Special Report: EUS Workshop

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Outcomes of a Short Expert Consultation on Epizootic Ulcerative Syndrome (EUS): Re-examination of Causal Factors, Case Definition and Nomenclature

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ABSTRACT

This paper presents the outcomes of a short expert consultation on epizootic ulcerative syndrome (EUS) held during the Fifth Symposium on Diseases in Asian Aquaculture (DAA V), Gold Coast, Australia in November 2002. The aims of the workshop were to review the body of knowledge on EUS, to provide an opportunity for experts to present mainstream and dissenting views on causal pathways and to re-examine issues relating to case definitions, the syndrome's name and fungal nomenclature. Workshop participants included five invited experts, two session moderators and DAA V attendees. It is now generally accepted that EUS is the same disease as mycotic granulomatosis (MG), red spot disease (RSD) and ulcerative mycosis (UM). In this paper, jointly developed after the workshop by participating experts and moderators, Japanese work on MG is reviewed and the findings related to work done on EUS in Australia, Southeast Asia, South Asia, the United Kingdom and UM in the United States of America. The majority of participating experts, supported by the weight of published evidence as well as ongoing research findings, held the mainstream view that EUS is essentially an aphanomycosis and that *Aphanomyces invadans* (= *A. piscicida*) is the only necessary infectious cause. Their arguments are juxtaposed with those of the minority of participating experts who asserted EUS is a polymicrobial infection, involving outbreak-

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specific viral, fungal and bacterial pathogens. A number of case definitions, appropriate for use in field surveys or for laboratory diagnosis, are proposed. The majority of experts supported a new name for the disease, 'epizootic granulomatous aphanomycosis' (EGA). It was further proposed that, in other than taxonomic contexts, the term *A. invadans* (= *A. piscicida*) be used in any initial reference to the putative causal fungal pathogen and that the name *A. invadans* be used thereafter. Key issues with a view to unifying the currently opposing views were identified including recommendation for further research work.

INTRODUCTION

The syndrome now called epizootic ulcerative syndrome (EUS) has caused major fish losses in many countries for over three decades and during that time has been given several colloquial names. It was first described in Japan in 1971 as *Aphanomyces* infection (Egusa and Masuda, 1971). The infection was found in other fishes and named mycotic granulomatosis (MG) based on histopathological findings (Miyazaki and Egusa, 1972). Since 1972, an epizootic cutaneous ulcerative syndrome in estuarine fishes in Australia has been termed red spot disease (RSD) (McKenzie and Hall, 1976). Similar conditions with dermal ulcerations and mortalities have occurred throughout Southeast and South Asia and the syndrome was given its present name in 1986 at the Consultation of Experts on Ulcerative Fish Diseases in Bangkok (FAO, 1986). In the United States, similar ulcerative lesions, designated ulcerative mycosis (UM) (Noga and Dykstra, 1986) have occurred in estuarine fishes along the east coast since 1978 (and perhaps before).

The literature and our knowledge of EUS through 1998 have been reviewed a number of times (Roberts *et al*., 1993; Chinabut, 1995; Roberts, 1997; Chinabut, 1998; Lilley *et al*., 1998). Egusa (1992) summarized the situation with MG in Japan while Noga (1993) assessed the findings regarding UM through the early 1990's. In the light of this information, it is now generally accepted that EUS is characterized by the presence of ulcerative, dermal lesions in which invasive fungal hyphae have elicited a granulomatous response. It is also generally accepted that despite extensive investigations in many affected countries in recent decades, no naturally occurring, epidemiologically similar but pathologically distinct cutaneous ulcerative syndrome has been found.

However, there remain two key areas of dispute among scientists, both relating to pathogenesis of the dermal ulcers. These differences were first brought into focus by the formal definition in 1994 of EUS as "a seasonal epizootic condition of freshwater and estuarine warm water fish of complex infectious aetiology characterized by the presence of invasive *Aphanomyces* infection and necrotising ulcerative lesions typically leading to a granulomatous response" (Roberts *et al*., 1994). Some scientists propose that a number of invasive fungal species, not necessarily including *Aphanomyces invadans* (= *A. piscicida*) in all cases, are involved in ulcer formation; others believe that only *A. invadans* is consistently present and responsible for the observed tissue destruction. Also disputed is whether or not various viruses or bacteria recovered from EUS cases have essential or merely opportunist roles in ulcer formation (Lio-Po, 1999; 2002).

These uncertainties and controversy have arisen for several reasons. The presence of many opportunistic organisms in the open ulcers on affected fish has complicated attempts to isolate a primary infectious agent. Contributing to this, until recently, has been a lack of

both molecular diagnostic techniques and a reproducible model for experimental induction. Moreover, a number of authors have failed to state the case definition for EUS used as a basis for their studies, while others failed to include histopathological findings, currently the recommended method for confirmatory diagnosis of EUS (OIE, 2003; Lilley *et al*., 1998). Finally, for many scientists, the problem has been exacerbated by difficulties accessing and accurately translating Japanese language publications on the topic.

In view of these uncertainties, it was deemed important to (a) discuss the current state of knowledge on EUS; (b) attempt to reach a consensus among experts on an appropriate case definition(s); and (c) establish consistency in naming both the putative fungal pathogen and the disease itself. Thus, an EUS Workshop was held on 25 November 2002 in conjunction with the Fifth Symposium on Diseases in Asian Aquaculture (DAA V) at the Gold Coast, Australia. Workshop participants included five invited experts, two session moderators and DAA V attendees (see Annex A).

This paper, jointly developed after the workshop by participating experts and moderators, summarizes the information presented at the expert consultation and its outcomes. To achieve this, Japanese work on EUS is reviewed extensively and the findings related to work done on EUS in Australia, Southeast Asia, South Asia, the UK and the USA. Issues relating to causation, case definitions and revised naming for EUS are then explored.

EUS AS AN APHANOMYCOSIS: JAPANESE STUDIES
(PRESENTED BY DR. K. HATAI)

A review of mycotic granulomatosis

Egusa and Masuda (1971) reported the first epizootic disease considered to be of fungal aetiology affecting cultured freshwater ayu (*Plecoglossus altivelis*) in Oita Prefecture, Kyushu Island. Although the bacterium *Aeromonas liquefaciens* was isolated from the ulcerative lesions, they were convinced that the disease was of a fungal aetiology based on a number of observations. Mortality was chronic with few fish dying each day beginning in early March and gradually increasing to a peak in late July and continuing through to September. A total of more than 500 kg of fish died. Clinical signs included localized swelling of the body wall, skin erosion and raised scales. In more advanced cases, the center of the lesion was necrotic, overlying skin and scales were missing and skeletal muscle exposed. The authors reported that the clinical signs of this disease were different from those of *Vibrio* infection. None of antibiotic treatments (e.g. sulfamonomethoxine, chloramphenicol, and nalidixic acid) applied were successful, suggesting that a bacterium was not the primary pathogen. Branched hyphae were consistently detected from tissue wet mounts under light microscopy from the skeletal muscle tissue of the ulcerated area of the lesions, with a diameter of 15-25 μm; and from the eroded external surface area of the lesion, with a thinner diameter of 5-7 μm. Histologically, hyphae were observed surrounded by epithelioid cells associated with fibrosis. Multi-nucleate giant cells (30-50 μm size) were relatively common. They also examined the zoosporangia but could not detect sexual stages. Information from this report was compared with reports of other Aphanomyces infections in other species such as in crayfish (Astacus astacus) and several species of tropical fish (*Lebistes reticulates, Anoptichythys jordani,* and cross breed between *Platypoecilus maculatus* and *Xiphophorus helleri*). It was concluded that the fungus belonged to the family Saprolegniaceae and the genus *Aphanomyces.*

More detailed pathology and the suggestion of a fungal aetiology were subsequently reported from naturally infected cultured goldfish (Miyazaki and Egusa, 1972), and ayu (Miyazaki and Egusa, 1973a). In goldfish, details of morphological changes of granuloma progression were described in tissues (e.g. gills, skin, skeletal muscle, brain and spinal cord) and internal organs (e.g. liver, spleen, pancreas, kidney, small intestine, gonads, and mesenteries). Observations on multi-nucleate giant cells at the lesion were also seen and varied depending on species affected and there appeared to be some geographic differences. The multi-nucleated cells were classified as two types: those which engulfed the fungal hyphae, which were larger in size with a maximum diameter of 90 μm and containing about 47 nuclei in the plane of the observed section; and those which did not. Fish from Oita, Miyazaki, and Tokyo areas exhibited multi-nucleate giant cells that engulfed hyphae (80-90% prevalence); while fish from Tokushima, Shiga, Nagano, and Tochigi Prefectures exhibited multi-nucleate giant cells without hyphae (80-100% prevalence).

Observations on behavior of the hyphae at different temperatures were also made. At 20°C, hyphae tended to invade deeper into the fish tissues, while at temperatures between 25°C and 30°C, hyphae actively grew outside the body surface around the lesions.

Miyazaki and Egusa (1973b, 1973c) further reported histopathological observations in other species such as bluegill (*Lepomis macrochirus*) and wild fish such as snakefish (*Channa argus*) from Chiba Prefecture, grey mullet (*Mugil cephalus*) from Kojima Bay, Okayama Prefecture, crucian carp (*Carassius auratus*), and trident goby (*Tridentiger obscurus*) from Lake Kasumigaura, Ibaragi Prefecture. Granulomatous inflammation was observed in all five species, consistent with earlier observations on goldfish and ayu. The general pathological features observed were dermatitis and myositis associated with deep penetrating granulomatous inflammation characterized by several layers of epithelioid cells surrounding the fungal hyphae; in some cases, multi-nucleate giant cells with or without fungal hyphae were detected, depending on species and geographic location. The disease was named ' mycotic granulomatosis' (MG).

Epidemiological studies conducted by Kumamaru (1973) from 1971 to 1972 in Lake Kasumigaura revealed that freshwater fish such as yellowfin goby (*Acanthogobius flavimanus*), grey mullet, oily gudgeon (*Sarcocheilichthys variegates*), trident goby and slender bitterling (*Acheilognathus lanceolatus*) were highly susceptible to MG; while carp (*Cyprinus carpio*), silver carp (*Hypophthalmichthys molitrix*) and eel (*Anguila japonica*) were not affected during the outbreaks.

Gross pathology and light microscopy

Egusa (1992) summarized the clinical signs of MG based on numerous published reports from Japanese studies from naturally infected fish (Egusa and Masuda, 1971; Miyazaki and Egusa, 1972; Miyazaki and Egusa, 1973a, b, c; Kumamaru, 1973; Hatai *et al*., 1977). These include localized swelling on the body surface, protruding scales, haemorrhage, scale loss, skin disintegration, exposure of underlying musculature and ulceration. Ulcers spread over a broad area and develop into a wide ulcer with exposed scarlet granuloma extending from several mm below the skin. A non-septate, branching form of fungal hyphae with a diameter of 10 to 25 μm and fungal granuloma were easily and consistently observed from tissue wet mount slide under light microscopy.

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Histopathology and clinical pathology

Histopathological observations made from both naturally infected fish (Egusa and Masuda, 1971; Miyazaki and Egusa, 1973a, b, c; Wada *et al*., 1994; Hatai *et al*., 1994; Hanjanvanit *et al*., 1997) and artificially infected fish (Hatai, 1977; Hatai, 1980; Hatai *et al*., 1994; Rha *et al*., 1996; Wada *et al*., 1996; Bondad-Reantaso *et al*., 1999b) showed similar pathological changes. Many branched aseptate hyphae were observed in the lesions. These hyphae were typically associated with a granulomatous response and extensive tissue necrosis. Marked inflammatory infiltration and formation of granulation tissue were also consistently observed in older lesions. Granulation tissue (a reparative response not to be confused with granulomas), comprising a fibrous network, regenerating capillaries and muscle fibers, mild haemorrhage and extensive inflammatory infiltration, developed to replace necrotic areas.

Mycology

Extensive mycological studies on MG were conducted beginning in the 1970's. The scope of the studies ranged from development of an artificial culture medium for MG fungus; effect of chemicals on mycelial growth; cultural, biological and biochemical characterization (Hatai and Egusa, 1978; Hatai and Egusa, 1979; Hatai *et al*., 1994, Bondad-Reantaso *et al*., 1999a); isolation of the fungus (Hatai, 1980); pathogenicity studies (Hatai *et al*., 1977; Hatai *et al*., 1984, Hatai *et al*., 1994; Rha *et al*., 1996; Wada *et al*., 1996; Bondad-Reantaso *et al*., 1999b); and description of the fungus (Hatai, 1980), named *Aphanomyces piscicida.* Fungal isolates recovered from ayu (Hatai, 1980), dwarf gourami (Hatai *et al*., 1994), ornamental fish (Hatai *et al*., 1994, Hanjanvanit *et al*., 1997; Rha *et al*., 1996) were in each case morphologically and culturally consistent with *A. piscicida.*

Japanese isolates were also provided to a number of scientists in different parts of the world and were used for comparative studies done by Lilley and Roberts (1997), Lilley *et al*. (1997b), and Blazer *et al*. (2002).

Artificial infection

A number of artificial infection experiments were carried out to reproduce the disease. The earliest was that of Hatai *et al*. (1977) who, after the successful isolation of the suspected fungi, injected cultured hyphae into the muscle of ayu and produced the same granuloma formation as seen in naturally infected fish. Hatai (1980) reproduced the disease by inoculating the hyphae of A. piscicida from ayu to a number of fish species (e.g. rosy bitterling (*Rhodeus ocellatus ocellatus*), carp, crucian carp, ayu, bluegill, goldfish (*C. auratus auratus*), rudd (*Scardinius erythrophthalmus*), rainbow trout (*Oncorhynchus mykiss*), eel, loach (*Misgurnus anguillicaudatus*), and catfish (*Parasilurus asotus*). In this study, rosy bitterling was highly susceptible, ayu, bluegill, crucian carp and goldfish were susceptible but the rudd and rainbow trout were less susceptible; and the carp, eel, loach and catfish were not susceptible. Hatai *et al*. (1994) used goldfish (*Carassius auratus auratus*) and Rha *et al*. (1996) used ayu as test animals to determine the pathogenicity of a strain of *Aphanomyces* isolated from an outbreak of disease associated with *Aphanomyces* infection among dwarf gourami (*Colisa lalia*) imported from Singapore, through zoospore injection. In another study, Wada *et al*. (1996) artificially infected ayu and carp with *Aphanomyces piscicida* using zoospore injection of *Aphanomyces piscicida* 1989 isolates (NJM 8997) from ayu. Bondad-Reantaso *et al*. (1999b)

conducted artificial infection using the methods of Wada *et al*. (1996) with *Aphanomyces* isolated from EUS-infected snakehead in Philippines in 1998, from MG-infected ayu in Japan in 1998 and EUS-infected mrigal in Bangladesh in 1999 to goldfish. All these experiments successfully reproduced the disease, re-isolated the fungus and the histopathological lesions were indistinguishable from those seen in natural cases.

Polymerase chain reaction (PCR)

On-going studies (Hatai, unpublished data) involve PCR methods using an ITS primer sequenced from *Aphanomyces piscicida*, NJM 0204 isolated from striped snakehead registered as accession number AY283640 in Genbank. A large number of isolates of *Aphanomyces* spp. from many fish collected from different countries were used in the study. These included 20 isolates of *Aphanomyces* recovered from EUS lesions in the following fish: grey mullet, yellow fin bream (from Australia); dwarf gourami (from Singapore); eel, snakehead (from Thailand); snakehead (from the Philippines); ayu, golden gourami (from Japan); menhaden (from the USA); *Aphanomyces astaci* from European crayfish (from England); 22 isolates of *Aphanomyces* not pathogenic to fish; 10 isolates of *Achlya* spp., 18 isolates of Saprolegnia spp., 4 isolates of *Lagenidium* spp. and 1 isolate of *Dicthyuchus.* Preliminary results showed that the 20 *Aphanomyces* pathogenic for fish tested positive for PCR, while the other non-pathogenic *Aphanomyces* isolates and other fungi tested negative.

Immunology

Studies on immunology include the reports of Bondad-Reantaso *et al*. (1999c), Sanpei *et al*. (1999), Kurata *et al*. (2000, 2002) and Kurata and Hatai (2002). These workers provided the earliest reports of a hemagglutinin in fish pathogenic peronosporomycete (Lilley *et al*., 2003); demonstrated a galactose-binding protein (GBP) from *A. piscicida* activated carp leukocytes which may be responsible for the inflammatory response unique to MG; and also provided some understanding how the host immune system (in this case carp which was proved not susceptible to MG) recognizes a microbial pathogen such as *A. piscicida.*

Other studies

Hatai *et al*. (1984) also studied the changes in blood constituents of natural and experimentally infected ayu, and their findings indicated that changes in the levels of various blood components may be characteristics of early stages of MG. Statistically significant differences in erythrocyte, hemoglobin, total cholesterol and glutamate pyruvate transaminase (GPT) were found between control and inoculated fish. Fish inoculated with *A. piscicida* SA7610 culture showed decreased levels of erythrocyte, hemoglobin, alkaline phosphatase (Al-P), leucine aminopeptidase (LAP) and total cholesterol; and increased levels of glutamate pyruvate transaminase (GPT) and blood urea-N (BUN).

Taxonomy

Hatai (1980) described a new species, *Aphanomyces piscicida*, based on the characteristics of the asexual reproductive stages of isolates. No oogonia were produced.

Aphanomyces piscicida was differentiated from *Aphanomyces* spp from other aquatic organisms (*i.e. A. laevis* of rainbow trout, *Aphanomyces* sp. of tropical fishes and *A. astaci* of crayfish).

In brief, diameter of hyphae range from 5-20 µm to 12-36 µm (measurements from samples soon after isolation). Hatai (1980) suggested that the diameter of the hyphae may change during many cultural passages and different cultural conditions. Zoosporangia of *A. piscicida* are simple, short, isodiametric or tapering with length ranging from 15 to 250 µm, most commonly 20-40 µm. A basal septum has not been observed between the zoosporangium and the hypha. Zoospore discharge is of achlyoid type, where the primary zoospores encyst at the tip of the zoosporangium, forming a cluster. Sometimes, zoospores leave the zoosporangium via lateral evacuation tubes.

Prior to sporulation, zoospores within sporangia are observed as ovoid shaped, lined up within the column as individual spores. At the tip of the sporangia, sporulated spores aggregate and become primary cysts. The number of zoospores generated from a sporangium is usually less than 10, but sometimes more than 20 zoospores are produced. A primary cyst excysts to become a secondary zoospore which posses two flagella of equal length. Zoospores are spherical, ranging in diameter from 5 to 23 µm, but commonly 8 to 9 µm. Germination commonly occurs from encysted secondary zoospores but, occasionally, primary cysts germinate at the tips of sporangia.

Other relevant studies in the area taxonomy include that of Nakamura *et al*. (1995) which determined the value of using ubiquinone systems as possible new taxonomic criteria for fungi belonging to the class Oomycetes; and Yuasa and Hatai (1996) on identifying some biochemical characteristics which can be used to differentiate between the fungal genera *Achlya, Aphanomyces,* and *Saprolegnia.*

Proposed re-naming and case definition

By fulfilling Koch's postulates based on injecting the putative pathogen, Japanese studies have produced strong evidence that *A. piscicida* is the primary pathogen of EUS. A new name is proposed – epizootic granulomatous aphanomycosis (EGA). A case of EGA is characterized by an epizootic fungal infection, ulcer formation is secondary, non-septate fungus and granulomas are always observed in the lesion, and the pathogen is a fungus of the genus *Aphanomyces*; confirmatory diagnosis is by PCR (Hatai, unpublished data).

EUS AS AN APHANOMYCOSIS: STUDIES IN AUSTRALIA, ASIA, UK AND USA
(PRESENTED BY DR. R. CALLINAN, DR. CV MOHAN AND DR. V. BLAZER)

Studies on EUS outside Japan followed detection of outbreaks in many other countries, beginning in the early 1970's in Australia and followed shortly thereafter by Southeast Asia, South Asia and the USA. The pattern of spread between and within countries was consistent with progressive dissemination of a single infectious agent. A number of comprehensive reviews of EUS were published in the 1990's (Roberts *et al*., 1993; Noga, 1993; Chinabut, 1995; Roberts, 1997; Chinabut, 1998; Lilley *et al*., 1998).

In the opinion of the above presenters, as these studies progressed, an indisputable body of evidence accumulated to support *A. invadans* (= *A. piscicida*) as the only necessary infectious cause of EUS. To illustrate this, features of EUS in which there is close agreement between Japanese studies and those done independently in distinctly different geographical locations – are presented in Table 1.

Table 1. Comparison of characteristic features of MG with RSD, EUS and UM.

	MG (Japan)	RSD (Australia)	EUS (Southeast Asia)	EUS (South Asia)	UM (USA)
Epizootic outbreaks in freshwater and estuarine fish populations	+	+	+	+	+
Characteristic macroscopic lesion is dermal ulcer	+	+	+	+	+
Characteristic histopathological lesion is granulomatous dermatitis and myositis associated with invasive fungal hyphae	+	+	+	+	+
Aphanomyces invadans (= *A. piscicida*) isolated	+	+	+	+	+
Histopathology not consistent with bacterial infection and no single bacterial species consistently recovered from lesions	+	+	+	+	+
Characteristic histopathological lesions reproduced by exposing fish to *A. invadans* (= *A. piscicida*)	+	+	+	+	+
Koch's postulates fulfilled by re-isolating *A. invadans* (= *A. piscicida*) from experimentally exposed fish	+	+	+	+	+

Additional findings of studies done outside Japan have had major implications for the issues under consideration in this paper and are summarized below.

Studies in UK

Lilley and Roberts (1997) provided convincing evidence that *A. invadans*, and not one or more other fungi, is responsible for much of the characteristic pathology of EUS. They injected zoospores from 58 fungal isolates intramuscularly into snakehead fish, *Channa striata*. These fungi comprised: *Aphanomyces* strains isolated in Asian countries and Australia from EUS-affected fish; saprophytic *Aphanomyces*, *Achlya* and *Saprolegnia* spp. from infected waters; and further saprolegniaceous fungi involved in other diseases of aquatic animals. Only the *Aphanomyces* strains isolated from fish affected by EUS, RSD or MG were able to grow invasively through the fish muscle and produce the distinctive EUS lesions. The snakehead-pathogenic strains were further distinguished from all the other fungi under comparison by their characteristic temperature-growth profile and inability to grow on certain selective fungal media.

In addition, Lilley *et al*. (1997a; 2003) used restriction fragment length polymorphism analyses of rDNA, sequencing the ITS1 region and random amplification of polymorphic DNA to confirm that 20 *A. invadans* isolates collected from EUS-affected fish in Asia and Australia, represented a single fish-pathogenic species. Furthermore, they showed that *A. invadans* was clearly distinct from a suite of other aquatic animal-pathogenic Saprolegniacieae and saprophytic Saprolegniaceae from EUS-affected countries. The results of the study indicated an extreme lack of genetic diversity between all the *A. invadans*

isolates, which the authors considered not only conspecific, but probably representing a single clonal genotype. On the basis of these findings, the authors suggested that the fungus achieved its colonisation of Australia, Asia and by implication USA, in one relatively rapid episode, consistent with reports of outbreak occurrence.

Studies in Australia

Two key field observations (Callinan, 1997) directed laboratory-based studies of EUS in estuarine fish in Australia. First, EUS prevalence in susceptible wild estuarine fish populations in the main channel of the Richmond River, NSW was highest at sampling sites close to junctions with tributaries containing acidified runoff water from acid sulfate soil (ASS) areas. Second, fish sampled from a wild, EUS-susceptible freshwater population, confined in a drainage canal and exposed naturally to runoff water (pH <4) from the surrounding ASS area following a rain event, showed severe necrotizing dermatitis.

Preliminary tank trials had shown that healthy, intact fish in aquaria exposed to *A. invadans* zoospores in the water did not develop EUS lesions and that prior damage to skin was necessary before lesions could be induced in fish. In follow-up tank trials, severe epidermal necrosis was induced in fingerling sand whiting *Sillago ciliata* sublethally exposed to ASS runoff (pH 3.1). Mild to moderate epidermal necrosis was induced in fish sublethally exposed to less acidic ASS runoff (pH 5.1). Typical EUS lesions, *i.e.* necrotizing granulomatous dermatitis and myositis associated with invasive non-septate fungal hyphae, were induced significantly more often than in controls when these fish were subsequently exposed to *A. invadans* zoospores. The fungus was recovered on culture from affected fish. These results had several important implications. They confirmed *A. invadans* as a primary infectious agent of EUS, and indicated that disruption of epidermal continuity may be a necessary precursor to fungal attachment and lesion induction (Callinan, 1997). They also confirmed that a sufficient cause for EUS comprised the putative necessary cause, *A. invadans*, together with an abiotic component cause, in this case chemically-induced epidermal damage. There was no evidence from these studies that other biotic agents, such as viruses or bacteria, were necessary causes.

Studies in India

Experimental laboratory infections. Co-habitation often failed to reproduce the disease. Under the recently completed IFS funded project (1999-2001, unpublished data), experimental infection studies were conducted to evaluate the disease susceptibility and sequential inflammatory response among different age groups of Indian major carps (*Catla catla, Labeo rohita, Cirrhinus mrigala*). Comparisons were made with corresponding age groups of susceptible snakeheads (*Channa* sp.), *Puntius* sp. and resistant common carp. Experimental infections were carried out using *Aphanomyces invadans* (Strain B 99C, provided by J.H. Lilley). Bath exposure to zoospores often failed to reproduce the disease. However, on a few occasions, small numbers of fish were successfully infected using this method of challenge. On the other hand, injection of spores under the dermis consistently reproduced the clinical and pathological features of the disease.

Size 7.25 x 10 inches

There is experimental evidence of variation in resistance to *A. invadans* infection among species and age classes. The fry and fingerlings of Indian major carp appear to be susceptible to *A. invadans* infection whereas yearlings appear to be resistant. In Indian major carp, there is also evidence for increased resistance with age but this does not appear to be the case for puntius and snakeheads. The cellular defense mechanisms against the fungus appear to be more well developed in yearlings of Indian major carp and advanced fingerlings and yearlings of common carp when compared to snakeheads and puntius of a similar age. In the more resistant species and age classes, few spores appear to germinate and the resulting fungal hyphae are confined and killed by very well developed epithelioid granulomata (Mohan, unpublished data)

Monoclonal antibody-based diagnosis. Recently, monoclonal antibodies against *Aphanomyces invadans* (Strain B 99C, provided by JH Lilley) have been developed. The monocloncal antibodies using a immunoperoxidase test have consistently reacted with fungal hyphae in tissue sections of experimentally infected fish, suspected EUS fish collected from different parts of India during 2002, and on retrospective EUS tissue samples of 1995 and 1997. This evidence further supports the view that a single fungal pathogen is involved in all EUS cases investigated.

A simple immunodot test on nitrocellulose paper has been developed and tested on several suspected EUS samples. In the immunodot test, consistent results are obtained if the sample for dotting is taken from below the ulcer followed by the sides and contra-lateral part of the ulcer. Samples dotted from the surface of the ulcer do not give consistent results, possibly suggesting that there are no fungal antigens in this area. The results of both immunoperoxidase and immunodot tests have been very consistent and agree very well with histopathology results (Gayathri *et al*., unpublished data).

Studies in Thailand

Kanchanakhan *et al*. (2002) induced EUS lesions in 20/20 juvenile snakehead fish (*Channa striata*) held at 20°C and injected intramuscularly with a rhabdovirus (strain T9412) followed by bath challenge with *A. invadans* spores. Although fish which received growth medium only by injection, followed by spore challenge, also developed EUS, significantly fewer (7/20) were affected. Rhabdovirus injection alone induced only small haemorrhagic lesions at the injection site and most had healed by the end of the experiment. A similar induction experiment conducted at 29°C failed to induce EUS. The authors concluded that one possible combination of events leading to EUS lesion induction in snakehead fish is low temperature and infection with rhabdovirus followed by and *Aphanomyces* challenge.

Studies in the USA

Experimental laboratory infections. Kiryu *et al*. (2002) were able to reproduce the lesions by both injection and bath exposure to *A. invadans* zoospores. In dose-response studies with menhaden (*Brevoortia tyrannus*) it was demonstrated that 31% of fish injected with as few as 1 zoospore developed characteristic EUS lesions within two weeks. The LD_{50} by injection was estimated to be only 10 zoospores per fish (Kiryu *et al*., 2003). By bath exposure to 100 zoospores ml^{-1}, 14% of exposed, untraumatized fish developed ulcers while 64% of those handled (net-stressed) developed ulcers (Kiryu *et al*. 2003).

These studies comply with Koch's postulates and demonstrate that the oomycete is a primary pathogen and is highly virulent. However, they also indicate that a greater infectivity rate is achieved when the epidermal layer has been noticeably compromised.

A summary of key arguments supporting EUS as an aphanomycosis is presented in Box 1 below.

Box 1. Key arguments supporting EUS as an aphanomycosis.

EUS as an aphanomycosis

Key arguments summarized

- Although it affects diverse fish species in many countries, EUS has consistent epidemiological and pathological features. No epidemiologically similar but pathologically distinct syndrome has been found.
- The 1994 case definition incorrectly states that EUS necessarily has a complex infectious aetiology. Aphanomyces invadans is the only necessary infectious causal agent and is the only pathogen involved in some, but not all, outbreaks.
- Histopathological evidence indicates that invasive fungal hyphae alone are responsible for the granulomatous inflammatory response and most, if not all of the tissue damage which are the characteristic and dominant features of EUS lesions.
- Provided rigorous attention is given to obtaining uncontaminated inocula and suitable culture conditions are used, A. invadans can be consistently recovered from progressing, but not resolving, EUS lesions.
- Typical lesions can be consistently reproduced and Koch's postulates can be consistently satisfied when susceptible fish species/life stages are exposed by a variety of routes to A. invadans zoospores or hyphae. Noticeable artificial disruption of the epidermal barrier is usually, but not always, necessary to induce infection.
- Intramuscular injection of zoospores of saprophytic Aphanomyces, Achlya and Saprolegnia spp., as well as saprolegniaceous fungi involved in other diseases of aquatic animals all failed to induce histopathological lesions consistent with EUS.
- Many environmental stressors, handling and other trauma, and possibly other infectious organisms may act as predisposing factors, making the fish more susceptible to natural and experimental infection with A. invadans.
- Compartive molecular studies of A. invadans isolates from several countries showed an extreme lack of genetic diversity consistent with a single clonal genotype, suggesting that the fungus achieved its wide colonization in one relatively rapid episode in the period 1970-1996.

EUS AS A POLYMICROBIAL INFECTION: AN ALTERNATIVE VIEW
(PRESENTED BY DR. I KARUNASAGAR)

In human and veterinary medicine the concept of polymicrobial disease is well accepted, and available evidence suggests EUS could be placed in this category. Specifically, EUS can be described as a polymicrobial disease precipitated by environmental insults, with an early viral stage, a subsequent mycotic stage and bacterial involvement at the ulcerative stage. Keeping the short generation time of bacteria in contrast to the fungi in mind, it would appear that following skin damage, the bacteria would precede the fungi in its entry and subsequent activity.

Brogden and Guthmiller (2002) state that polymicrobial diseases represent the clinical and pathological manifestations induced by multiple microorganisms. They are serious diseases whose causal agents are sometimes difficult to identify and difficult to treat. In animals or humans they can be induced by, for example, polymicrobial infections involving viruses and bacteria, polymicrobial infections involving fungi and parasites, and polymicrobial infections as a result of microbe-induced immunosuppression. There are five common underlying mechanisms in the pathogenesis:

- Physical, physiologic, or metabolic abnormalities and stress predispose the host to polymicrobial disease;
- One organism induces changes in a body surface that may favour colonization by other organisms;
- Microorganisms or their products can trigger proinflammatory cytokines to increase the severity of disease, reactivate latent infections, or favour the colonization of other microorganisms.
- Organisms may share determinants among each other allowing them the ability to damage tissue.
- One organism can alter the immune system, which allows the colonization of the host by other microorganisms.

Recognition of EUS as a polymicrobial disease may have been hindered by studies which have focused on single categories of putative pathogens, for example on fungi (Roberts *et al*., 1993; Willoughby and Roberts, 1994) or viruses (Kanchanakhan *et al*., 1999; Lio-Po *et al*., 2000). When it is considered that most EUS cases involve open ulcers it is only to be expected that there will be tissue invasion by opportunist pathogens such as bacteria. Accordingly, some of the tissue damage, sloughing and necrosis seen in EUS ulcers may be the result of highly proteolytic bacteria involved in liquefaction of tissue. In our studies, all bacterial isolates from ulcers on EUS-affected fish possessed the ability to produce several toxins and enzymes (Karunasagar and Karunasagar, 1994; Karunasagar *et al*., 1995).

Case definitions for a disease may be based on clinical signs or other findings and may not require that the primary cause of the disease has been identified. In the case of EUS, doubts remain regarding pathogenesis and conclusive identification of a primary infectious agent. For any microbial disease, Koch's postulates need to be satisfied and it can be argued that for EUS this has not been satisfactorily achieved. Consequently, an alternative view on EUS and its case definition are presented here.

Mycology

It is important to note that identification of fungal species in tissue sections, based on histopathology alone, cannot be conclusive. In addition, the isolation rate for *A. invadans* from cases of EUS has been variable although low isolation rates have been ascribed to the presence of dead mycelia in the lesions (Roberts *et al*., 1993; Callinan *et al*., 1995). In our laboratory, we attempted to isolate *A. invadans* from fish showing clinical signs consistent with EUS using the method of Willoughby and Roberts (1993); the fungus was recovered from only 5% of these fish.

Experimental reproduction of EUS lesions in various fish species has often been undertaken either through intramuscular injection of fish with a high number of viable zoospores or small pieces of fungal hyphae or by exposure of scarified fish to spores (Roberts *et al*., 1993; Hatai *et al*., 1994; Callinan, 1994). A high rate of lesion induction in fish without a breach of the physical barriers of the skin has not been demonstrated, although Kiryu et al. (2002) reported reproduction of lesions by both injection and bath exposure, with lesion induction in 14% of fish exposed by the latter route.

In this situation, defining EUS as an epizootic in which *A. invadans* is found, precludes further investigations on the involvement of other microorganisms in the epizootic. In an outbreak of ulcerative disease in fish in Pakistan, in two locations fed by the same river, experts identified two distinct diseases, though macroscopic lesions were similar: one as EUS because *A. invadans* was found and the other as an unidentified ulcerative disease because the fungus was not found. This example illustrates how an inappropriate case definition could lead to mislabeling of an outbreak. The fish-fungus relationship is less specific if we consider other fungi such as *Achlya, Saprolegnia* and other *Aphanomyces* that have been isolated from EUS-affected fish in different parts of Asia (Karunasagar, unpublished data).

To study the genetic diversity of *A. invadans* isolated from EUS-affected fish, we compared the RAPD patterns of two of our isolates and two isolates from Thailand (kindly provided by JH Lilley). Our results (unpublished data) indicate genetic diversity among the few (4) isolates that have been studied using four RAPD primers.

Bacteriology

Bacteria, particularly motile aeromonads have been associated with the surface of lesions in EUS (Llobrera and Gacutan, 1987; Lio-Po *et al*., 1992; Karunasagar and Karunasagar, 1994). Studies in our laboratory show a high prevalence of motile aeromonads in all lesions (n= 26), and motile aeromonads were also recovered from internal organs of ulcerated fish indicating systemic invasion (Karunasagar *et al*., 1995). Although the phenotypic diversity and variations in virulence suggest that the bacterial infection is secondary in nature, the role of bacteria at the ulcerative stage of the disease cannot be ignored, even though they are not the primary cause of EUS. Fish being very rich in non-protein nitrogenous substances, it is to be expected that these are readily utilized by bacteria and proliferate. This is due to their short generation time and ready nutrient availability without fungal flora being able to compete due to their longer generation time. The more complex proteins are available for the fungi to utilize after the non-protein simple nitrogenous substances are exhausted by

Size 7.25 x 10 inches

the bacterial flora. It is therefore logical to conclude that bacteria precede the fungi in their degradative activity an ulcer formation.

Box 2. Key arguments supporting EUS as a polymicrobial infection.

EUS as a polymicrobial infection

Key arguments summarized

- The 1994 case definition correctly states that EUS has a complex infectious aetiology but incorrectly limits invasive fungi to the genus *Aphanomyces*.
- A number of opportunistic, invasive fungi may be involved in EUS ulcer formation. Conventional histopathological examination alone cannot reliably distinguish *Aphanomyces invadans* hyphae from hyphae of some other non-septate fungi in tissue sections.
- In some studies, *A. invadans* has been recovered from only a small proportion of EUS cases. Other fungi such as *Achlya, Saprolegnia* and other *Aphanomyces* sp. have been isolated from EUS lesions in several countries and may have causal roles.
- Attempts to reproduce EUS and satisfy Koch's postulates using *A. invadans* infection have, in most reported studies, required artificial disruption of the epidermal barrier in susceptible fish species and life stages.
- Viral infections may initiate dermal lesions in natural outbreaks, while at least some of the tissue damage, sloughing and necrosis seen in EUS ulcers may be the result of highly proteolytic bacteria involved in liquefaction of tissue.
- Comparative molecular studies of *A. invadans* isolates from affected countries need to be repeated before firm conclusions can be drawn about their relatedness.

Virology

Virological studies can be carried out only in a few laboratories which have appropriate cell lines and access to an electron microscope. This could account for the paucity of studies on virological aspects of EUS. Of those studies which have been done, a range of viruses have been recovered (Frerichs *et al*., 1986; Frerichs, 1995; Kanchanakhan *et al*., 1999), and it may be too early to disregard their involvement in lesion induction in some outbreaks. Evidence suggests that *A. invadans* can more readily invade and induce lesions in fish with damaged, compared with intact skin (Callinan, 1997; Kiryu *et al*., 2002). The studies of Kanchanakhan *et al*. (1999) show that rhabdoviruses could be isolated only from samples collected during the early stages of outbreaks in snakeheads in Thailand. There is convincing experimental evidence that rhabdovirus infection can induce skin lesions which, in providing a portal of entry for *A. invadans*, results in EUS.

Some of the main problems in establishing a case definition lie in deciding when an animal actually can be considered to have the disease in question and what actually "causes" that disease.

In its broadest sense, disease can be defined as *any condition impacting on an animal which may be deleterious to animal or human health.* In the case of infectious disease, there is the

additional issue of deciding at what stage(s) in the entire process from initial infection through to the eventual outcome (e.g. recovery, disability or death) do we classify the animal as diseased. For example, do we consider a fish with EUS diseased only when it has gross lesions which are visible to the naked eye or is it more appropriate to say it is diseased once pathological changes can be detected by histopathology or some more indirect means such as an immunoassay? The issue is further complicated when carrier states exist – is a carrier considered to be diseased? It is because there are so many ways that we can consider an animal as being diseased, that scientists have developed the concept of having case definitions which is discussed in the next section.

Key arguments supporting EUS as a polymicrobial infection are presented in Box 2 above.

DEVELOPMENT OF A CASE DEFINITION

Disease and causation

Deciding on what actually "causes" a particular disease can also be a challenge. Following the realisation that many human epidemics were caused by infectious agents, the late 19th century saw the development of a deterministic view of causality, *i.e.* agent "X" produced disease "Y". Specificity of both cause and effect was implied. The development of the Henle-Koch's postulates reinforced this view and was helpful in formulating the link between microorganisms and disease:

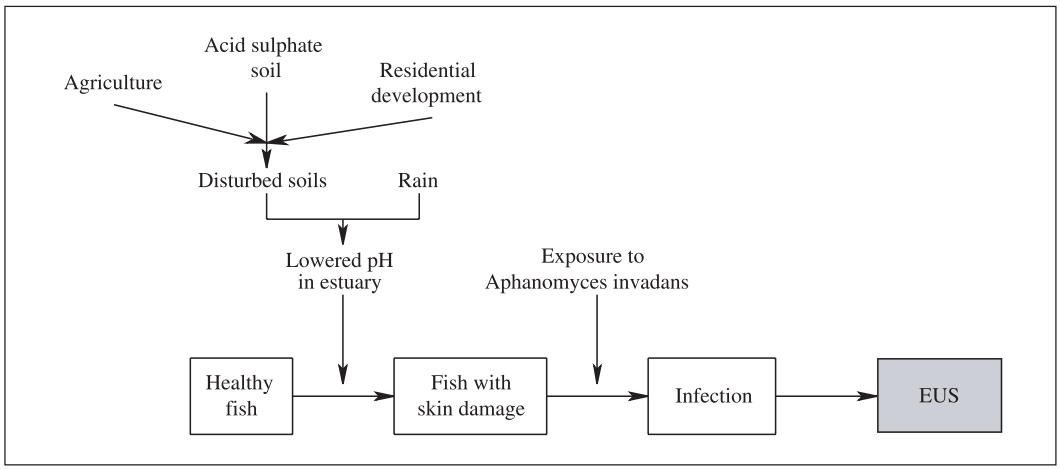
1. The agent must be present in every case of disease by isolation in pure culture.
2. The agent must not be present in other diseases.
3. Once isolated, the agent must be capable of inducing disease in experiments.
4. The agent must be recovered from the experimental disease produced.

As scientific understanding of disease processes developed through the 20th century, Henle-Koch's postulates were considered too restrictive in thinking about causality for many human diseases for a number of reasons including:

Multiple aetiologic factors	e.g. combination of many factors resulting in heart disease
Multiple effects of single factors	e.g. relationship of smoking to both cancers and heart disease
Carrier states	e.g. hepatitis B
Quantitative causal factors	e.g. amount and period of smoking
Non-agent factors	e.g. age, sex

This led to a broader definition of "cause" to embrace this expanded understanding and to facilitate epidemiological studies which might uncover less direct interventions to mitigate against disease. Thus, the concept of cause was expanded to include *an event, condition or characteristic that plays an essential role in producing an occurrence of the disease in question.*

For infectious diseases where a specific aetiologic agent can be recognised, we can combine the two concepts by nominating the agent as the "cause" and other factors which may also be necessary to produce disease in some instances as predisposing or enabling factors. For example, under this model, acidic water might be seen to predispose otherwise healthy fish to infection with *Aphanomyces invadans* by damaging the skin. Under this model, the evidence suggests it is the actual skin damage which leads to infection and subsequent development of lesions and other factors could be important in producing such skin damage in other situations. We can extend this view of causality even further to link events and factors into a web or chain of causation as indicated in the diagram below which attempts to explain what precipitates EUS outbreaks in some estuaries in eastern Australia where there are acid sulphate soils.



Establishing a case definition

A case definition is neither right nor wrong in terms of diagnosing a disease, it is simply an agreed set of rules which permits investigators to uniformly decide that a particular individual has or does not have a particular disease as defined. It is the "as defined" part that is important here. In addition, it may be appropriate to develop a set of rules that will define both suspect and confirmed cases. An example for mad cow disease (bovine spongiform encephalopathy or BSE) in the United Kingdom is shown in the box below.

Reported or suspect cases: cattle reported to MAFF (or to DANI in Northern Ireland) with clinical signs that might indicate BSE, and where the Veterinary Officer cannot rule it out.

Confirmed cases: those cases in which the diagnosis of BSE has been confirmed by histopathological examination of brain tissue or by electron microscopy examination for scrapie-associated fibrils (SAFs)[1].

By using these standardised criteria, the UK government has been able to keep reliable statistics on the course of the BSE epidemic over many years.

[1] From the official BSE inquiry - http://www.bseinquiry.gov.uk/report/volume16/chaptea2.htm#13383

This leads us to a more formal understanding of what a case definition is as shown in the box below.

***A case definition** is a set of standard criteria for deciding whether an individual study unit of interest has a particular disease or other outcome of interest. The study unit may be an individual animal or a group of animals such as a pond of shrimp, a cage of fish, an entire farm or a village.*

A useful approach to development of a case definition for aquatic animal diseases is given by Stephen and Ribble (1996).

An optimal case definition depends on criteria that can be applied to any potential case in the source population. In many instances, it will be difficult to define a set of criteria that will include all true cases of the disease of interest and exclude all similar, but unrelated conditions. Few cases will show the complete range of disease criteria and there will always be some non-cases which have some *criteria* (e.g. clinical signs) similar to those of the particular disease being investigated.

Some examples of case definitions which might be used when investigating white spot disease (WSD) in shrimp are given in Table 2. The choice of a particular case definition will depend on the objectives and methods used in the investigation. No matter what case definition is used, it will not be perfect. In fact, case definitions are subject to the same types of errors as screening and diagnostic tests in general, i.e. they are subject to random (lack of precision) and non-random (false negative and false positive) errors. For example, we know that some outbreaks of WSD can have some or most affected shrimp with no white spots. Thus, the first case definition below will result in some false negative results where the study unit is an individual animal. False negative results are due to lack of sensitivity, while false positive results are due to lack of specificity. In any test system, there is always a trade-off between sensitivity and specificity – as we increase one, there is a related decrease in the other.

Table 2. Examples of case definitions for white spot disease (WSD) in shrimp.

Study Unit	Case definition
Animal	A shrimp with one or more visible, discrete white patches on the inside of the carapace.
Animal	A shrimp which returns a positive PCR result for white spot syndrome virus.
Pond	A pond where one or more shrimp have one or more visible, discrete white patches on the inside of the carapace.
Pond	A pond where one or more shrimp return a positive PCR result for white spot syndrome virus.
Pond	A pond subject to emergency harvest because, in the opinion of the manager, there is a risk of mass mortality from white spot syndrome.

It is often useful to have definitions for a suspect case based on field observations (*i.e.*, history, clinical signs, gross pathology, etc.) and a *confirmed case* based on laboratory findings especially where it may take some time to confirm cases. Where a previously unrecognised and potentially serious syndrome is being investigated, it is advisable to initially use a very broad case definition (high sensitivity but lower specificity) to minimise the risk of missing any cases. In this instance, a revised classification can be applied later when time permits.

Development of a working case definition for EUS

At the workshop, a number of brief expert presentations were presented followed by a general discussion where the first three case definitions were proposed. The other three are additional possible case definitions.

Table 3. Draft case definitions for EUS.

Study Unit	Case definition
Animal	A fish with necrotising, granulomatous dermatitis and/or myositis and/or granulomas in internal organs with *A. invadans* (=*A. piscicida*) found within the lesion.
Animal	A fish with one or more granulomas with *A. invadans* (=*A. piscicida*) found within the lesion.
Animal	A fish with lesions in which *A. invadans* (=*A. piscicida*) can be found.
Animal	A fish with one or more surface lesions each of which could be described as a "red spot".
Pond	A pond with one or more fish meeting the selected case definition for an individual animal.
River	A river with one or more fish meeting the selected case definition for an individual animal.

An appropriate name for the disease described in the first three case definitions would simply be *aphanomycosis* while an appropriate name for the fourth would be *red spot*. If the consensus among experts is that EUS is a specific condition involving tissue damage due to *A. invadans* (= *A. piscicida*) regardless of the pre-disposing factors, then it could be called *aphanamycosis* as this implies that infection by one or more *Aphanomyces* sp., in this case *A. invadans* (= *A. piscicida*), is a necessary (although usually not a sufficient) cause.

All of the case definitions in Table 3 are legitimate. It should also be noted that the animal case definitions in Table 3 range from being very specific (but less sensitive) for the first through to very sensitive (but less specific) for the fourth.

How then should these case definitions be used? A particular case definition may be more appropriate depending on the objective of the application. For example, say we are interested in the early detection of *aphanomycosis* in an area because the disease has never been reported and we think it is exotic. In this situation, we are interested in early detection and would want to know about any fish which could possibly be a case, *i.e.*, we want a very sensitive case definition. We would probably choose the "red spot" definition to identify suspect cases and then subject these to laboratory examinations aimed at detecting *Aphanomyces piscicida* (= *A. piscicida*). If we found evidence of the fungus, we would then have a confirmed case.

DISCUSSION

It is now generally accepted that EUS, however it is named or defined, is a cutaneous ulcerative syndrome which has spread widely and caused serious losses in many freshwater and estuarine fish populations in Asia, Australia and the USA since the 1970s. It is also widely accepted that the unifying pathological feature of the ulcerative lesions, wherever outbreaks have been studied, is the presence of invasive fungal hyphae which have elicited a granulomatous response. Despite numerous investigations in most affected countries during this period, no other epidemiologically similar but pathologically distinct epizootic cutaneous ulcerative syndrome naturally affecting freshwater and/or estuarine fishes has been reported.

The majority of experts at the workshop held strongly to the view that *A. invadans* is a necessary cause, and in some situations possibly a sufficient cause, of EUS. They further asserted that a sufficient cause for most outbreaks requires, in addition to *A. invadans*, involvement of one or more biotic or abiotic component causes, such as virus infection or cutaneous trauma. Individual component causes, as distinct from the proposed necessary cause, *A. invadans*, are not involved in all outbreaks.

A minority of experts held a different view. They asserted that EUS is a polymicrobial infection with involvement of virus, fungus and bacteria, precipitated by environmental insults. They further suggested that invasive fungi other than *A. invadans* may be involved in some cases.

In this discussion, we explore ways of reconciling these positions.

Diagnosis

Correct diagnosis is a basic requirement of any disease investigation and is dependent on the case definitions used. Three levels of diagnosis (Levels I, II and III) have been defined to assist in the surveillance and control of aquatic animal disease in Asia (Bondad-Reantaso *et al*. 2001). Level I diagnosis can be made on the farm without any laboratory confirmation. Level II diagnosis requires some laboratory support, while Level III requires the use of advanced laboratory techniques. At the field level, suspect cases may be identified using macroscopic criteria. Currently, to confirm a case of EUS using OIE criteria, the first step is to demonstrate the presence of mycotic granulomas in a histological section (Level II diagnosis) and the second is to isolate *A. invadans* from internal tissues (Level II diagnosis) (Lilley *et al*., 1998; OIE, 2003).

A number of earlier studies on EUS, which focused on putative pathogens other than fungi, often lacked the histopathological observations essential to confirming EUS cases. The studies of Torres *et al*. (1992), Lio-Po *et al*. (1992); Cruz-Lacierda and Shariff (1995), Karunasagar *et al*. (1995), Leano *et al*. (1995), Iqbal *et al*. (1998), and Lio-Po *et al*. (2000, 2001) used only macroscopic lesions consistent with EUS to identify putative cases. By contrast, those studies focusing on fungi as potential primary pathogens for EUS used histopathological examination to confirm cases (see for example, Bondad-Reantaso *et al*., 1992; Callinan *et al*., 1995; Mohan and Shankar, 1995; Lumanlan-Mayo *et al*., 1997; Khan *et al*., 1998; Vishwanath *et al*., 1998); two other studies also used histopathology to determine health status of fish samples (Kanchanakhan *et al*., 2002; Pathiratne *et al*., 2002).

There is compelling evidence that the OIE diagnostic criteria are correct. In a recent study, monoclonal antibody based immunofluorescent staining was found to be more sensitive than conventional staining methods for detecting *A. invadans*. Such specific methods may allow early detection of the disease and have utility in confirming the case definition of EUS (Miles *et al*., 2003). In another study, monoclonal antibodies against *A. invadans* consistently reacted with fungal hyphae in tissue sections from experimentally infected fish, from naturally occurring cases collected in India during 2002, and on retrospective EUS cases collected in 1995 and 1997 (Gayathri *et al*., unpublished data). There are no reports associating the fungus with other diseases. However, to conclusively resolve this issue, immunohistochemical techniques must be applied to tissue sections from representative EUS and other ulcerative disease outbreaks, using current and archived material, from a variety of countries. Only in this way can the consistent and sole presence, or otherwise, of *A. invadans* in the mycotic granulomas of EUS be established.

Mycology

Although one of the controversies surrounding EUS has been the varied success in isolating the putative causal fungus, many workers have successfully and consistently isolated *A. invadans* from EUS affected fish. Since the first successful isolation of fungus in Japan (Hatai *et al* 1977; 1980), there followed successful isolations from naturally infected fish in other countries such as Australia, Bangladesh, Philippines, India, Thailand and the US (Fraser *et al*., 1992; Roberts *et al*., 1993; Paclibare *et al*., 1994; Chinabut *et al*., 1995; Willoughby and Roberts, 1994; Callinan, 1997; Bondad-Reantaso, 1999b; Blazer *et al*., 1999, 2002). Rigorous application of improved isolation methodology (Fraser *et al*., 1992; Willoughby and Roberts, 1994) and standardization of methods for growing and sporulating the fungus (Lilley *et al*., 1998) have greatly increased the rate of successful recovery and thereby greatly aided our understanding of EUS causation.

Disease reproduction and Koch's postulates

For an infectious organism to cause disease it must come into contact with and be able to invade a susceptible host. Host susceptibility may depend on numerous innate factors as well as environmental, nutritional or toxic influences that affect disease resistance. For over 100 years Henle-Koch's postulates have been used in evaluating the causal relationship of a new infectious agent to a clinical disease (Davis *et al*., 1980).

Application of Koch's postulates distinguishes a pathogenic from an adventitious microbe (Davis 1980). The criteria used are: (1) the organism is regularly found in the lesions of the disease; (2) it can be isolated in pure culture on artificial media; (3) inoculation of this culture produces a similar disease in experimental animals; and (4) the organism can be recovered from the lesions in these animals.

In relation to *A. invadans* as the causal infectious agent, there is strong evidence that the postulates have been satisfied. Histological examination of lesions consistent with EUS from all affected countries shows, beyond reasonable doubt, that one or more invasive fungi is responsible for most, if not all, the host response and tissue destruction. As noted above, numerous studies have now shown that *A. invadans* can be consistently recovered from progressing, as distinct from resolving, lesions provided rigorous attention is given to

Size 7.25 x 10 inches

obtaining uncontaminated inocula and suitable culture conditions are used (Lilley *et al*., 1998; Blazer *et al*., 2002).

Many studies have now fulfilled Koch's postulates and proved that *A. invadans* (= *A. piscicida*) is a primary infectious cause of EUS. These include the works of Hatai *et al*. (1977), Hatai (1980), Hatai *et al*. (1984), Hatai *et al*. (1994), Rha *et al*. (1996), Wada *et al*. (1996), Callinan (1997), Bondad-Reantaso *et al*. (1999b), Catap and Munday (2002), Kiryu *et al*. (2002) and Kiryu *et al*. (2003). However, there are apparent differences between studies in rates and severity of lesion induction (see for example Callinan, 1997; Lio-Po *et al*., 2002; Kiryu *et al*., 2003). These differences could be due, at least in part, to differences in susceptibility of exposed fish species and progressive loss of pathogenicity and/or virulence by *A. invadans* isolates maintained for different periods on artificial media.

Although a number of viruses, bacteria and putatively saprophytic fungi have been inconsistently recovered from EUS lesions, attempts to fulfill Koch's postulates and reproduce EUS lesions using these agents alone have been uniformly unsuccessful. For example, an *Aphanomyces* sp. (not *A. invadans*) first isolated from UM lesions in menhaden was not capable of inducing lesions (Noga, 1993) and it is now considered to be a saprophytic species (Blazer *et al*., 2002). Similarly, Lilley and Roberts (1997) failed to induce lesions histopathologically consistent with EUS by exposing fish to numerous other fungi, including isolates from EUS-endemic areas. However, as noted above, immunohistochemical or *in situ* hybridization studies are required to conclusively prove whether or not *A. invadans* is the only fungus involved in the granulomatous lesions characteristic of EUS.

Viruses isolated from EUS lesions include a birnavirus (Wattanavijarn *et al*., 1985) several rhabdoviruses (Frerichs *et al*., 1989; Kasornchandra *et al*., 1992; Kanchanakhan *et al*., 1998), reoviruses and a distinct group of type-C retroviruses (Frerichs *et al*., 1993). Frerichs *et al*. (1986) suggested a virus to be the causative agent of EUS. As noted above, infection experiments conducted using a rhabdovirus alone induced ulcers, but not EUS lesions, in exposed striped snakehead (Kanchanakhan *et al*., 2002); EUS lesions were induced only in fish exposed first to rhabdovirus, then *A. invadans.*

Numerous bacteria, including *Aeromonas, Vibrio* and *Plesiomonas* spp. have also been isolated from ulcerative lesions in fish (Iqbal *et al*., 1998; McGarey *et al*., 1991) and in some reports have been either proposed as the cause of EUS (Llobrera and Gacutan, 1987; Rahman *et al*., 2002) or capable of inducing lesions macroscopically resembling EUS (Lio-Po *et al*., 1992). Certainly a number of these bacteria, particularly *Aeromonas* and *Vibrio* sp. are known to cause skin lesions in fishes that may ulcerate (Thune *et al*., 1993) and they may thereby act as predisposing factors or "component causes". However, if by definition EUS lesions include a granulomatous response around invasive fungal hyphae, the lesions caused by bacteria could not be EUS lesions. .

Epidemiology

From an epidemiological perspective, and to accommodate the apparently multifactorial nature of EUS, Lilley *et al*. (1998) used the concepts of 'necessary cause', 'component cause' and 'sufficient cause'. Each combination of various 'component causes' which result in disease is known collectively as a 'sufficient cause' for that disease. However, it is

important to recognise that, under different circumstances, different combinations of ' component causes' may constitute 'sufficient cause' for a disease and these 'sufficient causes' for a particular disease have in common at least one 'component cause', known as 'necessary cause' which must always be present for that disease to occur.

The proponents of 'EUS as an aphanomycosis' consider *A. invadans* (=*A. piscicida*) to be the only necessary infectious cause for the disease. By contrast, proponents of 'EUS as a polymicrobial disease' suggest the condition has three necessary infectious causes: a virus (in some cases a rhabdovirus), a fungus (in some cases *A. invadans*) and a bacterium (such as an *Aeromonas* sp. or a *Vibrio* sp.) which act in sequence or in concert to induce lesions.

Both groups propose that one or more additional component causes are usually involved in outbreak causation. These include environmental insults which increase the probability that the necessary infectious cause(s) can infect the host and induce lesions. As examples, exposure to acidified water, trauma-induced epidermal damage or conditions that lead to immunosuppression have all been shown to increase susceptibility of fish to EUS.

Nomenclature/Taxonomy

The *Aphanomyces* sp. proposed as the cause of EUS has been formally described and named on two separate occasions. Hatai (1980) described *Aphanomyces piscicida* as a new species and the primary pathogen of MG. The description was based on morphological characteristics of the hyphae, zoosporangia, primary zoospore cysts and asexual reproduction. The agent was also compared with other similar species such as *A. laevis*, an *Aphanomyces* sp. of tropical fishes and *A. astaci*. Subsequently, Roberts *et al*. (1993), in an extensive survey of fish affected with EUS collected from outbreaks in countries through south and south-east Asia, described in detail the histopathology, pathogenicity and morphological and physiological characteristics of the isolated fungal species. Based on the findings of this study, Willoughby *et al*. (1995) proposed a new species, *Aphanomyces invaderis,* for the *Aphanomyces* causing EUS, and formally described its morphological and cultural characteristics using methods required by the International Code of Botanical Nomenclature (ICBN).

Subsequently, the Index of Fungi (1997) accepted *Aphanomyces invadans* as the valid name, as allowed under Article 60 of the ICBN (Greuter et al. 2000), given that the epithet *'invaderis'* was incorrect because it has no meaning in Latin (Dr. John David, CABI Bioscience, pers. comm.). The Index of Fungi (1998) listed *Aphanomyces piscicida* Hatai as not valid because of the lack of a Latin diagnosis designation of a type specimen at the time of its publication, which are required by Articles 36.1 and 37.1 of the ICBN.

Although *A. piscicida* was effectively described earlier than *A. invadans*, whenever the two names are regarded as synonyms the latter name must be adopted, given that the priority of names is determined by the date of valid publication (see Articles 6.3 and 11.4 of the ICBN).

CONCLUSIONS AND RECOMMENDATIONS

Alternative case definition(s) for EUS

ODA (1994) defined epizootic ulcerative syndrome or EUS as "a seasonal epizootic condition of freshwater and estuarine warm water fish of complex infectious aetiology characterised by the presence of invasive *Aphanomyces* infection and necrotising ulcerative lesions typically leading to a granulomatous response". In view of the foregoing expert discussions and based on the large body of scientific information generated during the last three decades, two groups of case definitions, applicable in different situations, are proposed below. Note that the lists are not exclusive and that each proposed case definition has its own sensitivity and specificity.

(a) Case definitions for screening programs, surveys, etc.:

- A fish with focal to locally extensive cutaneous ulceration
- A fish with focal to locally extensive cutaneous erythema or ulceration

(b) Case definitions for definitive diagnosis:

- A fish with necrotising granulomatous dermatitis and myositis associated with *Aphanomyces invadans* hyphae
- A fish with necrotising, granulomatous dermatitis and/or myositis and/or granulomas in internal organs with *A. invadans* (= *A. piscicida*) found within the lesion.
- A fish with necrotising, granulomatous dermatitis, myositis and/or granulomatous response in internal organs, associated with the presence of *A. invadans* (= *A. piscicida*) hyphae.
- A seasonal epizootic affecting fresh and brackishwater fish species involving a specific fungal pathogen *A. invadans* (= *A. piscicida*) characterised by necrotising surface ulcerative lesions and typical mycotic granulomatous response
- An epizootic fungal infection, where formation of ulcer is secondary, aseptic fungus and granulomas are always observed in the lesion, and the pathogen is a fungus of the genus *Aphanomyces;* confirmatory diagnosis is by PCR test.

Common names for the disease. **Two new common names are proposed:**

- Epizootic granulomatous aphanomycosis (EGA)
- Ulcerative aphanomycosis

Synonymy in the **Aphanomyces** *sp. causing the disease*

In view of the above, and since synonymy is a matter of opinion without formal nomenclatural requirements, we propose that, in other than purely taxonomic contexts, the *Aphanomyces* causing EUS, MG, RSD, UM, should be initially referred to as *A. invadans* (= *A. piscicida*) and thereafter as *A. invadans.*

Size 7.25 x 10 inches

Research and diagnostics

A key point of dispute, representing a significant data gap, which divides the 'EUS as an aphanomycosis' and 'EUS as polymicrobial infection' proponents is whether or not *A. invadans* is the only fungus causing the mycotic granulomas in EUS lesions. To resolve this issue, immunohistochemical or *in situ* molecular techniques must be applied to tissue sections from representative EUS and other ulcerative disease outbreaks, using current and archived material, from a variety of countries.

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Annex A. Participants (in alphabetical order) to the EUS Workshop, 26 November 2002, Gold Coast, Australia

Experts:
1. Vicki Blazer - USA
2. Richard Callinan - Australia
3. Kishio Hatai - Japan
4. Indrani Karunasagar - India
5. CV Mohan - India/Thailand

Workshop Moderators:
6. Chris Baldock - Australia
7. Melba Reantaso - Philippines/USA

Participants:
8. Abu Tweb Abu Ahmed - Bangladesh
9. Rachel Bowater - Australia
10. Nicky Buller - Australia
11. Elena Catap - Philippines
12. Supranee Chinabut - Thailand
13. Kwang-Sik Albert Choi - Korea RO
14. Flavio Corsin - UK/Italy
15. Sugantham Felix - India
16. M. Rosalind George - India
17. Brian Jones - Australia
18. Somkiat Kanchanakhan - Thailand
19. Coco Kokarkin - Indonesia/Australia
20. Susan Gibson- Kueh - Singapore
21. Tina Hawkesford - Australia
22. Mangalika Hettiarachchi - Sri Lanka
23. Ikuo Hirono - Japan
24. Ellen Ho - Singapore
25. Bob Lester - Australia
26. Thitiporn Laoprasert - Thailand
27. Seyed Saeed Mirzargar - Iran
28. Teruo Miyazaki - Japan
29. Kenton Morgan - UK
30. Barry Munday - Australia
31. Satya Nandal - Fiji
32. Sonia Somga - Philippines
33. Rohana Subasinghe - Italy
34. Kamonporn Tonguthai - Thailand
35. Richard Whittington - Australia
36. Jiang Yulin - China