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

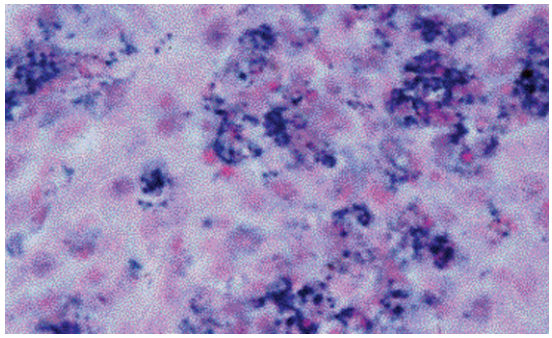

Figure 1. Detection of *Renibacterium salmoninarum* in fixed kidney tissue by IHC. (Photograph courtesy of Dr K.D. Thompson, University of Stirling).

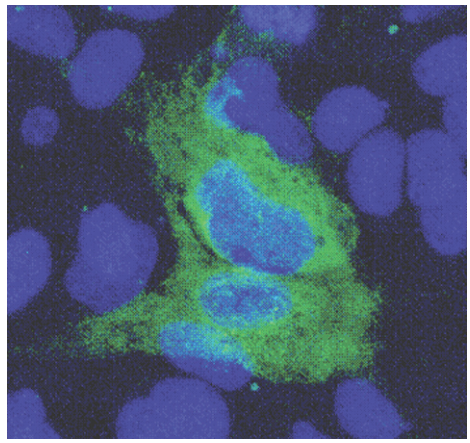

Figure 2. Detection of ISAV in SHK-1 infected cells by IFAT (Photograph courtesy of Dr K.D. Thompson, University of Stirling).

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.

REFERENCES

Adams, A. 1992. Sandwich enzyme linked immunosorbent assay (ELISA) to detect and quantify bacterial pathogens in fish tissue, pp. 177-184. *In* Stolen, J.S., Fletcher, T.C., Kaattari, S.L. and Rowley A.F. (eds.). Techniques in Fish Immunology Volume 2. SOS Publications.

Adams, A. 1999. Application of antibody probes in the diagnosis and control of fish diseases, pp. 1-12. *In* Karunasagar, I., Karunasagar, I. and Reilly, A. (eds.). Aquaculture and Biotechnology, Oxford and IHB Publishing Co. PVT. LTD.

Adams. A. 2004. Immunodiagnostics in Aquaculture. *Bull. Euro. Assoc. Fish Pathol*. 24, 33-37.

Adams, A. and de Mateo, M. 1994. Immunohistochemical detection of fish pathgens, pp. 133-144. *In* Stolen, J.S., Fletcher, T.C., Kaattari, S.L., Rowley A.F. (eds). Techniques in Fish Immunology. Volume 3. SOS Publications.

Adams, A. and Thompson, K.D. 1990. Development of an ELISA for the detection of *Aeromonas salmonicida* in fish tissue. *J. Aquat. Animal Health* 2: 281-288.

Adams, A and Thompson, K.D. 2006. Review: Biotechnology offers revolution to fish health management. *Trends in Biotechnology* 24: 201-205.

Adams, A., Thompson, K.D., Morris, D., Farias, C. and Chen, S.-C. 1995. Development and use of monoclonal antibody probes for immunohistochemistry, ELISA and IFAT to detect bacterial and parasitic fish pathogens. *J. Fish Shellfish Immunol.* 5:537-547.

Aoki, T., Hirono, I., de Castro, T. and Kitao, T. 1989. Rapid identification of *Vibrio anguillarum* by colony hybridization. *J. Appl. Ichthyol*. 5:67-73.

Berthe, F., Burreson, E. and Hine, M. 1999. Use of melecular tools for mollusc disease diagnosis. *Bull. Eur. Ass. Fish Pathol*. 19: 277-278.

Benmansour, A. and de Kinkelin, P. 1997. Live Fish Vaccines: History and Perspectives, pp. 279-289. *In* Gudding, R., Lillehaug, A., Midtlyng, P.J., Brown, F. (eds.). Fish Vaccinol. Dev. Biol. Stand. Karger, Basel.

Bondad-Reantaso, M.G. and Subasinghe, R. 2005. Aquaculture Health International, Issue 1, 4-5.

Boulot, V., Mialhe, E., Rogier, H., Paolucci, F. and Grizel, H. 1989. Immunodiagnosis of *Bonamia ostreae* (Ascetospora) infection of *Ostrea edulis* L. and subcellular identification of epitopes by monoclonal antibodies. *J. Fish Dis.* 12: 257-262.

Briggs, M., Funge-Smith, S., Subasinghe, R. and Phillips, M. 2004. Introductions and movement of *Penaeus vannamei* and *Penaeus stylirostris* in Asia and the Pacific. Food and Agricultural Organization of the United Nations, Regional Office for Asia and the Pacific. RAP Publication 2004/10. 99pp.

Carnegie R.B., Meyer, G.R., Blackbourn J., Cochennec-Laureau, N., Berthe, F.C.J. and Bower, S.M. 2003. Detection of the oyster parasite *Mikrocytos mackini* by PCR and fluorescent *in situ* hybridization, and a preliminary phylogenetic analysis using SSU rDNA. *Dis. Aquat. Org.* 54(3): 219-227

Cochennec, N., Hervio, D., Panatier, B., Boulo, V., Mialhe, E., Rogier, H., Grizel, H., Paolucci, F. 1992. A direct monoclonal antibody sandwich immunoassay for detection of *Bonamia ostreae* (Ascetospora) in hemolymph samples of the flat oyster *Ostrea edulis* (Mollusca: Bivalvia). *Dis. Aquat. Org.* 12: 129-134

Cochennec, N., Le Roux, F., Berthe, F., and Gerard, A. 2000. Detection of *Bonamia ostreae* based on small subunit ribosomal probe. *J. Invertebr. Patho*l. 76: 26-32

Corsin, F., Turnbull, J.F., Hao, N.V., Mohan, C.V., Phi, T.T., Phuoc, L.H., Tinh, N.T.N. and Morgan, K.L. 2001. Risk factors associated with White Spot Syndrome Virus infection in a Vietnamese rice-shrimp farming system. *Dis. Aquat. Org.* 47:1-12.

Costa, J. Z. 2005. PhD Thesis, University of Stirling.

Cowley, J.A., Dimmock, C.M., Wongteerasupaya, C., Boonsaeng, V., Panyim, S. and Walker, P.J. 1999. Yellow head virus from Thailand and gill-associated virus from Australia are closely related but distinct prawn viruses. *Dis. Aquat. Org.* 36: 153-157.

Cunningham, C.O. 2004. Use of molecular diagnostic tests in disease control: Making the leap from laboratory to field application. *In* Leung, K.-Y. (ed). Current trends in the study of bacterial and viral fish and shrimp diseases. *Molecular Aspects of Fish and Marine Biology* 3: 292-312. World Scientific Publishing Co.

Dupont, N.C., Wang, K. Wadhwa, P.D., Culhane, J.F. and Nelson, E.L. 2005. Validation and comparison of luminex multiplex cytokine analysis kits with ELISA: Determinations of a panel of nine cytokines in clinical sample culture supernatants. *J. Reprod. Immunol.* 66: 175-191.

Fournier, P-E. and Raoult, D. 2003. Comparison of PCR and Serology Assays for Early Diagnosis of Acute Q Fever. *J. Clin. Micro.* 41: 5094-5098.

FAO. 2002. The state of world fisheries and aquaculture - SOFIA 2002. FAO, Rome, Italy. 150 pp.

Frost, P. and Ness, A. 1997. Vaccination of Atlantic salmon with recombinant VP2 of infectious pancreatic Necrosis virus (IPNV), added to a multivalent vaccine, suppresses viral replication following IPNV challenge. *Fish and Shellfish Immunology* 7: 609-619.

Giavedoni, L.D. 2005. Simultaneous detection of multiple cytokines and chemokines from nonhuman primates using luminex technology. *J. Immunol. Methods* 301:89-101.

Grizel, H. and Héral, M. 1991. Introduction into France of the Japanese oyster *Crassostrea gigas*. *Journal du Conseil International pour l'Exploration de la Mer* 47 : 399-403

Gudding, R., Lillehaug, A. and Evensen, Ø. 1999. Recent developments in fish vaccinology . *Vet. Immunol. Immunopathol*. 72(1-2): 203-212.

Harlow, E. and Lane, D. 1988. Chapter 6: Monoclonal Antibodies. *In* Harlow, E. and Lane, D. (eds.). *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York.

Hastings, T., Olivier, G., Cusack, R., Bricknell, I., Nylund, Å., Binde, M., Munro, P. and Allan, C. 1999. Infectious salmon anaemia. *Bull. Eur. Assoc. Fish Pathol.* 19:286-286.

Hossain, M.S., Otta, S.K., Chakraborty, A., Sanath Kumar, H., Karunasagar, I. and Karunasagar, I. 2004. Detection of WSSV in cultured shrimps, captured rooders, shrimp postlarvae and water samples in Bangladesh by PCR using different primers. *Aquaculture* 237, 59-71.

Hossain, M.S., Chakraborty, A., Joseph, B., Otta, S.K., Karunasagar, I. and Karunasagar, I. 2001. Detection of new hosts for white spot syndrome virus of shrimp using nested polymerase chain reaction. *Aquaculture* 198:1-11.

Jorgensen, J.B., Johansen, L.H., Steiro, K. and Johansen, A. 2003. CpG DNA induces protective antiviral immune responses in Atlantic salmon (*Salmo salar* L.).J. Virol 77(21):11471-9.

Karunasagar, I., Nayak, B.B. and Karunasagar, I. 1997. Rapid detection of *Vibrio parahaemolyticus* from fish by polymerase chain reaction. pp. 119-122. *In* T.W. Flegel e*t al.* (eds.). Diseases in Asian Aquaculture III. FishHealth Section, Asian Fisheries Society, Manila.

Leong, J.C., Anderson, E., Bootland, L.M., Chiou, P.-W., Johnson, M., Kim, C., Mourich, D. and Trobridge, G. 1997. Fish vaccine antigens produced or delivered by recombinant DNA technologies. pp. 267-277. *In* R. Gudding, A. Lillehaug, P.J. Midtlyng, F. Brown (eds). *Fish Vaccinol. Dev. Biol. Stand.* Karger, Basel.

Le Roux, F., Audemard, C., Barnaud, A. and. Berthe, F.C.J. 1999. DNA probes as potential tools for the detection of *Marteilia refringens*. *Marine Biotechnology* 1 (6): 588-597.

Lightner, D.V. 1996. A Handbook of Shrimp Pathology and Diagnostic Procedures for Diseases of Cultured Penaeid Shrimp. World Aquaculture Society, Baton Rouge, LA, USA. 304 pp.

Lightner, D.V. 2005. Biosecurity in shrimp farming: pathogen exclusion through use of SPF stock and routine surveillance. *J. World Aqua. Soc.* 36: 229-248.

Lightner DV, Redman RM. 1998. Shrimp disease and current diagnostic methods. *Aquaculture* 164: 201–220.

Lin, J.-H., Yu, C.-C., Lin, C.-C., and Yang, H.-L. 2005. An oral delivery system for recombinant subunit vaccine to fish. *Dev. Biol Standards* 121:175-180.

Lo, C.F., Chang, Y.S., Cheng, C.T. and Kuo, G.H. 1998. PCR monitoring in cultured shrimp for white spot syndrome virus (WSSV) infection in growout ponds, pp. 281-286. *In* Flegel, T.W. (ed.). Advances in shrimp biotechnology. National Centre for Genetic Engineering and Biotechnology, Bangkok.

Lorenzen, N. and La Patra, S.E. 2005. DNA vaccines for aquacultured fish. *Rev. Sci. Tech. Off. Int. Epiz.* 24: 201-213.

Mannivanan, S., Otta, S.K., Karunasagar, I. and Karunasagar, I. 2002. Multiple viral infection of *Peneus monodon* shrimp postlarvae in an Indian hatchery. *Dis. Aquat. Org.* 48:233-236.

Markestad, A. and Grave, K. 1997. Reduction of antibacterial drug use in Norwegian fish farming due to vaccination. *In* Gudding, R., Lillehaug, A., Midtlyng, P.J. and Brown, F. (eds.). *Fish Vaccinol. Dev. Biol. Stand,* Karger, Basel. pp. 365.

McCafferty, J., Griffiths, A.D., Winter, G. and Chiswell, D.J. 1990. Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* 348:552-554.

Mialhe, E., Bachère, E., Boulo, V., Cadoret, J. P., Saraiva, J., Carrera, L., Rousseau, C., Cedeno, V., Calderon, J. and Colwell, R. R. 1995. Future of biotechnology-based control of disease in marine invertebrates. *Mol. Mar. Bio. and Biotech.* 4: 275-283.

Miles, D.J.C., Thompson, K.D., Lilley, J.H., and Adams, A. 2003. Immunofluorescence of the epizootic ulcerative syndrome pathogen, *Aphanomyces invadans*, using monoclonal antibodies. *Dis. Aquat. Org.* 55 (1): 77-84.

Mohan, C.V., Corsin, F., Thakur, P.C., Padiyar, P.A., Madhusudan, M. Turnbull, J.F., Hao, N.V. and Morgan, K.L. 2002. Usefulness of dead shrimp specimens to study the epidemiology of white spot syndrome virus (WSSV) and chronic bacterial infection. *Dis. Aquat. Org.* 50:1-8.

Morris, D.C., Morris, D.J. and Adams, A. 2002. Development of improved PCR to prevent false positives and false negatives in the detection of *Tetracapsula bryosalmonae*, the causative agent of Proliferative Kidney Disease. *J. Fish Dis.* 25(8): 483-490.

Overturf, K., LaPatra, S. and Powell, M. 2001. Real-time PCR for the quantitative analysis of IHNV in salmonids. *J. Fish Dis*. 24: 325-333.

Palmer-Densmore, M.L., Johnson, A.F. and Sabara, M.I. 1998. Development and evaluation of an ELISA to measure antibody responses to both the nucleocapsid and spike proteins of canine coronavirus. *J. Immunoassay* 19:1-22.

Pantoja, C.R., Song, X., Xia, L., Gong, H., Wilkenfeld, J., Noble, B. and Lightner, D.V. 2005. Development of a specific pathogen free (SPF) population of the Chinese fleshy prawn *Fenneropenaeus chinensis*. Part 1: Disease pre-screening and primary quarantine. *Aquaculture* 250:573-578.

Powell, J.L. and Loutit, M.W. 2004. Development of a DNA probe using differential hybridization to detect the fish pathogen *Vibrio anguillarum. Microbial Ecol.* 28: 365-373.

Puttinaowarat, S., Thompson, K.D. and Adams, A. 2000. Mycobacteriosis: detection and identification of aquatic *Mycobacterium* species. *Fish Vet. J.* 5:6-21.

Richards, R.H. 2005. Infectious salmon anaemia: development and standardisation of diagnostic methods and aspects of the epidemiology of ISA. Abstract, EC workshop, European Association of Fish Pathologists Conference , Copenhagen.

Romestand, B. and Bonami, J.R. 2003. A sandwich enzyme linked immunosorbent assay (s-ELISA) for the detection of MrNV in the giant freshwater prawn, *Macrobrachium rosenbergii* (de Man). *J. Fish Dis*. 26:71-75.

Rose, A.S., Ellis, A.E. and Adams, A. 1989. An assessment of routine *Aeromonas salmonicida* carrier detection by ELISA. *Bull. Euro. Assoc. Fish Pathol*. 9:65-67.

Smith, PD. 2000. Vaccines and vaccination- a widening choice. *Fish Farmer* 23(6): 45-53.

Sommerset, I., Krossoy, B., Biering, E. and Frost, P. 2005. Vaccines for fish in aquaculture. *Future Drugs, Expert Review of Vaccines* 4:89-101.

Starkey, W., Millar, R., Jenkins, M.E., Ireland, J.H., Muir, K. F. and Richards, R.H. 2004. Detection of piscine noda viruses using real time nucleic acid based sequence amplification (NASBA) *Dis. Aquat. Org.* 59:93-100.

Starkey, W.G., Smail, D.A., Bleie, H., Muir, K.F., Ireland, J.H., and Richards, R.H. 2006. Detection of infectious salmon anaemia virus by real-time nucleic acid sequence based amplification. *Dis. Aquat. Org.* 72:107-113.

Steiropoulos, N.A., Yuksel, S.A., Thompson, K.D., Adams, A. Ferguson, H.W. 2002. Detection of *Rickettsia*-like organisms (RLOs) in European sea bass (*Dicentrarchus labrax*, L.) by immunohistochemistry, using rabbit anti-*Piscirickettsia salmonis* serum. *Bull. Euro. Assoc. Fish Pathol*. 22: 428-432.

Stokes, N.A. and Burreson, E.M. 1995. A sensitive and specific DNA probe for the oyster pathogen *Haplosporidium nelsoni*. *J. Eukaryot. Microbiol*. 42: 350-357

Tang, K.F-J. and Lightner, D.V. 1999. A yellow head virus gene probe: application to *in situ* hybridization and determination of its nucleotide sequence. *Dis. Aquat. Org.* 35: 165-173.

Tang, K.F.J., Pantoja, C.R, Poulos, B.T., Redman, R.M. and Lightner, D.V. 2005. *In situ* hybridization demonstrates that *Litopenaeus vannamei*, *L. stylirostris* and *Penaeus monodon* are susceptible to experimental infections with infectious myonecrosis virus (IMNV).Dis. Aquat. Org. 63:261-265.

Thakur, P.C., Corsin, F., Turnbull, J.F., Shankar, K.M., Hao, N.V., Padiyar, P.A., Madhusudhan, M., Morgan, K.L. and Mohan, C.V. 2002. Estimation of prevalence of white spot syndrome virus (WSSV) by polymerase chain reaction in Penaeus monodon postlarvae at time of stocking in shrimp farms of Karnataka, India: a population-based study. *Dis. Aquat. Org*. 49: 235-243.

Thompson, K.D. and Adams, A. 2004. Current Trends in Immunotherapy and Vaccine Development for Bacterial Diseases of Fish (Chapter 13). *In* Leung Ka Yin (ed). Molecular Aspects of Fish and Marine Biology - *Vol. 3* Current trends in the study of bacterial and viral fish and shrimp diseases.

Thrusfield, M 1986. Veterinary Epidemiology. Blackwell Science Ltd, Oxford, London, pp. 47-75.

Umesha, K.R., Das, B.K.M., Naik, B.M., Venugopal, M.N., Karunasagar, I. and Karunasagar, I. 2006. High prevalence of dual and triple viral infections in black tiger shrimp ponds in India. *Aquaculture* 258:91-96.

Van Regenmortel, M.H.V. 1996. Mapping epitope structure and activity: from one-dimensional prediction to four-dimensional description of antigenic specificity. *Methods: a companion to Methods in Enzymology* 9: 465-472.

Vatsos, I.N., Thompson, K.D. and Adams, A. 2002. Development of an immunofluorescent antibody technique (IFAT) and *in situ* hybridisation to detect *Flavobacterium psychrophilum* in water samples. *Aqua. Res.* 33: 1087-1090.

Vatsos, I., Thompson, K.D and Adams, A. 2003. Starvation of *Flavobacterium psychrophilum* in broth, stream water and distilled water. *Dis. Aquat. Org*. 56: 115-126.

Wilson, T. and Carson, J. 2003. Development of sensitive, high-throughput one –tube RT-PCR-enzyme hybridisation assay to detect selected bacterial fish pathogens. *Dis. Aquatic Org.* 54: 127-134.

Yuce, A., Yucesoy, M., Genc, S., Sayan, M. and Ucan, E.S. 2001. Serodiagnosis of tuberculosis by enzyme immunoassay using A60 antigen. *Clin. Microbiol. Infect.* 7: 372-376.

Zhang, J.Y., Wu, Y.S. and Wang, J.G. 2004. Advance of phage display antibody library and its' implication prospect in aquaculture. *J. Fish. China* 28: 329–333.

Zhang, J.Y., Wang, J.G., Wu, Y.S., Li, M., Li, A.H. and Gong, X.L. 2006. A combined phage display ScFv library against *Myxobolus rotundus* infecting crucian carp, *Carassius auratus auratus* (L.), in China. *J. Fish Dis.* 29: 1-7.