards sustainability.

BMP should be developed for every link in the supply chain, from broodstock suppliers, to hatcheries, seed middlemen and farmers. Most sets of farm-level BMP developed for different farming systems address the following: pond location and construction; pond preparation; seed selection and stocking practices; improve pond management; pond bottom monitoring, water quality and shrimp health; improve biosecurity during production; record keeping; better management of health problems (especially concerning

the responsible use of antibiotics and chemicals); better collaboration with other resource users. BMP also emphasise the advantages of establishing farmer groups and associations (in India known as aquaclubs) in order to share costs (e.g., for shrimp seed testing), improve water management, increase awareness on the health status of neighbouring farms and through the appointment of voluntary extension workers with the role of assisting BMP implementation by other farmers in the group.

Although BMPs are indeed simple, well-know practices, most often these practices are not followed by farmers because of poor awareness or lack of the necessary conducive environment.

Disseminate Better Management Practices/Good Aquaculture Practices to all links in the supply chain

Building awareness on BMP among farmers and other links in the supply chain relies on an effective extension mechanism. This mechanism can take different forms, depending on the country, institutional context, production system, economics and other factors.

Most Asian countries have capillary extension services that can be effectively involved in BMP dissemination, provided that the right support and incentives are given by national and local authorities. Input suppliers and processors also play an important role. Feed suppliers in India are for example an important channel for dissemination of technical knowledge. The involvement of such stakeholders in BMP programs is therefore essential to create synergy rather than parallel efforts or worse, contradictory messages being given to producers.

In view of the large numbers of producers involved in shrimp farming, farmer to farmer extension is essential. As mentioned above, the development of a voluntary extension system has sometimes proven key to the dissemination of BMP messages in Vietnam (NACA/FSPS/MOFI, 2005). Voluntary extension workers are generally selected by the members of a farmer group based on their experience in shrimp farming and willingness to support other farmers in BMP implementation. Voluntary extension workers generally do not get financial compensation for their work. Their incentives to provide extension services consist of: rapid access to information because they are the first to be contacted for dissemination of new information; better awareness about shrimp health in neighbouring ponds; ability to reduce disease risk in neighbouring ponds and consequently in their own pond.

Extension activities are greatly supported and standardized through the development and dissemination of extension material. Several extension documents are now freely available in many local languages. Links to several extension documents freely available on the internet can be found on the NACA website (www.enaca.org). Extension material is generally simple in language and often organized in steps that farmers can follow to improve the sustainability of their farm operations and, with it, more effective shrimp health management.

In addition to extension manuals, BMP messages are also disseminated at farmers meeting held by (voluntary) extension workers and though regular pond visits. The active participation and sharing of experiences by farmers plays a very important role in

awareness building and dissemination of BMP messages. Successful dissemination of BMP messages can also be achieved using demonstration farms (Padiyar *et al.,* 2008) and through more structured Aquaculture Farmer Field Schools, where demonstration farms are used to conduct regular pond-side training courses (NACA/FSPS/MOFI 2005).

In a context in which scientific knowledge has to reach millions of producers, it is important to emphasise the need for novel and practical extension approaches to facilitate an effective and efficient flow of information (e.g., BMP messages) to the end users.

Develop legal documents

BMP can also be disseminated through the development of legal documents. As the principles contained in the CCRF were voluntarily taken up by several countries to develop their own fisheries legislation (R. Subasinghe, pers. comm.), similarly, the principles for responsible shrimp farming and the BMP for their implementation can also be incorporated into legislation. An example of this is represented by Viet Nam, where the Ministry of Fisheries promulgated a "Regulation on safe shrimp culturing zone and shrimp farm management" (MOFI, 2006). This regulation aims at supporting the implementation of the Aquaculture Development Article (Article 9) of the FAO CCRF and the International Principles for Responsible Shrimp Farming and regulates on the adoption of sustainable farming practices (defined in the regulation as GAP and CoC).

Other examples of the development of legal documents for the implementation of BMP messages include also, but are not limited to, India and Thailand, where the Marine Product Export Development Authority (MPEDA) and the Department of Fisheries (DoF) respectively, are playing a key role in promoting the sustainability of the shrimp farming sector.

The development of legal documents is also critical to regulating the implementation of strategies and systems which are developed to allow the implementation of BMP by farmers and other links in the supply chain.

Develop and implement strategies

The process of BMP dissemination and implementation needs supporting also through the development of specific strategies and systems. The development of national aquatic animal health strategies has obvious implications for effective shrimp health management. These strategies would include the development of a surveillance system, contingency plans, accurate health certification and others. Guidelines for the development of strategies related to aquatic animal health management have been developed by FAO and NACA (FAO/NACA, 2000) and are currently assisting efforts conducted in several Asian countries.

The development of an extension strategy also plays an important role towards harmonisation of BMP messages distributed to farmers and other stakeholders. In addition, although keeping records is the responsibility of farmers and other players in the supply chain, their efforts have to be assisted through the development of a traceability system capable of managing those records and provide full product traceability in compliance with the requirement of several importing countries (FAO, 2002).

Strategies and systems should be developed through broad stakeholder consultations to achieve general agreement and maximise the chances of compliance at all levels. The implementation of the strategies can also be assisted through the development of legal documents that can regulate in a more structured way stakeholders' compliance.

Develop infrastructure and human resources

The implementation of strategies and systems often requires that infrastructures and the necessary human resources to operate them are also put in place. Diagnostic laboratories capable of assessing shrimp seed quality (including the presence of dangerous pathogens) and the occurrence of shrimp diseases have to be established. These should be located and equipped as to respond to the demand of hatcheries and farmers, being capable of providing diagnosis ranging from Level 1 (gross signs) to Level 3 (using advanced diagnostic methods) as necessary. For example, White Spot Disease (WSD) management could be assisted though the establishment of PCR laboratories capable of assessing White Spot Syndrome Virus (WSSV) presence in broodstock and seed and of laboratories for the rapid detection of clinical WSD in proximity of farms (NACA/FSPS/MOFI, 2005).

Water quality facilities could also be established in assistance to farming communities and to provide effective monitoring of aquatic resources and the identification of events that may affect shrimp farming operations (e.g. red tides).

Centres for the analysis of data collected from surveillance system and for the development of accurate and rapid responses to health emergencies should also be established and equipped with the necessary epidemiological expertise.

Infrastructure to allow the management of the data collected as part of a traceability system would also need establishing. The equipment requirements and expertise required will depend on the structure and mechanism of implementation of the system, ranging from largely paper based systems with little IT requirements, to systems with a high degree of digitalization of information.

In addition to the above, capacity on BMP development and implementation should also be built among different players involved with the shrimp farming sector, including extension workers, local authorities, policy makers and others.

Implement Better Management Practices/Good Aquaculture Practices

Although BMP are often simple practices, their systematic adoption by farming communities and countries to manage shrimp health problems and to achieve widespread sustainable shrimp production has a relatively recent history. The first project aimed at identifying BMP targeted to shrimp health management was implemented in the year 2001 in the east coast of India with the support of MPEDA, NACA and the Australian Centre for International Agricultural Research (ACIAR). Since then, this approach towards sustainability has been adopted by several countries including Viet Nam, Thailand, Indonesia and Iran and it is expected to spread to many other countries in the Asian region among which China, Sri Lanka and the Philippines have already expressed interest.

Experiences from India

Since mid-1990s, the occurrence of shrimp diseases in India has been causing severe economic losses and disruption throughout the shrimp production chain. To address the rising concerns about the sustainability of the sector, in the year 2000, the Marine Products Export Development Authority (MPEDA) of the Government of India with the technical assistance of NACA and the support of ICAR and ACIAR began a project on "Shrimp disease and coastal management". The project started in 2001 with an extensive epidemiological study aimed at identifying the risk factors for key shrimp diseases and developing and disseminating better management practices to minimize farm-level risk factors for disease outbreaks and to address more broadly shrimp farming sustainability.

During the period 2002–2006 BMP were massively promoted through village demonstrations. The concept of cluster management was developed for shrimp farming through the development of farmer self help groups known as "aquaclubs". Efforts were made to link the aquaclubs to input suppliers like shrimp hatcheries and feed manufacturers. The concept of producing good quality seed under contract agreements (contract hatchery system) with aquaclubs was also introduced. A massive awareness campaign was carried out to promote BMP by using articles on local newspapers, documentaries on local television channels, posters, leaflets and farmers meeting.

Major achievements obtained by the program were

- By 2006, 29 Aquaclubs have been established in 5 Indian States (Andhra Pradesh, Gujarat, Karnataka, Orissa and Tamil Nadu). In 2005 in Andhra Pradesh alone, 635 farmers (930 ponds) produced 482 tons of BMP shrimp.
- Compared to surrounding non-BMP ponds, BMP ponds showed a 2-fold increase in production, a 65% reduction in disease occurrence, a 34% increase in shrimp size and a 68% improvement in survival.
- Economic analysis clearly demonstrated that farmers adopting BMP gained higher profits, partially because of the lower production costs owing to better feed management, and were able to produce quality and traceable shrimp without using banned chemicals.

For more information on the Indian experiences see also Padiyar *et al.* (2008).

Experiences from Viet Nam

Following an unprecedented growth in shrimp production that saw a 4-fold increase in yield in the 5 year period between 1998 and 2003 and increasing environmental, shrimp health and food safety issues rising from the spontaneous development of the industry and the occasional use of banned substances, the government of Viet Nam recognized the need to promote a more sustainable development of the sector. In addition to supporting the development of the International Principles for Responsible Shrimp Farming, projects were initiated to translate the principles into practices, which targeted better production, product quality and environmental and socio-economic sustainability.

In 2003, NACA and the Ministry of Fisheries (MOFI) with the support of the Danida-funded Fisheries Sector Programme Support (FSPS) began implementing a project to support the promotion of responsible shrimp farming at all levels and for all links in the production chain. Simple and practical BMPs were developed for broodstock traders, hatcheries, seed traders and farmers, addressing particularly the needs of less resourced small-scale farmers involved with semi-intensive shrimp farming systems. Ten sets of extension material were developed and disseminated.

Outcomes of the project included:

- Implementation of BMP for hatcheries was supported in six hatcheries and resulted in seed production up to 1.5 times higher and a price per unit seed of about 30-40% higher than non-BMP seed.
- BMP implementation was also supported in 7 pilot farming communities (655 direct beneficiaries).
- Farmer groups were established in all communes and group activities were supported by voluntary extension workers who contributed to BMP dissemination and supported BMP implementation by neighboring farmers
- Implementation led to a remarkably lower (almost half) risk of mortality, higher production (sometimes 4 times higher) and higher probability (sometimes double) of making a profit.
- Strengthened institutions involved with aquatic animal health management at the national and local level

For more information on the above experiences see also the NACA/FSPS/MOFI report (2005), Corsin *et al.,* (2005) and Khang *et al*., (2008).

In parallel to the NACA/FSPS/MOFI project, the National Fisheries Quality Assurance and Veterinary Directorate (NAFIQAVED) of MOFI also initiated efforts towards the implementation of GAP in intensive and semi-intensive farming systems. Experiences from the NACA/FSPS/MOFI project were gradually incorporated into NAFIQAVED's efforts. NAFIQAVED's experiences on shrimp health management and improved food safety were also very positive, with shrimp yields being doubled in GAP farms and increasing interest in the GAP program being expressed by farming communities and local authorities (NAFIQAVED, 2006).

Experiences from Thailand

Thailand began adopting a BMP approach to shrimp production in 2002. The objective of BMP implementation include disease prevention, reduction in drug and chemical use and presence of residues in harvested products, reduction of environmental impact and increase overall sustainability of the sector. The Thai Department of Fisheries (DoF) developed 3 sets of standards for good quality shrimp production in both farms and hatcheries, based on the extent of compliance by farmers and hatcheries. The standards of highest level are called CoC, following FAO CCRF. CoC standard is made of 11 management criteria which need to be inspected and complied with for CoC certificates to be issued. The second-level standards (*i.e*. GAP) require compliance to 7 management criteria. The lower,

basic standard is called Food Safety level and represents the minimum standard acceptable by the DoF. Food Safety standards are made of 3 criteria, namely requiring registration of the enterprise, lack of use of banned substances and compliance to traceability.

To support this 3 level certification program, 17 coastal aquaculture research and development centers, 5 coastal aquaculture stations, 4 institutes and 22 provincial offices have been assigned to provide training programs and technical and testing services for farmers. By the end of August 2006 there were 222 farms and 176 hatcheries certified CoC, while the number of GAP certified enterprises was 17750 farms and 700 hatcheries. Although the above data are an indication of the rapid expansion of the Thai program towards sustainable shrimp farming, still a number of issues require addressing.

- Because markets often do not pay premium prices for products from CoC or GAP farms, farmers allegedly have limited incentives to comply with this voluntary program and may do so primarily because of the strong government support to the program
- Funds for the implementation of the program are contributed largely by the Thai government and not by the supply chain, posing concerns on the long-term sustainability of the program and raising the need to involve more actively other players (*e.g.*, processors, retailers, etc.).

The Thai program shows how strong government commitment can lead to large-scale improvement in the sustainability of the shrimp farming sector.

Experiences from I.R. Iran

In response to a serious outbreak of WSD that struck the largest shrimp producing province of I.R. Iran in middle 2005 (Corsin *et al*., 2006), the Iranian Fisheries Organization (IFO) requested NACA to provide an emergency support team to identify the source of the outbreak and develop a strategy to control the outbreak and future WSD occurrences. As part of these efforts, BMP focusing primarily of shrimp health management were developed for broodstock management and hatchery and farm operations. BMP implementation during the 2005 outbreak appeared to control the spread and impact of the disease (unpublished) and BMP are currently been implemented to different extents in several shrimp farming provinces of I.R. Iran.

Identify market opportunities for BMP/GAP products

BMP implementation throughout the production chain would greatly benefit from higher prices or better market access being granted for BMP products. Although not essential, premium prices and market access have been reported to provide incentives to achieve farmers' compliance to better practices in the agriculture sector (Sonn, 2005). However, contrary to organic and fair-trade products which supply niche markets, the production of BMP products has yet to create the interest necessary to lead to an increased market price for BMP products, although farmers are indeed willing to pay higher prices for BMP seed (NACA/FSPS/MOFI, 2005) and higher prices have occasionally been offered for BMP shrimp (Mr Nguyen Tu Cuong, Director of NAFIQAVED, pers. comm.).

In view of the inclusive approach adopted during BMP promotion, with a strong interest in encouraging sustainable shrimp production in as many communities as possible, it is also possible that BMP will serve to farmers to keep their share of the market and that importing countries will increasingly move towards accepting only products what have been produced in a sustainable manner.

Develop standards and certification

Whatever the direction that BMP implementation will take in respect to markets, providing insurance of BMP implementation will most likely be a key step in this process.

As explained above, BMP are "indicative" practices aimed at improving the sustainability of shrimp farming. However, these practices can also be modified into more "normative" standards. Standards are often more rigorous, and quantifiable, ways to achieve sustainability. Although standards can be developed as completely separated from the process of certification (*e.g.,* ISO standards), often standards are linked to a certification system. The objective of a certification system is to provide insurance of compliance to the standards.

There are presently several examples of standards and certification for the production of allegedly more sustainable aquaculture products, although some of these are presently available only as drafts. Standards can be produced by the private sector (either producers or retailers), governments or intergovernmental/non-governmental organizations. Examples of standards produced by private organizations are Global Aquaculture Alliance (GAA), Safe Quality Food and Global GAP. Among the standards developed by government there are the GAP/CoC standards produced in Thailand and similar efforts currently being conducted, among others, in India and Viet Nam.

A key issue to be considered while examining certification for a certain set of sustainability standards is the credibility of that certification to the importers, retailers and consumers. So far, none of the above schemes seem to have gathered extensive credibility, potentially with the exception of retailers-led schemes which naturally have a link with the consumers.

Compliance to most of the above standards requires a relatively large amount of resources. Although some provisions for certifying farmer groups sometimes exist as part of these schemes, certification is often expensive and requiring a relatively high degree of literacy to keep all the necessary records to be audited. It is however important that standards addressing sustainability broadly can be complied with also by farmers with limited resources and literacy.

CONCLUSIONS

This manuscript describes how approaches towards sustainable shrimp production can effectively contribute to shrimp health management. It also highlights the need for broad stakeholder involvement and for providing the right incentives to producers such as reduced costs, improved production, access to information and diagnostic services and, if possible, higher market prices for BMP products.

Because of the successful experiences gathered so far, a similar approach to the one described above is currently been put in place in several country to target the sustainable production of a wide range of aquaculture commodities. This approach was also endorsed by the 17 member countries represented at the NACA Governing Council, showing the commitment of the Asia-Pacific region to this process.

Effectively engaging with the millions of aquaculture producers in the Asian region will not be a small task, but it is one that can only be achieved with the involvement and contribution of the many players involved in the supply chain, from producers to consumers.

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Limiting the Impact of Shrimp Diseases through the Implementation of better Management Practices: the Vietnamese experience

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ABSTRACT

Through a collaborative project between the Vietnamese Ministry of Fisheries, the Network of Aquaculture Centres in Asia-Pacific and the Fisheries Sector Programme Support funded by the Danish International Development Assistance, better management practices (BMPs) for shrimp farming were developed and disseminated to 5 provinces of Viet Nam. The implementation of BMPs was promoted to 655 households and monitored in 269 ponds distributed over 6 communes in 2 provinces. The BMPs were well accepted by farmers and implemented to different extents. Data collected at the end of the 2004 crop indicated that ponds in which BMPs were applied had a significantly lower risk of experiencing mortality, higher yields, and higher probability of making a profit. Testing of seed for white spot syndrome virus before stocking, as conducted by groups of farmers, showed remarkably positive outcomes. The results are discussed in the contexts of experiences gathered from other BMPs projects in the Asian region.

Khang, P.V., Corsin, F., Mohan,. C.V. and Phillips, M. 2008. Limiting the impact of shrimp diseases through the implementation of better management practices: the Vietnamese experience, pp. 433-440. *In* Bondad-Reantaso, M.G., Mohan, C.V., Crumlish, M. and Subasinghe, R.P. (eds.). Diseases in Asian Aquaculture VI. Fish Health Section, Asian Fisheries Society, Manila, Philippines. 505 pp.

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INTRODUCTION

The growth of the aquaculture sector over the past few decades and its ability to respond to the increasing global demand for fisheries products have been remarkable. Shrimp farming is a clear example of this outstanding development. During the last 10 years, shrimp production worldwide more than doubled. Although extraordinary, these aggregated data do not express the degree of growth of some countries like Viet Nam, where shrimp harvests showed a 4-fold increase in the 1998-2003 period only (FAO, 2006).

This rapid development came at a cost and the need for a more sustainable approach to improve shrimp health and quality while reducing the environmental impact of operation became clear to several stakeholders. A Consortium Program involving the Food and Agriculture Organization of the United Nations (FAO), the Network of Aquaculture Centres in the Asia-Pacific (NACA), the World Bank (WB) and World Wildlife Fund (WWF) was initiated in 1999 and was later joined by the United Nations Environment Program (UNEP). Through this program, principles for responsible shrimp farming were developed following a process of stakeholder consultations (FAO/NACA/UNEP/WB/WWF, 2006). At the same time, Better Management Practices (BMPs) to help farmers in the implementation of those principles were also developed and their implementation by farming communities promoted (NACA, 2006). A remarkable example came from India, where BMP adoption significantly contributed to improving shrimp yields and controlling the impact of shrimp diseases (Padiyar *et al.,* 2008).

Facing a compelling need to control shrimp diseases such as White Spot Disease (WSD), which constantly claimed a huge share of shrimp harvests, and the resulting quality problems associated with the use of banned chemicals and antibiotics, the Vietnamese Ministry of Fisheries (MOFI) took a leading role in promoting more sustainable approaches to shrimp farming. This was done by taking part in stakeholder consultations for the development of the Consortium Program principles and by supporting both directly and indirectly the implementation of BMP approaches.

Since 2003, MOFI, NACA and the Fisheries Sector Programme Support (FSPS) funded by the Danish International Development Assistance (DANIDA) began supporting the development and implementation of BMPs in small-scale shrimp farming systems in Viet Nam, with the target to improve both quality and quantity of shrimp harvests.

This paper describes the process of BMP development, dissemination and implementation and reports an assessment of the effect of BMP implementation on shrimp health and yield.

MATERIALS AND METHODS

Selection of study sites

The MOFI/NACA/FSPS project promoted BMP implementation and more sustainable approaches to shrimp health management at all levels (from the national-level to farmers). Activities were conducted primarily in 5 coastal provinces, namely Quang Ninh, Nghe An, Ha Tinh, Khanh Hoa and Ca Mau. In 2004, BMP implementation was supported and

monitored in 6 communes located in 2 of these provinces: Nghe An and Ha Tinh. Pilot communes were selected primarily by provincial fisheries authorities primarily because of their need for assistance and the recurrent shrimp health problems. In 5/6 communes, all the ponds in the commune were selected. Because of the large number of pond in one commune, 2 groups of farmers were selected with the assistance of commune authorities and following the criteria for commune selection. A total of 269 ponds were selected for BMP implementation.

BMP development

BMPs were developed to target improvements to the following aspects of sustainability:

- shrimp health by focusing on prevention, monitoring and more specific treatment of health problems;
- food safety through building awareness on banned substances and on practices to effectively replace the need for antibiotics;
- environment protection by limiting the amounts of wastes discharged in the environment;
- social equity by increasing communication between farmers and other resource users so that the needs of all stakeholders could be addressed.

In addition to the above, strong focus was given to promoting the establishment of farmer groups. Main incentives for farmers to work as groups included:

- reduced costs associated with seed testing, purchasing of inputs (seed, feed, etc.);
- improved collaboration and sharing of experiences between farmers;
- increased awareness on shrimp health problems occurring within the group;
- improved management of water supply and discharge;
- stronger link with government extension services and officials, which liaise with 1 unit (the group) instead of liason with individual farmers;
- better bargaining power when selling harvested products.

BMPs were designed to be simple, practical, affordable and cost-effective. Special attention was given to addressing the needs of small scale producers.

The process of BMP development started with the establishment of an expert team composed by international, regional and national shrimp health specialists. The team conducted farm visits and used BMP documents and other technical manuals to draft 4 sets of BMP to address: pond preparation, seed selection and stocking, pond management under normal conditions, and shrimp health management. BMPs were then discussed with local authorities and farmers and their comments included as appropriate.

BMP dissemination

BMPs were disseminated using a wide range of methods. A total of 4 sets of leaflets were developed and disseminated to all the farmers in the pilot communes. Leaflets were later incorporated into a single booklet which was further disseminated to the pilot farmers. Pond books, primarily aimed at allowing record keeping but also containing key BMP messages, were also distributed to all the farmers. Farmer meetings at which

BMPs were presented and discussed were held in every commune before and during the production cycle at an average of 3-4 meetings per commune. All ponds were visited at least on a fortnightly basis and more often with rising health problems. During pond visits, pond-specific BMPs were disseminated. While visiting farming sites, effort was put to interact particularly with voluntary extension workers. These were farmers selected by a group for their higher technical knowledge and willingness to share their expertise with other farmers. Voluntary extension workers conducted additional BMP dissemination sessions through their interactions with other farmers. Indirect BMP dissemination was also achieved by conducting training courses for government extension workers and involving them in project activities. BMP dissemination was also conducted at the end of the production cycle.

Supporting BMP implementation

Although limited, some support towards BMP implementation was also given to farmers in the pilot communes. Free WSSV-testing of seed batches was offered through a provincial testing laboratory. Magnifying lenses to assess post-larval quality, kits for testing water pH and alkalinity, densitometers and thermometers were distributed to voluntary extension workers. Pond books and extension material were also provided free of charge to farmers. The total cost of the support given to the pilot communes amounted to an estimated US$1 dollar per farmer for the whole production cycle.

Monitoring BMP implementation and effect

Management practices adopted by farmers were measured using an interview-based questionnaire delivered at the end of the crop; data collected only on the first crop conducted in 2004. The questionnaire also collected data on the occurrence of shrimp health problems, size of harvest and perceived profit made from the crop. Questionnaires were delivered to either the farmer or a member of the household who contributed to the management of the pond. Data were collected on all the 269 ponds in the 6 pilot communities.

Statistical analysis

Data collected using the survey questionnaire were used to assess whether specific BMP had been implemented. Three BMP messages, representing BMP implementation and the ease of measurement, were selected for analysis, as follows:

- Remove waste soil before stocking (measured directly as categorical (dichotomous) variable).
- Do not plough a pond with acid soil [measured combining information on soil type (categorical) and on whether the pond had been ploughed or not (categorical, dichotomous)].
- Test shrimp seed for White Spot Syndrome Virus (WSSV) and stock only negative seed (measured by combining information of WSSV testing and on whether WSSV positive seed had been stocked).

Crop success was measured using 3 outcome variables, namely:

- occurrence of shrimp mortality (categorical, dichotomous)
- shrimp yield per hectare (continuous)
- perceived profit made from the crop (categorical with 3 levels: loss, break-even, profit)

Statistical analysis was conducted using *Chi*-square and t-test for categorical and continuous outcomes respectively (Minitab). Relative Risk (RR) was used to quantify the strength of association between BMP implementation and the occurrence of mortality.

RESULTS

BMP implementation was significantly associated with a lower risk of experiencing shrimp mortality, higher shrimp yields per hectare and higher probability of making a profit (Tables 1-3).

Table 1. Association between lack of BMP adoption and the occurrence of shrimp mortality

BMPs not followed	RR of experiencing mortality	p-value
Remove waste soil	1.36	0.001
Do not plough acid soil ponds	1.36	0.022
Test seed for WSSV	1.74	< 0.001

Table 2. Association between BMP adoption and shrimp yield per hectare

BMP followed	Kg/ha BMP	Kg/ha non-BMP	p-value
Removing soil	385	123	< 0.001
Do not plough acid soil ponds	73	42	0.023
Test seed for WSSV	452	112	< 0.001

Table 3. Association between BMP adoption and the perceived profit made from the crop. Percentages indicate the proportion of ponds in which a certain BMP was adopted in the 3 categories of ponds, i.e. where loss, break-even or profit was made.

BMP followed	Loss	Even	Profit	P-value
Removing soil	45%	65%	70%	0.001
Acid no plough	16%	50%	47%	0.006
Test PL for WSSV	30%	29.4%	59%	< 0.001

DISCUSSION

These results clearly indicate that simple and practical BMPs can be effectively disseminated to communities of small-scale shrimp farmers and can significantly contribute to improving shrimp health and yields.

The effect of WSSV testing was particularly remarkable, with yields 4 times higher in ponds where WSSV negative seed was stocked. This is particularly outstanding especially in view of the fact that ponds stocking WSSV-negative seed were compared with ponds stocking un-tested seed, which could also have been negative. Corsin *et al.* (2005) reviewed the risk factors associated with WSD, indicating that testing of seed for WSSV was not always reported to be effective as a practice to control the disease, especially in open systems, which adopt heavy water exchange. Although the farming system used for BMP implementation in this project was an open system, we believe that the risk for WSD was reduced not only by the mere WSSV testing of the seed, but by a combination of other factors. Tested seed was generally stocked by groups of neighboring farmers, therefore reducing the chances of experiencing WSD in the whole group, consequently reducing also the probability that any pond in the group could be infected by a neighboring pond. In addition, the BMP used in analysis were only some of the BMP being disseminated. Therefore, it is reasonable to think that farmers, in addition to the BMP analysed (*e.g.,* testing seed for WSSV), also adopted other practices that reduced the risk of experiencing WSD. The same argument should be applied when interpreting the effect of all the BMPs used in analysis, therefore highlighting that the main finding of this study was not quantifying the effect of BMP implementation, but identifying that BMP implementation represents a practical and cost effective approach towards shrimp health management in Viet Nam.

Although in this study only univariate analytical methods were used (*i.e.* the association between BMPs and outcome was not adjusted for the effect of other variables), preliminary multivariable analyses confirmed the direction of the results presented above, indicating that the observed association between BMP implementation and the outcome variable was not a result of the confounding effect of other variables (*e.g.,* degree of intensification, etc.).

CONCLUSION

The results presented in this paper show that the implementation of BMPs may represent the way forward for controlling health problems and improving production in small-scale shrimp farming systems in Viet Nam. This can be achieved while improving the quality of the harvested products and limiting the environmental impact of farming operations.

As a follow up to the project described in this paper, several efforts were conducted in Viet Nam towards the implementation of more sustainable practices for shrimp farming. Similar results were achieved by those efforts, confirming the usefulness of adopting BMP approaches.

Although promising, several challenges still lie ahead. The shrimp farming sector in Viet Nam is dominated by hundreds of thousands of farmers, which are difficult to reach with extension (or BMP) messages. The legal and institutional frameworks to allow BMP implementation still need improvement. In addition, farmers of BMP shrimp do not get higher prices for their products, and are therefore deprived of an important incentive for BMP implementation.

These challenges, however, are fully recognized by the Government of Viet Nam, which is continuing to take steps towards the responsible production of shrimp and other aquaculture commodities.

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Transient Expression of a Heterologous Gene Driven by Promoters Isolated from Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) of Shrimp

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ABSTRACT

Two putative promoter elements of the infectious hypodermal and hematopoietic necrosis virus (IHHNV) of shrimp were cloned and their abilities to drive heterologous gene expression in shrimp and bacteria were determined. These promoters (herein referred to as P2 and P61) are upstream of the Left (non-structural protein) and Right (capsid protein) open-reading frames (ORFs) contained within the IHHNV genome, respectively. The P2 and P61 promoters were cloned upstream of the firefly Luciferase gene (*Luc*) in the pGL3-Basic (pGL3-B; contains no regulatory element) and pGL3-Enhancer (pGL3-E; contains the SV40 enhancer element) promoter trapping vectors. Luciferase activity was assayed in recombinant bacteria containing these constructs as well as in shrimp tail muscle taken from animals injected intramuscularly with plasmid DNA. In bacteria, *Luc* expression driven by the P61 promoter in pGL3-B was greater than that from the P2 promoter. The SV40 enhancer element contained in pGL3-E based constructs increased bacterial expression driven by the P61 marginally, while expression under P2 control was inhibited. In shrimp, there was no significant difference in luciferase expression driven by these two promoters. The SV40 enhancer element suppressed P61driven Luciferase expression in shrimp. Our data suggest that both P2 and P61 are constitutive promoters, and that these promoters can drive gene expression in prokaryotes and eukaryotes. Having shrimp virus promoters available to crustacean molecular biologists provides new tools for studies in the functional genomics in shrimp.

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INTRODUCTION

Shrimp bioengineering and basic molecular biological research on shrimp have been hampered by the lack of effective tools. There are no shrimp immortalized cell lines, effective promoters for expression of heterologous genes, transposons, regulatory genes, enhancers, or any of the myriad other tools necessary for effective directed manipulation in whole shrimp. Transgenic shrimp have been developed with embryo manipulation (Lu and Sun, 2005), but many of the more directed structure /function research that relies on rapid experiments in shrimp have lagged due to lack of appropriate molecular tools. This study characterizes two shrimp viral promoters that will add to the arsenal of tools for shrimp molecular biological manipulation, perhaps enabling the field to produce an immortalized cell line, identify viral disease control strategies, and provide other benefits that can be derived from the application of modern biotechnological research to this field.

Infectious hypodermal and hematopoietic necrosis virus (IHHNV) is one of the most important viral pathogens that infect penaeid shrimp worldwide (Lightner *et al*., 1996). The virus was first detected in Hawaii in 1981, where it caused lethal disease (up to 90% mortality) in juvenile blue shrimp (*Penaeus stylirostris*) (Bell and Lightner, 1983; Lightner *et al*., 1983). IHHNV is infectious to many other *Penaeus* species (Kalagayan *et al*., 1991; Primavera and Quinitio, 2000). In *P. vannamei* and *P. monodon*, IHHNV causes "Runt Deformity Syndrome" (RDS), which is characterized by reduced growth rates and a variety of cuticular deformities of the rostrum, antenna, thorax, and abdomen (Kalagayan *et al*., 1991; Primavera and Quinitio, 2000).

IHHNV virions are icosahedral, non-enveloped, and measure 22 nm in diameter. The virus contains a linear single-stranded DNA of ~4.1 kb in size (Bonami *et al*., 1990). There are three (Left, Middle, and Right) large open reading frames (ORFs) in the IHHNV genome (Figure 1). The Left ORF most likely represents a non-structural protein, since it contains replication initiator motifs, NTP-binding, and helicase domains (Shike *et al*., 2000). The Right ORF encodes the capsid protein, while the function of the Middle ORF is not known. There are two putative promoters located upstream of the Left (designated

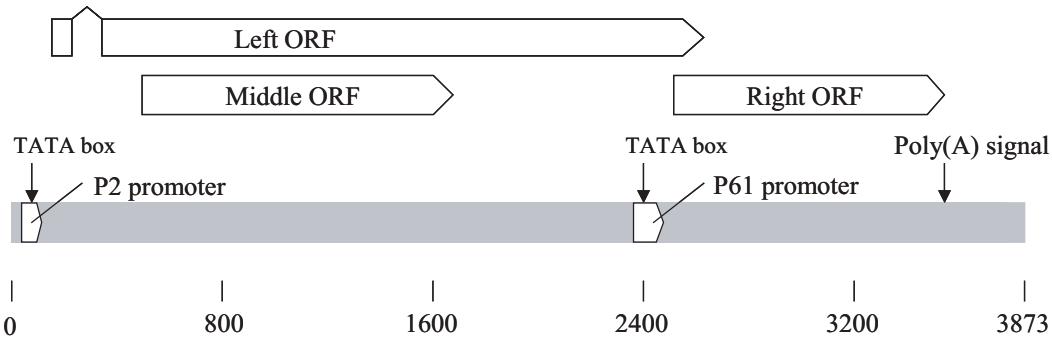


Figure 1. IHHNV genome organization based on the sequence of GenBank accession number AF273615. P2 and P61, the two promoters in this study, are located as indicated. Numbers correspond to the nucleotide number of the sequence ORF.

P2) and the Right (designated P61) ORFs. The genome organization of IHHNV is similar to the densoviruses of the genus *Brevidensovirus* in the family *Parvoviridae* (Shike *et al.*, 2000).

To characterize the IHHNV promoters, the putative P2 and P61 regions were cloned upstream of the firefly luciferase gene (*Luc*) in two transcription reporter vectors, pGL3-Basic and pGL3-Enhancer. These vectors differ in that the latter carries the SV40 enhancer sequence downstream of the reporter gene. The recombinant bacteria were assayed for luciferase activity. The plasmid DNA of P2 and P61 promoter constructs were then used to transfect shrimp tail muscle tissue to determine if *in vivo* luciferase transient expression was observed. The IHHNV promoters were demonstrated to be capable of driving expression of a heterologous gene in both a prokaryotic and its native eukaryotic host (shrimp).

MATERIALS AND METHODS

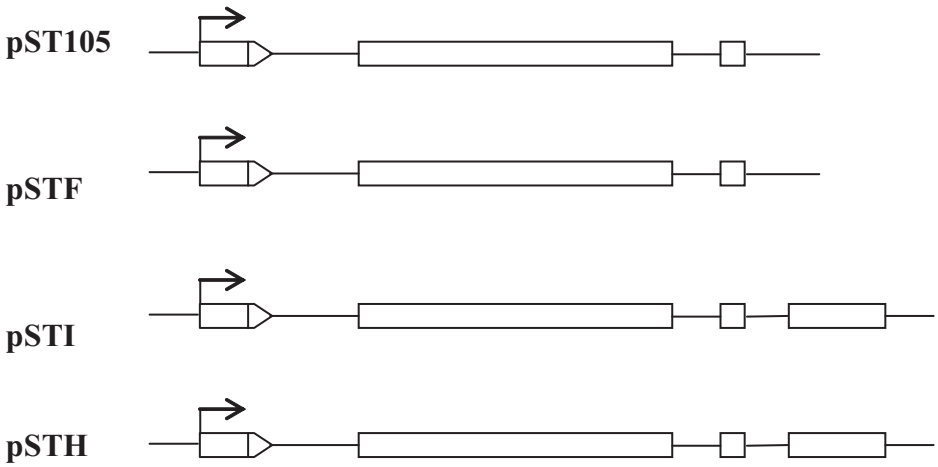
Cloning of IHHNV promoters

The putative IHHNV P61 promoter region (GenBank Accession No: AF273215) was amplified by PCR using the primers: IHHNP61F, 5'GGTACCTCCA GCTGATGGTA AAGCT3' (nucleotides 2347-2371) and IHHNP61R, 5'TTCGTATTCT TGGAAGAGTC CTAG3' (nucleotides 2488-2512) as forward and reverse primers, respectively. The P61 amplicon was cloned in vector PCR2.1-TOPO (Invitrogen, Carlsbad, CA) and reamplified by PCR to generate *Sac*I and *Nhe*I endonuclease restriction sites on the 5' and 3' termini of the amplicon, respectively. To synthesize the putative IHHNV P2 promoter region (nucleotides 21-110) flanked by *Sac*I and *Nhe*I sites, 108-mer sense and antisense oligonucleotides were synthesized (Midland Certified Reagent Co., Midland, TX). The sense and antisense oligonucleotides were annealed and then digested with *Sac*I and *Nhe*I for insertion into the luciferase reporter vectors.

Cloning of luciferase reporter gene

Plasmids constructed for this study were based on pGL3-Basic and pGL3-Enhancer luciferase transcription trapping vectors (Promega, Madison, WI). These vectors contain a modified coding region for firefly (*Photinus pyralis*) luciferase (*Luc*) that has been optimized for evaluating transcriptional activity of unknown promoters in transfected eukaryotic cells. Vector pGL3-Enhancer differs from the pGL3-Basic in that it contains a SV40 eukaryotic enhancer element downstream of the *Luc* gene (Fig. 2). The P2 and P61 promoters were inserted between the *Sac*I and the *Nhe*I sites of pGL3-Basic and pGL3-Enhancer vector upstream of the *Luc* gene using the DNA Ligation kit "Mighty Mix" (Takara, Shiga, Japan) according to the manufacturer's recommendations then transformed into *Escherichia coli* cells (Strain JM109, Invitrogen). Plasmid DNA was isolated from recombinant clones and sequenced for verification. The pGL3-Basic derived clones containing the P2 and P61 promoter were designated pSTI05 and pSTF, respectively, and the pGL3-Enhancer derived clones containing the P2 and P61 promoter were designated as pSTI and pSTH, respectively (Figure 2).

Figure 2. P2 and P61 IHHNV promoter cloning: (A) vector pGL3-Basic, resulting in plasmids pSTI05 and pSTF, and (B) into vector pGL3-Enhancer, resulting in plasmids pSTI and pSTH.



Luciferase assay in recombinant bacteria

Luciferase expression was measured in recombinant bacteria containing P2 and P61 promoter constructs. Triplicate sets of log phase bacterial cultures carrying the control plasmid pUC19 or reporter plasmids (pGL3-Basic, pGL3-Enhancer, pSTI05, pSTF, pSTI, and pSTH) were obtained by growing 100-fold diluted stationary phase cultures in Luria-Bretani (LB) medium containing 100 µg/mL ampicillin at 37ºC in a shaker for 2.5 hours. Bacterial cells (1.5 mL) were pelleted, then resuspended in 0.1 mL bacterial treatment buffer (100 mM $KHPO_4$ (pH 7.8), 2 mM EDTA, 1 µM PMSF (Sigma-Aldrich, St. Louis, MO), and 1 µM benzamidine-HCl (Sigma-Aldrich)). Resuspended bacteria were frozen at -80ºC for 5 min and then thawed in water at room temperature for 5 min. Then 0.3 mL bacterial lysis buffer (Cell Culture Lysis Reagent; Promega, Madison, WI) supplemented with 1.25 mg/mL lysozyme (Research Organics, Cleveland, OH), 2.5 mg/mL BSA (Fisher Scientific, Newark, DE), and 1 µM PMSF, and 1 µM benzamidine-HCl was added and the cells were briefly vortexed and incubated at room temperature for a further 10 min. Cellular debris was removed by centrifugation and 50 µL of the supernatant assayed immediately for luciferase activity while the remaining supernatant was used for total protein determination following Lowry's protocol (Lowry *et al*., 1951) with BSA as protein in the calibration series. The luciferase assay was performed using the Bright-Glo assay system (Promega) according to the manufacturer's protocol. Luminescence measurements were done in white, flat bottom 96-well microplates (Greiner, Bio-One, Longwood, FL) on a Tecan SpectraFluor Plus in the luminometer mode (XFLUOR4 Version 4.50; Tecan US, Research Triangle Park, NC). Readings were converted into luciferase equivalents via a calibration curve prepared by serial dilution of luciferase (Quantilum recombinant luciferase; Promega) into bacterial extract from bacterial cells carrying the control pUC19 plasmid. Luciferase activity in bacteria was normalized to the total protein content and data presented in parts per million.

Luciferase assay in shrimp (*Penaeus vannamei*)

Specific Kona line pathogen-free *P. vannamei* shrimp were purchased from Marine Resources Research Institute, South Carolina Department of Natural Resources (Charleston, SC) and kept at approximately 24ºC using artificial seawater prepared by dissolving Instant Ocean (Aquarium Systems, Mentor, OH) in tap water. Shrimp averaging ~1.25 g were injected in the 4th abdominal segment of the tail muscle with 25-30 µL of a freshly prepared suspension consisting of 10 µg plasmid DNA in 20% glycerol and 0.9% NaCl. Animals were sacrificed at 70 to 72 hr post-injection, immediately frozen in liquid N_2, and then stored at -80ºC until assayed. Approximately 0.25 mg tissue, preferentially including the site of injection, was cut from each shrimp and manually homogenized on ice in a microcentrifuge tube in 200 µL Cell Culture Lysis Buffer (Promega). Tissue debris was removed by centrifugation and 50 µL of the resulting shrimp extract immediately assayed for luciferase activity using the Bright-Glo assay system as described above. The remainder of the tissue extract was used for protein determination according to Lowry (Lowry *et al*., 1951) as described above. A luciferase calibration curve was prepared by using a serial dilution of commercially available luciferase (Quantilum recombinant luciferase, Promega) prepared in tissue extract from sham-injected shrimp. Luciferase activity was normalized to total protein content and presented in parts per billion. The results of two experiments were combined for analysis and data points from shrimp that did not show luciferase activity higher than the background signal plus two standard deviations were excluded from further analysis.

RESULTS AND DISCUSSION

The major impetus for this research was to improve the tools available for expression of heterologous proteins in shrimp. In order to fully characterize these shrimp viral promoters, expression in both shrimp and other eukaryotes as well as in prokaryotic systems needs to be evaluated. In this study, we evaluated both bacteria and shrimp as hosts for exogenous protein expression. Such information would help in future application of these promoters to both shrimp studies and studies in other systems. Expression in other systems, especially bacterial ones, would not be optimal but rather an indication of functionality. Validation of promoters that work in shrimp is a first addition to tools necessary for effective crustacean molecular engineering. Future work will need to be done to optimize the promoters for expression in crustacean hosts as well as to locate other tools like transposons and enhancer elements that are optimally functional in shrimp.

Luciferase expression in bacteria

The promoter activity was determined in *Escherichia coli* JM109 transformed with the luciferase reporter / promoter constructs. The results show that both P2 and P61 promoters are functional in bacteria and that these promoters were influenced by the presence of the SV40 enhancer element. Reporter protein expression driven by the P61 promoter was approximately 1.3 and 4.5-fold higher than the P2 promoter in the pGL3-Basic and Enhancer vectors, respectively (Figure 3). The SV40 enhancer element lowered the activity of the P2 promoter more than twofold to a value lower than the control (pGL3-Enhancer

without a promoter; Figure 3). Since it is unlikely that the eukaryotic SV40 transcriptional enhancer is active in prokaryotes, the significance of differences in expression between the pGL3-Basic and pGL3-Enhancer-based plasmids is unknown.

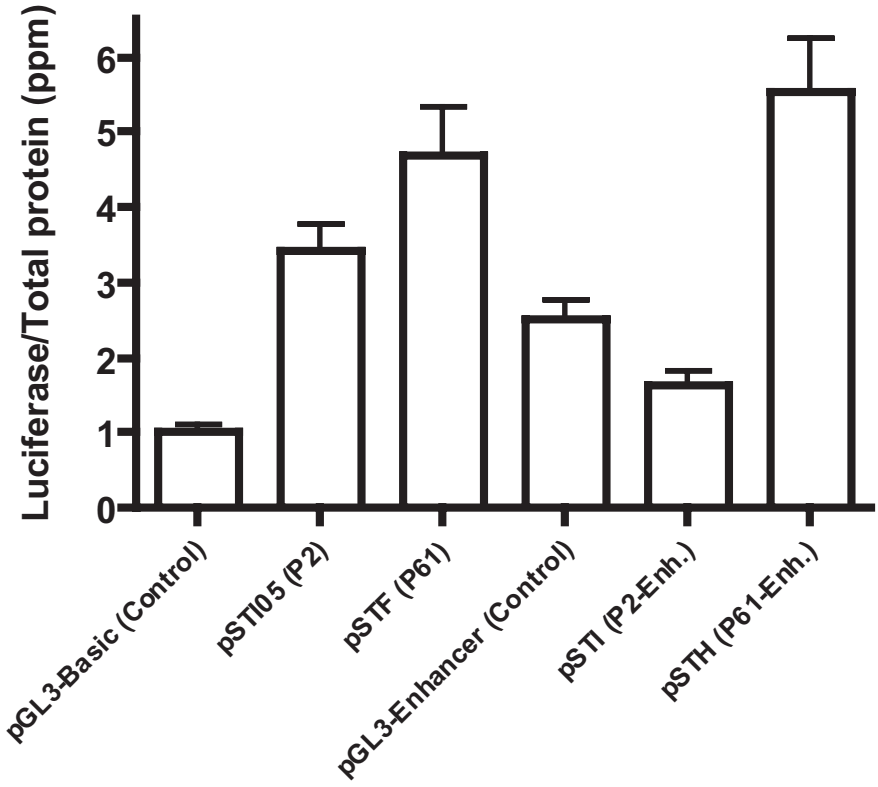


Figure 3. Expression of luciferase (Luc) in recombinant Escherichia coli. Luc expression corrected for background signal by comparison to a plasmid lacking the Luc coding sequence. Error bars represent standard error of the mean.

Luciferase expression in shrimp tail muscle

The transient expression of luciferase in shrimp tail muscle was highly variable (Figure 4). Both the P2 and P61 promoters were functional in shrimp, but the presence of SV40 enhancer element had no significant effect on the P2 promoter and a negative effect on the P61 promoter. Although the mean *Luc* expression value when driven by the P61 promoter was higher than the corresponding mean value of the P2 promoter, there was no significant difference. This is possibly due to observed high individual shrimp variability. Nevertheless, significant numbers of shrimp exhibited luciferase activity when injected with the IHHNV promoter driven *Luc* constructs, clearly demonstrating promoter activity as predicted (Shike *et al*., 2000).

Luc expression in shrimp was more than 10,000-fold lower compared to the *Luc* expression in bacteria even though bacteria are not natural hosts for IHHNV. The difference is probably due to variable efficiency of transformation in bacteria compared to *in vivo* transfection (shrimp). In shrimp, part of the transfection suspension spreads throughout

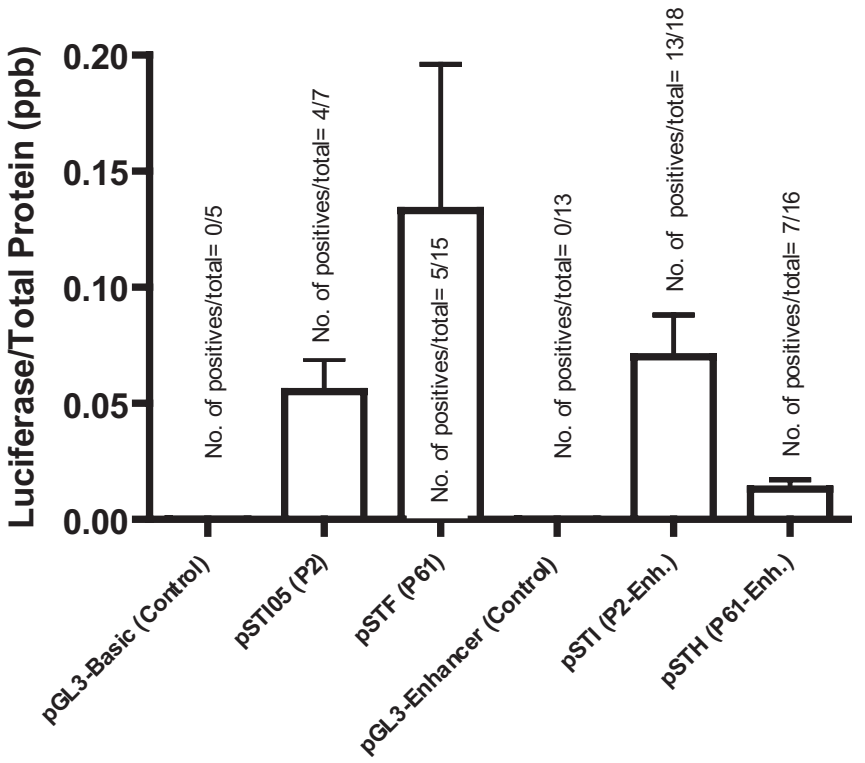


Figure 4. Transient expression of luciferase in shrimp tail muscle 3 days post-transfection. Only samples with a signal greater than background + 2 standard deviations were considered positive. Error bars represent standard error of the mean.

the body immediately upon injection. This was demonstrated by adding a fluorescent protein dye, C-phycocyanin (Martek Biosciences, Columbia, MD), to the injection mix. The dye rapidly spread throughout the shrimp body within two minutes (data not shown). As we collected tissue surrounding the site of injection for luciferase assay, potentially all transfected shrimp cells are not included. In addition, the amount of vector used for transfection, on a target tissue weight basis, and transfection efficiency were much lower than recombinant bacteria.

Our data showed that P2 and P61 are constitutive promoters capable of driving gene expression in both a prokaryote and a eukaryote. The differences in expression between these two promoters in each host system might be due to the difference in recognition of these promoter elements by the cellular transcriptional machinery of the host. In IHHNV, the P2 promoter drives the expression of the non-structural gene (NS-1), whereas P61 drives the expression of the structural gene (Shike *et al*., 2000). Promoters for viral capsid genes are generally stronger than those for non-structural genes. It is possible that during IHHNV replication the P2 promoter transcribes the NS-1 gene first, and that the P61 promoter is then transactivated by the NS-1 protein. In mammalian parvoviruses, such as the minute virus of mice and the rodent parvovirus H-1, there is a temporal order of expression for the structural and non-structural gene promoters. In these viruses, the non-

structural protein is expressed first, and then this non-structural protein transactivates the promoter for the structural gene (Doerig *et al*., 1988; Rhode, 1985). Transactivation of a structural gene promoter by the viral non-structural protein has also been reported in *Aedes aegypti* Densonucleosis virus (Afanasiev *et al*., 1994) and *Junonia coenia* Densonucleosis virus (Giraud *et al*., 1992). The IHHNV promoters will be useful for constructing vectors for the expression of homologous or heterogonous gene(s) in shrimp and potentially other prokaryotic and eukaryotic hosts. This initial research on the IHHNV promoters sets the stage for further characterization of their function at the molecular level in shrimp tissues as well as in exploring their utilities in other eukaryotic systems.

ACKNOWLEDGEMENTS

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Molecular Typing and Antimicrobial Susceptibility of *Vibrio parahaemolyticus* Strains

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ABSTRACT

The aim of the present study was to use three PCR-based techniques for the analysis of genetic variability among *Vibrio parahaemolyticus* strains isolated from the Philippines. Seventeen strains of *V. parahaemolyticus* isolated from shrimps and from the environments where these shrimps are being cultivated were analyzed by RAPD, ERIC and REP-PCR. Antimicrobial susceptibility of these strains to selected compounds was investigated using broth microdilution method. Results of this work and analysis of similarity among strains using Dice coefficient and unweighted average pair group method have demonstrated genetic variability within the *V. parahaemolyticus* strains. The RAPD, ERIC and REP-PCR were found to be suitable typing methods for *V. parahaemolyticus*. They have good discriminative ability and can be used as rapid means of comparing these strains for epidemiological investigation. However, the REP-PCR analysis yielded a relatively small number of products suggesting that the REP sequences may not be widely distributed in the *V. parahaemolyticus* genome. Results of antimicrobial susceptibility revealed that resistance among the strains was rare. In conclusion, RAPD, ERIC and REP-PCR techniques are useful methods for molecular typing of *V. parahaemolyticus* strains. To our knowledge this is the first study of this kind carried out on *V. parahaemolyticus* strains isolated from the Philippines.

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INTRODUCTION

Vibrio parahaemolyticus belongs to the expanding group of water and food-borne pathogens. It is widely distributed in the aquatic environment and is increasingly regarded as an important pathogen of shrimp causing significant economic problems within the aquaculture industry worldwide (Lightner, 1996; Goarant *et al.*, 1999). In recent years, *V. parahaemolyticus* has been implicated as an opportunistic pathogen, mainly causing gastroenteritis in humans due to consumption of contaminated seafood (DePaola *et al*., 2003). These isolates tend to possess the tdh or trh genes which have only been identified in a minority of the environmental isolates. Therefore, due to increasing reports of disease outbreaks in humans and with the great economic loss in aquaculture, the correct identification and classification of this organism is of great importance.

The development of molecular biology has allowed the typing of organisms on the basis of analysis of their nucleic acids. Molecular typing techniques are specifically useful for microbial epidemiological purposes as they can give information on the genetic relatedness of strains, the source of infection and detection of particularly virulent strains, as well as the study of the geographical and host distribution of possible variants of a specific pathogen (Olive and Bean, 1999). Included are several PCR-based methods such as the Random Amplified Polymorphic DNA PCR (RAPD-PCR), Enterobacterial Repetitive Intergenic Consencus Sequence PCR (ERIC-PCR) and Repetitive Extragenic Palindromic PCR (REP-PCR). The RAPD method involves the use of short random sequence primers, usually 9 to 10 nucleotides long, and low-stringency primer annealing conditions to amplify arbitrary fragments of template DNA. The single primer anneals anywhere on the genome where a near-complementary sequence exists, and amplification occurs if two priming sites are sufficiently close (Welsh and McClelland, 1990). On the other hand, ERIC and REP-PCR methods, respectively, utilize primers complementary to specific sequences in the bacterial genome. ERIC sequences are 126 bp long and appear to be restricted to transcribed regions of the genome, whereas the REP sequences consist of a highly conserved 33 bp inverted repeat sequence (Versalovic *et al.,* 1991).

The main aim of this study was to use and compare three PCR-based techniques for the analysis of genetic variability among *V. parahaemolyticus* strains isolated from the Philippines. These strains were recovered from shrimp (*Penaeus monodon*) and from the environments where these shrimps are being cultivated. RAPD-PCR, ERIC-PCR and REP-PCR were used to establish the DNA fingerprints of *V. parahaemolyticus* strains, with the aim of evaluating the applicability of these techniques in epidemiological studies. In addition, the antimicrobial susceptibility profile and the presence of extended-spectrum β-lactamases (ESBLs) among *V. parahaemolyticus* strains were determined. To our knowledge this is the first study of this kind carried out on *V. parahaemolyticus* strains isolated from the Philippines.

MATERIALS AND METHODS

Bacterial strains

Seventeen *V. parahaemolyticus* strains were analyzed in this study including a strain from the USA and two American Type Culture Collection (ATCC) reference strains. These strains, identified by phenotypic characterisation using classical tube and plate tests (West and Colwell, 1984) and API 20E system (BioMerieux, France), matched perfectly the *V. parahaemolyticus* species description. In addition, identification was confirmed by DNA hybridisation using a thermolabile haemolysin (*tlh*) gene probe (McCarthy *et al*. 1999). The sources of these strains are shown in Table 1. Stock cultures were maintained frozen at -80ºC in tryptone soy broth (Difco, Madrid, Spain) with 15% glycerol.

Table 1. The sources of *V. parahaemolyticus* strains used in this study.

Strain Code	Origin	Year	Donor
Phillipine isolates			
MF-0107-1	Creek water	2001	SEAFDEC
MF-0107-4	Pond water	2001	SEAFDEC
MF-0107-7	Reservoir water	2001	SEAFDEC
CLM3-0108	Crab haemolymph	2001	SEAFDEC
IA3	Pond water	2002	SEAFDEC
IA13	Creek water	2002	SEAFDEC
IA16	Creek water	2002	SEAFDEC
IM12	Source water	2002	SEAFDEC
03S1a-04-5	Rearing water of *P. monodon*	2003	SEAFDEC
03S3-01-2	P. monodon	2003	SEAFDEC
03S3-01-4	P. monodon	2003	SEAFDEC
03S3-07-2	Crab haemolymph	2003	SEAFDEC
03S3-07-5	P. monodon	2003	SEAFDEC
03S3-07-6	P. monodon	2003	SEAFDEC
Reference strains			
IV – 86	Chesapeake Bay, USA	1986	R.R. Colwell
ATCC 43996	Cockles, England	1972	ATCC
ATCC 17802^T	Shirasu food poisoning, Japan	1951	ATCC

SEAFDEC, Southeast Asian Fisheries Development Center, Tigbauan (Philippines)(Dr. C. Lavilla-Pitogo collection); R.R. Colwell, Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore (MD), USA; ATCC, American Type Culture Collection, Manassas (VA), USA.

Isolation of DNA

Isolation of DNA was performed using InstaGene Matrix (Bio-Rad, Madrid, Spain). Strains were routinely grown on Trypticase soy agar (TSA, Oxoid Ltd., Madrid, Spain) plates with 1% NaCl at 25ºC for 24 to 48 hr, after which colonies were scraped off and suspended in 1 ml of autoclaved water and centrifuged at 13,200 x g for 1 min. The supernatants were

removed and the remaining pellets were resuspended in 200µl of InstaGene Matrix and incubated at 56ºC for 30 min. They were then vortexed at high speed for 10 sec and boiled in a water bath for 8 min. The lysates were vortexed again at high speed and centrifuged at 13,200 x g for 3 min. The InstaGene DNA preparations were stored at -20ºC until used for PCR amplifications.

RAPD-PCR typing

The RAPD-PCR amplifications were performed using Ready-to-Go RAPD analysis beads (Amersham Biosciences) as previously described (Romalde *et al*., 1999). Six distinct random 10-mer primers (Amersham Pharmacia Biotech) were used: primers P1 (GGTGCGGGAA), P2 (GTTTCGCTCC), P3 (GTAGACCCGT), P4 (AAGAGCCCGT), P5 (AACGCGCAAC), and P6 (CCCGTCAGCA). Amplifications were performed in a T-Gradient thermocycler (Biometra) programmed as follows: an initial denaturation step at 95ºC for 5 min followed by 30 cycles of denaturation (95ºC for 1 min), annealing (35ºC for 1 min), and extension (72ºC for 2 min), with a final extension step at 72ºC for 5 min.

ERIC-PCR typing

The ERIC-PCR amplifications were performed using Ready-to-Go PCR analysis beads (Amersham Biosciences). A pair of 22-mer primers (Sigma): ERIC 1R (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC 2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3') were used as previously reported by Versalovic *et al.* (1991). After bead rehydratation, each 25 µl ERIC-PCR reaction mixture contained 2.5 U of *Taq* polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each deoxynucleoside triphosphate, 20 ng/µl of the respective primer, and 1 µl of DNA solution. Amplifications were carried out in a T-Gradient thermocycler using an initial denaturation step at 95ºC for 5 min followed by 35 cycles of denaturation (92ºC for 45 secs), annealing (52ºC for 1 min), and extension (70ºC for 10 min), with a final extension step at 70ºC for 20 min.

REP-PCR typing

The same Ready-to-Go PCR analysis beads were used for REP-PCR amplifications. For REP-PCR the following 18-mer primers (Sigma) were utilized: REP 1D (5'-NNN RCG YCG NCA TCM GGC-3') and REP 2D (5'-RCG YCT TAT CMG GCC TAC -3'), where M is A or C, R is A or G, Y is C or T, and N is any nucleotide (Stern *et al*., 1984). Each 25 µl REP-PCR reaction mixture contained the same components as with ERIC-PCR mixture except that here REP primers were used. Amplifications were also performed in a T-Gradient thermocycler programmed as follows: an initial denaturation step at 95ºC for 7 min followed by 35 cycles of denaturation (92ºC for 45 secs), annealing (40ºC for 1 min), and extension (72ºC for 8 min), with a final extension step at 72ºC for 15 min.

Gel electrophoresis

The RAPD, ERIC and REP-PCR products were separated by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide (Bio-Rad). A negative control, consisting

of the same reaction mixture but with distilled water instead of DNA template DNA, was included in each run. The gels were photographed under UV light. A 50- to 2000-bp ladder (Sigma, St. Louis, Mo.) was used as a molecular mass marker.

To determine significant differences in the patterns, the reproducibility of results was assessed by repetition of at least three independent RAPD, ERIC and REP-PCR assays.

Computer data analysis

All the gels were also scanned and the images were captured by a Gel Doc-2000 gel documentation system (Bio-Rad). The data analysis was performed by using Diversity Database software (Bio-Rad), and the computed similarities among strains were estimated by means of the Dice coefficient (S_d) (Dice, 1945). Dendrograms were produced on the basis of the unweighted average pair group method (UPGMA) (Sneath and Sokal, 1973).

Annealing temperature gradient-PCR

To confirm the presence of ERIC and REP sequences in the genome of the investigated strains, representative samples were subjected to PCR amplification using 10 different annealing temperature gradients. The method used was patterned with the work of Gillings and Holley (1997). If ERIC and REP sequences are not present on the genome, bands will fail to amplify at higher temperatures, specifically the T_m of ERIC and REP primers.

For ERIC-PCR amplifications the annealing temperature gradient ranged from 52.3 to 65.6ºC, whereas for REP-PCR a temperature gradient between 40.5 and 63.4ºC was employed.

ANTIMICROBIAL SUSCEPTIBILITY TESTING

A broth microdilution method (VetMIC™, National Veterinary Institute (SVA), Uppsala, Sweden) was used for susceptibility testing of the 14 *V. parahaemolyticus* strains isolated from the Philippines. The standards of the National Committee for Clinical Laboratory Standards (NCCLS) M31-A were followed with some modifications (NCCLS, 1999). The direct inoculum method, with Mueller-Hinton broth (MHB, Difco, MD, USA) as test medium, was used and the microdilution panels were incubated at 28°C for 18 h. For isolates that did not grow, 2% NaCl was added to MHB. *Escherichia coli* ATCC 25922 was included as a quality control strain

Minimum inhibitory concentration (MIC) was registered as the lowest concentration of an antimicrobial agent completely inhibiting bacterial growth. As no criteria were available for these bacteria, the Swedish Veterinary Antimicrobial Resistance Monitoring (SVA, Uppsala, Sweden) susceptibility criterion for enterobacteriaceae was used (SVARM, 2002). The MIC results were interpreted using the following resistance breakpoints (mg/L): ampicillin: >8; ceftiofur: >2; gentamicin: >8; neomycin: >8; streptomycin: >32; enrofloxacin: >0.5; florfenicol: >16; oxytetracycline: >8; and trimethoprim-sulphamethoxazole (TMPS) : >2/38.

Detection of extended-spectrum β-lactamases (ESBLs)

Strains resistant to ampicillin (n=12) were tested for the presence of ESBLs by combination disc diffusion method. Discs of cefpodoxime (10 μg), ceftazidime (30 μg), cefotaxime (30 μg) and imipenem (10 μg) were used in the detection, along with discs in combination with clavulanic acid: cefpodoxime (10/1 μg), ceftazidime (30/10 μg), cefotaxime (30/10 μg) (Oxoid, Hampshire, England). The tests were conducted according to NCCLS standard M100-S9 with some modifications (NCCLS, 1995). Mueller-Hinton agar (Oxoid, Hampshire, England) was used and the plates were incubated at 28°C for 18 h. An ESBLs-producing organism was confirmed if there was a ≥5 mm increase in zone diameter for the antimicrobial agent tested in combination with clavulanic acid compared with the zone when tested without clavulanic acid.

RESULTS

RAPD fingerprinting

RAPD analysis of the *V. parahaemolyticus* strain MF-0107-1 was initially performed using each of the six primers provided in the commercial kit. Only two of them, oligonucleotides P4 and P6, generated reproducible patterns with an appropriate number of amplified products suitable for an accurate analysis. Analysis with the remaining primers did not provide a good amplification of the strain or only gave a small number of PCR products. Primers P4 and P6 were then used to analyse all the 17 strains of *V. parahaemolyticus*. A set of reproducible bands produced for a particular primer was defined as a pattern or profile. The reproducibility of the RAPD method was tested by repeating the RAPD assays at least three times for each primer tested. Results revealed that apart from some minor variations in the band intensity, no significant differences were observed between the profiles obtained, which demonstrated the reproducibility of the method.

The nine distinct patterns obtained with primer 4 , designated RAPD types A to I exhibited between 2 and 6 bands ranging from 300 to 1,500 bp in size (Figs 1 and 5). Three strains recovered from water were shown to form RAPD profile A, while three isolates from *P. monodon* and the type strain ATCC 17802 comprised profile F. Reference strain ATCC 43996 clustered together with three isolates from different sources forming profile G. The remaining profiles contained a unique strain. Regardless of the RAPD profile, all the strains shared a common band at 750 bp (Figure 1).

When primer 6 was used, six distinct patterns were observed and were designated RAPD types A to F, comprising of two to four bands with sizes ranging between 300 and 2,000 bp (Figures 2 and 6). Eight strains from aquatic environment formed RAPD types A and D, while four strains from *P. monodon* formed profile F. Profile E was composed of two strains coming from aquatic environment and from *P. monodon*. Finally, profiles B and C contained a unique strain. Using primer 6, all strains showed a common band at approximately 600 bp (Figure 2).

The results of the analysis of similarity among the different profiles with the Diversity database software employing the S_d and the UPGMA allowed us to identify at least 3 genetic groups or clusters (I-III), with at least 70% similarity level. Thus, with primer

4, the three genetic groups were defined at S_d values of 70% for cluster I (profiles A, C, H, I), 99% for cluster II (profile F), and 90% for cluster III (patterns B and G) (Figure 5). At 80% similarity level all the strains in cluster I were from aquatic environments. The second cluster was composed of two strains from *P. monodon* that are genetically identical sharing the same common bands at 750, 800 and 1,000 bp. Lastly cluster III was composed of strains coming from both aquatic environment and *P. monodon.* The RAPD patterns of all remaining strains were diverse, with similarities below 70% and were considered genetically unrelated by this method.

However, when primer 6 was used three genomic clusters with S_d values ranging from 85 to 99% were formed (Figure 6). Cluster I has an 85% similarity and was formed by RAPD profiles D and E. Majority of the strains (4/7) from this cluster were recovered from aquatic environment. Clusters II (profile A) and III (profile F) were composed of strains that have an identical RAPD type. Both clusters had a 99% similarity. Cluster II was composed of strains coming from aquatic environments whereas cluster III was composed of strains recovered from affected *P. monodon*. In addition, genetic clusters I and II showed a close relationship with a similarity of 80%. Lastly, RAPD types B and C were more diverse with similarities below 50% and were considered genetically unrelated to any of the three clusters.

ERIC-PCR fingerprinting

The fingerprints of *V. parahaemolyticus* strains consisted of 6 to 8 amplification bands, ranging in size from 50 to 2,500 bp (Figure 3). All strains were typeable by ERIC-PCR. The same fingerprints were observed when ERIC-PCR was repeated at least three times, demonstrating the reproducibility of this technique. Almost each strain gave a different ERIC profile although a common band was observed in all 17 strains around 500 bp (Figure 3). Analysing the similarity among the different profiles with the Diversity database software identified three genomic clusters; although there are only few strains that formed these clusters as majority of the strains were genetically unrelated (Figure 7). The first group included strains CLM3-0108 and reference strain ATCC 43996 with 85% similarity. The second cluster consisted of strains IA13 and 03S1a-04-5 with a S_d of 90%. Both strains were recovered from the aquatic environment. Lastly, the third cluster with 80% similarity was composed of strains MF-0107-4 and 03S3-01-2 recovered from pond water and *P. monodon*, respectively. The remaining strains were distinct with similarity of lower than 80%.

REP-PCR fingerprinting

Analysis of the strains with REP-PCR yielded one to four bands depending on the strains (Figure 4). The size of these bands ranged from 150 to 2,000 bp and it was evident that the majority of the strains exhibited patterns with small number of REP-PCR products. As with ERIC-PCR, each strain gave a REP-PCR profile that was different from each other. However, all strains demonstrated a common band at approximately 400 bp (Figure 4). The REP-PCR amplifications were also repeated at least three times. Results revealed that some of the minor light amplification bands were inconsistent making the analysis more difficult.

Similar to ERIC-PCR, only a few strains formed clusters as most strains had unique RAPD profile. Nevertheless, three genomic clusters were identified among the 17 strains of *V. parahaemolyticus* using the Dice coefficient and the UPGMA (Figure 8). The first cluster with 99% similarity was composed of only two strains (MF-0107-4 and MF-0107-7) isolated from the aquatic environment and strain number 03S3-01-2 from *P. monodon*. The second genomic group was composed of two strains (IA13 and IM12) that were both recovered from creek water. The two strains were considered to be genetically related as demonstrated by a 90% similarity. The first and second group showed a closer relationship, with a similarity of 85%. Lastly, the third cluster with a S_d of 90% was composed of strains (03S3-07-5 and 03S3-07-6) isolated from *P. monodon.* The REP-PCR patterns of the majority of the strains demonstrated similarities below 90%.

Annealing temperature gradient-PCR

For both ERIC and REP-PCR, band formation was observed at the highest temperature gradient (T_m) (data not shown). This confirmed the presence of ERIC and REP sequences in the genome of the investigated strains.

ANTIMICROBIAL SUSCEPTIBILITY TESTING

The results of MIC determination and antimicrobial susceptibility revealed that 12/14 (86%) of *V. parahaemolyticus* were resistant to ampicillin. In the case of the remaining antimicrobials, all strains were susceptible. In addition, none of the ampicillin-resistant strains were ESBLs producing and all were susceptible to imipenem.

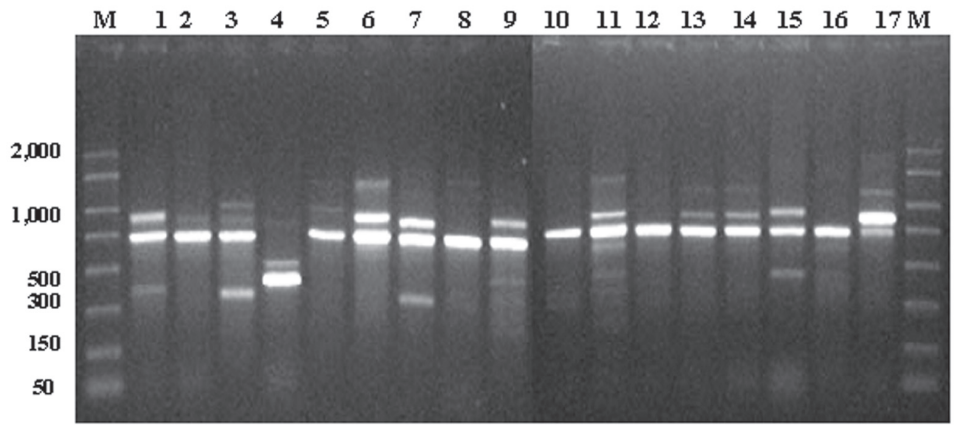

Figure 1. RAPD fingerprints obtained for the *V. parahaemolyticus* strains with primer 4. Lanes: M, AmpliSize Molecular Ruler (50-2000-bp; Sigma); 1, MF-0107-1; 2, MF-0107-4; 3, MF-0107-7; 4, CLM3-0108; 5, IA3; 6, IA13; 7, IA16; 8, IM12; 9, 03S1a-04-5; 10, 03S3-01-2; 11, 03S3-01-4; 12, 03S3-07-2; 13, 03S3-07-5; 14, 03S3-07-6; 15, IV-86; 16, ATCC 43996; 17, ATCC 17802^T. The molecular sizes (in base pairs) are indicated on the left.

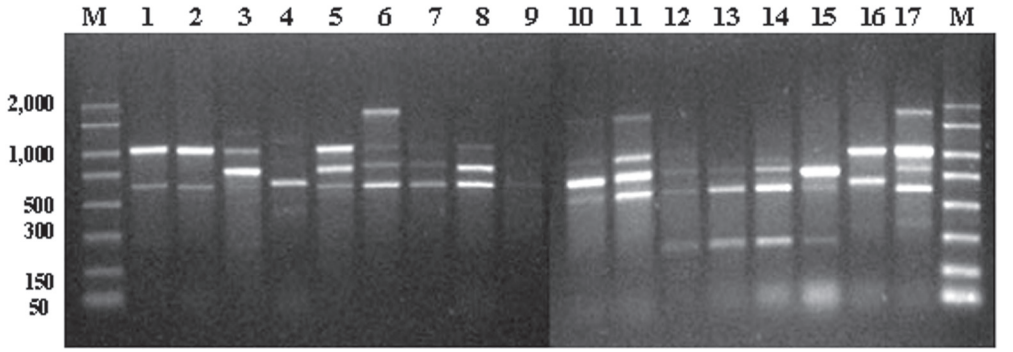

Figure 2. RAPD fingerprints obtained for the *V. parahaemolyticus* strains with primer 6. Lanes as in Figure 1. The molecular sizes (in base pairs) are indicated on the left.

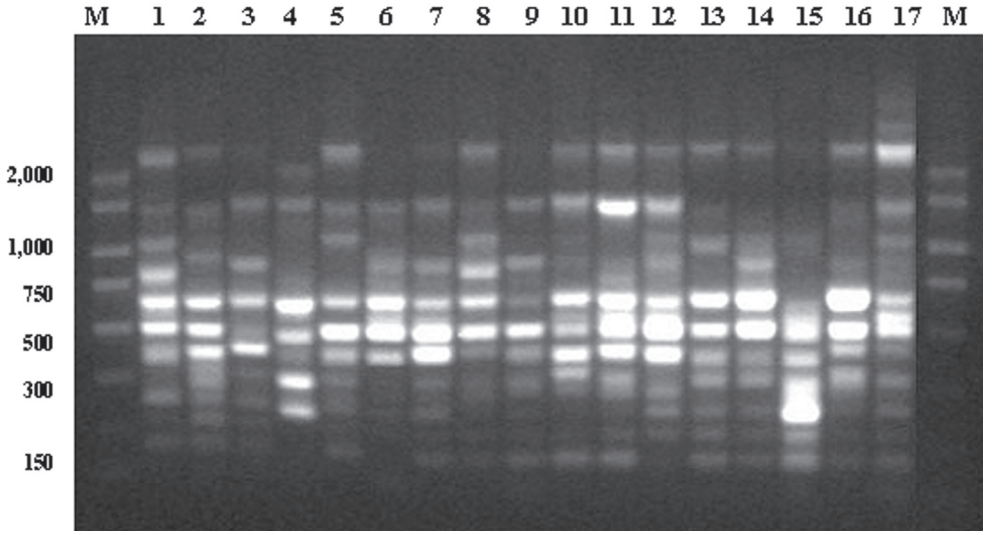

Figure 3. ERIC-PCR fingerprints obtained for the *V. parahaemolyticus* strains. Lanes as in Figure 1. The molecular sizes (in base pairs) are indicated on the left.

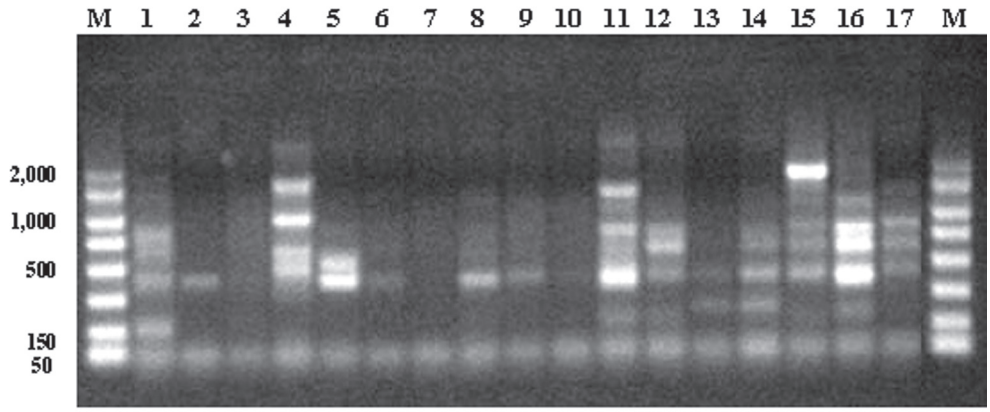

Figure 4. REP-PCR fingerprints obtained for the *V. parahaemolyticus* strains. Lanes as in Figure 1. The molecular sizes (in base pairs) are indicated on the left.

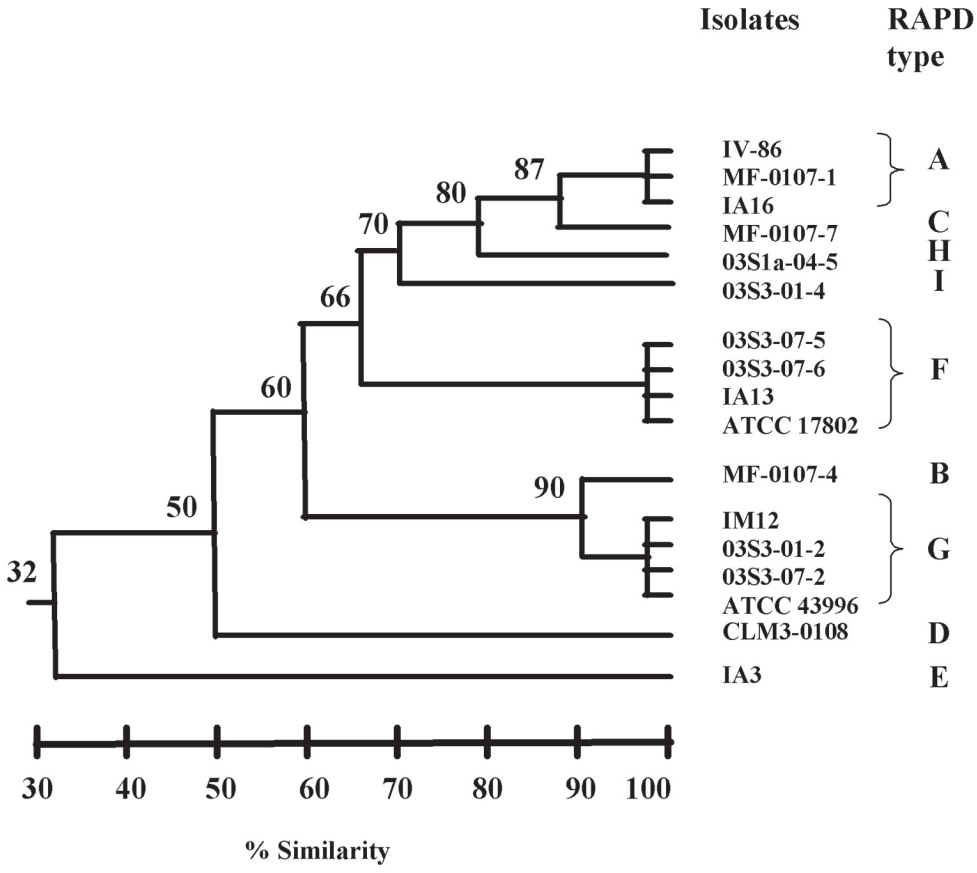

Figure 5. Dendrogram established by the Diversity Database software package (Bio-Rad) using the Dice similarity coefficient and UPGMA on the basis of the RAPD profiles of *V. parahaemolyticus* strains obtained with primer 4.

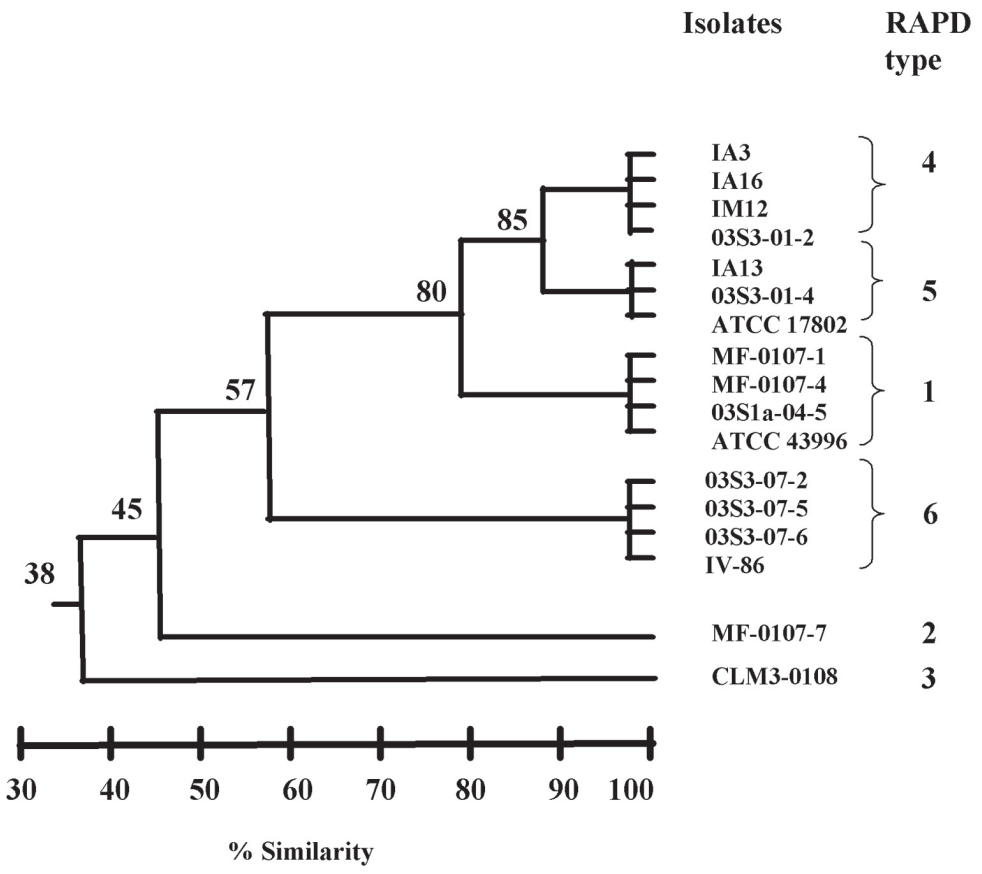

Figure 6. Dendrogram established by the Diversity Database software package (Bio-Rad) using the Dice similarity coefficient and UPGMA on the basis of the RAPD profiles of *V. parahaemolyticus* strains obtained with primer 6.

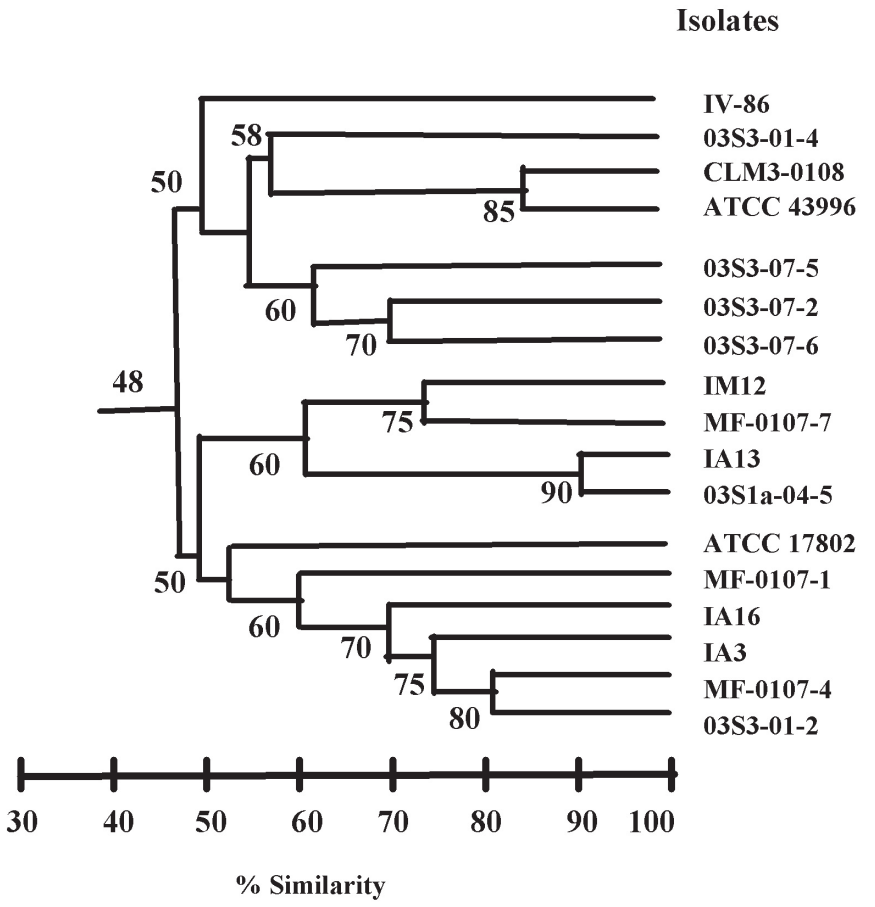

Figure 7. Dendrogram established by the Diversity Database software package (Bio-Rad) using the Dice similarity coefficient and UPGMA on the basis of the ERIC-PCR profiles of *V. parahaemolyticus* strains.

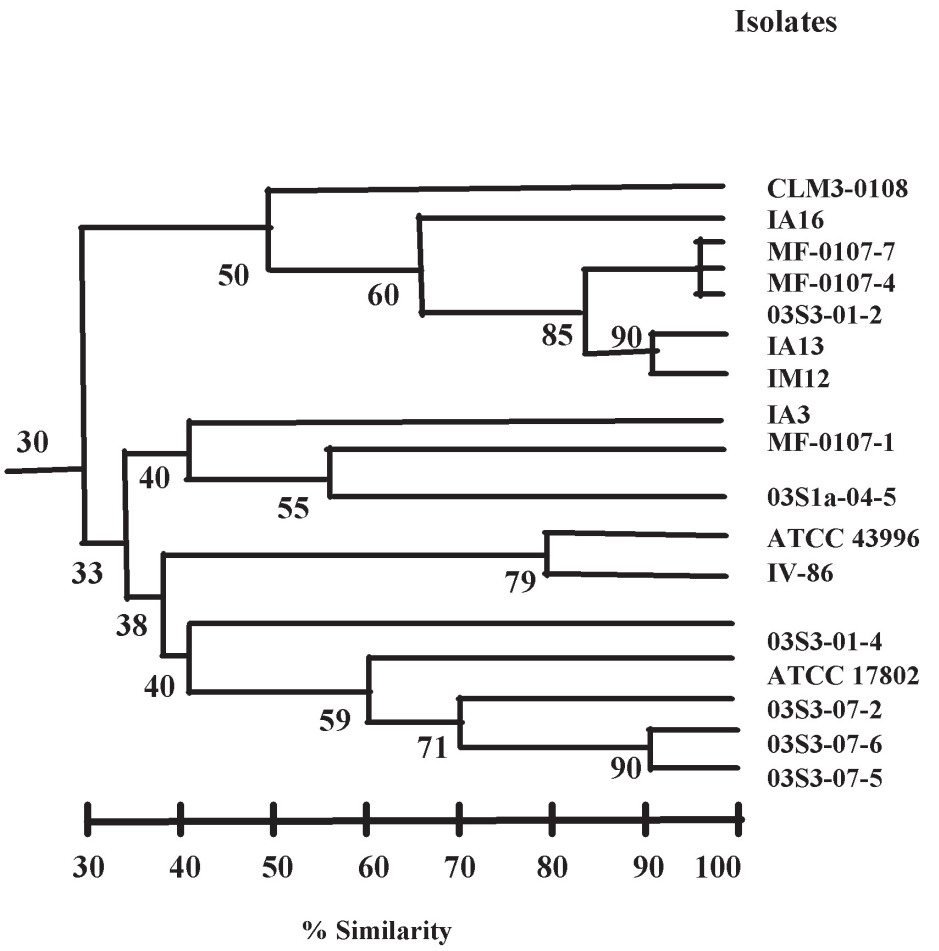

Figure 8. Dendrogram established by the Diversity Database software package (Bio-Rad) using the Dice similarity coefficient and UPGMA on the basis of the REP-PCR profiles of *V. parahaemolyticus* strains.

DISCUSSION

Results of several epidemiological studies on *V. parahaemolyticus* infections have supported the genetic similarity between clinical and environmental strains (Kelly and Stroh, 1998; Marshall *et al.*, 1999), revealing that *V. parahaemolyticus* acquired infections in humans were only reported when the organism could be detected in the local environment (Kelly and Stroh, 1998). This was particularly true when the water temperatures were greater than 14ºC. This is of great importance in tropical countries such as the Philippines, wherein environmental temperatures are usually high.

Results of RAPD fingerprinting with the appropriate primers revealed that some of the patterns obtained could be related to the origin of the strains, which indicated its potential use in epidemiological studies of this organism. Analysis of the similarity of the RAPD patterns using the Dice coefficient and UPGMA has revealed the high genetic variation among strains. As shown in the dendrograms, only few strains clustered with a similarity of 90 to 99%. In a study done by Szczuka and Kaznowski (2004), strains with similarities below 90% were considered genetically unrelated. We could say that based on similarity using the Dice coefficient, our *V. parahaemolyticus* strains are genetically diverse wherein majority have similarity coefficient of below 90%. This result agreed with other studies confirming the genetic diversity among *V. parahaemolyticus* strains (Goarant *et al.,* 1999; Sudheesh *et al.,* 2002). It was found that, regardless of the RAPD profiles observed, all strains showed a common band of 750 bp or 600 bp using both P4 and P6 respectively. These fragments would be favourable traits for the development of genetic amplification and hybridization assays for diagnostic purpose (Dalla Valle *et al*., 2002), which can be important to verify *V. parahaemolyticus* strains that are relatively difficult to identify.

Both ERIC and REP methods gave almost a unique profile for each strain. Comparison of the similarities among the different patterns using S_d and UPGMA confirmed the genetic heterogeneity among *V. parahaemolyticus* strains, as the majority of the strains gave a similarity value below 90%. Although these two methods did not allow the establishment of well defined genetic clusters because of their high discriminatory power such techniques could be useful to follow the spreading of bacterial strain responsible for a particular outbreak. In addition, a common band of 500 bp and 400 bp was shown by all the strains using ERIC and REP PCR methods, respectively. This could be useful for the tracing of the point source of infection.

As shown by Marshall *et al.* (1999), Sudheesh *et al.* (2002) and Szczuka and Kaznowski (2004), it was found that both RAPD and ERIC-PCR gave more reproducible results compared with REP-PCR. The presence of the repeatable fingerprints in ERIC and REP-PCR suggested the presence of these repetitive consensus sequences (ERIC and REP) in *V. parahaemolyticus*. However, Gillings and Holley (1997) have revealed that besides the presence of ERIC, the formation of fingerprints could be due to random amplification. Our results showed band amplifications at higher temperatures (T_m of ERIC and REP) confirming the presence of these sequences on the genome of *V. parahaemolyticus* strains.

Antibiotics resistance among *V. parahaemolyticus* strains was less prevalent in this study. The only resistance observed was to ampicillin (12/14). This finding supported

several reports on the increasing β-lactam resistance among vibrios from different sources (Zanetti *et al*., 2001; Molina-Aja *et al.,* 2002). This study also determined that *V. parahaemolyticus* strains were susceptible to the majority of antimicrobials tested, indicated by the susceptibility of all the strains to ceftiofur, aminoglycosides, florfenicol, TMPS, enrofloxacin and oxytetracycline. However, an increasing number of cases of resistance to these antimicrobials have been reported (Molina-Aja *et al.,* 2002).

Extended-spectrum β-lactamases (ESBLs) are enzymes that mediate resistance to extended-spectrum (third-generation) cephalosporins. The presence of ESBLs was reported in enterobacteriaceae and *Pseudomonas aeruginosa* (Carter *et al.,* 2000; Gheldre *et al.,* 2003). In this study, production of ESBLs among *V. parahaemolyticus* strains could not be demonstrated.

ACKNOWLEDGEMENTS

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Pond Health Management of Black Tiger Shrimp *Penaeus monodon* (Fabricius) using Bacterial Products

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ABSTRACT

The pond health management (e.g. sediment condition and water quality and growth performance) of the black tiger shrimp *Penaeus monodon* were studied in brackishwater ponds using bacterial products (probiotics) in the tropics. The improvement of organic matter and total sulfur (TS) of pond sediment was observed during the culture period. Organic matter content in the ponds was low (3.95±0.56%) probably due to decomposition activity by bacteria during mineralization process. The lower (1.58±0.33%) concentration of TS in the culture pond sediments suggested that heterotrophic bacteria utilized the superficial soil sulfate compounds, which converts into sulfur and its related compounds. The water quality condition such as dissolved oxygen (6.8-10.3 mg/l), pH (7.22-8.44), temperature (30-32ºC), salinity (16.0-28.2‰), total suspended solids (0.10-0.17 g/l), biological oxygen demand (0.15-0.25 mg/l), chlorophyll *a* (50-250 mg/m^3), NO_3^- (0.002-0.01 mg/l) and $PO_4^=$ (0.0-0.16 mg/l) were within suitable range for shrimp growth and did not cause stress. Concentration of ammonium (NH_4^+) was high (0.28-2.0 mg/l) during the culture period. Nevertheless, daily growth rate of shrimp was found 0.20 g/day in 116 days culture period. The higher concentrations of major macronutrients such as Ca, Na, Mg and K in pond sediments could be attributed partly to nutrient loading and accumulation from soil pore water during drying over time and pond age. None of the elements accumulated in the ponds reached harmful concentrations for pond health and the cultured shrimp species.

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INTRODUCTION

The culture potential of black tiger shrimp *P. monodon* is well known due to its production performance and economic profitability. The growth, production and survival of any culture species like shrimp depends on the culture system practiced (e.g. extensive and semi- intensive). The physico-chemical factors of the culture pond and their individual or synergetic effects play an important role on shrimp production and pond ecology. The ecosystem and biota of the culture ponds may also influence the production performance of shrimp culture. Studies suggested that the growth and survival of shrimps are affected by temperature, salinity and dissolved oxygen concentration (Subrahmanyam, 1973; Verghese *et al.,* 1975, 1982; Liao, 1977). The growth of shrimp also depends on the water management followed by the depth of pond water and quality of supplementary feed. Millamena (1990) found that the survival rate of *P. monodon* post-larvae is directly influenced by organic content and dissolved oxygen concentration in the culture ponds.

Several studies have aimed to increase production of tiger shrimp through manipulating of stocking density, fertilization, artificial feeding, opening of new lands for culture and combination of other species into the culture system (Verghese *et al*., 1975; Chakraborti *et al*., 1985). Currently, in advanced aquaculture technology systems, farmers used water reservoirs for sedimentation, green water, microbial products (i.e. probiotics) and compressed air line for bottom aeration together with paddle wheels (Shishehchian, 2000). Biomanipulators (i.e. tilapia, milkfish and sea bass) were also stocked inside net cages in the reservoirs; while some of the species especially tilapia can directly help shrimp when they produce enzymes or slime that inhabit the growth of luminous bacteria (SEAFDEC, 2000). Recent development in aquaculture science has improved vastly in certain areas of tiger shrimp production but the results still remain inconsistent. Therefore, in this context, an investigation was undertaken to observe the ecological factors with the growth of *P. monodon* in the culture pond using microbial products and compared with the other studies elsewhere.

MATERIALS AND METHODS

The study area is situated in the District Perak, west coast of Malaysia (5° 45' N and 101° 37'E). The ponds were about 8 years old and considered aged ponds. Three culture ponds were selected initially, but White Spot Syndrome Virus (WSSV) infected two ponds during the middle of the production cycle period leaving only one pond remaining uninfected. Therefore, sampling was carried out in one pond (4673 m^2) until end of 116 days culture period. Since the pond is fully managed by the owner, the present study is considered as a case study, which compared with the other parallel study using different pond management system. All data were collected between April 2001 and July 2001 during the whole culture period. The culture management and preparation of sampling pond was described in Abu Hena *et al.* (2003). Water quality parameters were measured *in situ* every three-week interval. Dissolved oxygen (DO) was measured using DO meter (YSI model 57), water salinity and temperature by SCT meter (YSI model 33), water pH by pH meter (EDT

model FE 253), and transparency by Secchi disk in nearest cm. Ekman grab sampler was used for collection of soil samples. Three samples were collected in a diagonal direction (corner to corner) from each pond by using a small boat. Samples were brought back to laboratory for further analysis within 2-4 hrs. In the laboratory, soil samples were dried in room temperature and powdered. Later on, it was sieved through 200 μm mesh screen. Organic matter of soil was detected by ignition method (Boyd, 1995a). Soil texture was analyzed following procedure described by Bouyoucos (1962). The total sulfur was analyses following the method by Tandon (1990) and macro-micro nutrients by Allen (1972) using ELAN 6000 ICPMS.

RESULTS AND DISCUSSION

The details of the physico-chemical factors and nutrients of culture pond water are given in Table 1. Water quality is the most important limitation of the commercial viability of aquaculture operation, especially in semi-intensive culture condition (Boyd and Watten, 1989). The physico-chemical factors of water were not significantly different within the ponds using probiotics and none probiotics throughout the culture period. The concentrations of water nutrients increased with time and reached maximum stage at the end of culture period. Ammonium (NH_4^+) concentration was found higher in the culture pond using probiotics (Table 1) when compared with previous study (Abu Hena *et al*., 2003). Higher stocking density of shrimp and their metabolic products, and/or partly microbial activity is the probable cause. As expected ammonium concentrations increased throughout the grow-out period as the shrimp biomass increased. Compared with other reported study, the pond water nutrients of the presently studied pond are similar to or comparable with other culture ponds of *P. monodon* in Australia (Burford, 1997) with the value for NO_3^- (0.08 ppm), NH_4^+ (0.30 ppm) and $PO_4^=$ (0.05 ppm). This comparison suggests that the expected improvement of water quality was not apparent in the pond using microbial products. The result of present study was in agreement with the statements of Boyd and Gross (1998). Shariff *et al*. (2001) also observed that the improvement of overall pond water parameters was not achieve between treated and untreated ponds with microbial products and the mechanisms by which the bacteria improved survival is unknown.

The textural classes of the soil samples and different physical and chemical variables of soil are shown in Table 2. The soil of the pond using probiotics was sandy. Soil texture is an important factor in the construction of pond, and shrimp farmers tend to favor sites with high clay content. The wet pH values were not significantly different between the sampling ponds. The dry soil pH was not found significantly different among the presently studied ponds through out the culture period (Table 2) and almost near to the wet pH values. Culture pond soils become aerobic when dried. In acidic soils the exchangeable aluminum concentrations controlled pH value, while calcium carbonate and other minerals in neutral and slightly alkaline soils. Therefore, dry soil pH measured in distilled water may be higher or lower than the pH values of wet pond soils (Munsiri *et al*., 1995). The dry pH values in this study (Table 2) were comparable (7.1-8.6) with the study ponds by Ritvo *et al*. (1998) and higher than found (3.68-6.2) by Das *et al*. (2002).

Table 1. The range of water quality parameters of culture ponds with probiotics and without probiotics (Source; Abu Hena et al., 2003).

Parameters (Range)	This study	Pond without probiotics[1]
DO (mg/l)	6.8-10.3	7.2-12.2
Temperature (ºC)	30.0-32.0	26.2-32.0
pH	7.22-8.44	7.53-8.17
Salinity (‰)	16.0-28.2	19.0-27.0
Transparency (cm)	16.0-37.0	20.0-70.0
Total suspended solid (g/l)	0.10-0.17	0.08-0.18
BOD_3 (mg/l)	0.15-0.65	4.80-7.90
Chlorophyll *a* (mg/m^3)	50.0-250.0	30.0-240.0
NO_3^- (ppm)	0.01-0.02	0.01-0.04
NH_4^+(ppm)	0.28-1.99	0.14-0.41
$PO_4^=$(ppm)	0.01-0.16	0.01-0.11
TS (ppm)	1010-2486	1205-4375

[1]Abu Hena *et al.* (2003)

Table 2. Range of concentration for different physical and chemical variables of soil from shrimp culture ponds (Source: Abu Hena, 2005).

Variable	This study	Pond without probiotics[1]
Sand (%)	86.12-89.24	5.48-7.56
Silt (%)	4.51-5.89	36.25-38.24
Clay (%)	5.99-7.01	54.10-57.15
pH (wet)	7.37-9.32	7.87-8.51
pH (dry)	7.65-8.79	7.92-8.33
Cation exchange capacity (meq/100 g)	4.22-6.56	7.55-22.06
Organic matter (%)	2.70-5.47	4.93-9.93
Organic carbon (%)	1.42-2.88	2.59-5.22
Total carbon (%)	2.20-6.82	5.11-7.55
Total sulfur (% dry wt)	0.77-3.63	1.98-2.48

The cation exchange capacity (CEC) of the presently investigated ponds is shown in Table 2. Cation exchange capacity is the ability of colloids in a soil to adsorb cations (Boyd, 1995a). The soils from probiotic and non-probiotic ponds differ markedly in CEC and the ranges were between 4.22-6.56 and 7.55-22.06-meq/100 g, respectively. This difference may be due to natural variation of soil textural composition between the two ponds. Munsiri *et al.* (1995) stated that cation exchange properties of soils result from clay and organic matter fractions. The range of clay percentage of the presently studied ponds was 5.99-7.01% and 54.10-64.58%, whereas the organic matter was 2.70-5.47% and 4.17-9.27% for the shrimp ponds using probiotics and non-probiotics, respectively. The soil contained 4.5-6.0% organic matter and 30-50% clay, and the comparatively high CEC of soil resulted from organic matter (Munsiri *et al.,* 1995), which support the present finding (Table 2). McNutt (1981) stated that sediment containing 40% clay and 5% organic matter has a CEC of 14.1 meq/100 g. This value is within the range of CEC values reported for non-probiotic pond. Organic matter concentration of soils was found significantly higher (>7.0%) in the pond with no probiotics compared to the ponds with probiotics (>3.0%). The culture pond soils using probiotics contain lower percentage of organic matter and total carbon probably due to decomposition of organic materials during the mineralization process (Moriarty, 1986). The mean value of soil TS (1.58±0.33% dry wt) decreased in the culture pond using probiotics, while it was higher in non-probiotic pond (unpublished data). The lower amount of soil TS in the culture pond with probiotics may be due to utilization sulfate compounds by heterotropic bacteria (Boyd, 1990, 1995b), which converted to sulfur and its related compounds into the pond environment. This may be the case for the presently investigated pond using probiotics. The sulfur oxidizing bacteria in the ponds treated with microbial products suggests efficient conversion of H_2S to sulfur compounds (Devaraja *et al*., 2002) and increased the TS of pond water.

The concentrations of macro-micronutrient of pond soils are presented in Table 3. The concentrations of Ca, Mg, K and Na were found significantly higher in the culture pond using probiotics than non-probiotic pond (Table 3). The observed higher concentrations of major macronutrients in soil could probably be attributed to nutrient loading from water, feed, uneaten feed, faeces and accumulation from soil pore water during drying over time and pond age. Ritvo *et al*. (1998) stated that the tentative explanation for the higher content of major cations in the incoming water could be due to precipitation. It is surprising to record that the concentrations of soil Mg in probiotic pond (16541.7±3007.8 ppm) was almost uniform probably due to the application of dolomitic agricultural limestone (8132 kg/t) into the pond. This concentration was very high according to the classification of soil characteristics done by Boyd *et al*. (1995b) for culture pond. The concentration of other elements was medium characteristic for K (567.1±103.23 ppm) and Ca (3961.9±705.3 ppm), and very low characteristic for Na (1303.7±78.47 ppm) in the pond using probiotics. Soils from non-probiotic pond have very low characteristic for Ca, Mg and Na, whereas concentration of K was medium (836.1±39.2 ppm) according to Boyd *et al.* (1995b) classification. The overall concentrations of Ca, Mg, K and Na did not increase significantly throughout the culture period in both ponds. It indicates that in these ponds, part of nutrient load was either converted to organic form (Ritvo *et al*., 1998), or major macronutrients was not washed out during water exchange.

Table 3. Range of concentration for different chemical variables of soil from shrimp culture ponds (Abu Hena *et al.,* 2003).

Variable (ppm)	This study	Pond without probiotics[1]
Calcium	1969-81510	206-586
Magnesium	4900-28412	231-445
Potassium	360-1240	466-1060
Sodium	946-1545	142.0-342.8

Daily mean growth rate of culture shrimps were observed at 0.20 g/day and 0.23 g/day for the culture pond using probiotics and non-probiotics, respectively. The lower daily growth rate of shrimps in probiotic pond probably due to several interrelated factors, i.e. water quality, natural food availability, stocking density and others environmental forces. A lower growth rate of shrimps was also reported as stocking density increased (Apud *et al*., 1981). Probably it may be the case for probiotic pond with the stocking density of 48 PL/m^2. Apud *et al*. (1981) also concluded that the amount and quality of food was adequate for the survival of shrimps, but probably not suitable for promoting faster growth when ponds are stocked at higher densities of PL. Compare to the reported studies elsewhere the growth rate of present studied pond (0.20 g/day) was relatively lower (Liao, 1977; Chen *et al*., 1989; Lumare *et al*., 1993) probably due to variability in the pond ecosystem and culture management.

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Appendices

Appendix 1

List of Reviewers for DAA VI Proceedings

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Appendix 2

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Appendix 3

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