CHAPTER 2.3.2.

EPIZOOTIC ULCERATIVE SYNDROME

1. Scope

Epizootic ulcerative syndrome (EUS) is considered to be an infection with the oomycete known as *Aphanomyces invadans* or *A. piscicida* and characterised histologically by penetrating hyphae surrounded by granulomatous inflammation. It is an epizootic condition of wild and farmed freshwater and estuarine fish.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent, agent strains

EUS is a seasonal epizootic condition of great importance in wild and farmed freshwater and estuarine fish. It has a complex infectious aetiology and is clinically characterised by the presence of invasive *Aphanomyces* infection and necrotising ulcerative lesions, typically leading to a granulomatous response. EUS is also known as red spot disease (RSD), mycotic granulomatosis (MG) and ulcerative mycosis (UM). In 2005, scientists proposed that EUS should be named as epizootic granulomatous aphanomycosis or EGA (1). However the term EUS has been used by most scientists. The oomycete that causes EUS is known as *Aphanomyces invadans* or *A. piscicida.* So far only one genotype of the EUS oomycete recognised that stresses the wide spread of the EUS since the first outbreak in1971 in Japan and the recent outbreak in 2008 Zambia. 24 countries within 4 continents, Northern America, Southern Africa, Asia and Australia, have been recorded the present of the EUS (10). Parasites and rhabdoviruses have also been associated with particular outbreaks, and secondary Gram-negative bacteria invariably infect EUS lesions.

The genera *Aphanomyces* is a member of a group of organisms commonly known as the water moulds. Although long regarded as a fungus because of its characteristic filamentous growth, this group, the Oomycetida, is not a member of the Eumycota, but is classified with diatoms and brown algae in a group called the Stramenopiles or Chromista.

2.1.2. Survival outside the host

How *A. invadans* survives outside the host is still unclear. If the motile zoospore cannot find suitable substrates, it will encyst. There is no suitable method to recover or isolate the encysted zoospore in EUS-infected fish ponds. How long the encysted spore can survive in water or on a non-fish substrate is still unclear. In an *in-vitro* experiment, the encysted zoospore survived for at least 19 days (19).

2.1.3. Stability of the agent (effective inactivation methods)

Aphanomyces invadans grows best at 20–30°C; it does not grow *in-vitro* at 37°C. Water salinity over 2 parts per thousand (ppt) can stop spread of the agent. Preparing fish ponds by sun-drying and liming are effective disinfection methods for EUS. Similar to other oomycetes or water molds, general disinfection chemicals effectively destroy any *A. invadans* that might contaminate farms, fish ponds or fishing gear.

2.1.4. Life cycle

Aphanomyces invadans (Saprolegniales, Oomycetes) has an aseptate fungal-liked mycelia structure. This oomycete has two typical zoospore forms. The primary zoospore consists of round cells that develop inside the sporangium. The primary zoospore is released to the tip of the sporangium where it forms a spore cluster. It quickly transforms into the secondary zoospore, which is reniform with laterally biflagellate cells and can swim freely in the water. The secondary zoospore remains motile for a period that depends on the environmental conditions and presence of the fish host or substratum. Typically, the zoospore encysts and germinates to produce new hyphae, although further tertiary generations of zoospores may be released from cysts (polyplanetism) (16).

2.2. Host factors

2.2.1. Susceptible host species

EUS causes disease and mortality in farmed and wild fish, worldwide. Around 76 species of fish have been confirmed by histological diagnosis to be naturally affected by EUS as shown in Table 2.1. Suspect cases of natural infection with *A. invadans* in species other than those listed should be referred immediately to the appropriate OIE Reference Laboratory, whether or not clinical signs are associated with the findings. Some fish, such as common carp (*Cyprinus capio*), Nile tilapia (*Oreochromis niloticus*) and milk fish (*Chanos chanos*), are considered to be resistant to EUS (16).

Table 2.1. *Fish species susceptible to EUS*

Scientific name	Common name	Scientific name	Common name
Acanthopagrus australis	yellowfin sea bream	*Marcusenius macrolepidotus*	bulldog
Acanthopagrus berda	black bream	*Melanotaenia splendida*	rainbow fish
Ambassis agassiz	chanda perch	*Micralestes acutidens*	silver robber
Ameiurus melas	black bullhead	*Mugil cephalus*	grey mullet or striped mullet
Amniataba percoides	striped grunter	*Mugilidae* (*Mugil* spp.*; Liza* spp.)	mullets
Anabas testudineus	climbing perch	*Nematalosa erebi*	bony bream
Arius sp.	fork-tailed catfish	*Oreochromis andersoni*	three-spoted tilapia
Aseraggodes macleayanus	narrow banded sole	*Oreochromis machrochir*	greenhead tilapia
Barbus peludinosus	straightfin barb	*Osphronemus goramy*	giant gourami
Barbus poechii	dashtail barb	*Oxyeleotris lineolatus*	sleepy cod
Barbus thamalakanensis	Thamalakane barb	*Oxyeleotris marmoratus*	marble goby
Barbus unitaeniatus	longbeard barb	*Petrocephalus catostoma*	churchill
Bidyanus bidyanus	silver perch	*Platycephalus fuscus*	dusky flathead
Brevoortia tyrannus	Atlantic menhaden	*Plecoglossus altivelis*	ayu
Brycinus lateralis	striped robber	*Psettodes* sp.	spiny turbot
Catla catla	catla	*Puntius gonionotus*	silver barb
Channa striatus	striped snakehead	*Puntius sophore*	pool barb
Cirrhinus mrigala	mrigal	*Rohtee* sp.	keti-Bangladeshi
Clarias gariepinus	sharptooth African catfish	*Sargochromis carlottae*	rainbow bream
Clarias ngamensis	blunt-toothed African catfish	*Sargochromis codringtonii*	green bream
Clarius batrachus	walking catfish	*Sargochromis giardi*	pink bream
Colisa lalia	dwarf gourami	*Scatophagus argus*	spotted scat
Esomus sp.	flying barb	*Schilbe intermedius*	silver catfish
Fluta alba	swamp eel	*Schilbe mystus*	African butter catfish
Glossamia aprion	mouth almighty	*Scleropages jardini*	saratoga
Glossogobius giuris	bar-eyed goby	*Selenotoca multifasciata*	striped scat
Glossogobius sp.	goby	*Serranochromis angusticeps*	thinface largemouth
Hepsetus odoe	African pike	*Serranochromis robustus*	Nembwe
Hydrocynus vittatus	tigerfish	*Sillago ciliata*	sand whiting
Ictalurus punctatus	channel catfish	*Siluridae* (*genus*)	wells catfish
Kurtus gulliveri	nursery fish	*Strongylura kreffti*	long tom
Labeo cylindricus	red-eye labeo	*Therapon* sp.	therapon
Labeo lunatus	upper Zambezi labeo	*Tilapia rendalli*	redbreast tilapia
Labeo rohita	rohu	*Tilapia sparrmanii*	banded tilapia
Lates calcarifer	barramundi or sea bass	*Toxotes chatareus*	common archer fish
Leiopotherapon unicolor	spangled perch	*Toxotes lorentzi*	primitive acher fish
Lepomis macrochirus	bluegill	*Trichogaster pectoralis*	snakeskin gourami
Lutjanus argentimaculatus	mangrove jack	*Trichogaster trichopterus*	three-spot gourami

2.2.2. Susceptible stages of the host

The susceptible life stages of the fish are usually juvenile and young adults. There is no report of EUS being found in fish fry or fish larvae.

An experimental injection of *A. invadans* into the yearling life stage of Indian major carp, catla, rohu and mrigal, revealed resistance to EUS (28), even though they are naturally susceptible species. Experimental infections demonstrated that goldfish are susceptible (13, 14), but common carp (32) and Nile tilapia (15) are resistant.

2.2.3. Species or subpopulation predilection (probability of detection)

EUS can be readily detected in diseased fish specimens collected from EUS-infected areas using histological techniques. However, *Aphanomyces* can be isolated only from fish with mild or moderate clinical signs of EUS, exhibiting red spots or small ulcers.

2.2.4. Target organs and infected tissue

The motile zoospore plays an important role in the spread of the disease. Once the motile spore attaches to the skin of the fish, the spore will germinate under suitable conditions and its hyphae will invade the fish skin, muscular tissue and reach the internal organs. Fish skeleton muscle is the target organ and exhibits major EUS clinical signs with mycotic granulomas.

2.2.5. Persistent infection with lifelong carriers

There is no information to indicate that fish can be lifelong carriers of *A. invadans*. Generally, most infected fish die during an outbreak. Although some mild or moderate EUS–infected fish could recover, they are unlikely to be lifelong carriers.

2.2.6. Vectors

No data available.

2.2.7. Known or suspected wild aquatic animal carriers

No data available.

2.3. Disease pattern

2.3.1. Transmission mechanisms

EUS is transmitted horizontally. The *Aphanomyces* zoospores can be horizontally transmitted from one fish to another through the water supply. It is believed that only the zoospores are capable of attaching to the damaged skin of fish and germinating into hyphae. If the zoospores cannot find the susceptible species or encounter unfavourable conditions, they can form secondary zoospores. The secondary zoospores can encyst in the water or pond environment waiting for conditions that favour the activation of the spores. How the *Aphanomyces* pathogen or its spores survive after the outbreak is still unclear as outbreaks usually occur about the same time every year in endemic areas.

2.3.2. Prevalence

The prevalence of EUS in the wild and in aquaculture farms is high in endemic areas that share the same water way or system. Uncontrolled water exchange in fish farms in endemic areas will result in EUS outbreaks in most of the farms that culture susceptible fish species.

2.3.3. Geographical distribution

EUS was first reported in farmed freshwater ayu (*Plecoglossus altivelis*) in Oita Prefecture, Kyushu Island, Japan in 1971 (9). It was later reported in estuarine fish, particularly grey mullet (*Mugil cephalus*) in eastern Australia in 1972 (11, 23). EUS has extended its range through Papua New Guinea into South-East and South Asia, and into West Asia, where it has reached Pakistan (16, 30). Outbreaks of ulcerative disease in menhaden (*Brevoortia tyrannus*) in the United States of America (USA) had the same aetiological agent as the EUS in Asia (3, 18, 31). The first confirmed outbreaks on the African continent occurred in 2007 in Botswana, Namibia and Zambia, and were connected to the Zambezi-Chobe river system. EUS has been reported from 24 countries in four continents: North America, Southern Africa, Asia and Australia (10).

2.3.4. Mortality and morbidity

When EUS spreads into a fish culture pond, such as a snakehead fish pond, high morbidity (>50%) and high mortality (>50%) might be observed in those years that have a long cold season, with water temperatures between 18 and 22°C. Some infected fish may recover when the cold period is over.

2.3.5. Environmental factors

EUS occurs mostly during periods of low temperatures or 18–22°C and after periods of heavy rainfall (4). These conditions favour sporulation of *A. invadans* (22), and low temperatures have been shown to delay the inflammatory response of fish to oomycete infection (5, 7). In some countries, outbreaks occur in wild fish first and then spread to fish ponds. Normally, a bath infection of *A. invadans* in healthy susceptible fish species does not result in clinical signs of disease. The *Aphanomyces* oomycete needs predisposing factors that lead to skin damage, such as parasites, bacteria or virus infection or acid water, to initiate the clinical signs of EUS disease (16).

EUS has been reported from 24 countries on four continents. Movements of live ornamental fish from EUS-infected countries might spread the disease as was the case with the outbreak in Sri Lanka (2). Flooding also caused the spread of EUS in Bangladesh and Pakistan (16). Once an outbreak occurs in rivers/canals, the disease can spread downstream as well as upstream where the susceptible fish species exist. Ensuring that water from infected rivers does not come into contact with fish culture ponds could prevent the spread of EUS.

2.4. Control and prevention

2.4.1. Vaccination

There is no protective vaccine available. However, snakehead fish that had been immunised with a crude extract of the *A. invadans* elicited humeral immune response as detected by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) and Western blot analysis (29).

2.4.2. Chemotherapy

There is no effective treatment for EUS-infected fish in the wild and in aquaculture ponds. To minimise fish losses in infected fish ponds water exchange should be stopped and lime or hydrated lime and/or salt should be applied (16). Attempts at using green water, ash, lime, and neem seeds or branches (*Azadirachta indica*) for prophylactic treatments of the EUS-infected fish in fish ponds gave variable results (Inland Aquatic Animal Health Research Institute(AAHRI], Thailand, internal report, 2001).

2.4.3. Immunostimulation

Preliminary experiments showed that intraperitoneal injection of the immunostimulant, Salar-bec (containing 300 g kg^{-1} vitamin C, 150 g kg^{-1} vitamin B and trace quantities of vitamins B1, B2, B6 and B12), into snakehead fish can increase serum inhibition both germination and growth of the zoospore *in vitro*. Snakehead fish fed on normal pellet feed and Salar-bec-supplemented feed still exhibited clinical signs of EUS after challenge with *A. invadans*. However, snakehead fish that received the immunostimulant, Salar-bec, showed a relative per cent survival of 59.2% higher than the control group that received normal feed (24).

2.4.4. Resistance breeding

No data available.

2.4.5. Restocking with resistant species

Some important culture species, including Nile tilapia, milk fish and Chinese carp, have been shown to be resistant to EUS and could be cultured in endemic areas.

2.4.6. Blocking agents

No data available.

2.4.7. Disinfection of eggs and larvae

Routine disinfection of fish eggs and larvae against water molds is equally effective against *A. invadans*. It should be noted that there is no report of the presence of *A. invadans* in fish eggs or larvae.

2.4.8. General husbandry practices

Control of EUS in natural waters is probably impossible. In outbreaks occurring in small, closed water-bodies or fish ponds, liming water with agricultural limes and improving water quality, together with removal of infected fish, is often effective in reducing mortalities and controlling the disease. Ensuring no leakage of water from EUS-infected areas into fish ponds is a normal practice that easily prevents the spread of EUS into farms. As for the issue of farmer safety, sodium chloride or salt and agricultural lime are the best chemicals for treating or preventing the spread of EUS.

3. Sampling

3.1. Selection of individual specimens

Scoop net, cast net or seine net represent the best choices for catching EUS-infected fish in natural waters or in fish ponds. For outbreak investigations, diseased fish with ulcerative lesions or red spots on the body should be sampled.

3.2. Preservation of samples for submission

Fish specimens should be transported to the laboratory live or in ice-cooled boxes for further diagnosis. Fish collected from remote areas should be anesthetised and can be fixed in normal 10% formalin or 10% phosphate-buffered formalin for at least 1–2 days. The fixed specimens are then transferred to double-layer plastic bags with formalin-moistened tissue paper. The bags are sealed and sent to the laboratory in semi-dry conditions.

3.3. Pooling of samples

Ten diseased fish specimens are sampled from the EUS-infected site. Diagnosis is achieved using the histological technique and oomycete isolation on individual fish or a group of a few fish.

3.4. Best organs or tissues

Fish with minor clinical signs are recommended and the muscle tissue next to or underneath the ulcer is best for oomycete isolation. The best tissue for histopathological examination is muscle tissue at the edge of the ulcers.

3.5. Sample/tissues that are not suitable

Severely diseased or dead fish are not suitable for oomycete isolation.

4. Diagnostic methods

Diagnosis of EUS is based on clinical signs and confirmed by histopathology. Diagnosis of EUS in clinically affected fish may be achieved by histopathology or by oomycete isolation. Positive diagnosis of EUS is made by demonstrating the presence of mycotic granulomas in histological sections or isolation of *A. invadans* from internal tissues.

4.1. Field diagnostic methods

EUS outbreaks have been associated with mass mortality of various species of freshwater fish in the wild (including rice-fields, estuaries, lakes and rivers) and in farms during periods of low temperatures and after periods of heavy rainfall.

4.1.1. Clinical signs

Fish usually develop red spots or small to large ulcerative lesions on the body.

4.1.2. Behavioural changes

The early signs of the disease include loss of appetite and fish become darker. Infected fish may float near the surface of the water, and become hyperactive with a very jerky pattern of movement.

4.2. Clinical methods

4.2.1. Gross pathology

Red spots may be observed on the body surface, head, operculum or caudal peduncle. Large red or grey shallow ulcers, often with a brown necrosis, are observed in the later stages. Large superficial lesions occur on the flank or dorsum. Most species other than striped snakeheads and mullet will die at this stage. In highly susceptible species, such as snakehead, the lesions are more extensive and can lead to complete erosion of the posterior part of the body, or to necrosis of both soft and hard tissues of the cranium, so that the brain is exposed in the living animal.

4.2.2. Clinical chemistry

No information available.

4.2.3. Microscopic pathology

Early EUS lesions are caused by erythematous dermatitis with no obvious oomycete involvement. *Aphanomyces invadans* hyphae are observed growing in skeletal muscle as the lesion progresses from a mild chronic active dermatitis to a severe locally extensive necrotising granulomatous dermatitis with severe floccular degeneration of the muscle. The oomycete elicits a strong inflammatory response and granulomas are formed around the penetrating hyphae. Lesion scrapes from fish body or ulcers generally show secondary fungal, bacterial and/or parasitic infections.

4.2.4. Wet mounts

Not suitable for EUS diagnosis.

4.2.5. Smears

Not suitable for EUS diagnosis.

4.2.6. Electron microscopy/cytopathology

Not suitable for EUS diagnosis.

4.3. Agent detection and identification methods

4.3.1. Direct detection methods

4.3.1.1. Microscopic methods

The squash preparation can be carried out as follows:

i) Remove ulcer surface using a sharp scalpel blade.

ii) Cut the muscular tissue at the edge of the ulcer.

iii) Place the pieces of tissue on a cutting board then make thin slices using a sharp scalpel blade.

iv) Place the thinly sliced tissue between two glass slides and squeeze gently with fingers.

v) Remove one of the glass slides and cover the tissue with a cover-slip. View under a light microscope to find the nonseptate hyphae structure of *A. invadans* (12–25 µm in diameter).

4.3.1.1.1. Wet mounts

Not suitable for EUS diagnosis

4.3.1.1.2. Smears

Not suitable for EUS diagnosis t

4.3.1.1.3. Fixed sections

Sampling procedure

i) Sample only live or moribund specimens of fish with clinical lesions.

ii) Take samples of skin/muscle (<1 cm^3), including the leading edge of the lesion and the surrounding tissue.

iii) Fix the tissues immediately in 10% formalin. The amount of formalin should be 10 times the volume of the tissue to be fixed.

Histological procedure

Processing the fixed tissue involves dehydration through ascending alcohol grades, clearing in a wax-miscible agent and impregnation with wax. The blocks of fish tissue are cut at about 5 µm and mounted on a glass slide. Before staining, the section must be completely de-waxed and stained in haematoxylin and eosin (H&E) (6). H&E and general fungus stains (e.g. Grocott's stain) will demonstrate typical granulomas and invasive hyphae.

4.3.1.2. Agent isolation and identification

4.3.1.2.1 Isolation of Aphanomyces invadans *from internal tissues*

The following are two methods of isolation of *A. invadans* or *A. piscicida* adapted from references 16 and 33.

Method 1: Moderate, pale, raised, dermal lesions are most suitable for oomycete isolation attempts. Remove the scales around the periphery of the lesion and sear the underlying skin with a red-hot spatula so as to sterilise the surface. Using a sterile scalpel blade and sterile fine-pointed forceps, cut through the stratum compactum underlying the seared area and, by cutting horizontally and reflecting superficial tissues, expose the underlying muscle. Ensure the instruments do not make contact with the contaminated external surface and thereby contaminate the underlying muscle. Using aseptic techniques, carefully excise pieces of affected muscle, approximately 2 mm^3, and place on a Petri dish containing glucose/peptone (GP) agar (see Table 4.1) with penicillin G (100 units ml^{-1}) and streptomycin (100 µg ml^{-1}). Seal plates, incubate at room temperature or at 25°C and examine daily. Repeatedly transfer emerging hyphal tips on to fresh plates of GP agar with antibiotics until cultures are free of contamination.

Method 2: Lesions located on the flank or tail of fish <20 cm in length can be sampled by cutting the fish in two using a sterile scalpel, and slicing a cross-section through the fish at the edge of the lesion. Flame the scalpel until red-hot and use this to sterilise the exposed surface of the muscle. Use a small-bladed sterile scalpel to cut out a circular block of muscle (2–4 mm^3) from beneath the lesion and place it in a Petri dish of GP medium (see Table 4.1) with 100 units ml^{-1} penicillin G and 100 µg ml^{-1} streptomycin. Instruments should not contact the contaminated external surface of the fish. Incubate inoculated media at approximately 25°C and examine under a microscope (preferably an inverted microscope) within 12 hours. Repeatedly transfer emerging hyphal tips to plates of GP medium with 12 g $litre^{-1}$ technical agar, 100 units ml^{-1} penicillin G and 100 µg ml^{-1} streptomycin until axenic cultures are obtained. The oomycete isolate can also be maintained at 25°C on GY agar (see Table 4.1) and transferred to a fresh GY agar tube once every 1–2 weeks (12).

4.3.1.2.2. Identification of Aphanomyces invadans

Aphanomyces invadans does not produce any sexual structures and should thus not be diagnosed by morphological criteria alone. However, the oomycete can be identified to the genus level by inducing sporogenesis and demonstrating typical asexual characteristics of *Aphanomyces,* as described in ref. 16. *A. invadans* is characteristically slow growing in culture and fails to grow at 37°C on GPY agar (Table 4.1). Detailed temperature–growth profiles are given in ref. 20. Two procedures that can be used to confirm *A. invadans* are bioassay and polymerase chain reaction (PCR) amplification of the rDNA of *A. invadans*.

4.3.1.2.3 Inducing sporulation in Aphanomyces invadans *cultures*

The induction of asexual reproductive structures is necessary for identifying oomycete cultures as members of the genus *Aphanomyces.* To induce sporulation, place an agar plug (3–4 mm in diameter) of actively growing mycelium in a Petri dish containing GPY broth and incubate for 4 days at approximately 20°C. Wash the nutrient agar out of the resulting mat by sequential transfer through five Petri dishes containing autoclaved pond water (Table 4.1), and leave overnight at 20°C in autoclaved pond water. After about 12 hours, the formation of achlyoid clusters of primary cysts and the release of motile secondary zoospores should be apparent under the microscope.

4.3.1.2.4. Bioassay

Fish can be experimentally infected by intramuscularly injecting a 0.1 ml suspension of 100+ motile zoospores in EUS-susceptible fish (preferably *Channa striata* or other susceptible species) at 20°C, and demonstrating histological growth of aseptate hyphae, 12–25 µm in diameter, in the muscle of fish

sampled after 7 days, and of typical mycotic granulomas in the muscle of fish sampled after 10–14 days.

4.3.1.2.5. Antibody-based antigen detection methods

Polyclonal antibodies against *A. invadans* or *Aphanomyces* saprophyte showed cross reactivity to each other using protein gel electrophoresis and Western blot analysis and immunohistochemistry. (21). However specific monoclonal antibody against *A. invadans* developed later was found to have high specificity and high sensitivity to the *Aphanomyces* pathogens of the EUS using immunofluorescence. This monoclonal antibody could detect the *Aphanomyces* hyphae at the early stage of the EUS infection (25).

4.3.1.2.6. Molecular techniques

4.3.1.2.6.1. Polymerase chain reaction amplification of the DNA of A. invadans

DNA preparation from A. invadans isolate

DNA is extracted from an actively growing colony of *A. invadans* culture in GY broth at about 4 days or when young mycelia reach 0.5–1.0 cm in diameter. The mycelia are transferred to sterile 100-mm Petri dishes, washed twice with PBS and then placed on tissue paper for liquid removal. Hyphal tips (~50–250 mg) are excised with a sterile scalpel blade and transferred to a 1.5 ml microcentrifuge tube for DNA extraction. Commercial DNA extraction kits have been used successfully (27, 31).

DNA preparation from EUS-infected tissue

Small pieces of EUS-infected tissue 25–50 mg are suitable for DNA extractions (26).

Diagnostic PCR technique

Two recent published techniques have been found more specific to *A. invadans* than the previous one (14).

Method 1: The species-specific forward primer site is located near the 3' end of the SSU (small subunit) gene and a species-specific reverse primer site is located in the ITS1 region for Ainvad-2F (5'-TCA-TTG-TGA-GTG-AAA-CGG-TG-3') and Ainvad-ITSR1 (5'-GGC-TAA-GGT-TTC-AGT-ATG-TAG-3'). The PCR mixture contained 25 pM of each primer, 2.5 mM each deoxynucleoside triphosphate, 0.5 U of Platinum *Taq* DNA polymerase and 20 ng of genomic DNA (either from an *Aphanomyces* isolate or from infected tissue) for a total volume of 50 µl. DNA is amplified in a thermocycle machine under the following cycle conditions: 2 minutes at 95°C; 35 cycles, each consisting of 30 seconds at 95°C, 45 seconds at 56°C, 2.5 minutes at 72°C; and a final extension of 5 minutes at 72°C. The PCR product is analysed by agarose gel electrophoresis and the target product is 234 bp (31).

Method 2: The species-specific primer sites are located in the ITS1 and ITS2 regions. The forward primer is ITS11 (5'-GCC-GAA-GTT-TCG-CAA-GAA-AC-3') and the reverse is ITS23 (5'-CGT-ATA-GAC-ACA-AGC-ACA-CCA-3'). The PCR mixture contains 0.5 µM of each primer, 0.2 mM each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 0.6 U of *Taq* DNA polymerase and 20 ng of genomic DNA (from an *Aphanomyces* isolate) for a total volume of 25 µl. The DNA is amplified under the following cycle conditions: 5 minutes at 94°C; 25 cycles, each consisting of 30 seconds at 94°C, 30 seconds at 65°C, 1 minute at 72°C; and a final extension of 5 minutes at 72°C. The PCR product is analysed by agarose gel electrophoresis and the target product is 550 bp. PCR amplification using the DNA template from the infected tissue is similar to the above protocol except that 5 ng of the DNA template is used for 35 cycles (27).

All *A. invadans* isolates found so far belong to a single genotype, and this facilitates identification. Alternatively, sequencing of the PCR products can be performed and the results can be compared with the sequence deposited in the public gene data banks. *A. invadans* has similar characteristics to *A. astaci,* the aetiological agent of crayfish plague. Both pathogenic oomycetes can be differentiated using molecular tools (8, 17, 27, 31).

4.3.1.2.6.2. Fluorescent peptide nucleic acid in-situ *hybridisation* (*FISH*)

A fluorescent peptide nucleic acid *in-situ* hybridisation (FISH) technique has demonstrated a high specificity for *A. invadans.* The technique can directly detect the mycelia-like structure of the oomycete in thinly sliced tissues of affected organs of the susceptible fish. The fluorescein (FLU) probe designed to hybridise the small subunit of the rRNA *A. invadans* (bp 621 to 635; GenBank acc. AF396684) is 5'-FLU-GTA-CTG-ACA-TTT-CGT-3' or Ainv-FLU3.

The EUS-affected tissue is fixed and hybridised as soon as possible after the fish are collected to minimise RNA degradation. Tissue (~20 mg) is dissected from the periphery of the lesions with sterile scalpel blades and placed in individual wells of a 24-well microtitre plate. One ml ethanol-saline fixative (44 ml of 95% ethanol, 10 ml of deionised H_2O, and 6 ml of 25 × SET buffer [3.75 M NaCl, 25 mM EDTA (ethylene diamine tetra-acetic acid), 0.5 M Tris/HCl, pH 7.8]) containing 3% polyoxyethyl-enesorbitan monolaurate (Tween 20) is added to enhance tissue permeabilization. The microtitre plate is gently agitated at room temperature on an orbital shaker (30 rpm) for 1.5 hours. The fixed tissues are rinsed (twice for 15 minutes each time) with 0.5 ml of hybridisation buffer (5 × SET, 0.1% [v/v] Igepal-CA630 and 25 µg ml^{-1} poly[A]) containing 3% Tween 20. The hybridisation buffer is removed, and the tissues are resuspended in 0.5 ml of hybridisation buffer containing 3% Tween 20 and 100 nM Ainv-FLU3 probe. "No-probe" control specimens are incubated with 0.5 ml of hybridisation buffer/3% Tween 20. All tissues are incubated at 60°C for 1 hour in the dark. Following incubation, the tissues are rinsed twice with 1 ml of pre-warmed (60°C) 5 × SET buffer containing 3% Tween 20 to remove residual probe. The tissue specimens are mounted onto poly-L-lysine-coated microscope slides. One drop of the light anti-fade solution is placed on the specimens, which are then overlaid with a cover-slip. Analyses are performed by light and epifluorescence microscopy. The camera and microscope settings for epifluorescent analyses are held constant so that comparative analyses of relative fluorescence intensity can be made between probed and non-probed specimens. The fluorescent oomycete hyphae appear as green fluorescence against the dark tissue background. The above detailed protocols are published by Vandersea *et al*. (31). Using the FISH technique, *A. invadans* can be visualised very well in thinly sliced tissue compared with freshly squashed tissue.

Table 4.1. *Media for isolation, growth and sporulation of Aphanomyces invadans cultures*

GP (glucose/peptone) medium	GPY (glucose/peptone/ yeast) broth	GPY agar	GY agar	Autoclaved pond water
3 g $litre^{-1}$ glucose 1 g $litre^{-1}$ peptone 0.128 g $litre^{-1}$ $MgSO_4.7H_2O$ 0.014 g $litre^{-1}$ KH_2PO_4 0.029 g $litre^{-1}$ $CaCl_2.2H_2O$ 2.4 mg $litre^{-1}$ $FeCl_3.6H_2O$ 1.8 mg $litre^{-1}$ $MnCl_2.4H_2O$ 3.9 mg $litre^{-1}$ $CuSO_4.5H_2O$ 0.4 mg $litre^{-1}$ $ZnSO_4.7H_2O$	GP broth + 0.5 g $litre^{-1}$ yeast extract	GPY broth + 12 g $litre^{-1}$ technical agar	1% glucose, 0.25% yeast extract, 1.5% agar	Sample pond/lake water known to support oomycete growth. Filter through Whatman 541 filter paper. Combine one part pond water with two parts distilled water and autoclave. pH to 6–7.

4.3.1.2.7. Agent purification

Maintaining *A. invadans* in the axenic culture is necessary. As it is characteristically slow growing, it easily becomes contaminated with other micro-organisms, such as bacteria and other fast-growing oomycetes and fungi. Attempts to purify or isolate *A. invadans* from contaminated cultures usually fail.

4.3.2. Serological methods

Serological methods for detection and identification of *A. invadans* in EUS specimens are not practical. If necessary, the monoclonal antibody offers a better specificity and sensitivity than polyclonal antibody for serological detection or identification of *A. invadans* in diseased specimens or in pathogen isolates.

5. Rating of tests against purpose of use

The methods currently available for surveillance, detection, and diagnosis of EUS are listed in Table 5.1. The designations used in the Table indicate: a = the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity; b = the method is a standard method with good diagnostic sensitivity and specificity; c = the method has application in some situations, but cost, accuracy, or other factors severely limits its application; and d = the method is presently not recommended for this purpose. These are somewhat subjective as suitability involves issues of reliability, sensitivity, specificity and utility. Although not all of the tests listed as category a or b have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Table 5.1. *Methods for targeted surveillance and diagnosis*

Method	Targeted surveillance			Presumptive diagnosis	Confirmatory diagnosis
	Fish fry	Juveniles	Adults		
Gross signs	c	c	c	b	c
Direct LM; observation of the oomycete hyphae in tissues, fresh squash	d	d	d	b	b
FISH; observation of the oomycete hyphae in tissues	d	d	d	a	a
Histopathology	c	c	c	a	a
Isolation of *A. invadans* and confirmatory identification by bioassay or PCR	d	d	d	a	a
PCR of tissue extracts	d	d	d	a	a
Sequence analysis	d	d	d	d	a
Transmission EM of tissues	d	d	d	d	d

LM = light microscopy; FISH = fluorescent peptide nucleic acid *in-situ* hybridisation;
PCR = polymerase chain reaction; EM = electron microscopy.

6. Test(s) recommended for targeted surveillance to declare freedom from epizootic ulcerative syndrome

The test for targeted surveillance to declare freedom from EUS is examination of gross signs. Targeted surveillance is conducted twice a year to cover the range of seasonal variation, at least once during the season that favours EUS occurrence or when water temperatures are about 18–22°C or below 25°C. Biosecurity measures should be implemented to maintain disease-free status in controlled aquaculture facilities or compartments.

Using the gross sign test for targeted surveillance, a large number of the fish should be examined without killing them. Fish on farms, in compartments or in natural water bodies should be sampled carefully using suitable gear or nets. The suitable numbers of fish specimens examined should be based on details described in the OIE *Guide for Aquatic Animal Health Surveillance* (2009).

Once fish show similar gross signs to EUS, they should be categorised as suspect EUS fish, and the location/farm/compartment/zone should be considered suspect. Suspect specimens should be further tested using the methods listed under presumptive diagnosis followed by confirmative diagnosis as described in the Table 5.1.

7. Corroborative diagnostic criteria

7.1. Definition of suspect case

A suspect case of EUS disease is defined as the presence of typical clinical signs, a single or multiple red spot(s) or ulcer(s) on the body, in a population of susceptible fish at water temperatures between 18 and 25°C OR the presence of branching non-septate oomycete hyphae in a muscle squash preparation OR the isolation of slow-growing *Aphanomyces* without further identification of the agent.

7.2. Definition of confirmed case

A confirmed case of EUS is defined as a suspect case that has produced typical mycotic granulomas in affected tissues or organs OR that has been identified as positive by the PCR or FISH detection techniques OR that *Aphanomyces invadans* has been isolated and confirmed by either bioassay, PCR. or sequence analysis.

8. References

1. BALDOCK F.C., BLAZER V., CALLINAN R., HATAI K., KARUNASAGAR I., MOHAN C.V. & BONDAD-REANTASO M.G. (2005). Outcomes of a short expert consultation on epizootic ulcerative syndrome (EUS): Re-examination of causal factors, case definition and nomenclature. *In:* Diseases in Asian Aquaculture V, Walker P., Laster R. & Bondad-Reantaso M.G., eds. Fish Health Section, Asian Fisheries Society, Manila, Philippines, 555–585.

2. BALASURIYA, L.K.S.W. (1994). Epizootic ulcerative syndrome in fish in Sri Lanka, country status report. *In:* Proceeding of the ODA Regional Seminar on Epizootic Ulcerative, Robert R.J., Campbell B. & MacRae I.H., eds. Aquatic Animal Health Research Institute, Bangkok, Thailand, pp 39–47.

3. BLAZER V.S., VOGELBEIN W.K., DENSMORE C.L., MAY E.B., LILLEY J.H. & ZWERNER D.E. (1999). Aphanomyces as a cause of ulcerative skin lesions of menhaden from Chesapeake Bay tributaries. *J. Aquat. Anim. Health,* **11**, 340–349.

4. BONDAD-REANTASO M.G., LUMANLAN S.C., NATIVIDAD J.M. & PHILLIPS M.J. (1992). Environmental monitoring of the epizootic ulcerative syndrome (EUS) in fish from Munoz, Nueva Ecija in the Philippines. *In:* Diseases in Asian Aquaculture 1, Shariff M., Subasinghe R.P. & Arthur J.R., eds. Fish Health Section, Asian Fisheries Society, Manila, The Philippines, 475–490.

5. CATAP E.S. & MUNDAY B.L. (1998). Effects of variations of water temperature and dietary lipids on the expression of experimental epizootic ulcerative syndrome (EUS) in sand whiting, Sillago ciliata. *Fish Pathol.,* **33**, 327–335.

6. CHINABUT S. & ROBERTS R.J. (1999). Pathology and Histopathology of Epizootic Ulcerative Syndrome (EUS). Aquatic Animal Health Research Institute, Department of Fisheries, Royal Thai Government, Bangkok, Thailand, 33 pp. ISBN 974-7604-55-8.

7. CHINABUT S., ROBERTS R.J., WILLOUGHBY G.R. & PEARSON M.D. (1995) Histopathology of snakehead, *Channa striatus* (Bloch), experimentally infected with the specific *Aphanomyces* fungus associated with epizootic ulcerative syndrome (EUS) at different temperatures. *J. Fish Dis.*, **18**, 41–47.

8. DIÉGUEZ-URIBEONDO J., GARCIA M.A., CERENIUS L., KOZUBÍKOVÁ E., BALLESTEROS I., WINDELS C., WEILAND J., KATOR H., SÖDERHÄLL K. & MARTÍN M.P. (2009). Phylogenetic relationships among plant and animal parasites, and saprotrophs in *Aphanomyces* (Oomycetes). *Fungal Genetics and Biology*, **46**, 365–376.

9. EGUSA S. & MASUDA N. (1971). A new fungal disease of Plecoglossus altivelis. *Fish Pathol.,* **6**, 41–46.

10. FAO. (2009). Report of the international emergency disease investigation task force on a serious fish disease in Southern Africa, 18-26 May 2007, FAO, Rome, Italy, 70 pp.

11. FRASER G.C., CALLINAN R.B. & CALDER L.M. (1992). *Aphanomyces* species associated with red spot disease: an ulcerative disease of estuarine fish from eastern Australia. *J. Fish Dis.*, **15**, 173–181.

12. HATAI K. & EGUSA S. (1979). Studies on pathogenic fungus of mycotic granulomatosis III. Development of the medium for MG-fungus. *Fish Pathol.,* **13**, 147–152.

13. HATAI K., EGUSA S., TAKAHASHI S. & OOE K. (1977). Study on the pathogenic fungus of mycotic granulomatosis – I. Isolation and pathogenicity of the fungus from cultured-ayu infected with the disease. *Fish Pathol.,* **12**, 129–133.

14. HATAI K., NAKAMURA K., AN RHA S., YUASA K. & WADA S. (1994). *Aphanomyces* infection in dwarf gourami (*Colisa lalia*). *Fish Pathol.,* **29**, 95–99.

15. KHAN M.H., MARSHALL L., THOMPSON K.D., CAMPBELL R.E. & LILLEY J.H. (1998). Susceptibility of five fish species (Nile tilapia, rosy barb, rainbow trout, stickleback and roach) to intramuscular injection with the Oomycete fish pathogen, *Aphanomyces invadans*. *Bull. Eur. Assoc. Fish Pathol.,* **18**, 192–197.

16. LILLEY J.H., CALLINAN R.B., CHINABUT S., KANCHANAKHAN S., MACRAE I.H. & PHILLIPS M.J. (1998). Epizootic ulcerative syndrome (EUS) technical handbook. Aquatic Animal Health Research Institute, Bangkok, Thailand.

17. LILLEY J.H., HART D., PANYAWACHIRA V., KANCHANAKHAN S., CHINABUT S., SÖDERHÄLL K. & CERENIUS L. (2003). Molecular characterization of the fish-pathogenic fungus Aphanomyces invadans. *J. Fish Dis.,* **26**, 263–275.

18. LILLEY J.H., HART D., RICHARDS R.H., ROBERTS R.J., CERENIUS L. & SODERHALL K. (1997). Pan-Asian spread of single fungal clone results in large scale fish kills. *Vet. Rec.*, **140**, 653–654.

19. LILLEY J.H., PETCHINDA T. & PANYAWACHIRA V. (2001). *Aphanomyces invadans* zoospore physiology: 4. *In vitro* viability of cysts. *The AAHRI Newsletter*, **10**, 1–4.

20. LILLEY J.H. & ROBERTS R.J. (1997). Pathogenicity and culture studies comparing the *Aphanomyces* involved in epizootic ulcerative syndrome (EUS) with other similar fungi. *J. Fish Dis.*, **20**, 135–144.

21. LILLEY J.H., THOMPSON K.D. & ADAMS A. (1997). Characterization of *Aphanomyces invadans* by electrophoretic and Western blot analysis. *Dis. Aquat. Org*., **30**, 187–197.

22. LUMANLAN-MAYO S.C., CALLINAN R.B., PACLIBARE J.O., CATAP E.S. & FRASER, G.C. (1997). Epizootic ulcerative syndrome (EUS) in rice-fish culture systems: an overview of field experiments 1993-1995. *In:* Diseases in Asian Aquaculture III, Flegel T.W. & MacRae I.H., eds. Fish Health Section, Asian Fisheries Society, Manila, The Philippines, 129–138.

23. MCKENZIE R.A. & HALL W.T.K. (1976). Dermal ulceration of mullet (*Mugil cephalus*). *Aust. Vet. J.,* **52**, 230–231.

24. MILES D.J.V., POLCHANA J., LILLEY J.H., KANCHANAKHAN S., THOMPSON K.D. & ADAMS A. (2001). Immunostimulation of striped snakehead *Channa striata* against epizootic ulcerative syndrome. *Aquaculture*, **195**, 1–15.

25. MILES D.J.C., THOMPSON K.D., LILLEY J.H. & ADAMS A. (2003). Immunofluorescence of the epizootic ulcerative syndrome pathogen, *Aphanomyces invadans*, using a monoclonal antibody. *Dis. Aqaut. Org*., **55**, 77–84.

26. PHADEE P., KURATA O. & HATAI K. (2004). A PCR method for the detection of *Aphanomyces piscicida*. *Fish Pathol.,* **39**, 25–31.

27. PHADEE, P., KURATA, O., HATAI K., HIRONO I. & AOKI T. (2004). Detection and identification of fish-pathogenic *Aphanomyces piscicida* using polymerase chain reaction (PCR) with species-specific primers. *J. Aquat. Anim. Health*, **16**, 220–230.

28 PRADHAN P.K., MOHAN C.V., SHANKAR K.M., KUMAR B.M. & DEVARAJA G. (2007). Yearlings of Indian major carps resist infection against the epizootic ulcerative syndrome pathogen, *Aphanomyces invadans*. *Current Science***,** **92**, 1430–1434.

29. THOMPSON K.D., LILLEY J.H., CHINABUT S. & ADAMS A. (1997). The antibody response of snakehad, *Channa striata* Bloch, to Aphanomyces invadans. Fish & Shellfish Immunology, **7**, 349-353.

30. TONGUTHAI K. (1985). A preliminary account of ulcerative fish diseases in the Indo-Pacific region (a comprehensive study based on Thai experiences). National Inland Fisheries Institute, Bangkok, Thailand, 39 pp.

31. VANDERSEA M.W., LITAKER R.W., YONNISH B., SOSA E., LANDSBERG J.H., PULLINGER C., MOON-BUTZIN P., GREEN J., MORRIS J.A., KATOR H., NOGA E.J. & TESTER P.A. (2006). Molecular assays for detecting *Aphanomyces invadans* in ulcerative mycotic fish lesions. *Appl. Environ. Microbiol.,* **72**, 1551–1557.

32. WADA S., AN RHA S., KONDOH T., SUDA H., HATAI K. & ISHII H. (1996). Histopathological comparison between ayu and carp artificially infected with *Aphanomyces piscicida. Fish Pathol.,* **31**, 71–80.

33. WILLOUGHBY L.G. & ROBERTS R.J. (1994). Improved methodology for isolation of the Aphanomyces fungal pathogen of epizootic ulcerative syndrome (EUS) in Asian fish. *J. Fish Dis.,* **17**, 541–543.

*
* *

NB: There is an OIE Reference Laboratory for Epizootic ulcerative syndrome (see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).