INFECTION WITH *APHANOMYCES INVADANS* (EPIZOOTIC ULCERATIVE SYNDROME)

1. Scope

Infection with *Aphanomyces invadans* means all infections caused by the oomycete *A. invadans* of the Genus *Aphanomyces* and Family *Leptolegniaceae*.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

Infection with *A. invadans* is most commonly known as epizootic ulcerative syndrome (EUS). It has also been known as red spot disease (RSD) (Mckenzie & Hall, 1976), mycotic granulomatosis (MG) (Egusa & Masuda, 1971; Hanjavanit, 1997) and ulcerative mycosis (UM) (Noga & Dykstra, 1986). The disease is caused by the oomycete *Aphanomyces invadans*.

Infection with *A. invadans* is a seasonal epizootic condition of great importance in wild and farmed freshwater and estuarine fish. It is clinically characterised by the presence of invasive, non-septate hyphae in skeletal muscle, usually leading to a granulomatous response. Infections with *A. invadans* have spread widely since the first outbreak in 1971 in Japan and to date a high degree of genetic homogeneity is observed for this species based on publicly available genome sequences (Dieguez-Uribeondo *et al.*, 2009; European Food Safety Authority [EFSA] 2011a; Huchzermeyer *et al.*, 2018; Iberahim *et al.*, 2018; Lilley *et al.*, 2003). Other pathogenic viruses (mostly rhabdoviruses), bacteria (mainly **Aeromonas h**ydrophila), fungi, oomycetes and parasites are routinely co-isolated from *A. invadans*-infected fish (Iberahim *et al.*, 2018).

Aphanomyces invadans is within a group of organisms commonly known as the water moulds. Although long-regarded as fungi because of their characteristic filamentous growth, this group, the Oomycota, is not considered a member of the Eumycota (true fungi) but is classified with diatoms and brown algae in a group called the Heterokonta or Stramenopiles within the Kingdom Chromista (Cavalier-Smith & Chao 2006; Tsui *et al.*, 2009). Junior synonyms of *A. invadans* include Aphanomyces piscicida and Aphanomyces invaderis.

2.1.2. Survival and stability in processed or stored samples

There is limited published data on the stability of the pathogen in host tissues. It is not clear whether the pathogen continues to grow for some time following the death of the host (Oidtmann, 2012).

Aphanomyces invadans cultures can be maintained for extended periods in glucose phosphate broth (6 weeks at 10°C), agar slopes and sodium phosphate buffer (over 6 months at 20°C) (Lilley *et al.*, 1998).

2.1.3. Survival and stability outside the host

How *A. invadans* survives outside the host is unclear (Oidtmann, 2012). It is assumed that the motile zoospores, which are released from an infected fish, will encyst when unsuccessful in finding a suitable substrate to grow on (Oidtmann, 2012). Encysted zoospores of *A. invadans* are capable of releasing a new zoospore generation instead of germinating in a process called repeated zoospore emergence (Dieguez-Uribeondo *et al.*, 2009). There is no suitable method to recover or isolate the encysted zoospore from affected fish ponds (Afzali *et al.*, 2013). How long the encysted spore can survive in water or on a non-fish substrate is unclear. In an *in-vitro* experiment, the encysted zoospore survived for at least 19 days (Lilley *et al.*, 2001).

For inactivation methods, see Section 2.4.5.

2.2. Host factors

2.2.1. Susceptible host species

[Note: an assessment of species that meet the criteria for listing as susceptible to infection with *A. invadans* in accordance with Chapter 1.5. has not yet been completed]

Table 2.1. Fish species susceptible to infection with Aphanomyces invadans

Family	Scientific name	Common name
Alestidae	*Brycinus lateralis*	striped robber
	Hydrocynus vittatus	tigerfish
	Micralestes acutidens	silver robber
Ambassidae	*Ambassis agassizii*	chanda perch
Apogonidae	*Glossamia aprion*	mouth almighty
Ariidae	*Arius sp.*	fork-tailed catfish
Belonidae	*Strongylura kreffti*	long tom
Centrarchidae	*Lepomis macrochirus*	bluegill
	Micropterus salmoides	largemouth black bass
Channidae	*Channa marulius*	great snakehead fish
	Channa striatus	striped snakehead
Cichlidae	*Coptodon rendalli*	redbreast tilapia
	Oreochromis andersoni	three-spoted tilapia
	Oreochromis machrochir	greenhead tilapia
	Sargochromis carlottae	rainbow bream
	Sargochromis codringtonii	green bream
	Sargochromis giardi	pink bream
	Serranochromis angusticeps	thinface largemouth
	Serranochromis robustus	Nembwe
	Tilapia sparrmanii	banded tilapia
Clariidae	*Clarias gariepinus*	sharptooth African catfish
	Clarias ngamensis	blunt-toothed African catfish
	Clarius batrachus	walking catfish
Clupeidae	*Alosa sapidissima*	American shad
	Brevoortia tyrannus	Atlantic menhaden
	Nematalosa erebi	bony bream
Cyprinidae	*Barbus paludinosus*	straightfin barb
	Barbus poechii	dashtail barb
	Barbus thamalakanensis	Thamalakane barb
	Barbus unitaeniatus	longbeard barb
	Carassius auratus	goldfish
	Catla catla	catla
	Cirrhinus mrigala	mrigal
	Esomus sp.	flying barb
	Labeo cylindricus	red-eye labeo
	Labeo lunatus	upper Zambezi labeo
	Labeo rohita	rohu
	Puntius gonionotus	silver barb
	Puntius sophore	pool barb
	Rohtee sp.	keti-Bangladeshi
Eleotridae	*Oxyeleotris lineolatus*	sleepy cod
	Oxyeleotris marmoratus	marble goby

Family	Scientific name	Common name
Gobiidae	*Glossogobius giuris*	bar-eyed goby
	Glossogobius sp.	goby
	Tridentiger obscures obscures	dusky tripletooth goby
Helostomatidae	*Helostoma temmincki*	kissing gourami
Hepsetidae	*Hepsetus odoe*	African pike
Ictaluridae	*Ameiurus melas*	black bullhead
	Ameiurus nebulosus	black bullhead
	Amniataba percoides	striped grunter
	Ictalurus punctatus	channel catfish
Kurtidae	*Kurtus gulliveri*	nursery fish
Latidae	*Lates calcarifer*	barramundi or sea bass
Lutjanidae	*Lutjanus argentimaculatus*	mangrove jack
Melanotaeniidae	*Melanotaenia splendida*	rainbow fish
Mormyridae	*Marcusenius macrolepidotus*	bulldog
	Petrocephalus catostoma	churchill
Mugilidae	*Mugilidae (Mugil spp.; Liza spp.)*	mullets
	Mugil cephalus	grey mullet or striped mullet
	Mugil curema	white mullet
	Myxus petardi	mullet
Osmeroidei	*Plecoglossus altivelis*	ayu
Osphronemidae	*Colisa lalia*	dwarf gourami
	Osphronemus goramy	giant gourami
	Trichogaster pectoralis	snakeskin gourami
	Trichogaster trichopterus	three-spot gourami
Osteoglossidae	*Scleropages jardini*	saratoga
Percichthyidae	*Maccullochella ikei*	freshwater cod
	Maccullochella peelii	Murray cod
	Macquaria ambigua	golden perch
	Macquaria novemaculeata	Australian bass
Platycephalidae	*Platycephalus fuscus*	dusky flathead
Psettodidae	*Psettodes sp.*	spiny turbot
Salmonidae	*Oncorhynchus mykiss*	rainbow trout
Scatophagidae	*Scatophagus argus*	spotted scat
	Selenotoca multifasciata	striped scat
Schilbeidae	*Schilbe intermedius*	silver catfish
	Schilbe mystus	African butter catfish
Sciaenidae	*Bairdiella chrysoura*	drums or croakers
	Pogonias cromis	black drum
Sillaginae	*Sillago ciliata*	sand whiting
Siluridae	*Silurus glanis*	wels catfish
Soleidae	*Aseraggodes macleayanus*	narrow banded sole
Sparidae	*Acanthopagrus australis*	yellowfin sea bream
	Acanthopagrus berda	black bream
	Archosargus probatocephalus	sheepshead
Synbranchidae	*Fluta alba*	swamp eel
Terapontidae	*Anabas testudineus*	climbing perch
	Bidyanus bidyanus	silver perch
	Leiopotherapon unicolor	spangled perch
	Scortum barcoo	Barcoo Grunter
	Therapon sp.	therapon
Toxotidae	*Toxotes chatareus*	common archerfish
	Toxotes lorentzi	primitive acherfish

2.2.2. Species with incomplete evidence for susceptibility [under study]

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with *A. invadans* according to Chapter 1.5 of the *Aquatic Code* are: [under study]

2.2.3. Likelihood of infection by species, host life stage, population or subpopulations

Subadult and adult fish are usually described as the susceptible life stages to natural outbreaks of EUS (FAO, 2009). However, there are reports of infection with *A. invadans* being found in early life stages (fish fry or fish larvae) (Baldock *et al.*, 2005; EFSA 2011a). While the size of the fish does not determine an EUS outbreak (Cruz-Lacierda & Shariff, 1995), younger fish seem to be more prone to EUS compared with adult fish (Gomo *et al.*, 2016; Pagrut *et al.*, 2017).

An experimental injection of *A. invadans* into the yearling life stage of catla (*Catla catla*), rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*), revealed resistance to *A. invadans* (Pradhan *et al.*, 2007), even though they are naturally susceptible species. Experimental infections demonstrated that goldfish (*Carassius auratus*) are susceptible (Hatai *et al.*, 1977; 1994), but common carp (*Cyprinus carpio*) (Wada *et al.*, 1996), Nile tilapia (*Oreochromis niloticus*) (Khan *et al.*, 1998) and European eel (*Anguilla anguilla*), (Oidtmann *et al.*, 2008) are considered resistant.

2.2.4. Distribution of the pathogen in the host

During the course of an infection with *A. invadans*, the free-swimming zoospore attaches to the skin of a fish host, encysts and germinates to develop hyphae invading and ramifying through host tissues (Kiryu *et al.*, 2003; Lilley *et al.*, 1998). The hyphal invasion and associated pathology are not confined to the region of dermal ulcers. The hyphae readily invade the body cavity and produce mycotic granulomas in all the visceral organs (Vishwanath *et al.*, 1998). In fish either suspected or confirmed to be infected with *A. invadans*, hyphae have also been observed in kidney, liver, spleen, pancreatic tissue, gut, parietal peritoneum, swim bladder, gonads, spinal cord, meninges, vertebrae, inter-muscular bones, the mouth region, and the orbits (Chinabut & Roberts, 1999; Vishwanath *et al.*, 1998; Wada *et al.*, 1996).

2.2.5. Aquatic animal reservoirs of infection

Generally, most infected fish die during an outbreak. Although some fish with mild or moderate infections could recover, they are unlikely to be lifelong carriers of *A. invadans*.

2.2.6. Vectors

No data available.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

The prevalence of infection with *A. invadans* in the wild and in aquaculture farms may be high (20–90%), in endemic areas with high levels of mortality observed. Mortality patterns appear to be seasonal and can vary substantially (Herbert *et al.*, 2019).

2.3.2. Clinical signs, including behavioural changes

Fish usually develop red spots or small-to-large ulcerative lesions on the body. The occurrence of skin lesions and ultimately mortality varies according to fish species. Fish presenting with lesions are usually weak, appear darker in colour, have a reduced appetite, are immobile and may float at the surface of the water. Generally infected fish are encountered in shallow water and present a retarded ability to escape capture occasionally followed by short lived bouts of hyperactivity characterised by jerky movements (Huchzermeyer *et al.*, 2018; Iberahim *et al.*, 2018).

2.3.3 Gross pathology

Early-stage lesions or mildly infected fish are characterised by red spots observed on the lateral body surface, head, operculum or caudal peduncle of the infected fish. Scales of infected fish are often protruding or lost. In severe cases, swollen haemorrhagic areas, massive inflammation and large

deep ulcers exposing the underlying necrotic muscle tissue are observed (Huchzermeyer *et al.*, 2018; Iberahim *et al.*, 2018). In advanced stages of the disease, the severity of the disease results in death of the fish (Hawke *et al.*, 2003; Iberahim *et al.*, 2018).

2.3.4. Modes of transmission and life cycle

Aphanomyces invadans has an aseptate fungal-like 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 a reniform, laterally biflagellate cell 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 (repeated zoospore emergence or polyplanetism) (Lilley *et al.*, 1998). The *A. invadans* zoospores can be horizontally transmitted from one fish to another through the water supply. It is believed that only the secondary zoospores or free-swimming stage zoospores are capable of attaching to the damaged skin of fish and germinating into hyphae. If the secondary zoospores cannot find the susceptible species or encounter unfavourable conditions, they can encyst in the pond environment. The cysts may wait for conditions that favour their transformation into tertiary generations of zoospores that are also in the free-swimming stage. The encysting property of *A. invadans* may play an important role in the cycle of outbreaks in endemic areas.

2.3.5. Environmental factors

Under natural conditions, infection with *A. invadans* has been reported at water temperatures in the range 10–33°C (Bondad-Reantaso *et al.*, 1992; Hawke *et al.*, 2003) often associated with massive rainfall (Bondad-Reantaso *et al.*, 1992). These conditions favour sporulation of *A. invadans* (Lumanlan-Mayo *et al.*, 1997), and temperatures of 17–19°C have been shown to delay the inflammatory response of fish to oomycete infection (Catap & Munday, 1998, Chinabut *et al.*, 1995). 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 presence of other pathogens (viruses, bacteria or ectoparasites, skin damage, water temperature (between 18 and 22°C), low pH (6.0–7.0) and low oxygen concentration in the water have all been hypothesised as predisposing factors for infection or factors influencing the expression of the disease (Oidtmann, 2012; Iberahim *et al.*, 2018).

Movements of live ornamental fish from countries from which infection with *A. invadans* is confirmed may spread the disease as was the case with the outbreak in Sri Lanka (Balasuriya, 1994). Flooding also caused the spread of infection with *A. invadans* in Bangladesh and Pakistan (Lilley *et al.*, 1998). Once an outbreak occurs in rivers/canals, the disease can spread downstream as well as upstream where the susceptible fish species exist.

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. Under laboratory conditions the optimal growth temperature range for *A. invadans* is 19–22°C, while under natural conditions *A. invadans* seems to be more robust (Hawke *et al.*, 2003).

2.3.6. Geographical distribution

Infection with *A. invadans* was first reported in farmed freshwater ayu (Plecoglossus altivelis) in Asia in 1971 (Egusa & Masuda, 1971). It was later reported in estuarine fish, particularly grey mullet (Mugil cephalus) in eastern Australia in 1972 (Fraser *et al.*, 1992; Mckenzie & Hall, 1976). Infection with *A. invadans* has extended its range into South-East and South Asia, and into West Asia (Lilley *et al.*, 1998; Tonguthai, 1985). Outbreaks of ulcerative disease in menhaden (Brevoortia tyrannus) in North America had the same aetiological agent as the disease observed in Asia (Blazer *et al.*, 1999; Lilley *et al.*, 1997a; Vandersea *et al.*, 2006). The first confirmed outbreaks of infection with *A. invadans* on the African continent occurred in 2007, and were connected to the Zambezi-Chobe river system (Andrew *et al.*, 2008; FAO, 2009; Huchzermeyer & Van der Waal, 2012; McHugh *et al.*, 2014). In 2010 and 2011, infection with *A. invadans* appeared in wild freshwater fish in Southern Africa and in wild brown bullhead fish in North America. Infection with *A. invadans* has been reported from more than 20 countries in four continents: North America, Southern Africa, Asia and Australia.

See WAHIS (https://wahis.woah.org/#/home) for recent information on distribution at the country level.

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

There is no protective vaccine available.

2.4.2. Chemotherapy including blocking agents

There is no effective treatment for *A. invadans*-infected fish in the wild and in aquaculture ponds.

2.4.3. Immunostimulation

Experimentally infected snakehead fish fed a vitamin-supplemented feed exhibited clinical signs of infection with *A. invadans* but had higher survival than controls (Miles *et al*., 2001).

2.4.4. Breeding resistant strains

No data available.

2.4.5. Inactivation methods

To minimise fish losses in infected fish ponds water exchange should be stopped and lime or hydrated lime and/or salt should be applied (Lilley *et al*., 1998). Preparing fish ponds by sun-drying and liming are effective disinfection methods for *A. invadans* (EFSA 2011b; Kumar *et al*., 2020; Oidtmann, 2012). Similar to other oomycetes or water moulds, general disinfection chemicals effectively destroy *A. invadans* that might contaminate farms, fish ponds or fishing gear (Iberahim *et al*., 2018).

2.4.6. Disinfection of eggs and larvae

There are no published protocols for *A. invadans* disinfection.

2.4.7. General husbandry

Control of *A. invadans* in natural waters is probably impossible. In outbreaks occurring in small, closed water bodies or fish ponds, treating water with agricultural limes and improving water quality, together with removal of infected fish, is often effective in reducing mortalities and controlling the disease. Preventing entry of water from *A. invadans*-infected water bodies into fish ponds can prevents spread of the disease into farms. Sodium chloride or salt and agricultural lime are safe and effective chemicals for treating or preventing the spread of *A. invadans*.

3. Specimen selection, sample collection, transportation and handling

3.1. Selection of populations and individual specimens

Scoop net, cast net or seine net represent the best choices for catching diseased fish in natural waters or in fish ponds (FAO 2009).

Fish with characteristic EUS-like lesions should be sampled from affected populations

3.2. Selection of organs or tissues

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 skeletal muscle is the target organ and exhibits major clinical signs of infection with *A. invadans* with mycotic granulomas (Iberahim *et al*., 2018). Samples should not be taken from the middle of large lesions as these are likely to be devoid of visible and viable hyphae. Instead, samples should be taken from the leading edge of the infected area or lesion and where possible, multiple samples should be taken from an infected individual to obtain viable hyphae. Fungal hyphae can be

seen in tissue squash mounts and histological sections at the leading edge of the infected area. Attempting to culture *A. invadans* from severe ulcers is often constrained because of contaminating bacteria, but still should be attempted. Polymerase chain reaction (PCR) on tissue taken from the leading edge of the ulcer also should be attempted.

Standard sample collection, preservation and processing methods for molecular techniques can be found in Section B.2.5. of Chapter 2.3.0 *General information* (diseases of fish).

3.3. Samples or tissues not suitable for pathogen detection

Samples should not be taken from the middle of large lesions as these are likely to be devoid of visible and viable hyphae.

3.4. Non-lethal sampling

None available.

3.5. Preservation of samples for submission

Fish specimens should be transported to the laboratory live or in ice-cooled boxes for further diagnosis. Samples must not be frozen as *A. invadans* is killed by freezing. 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.

For guidance on sample preservation methods for the intended test methods, see Chapter 2.3.0 *General information* (diseases of fish).

3.5.1. Samples for pathogen isolation

The success of pathogen isolation depends strongly on the quality of samples (time since collection and time in storage). Fresh specimens should be kept on ice and preferably sent to the laboratory within 24 hours of collection. To avoid degradation of samples use alternative storage methods only after consultation with the receiving laboratory. Multiple samples should be taken from each lesion to increase the chances of obtaining viable hyphae.

3.5.2. Preservation of samples for molecular detection

Tissue samples for PCR testing should be preserved in 70–90% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1.

3.5.3. Samples for histopathology, immunohistochemistry or *in-situ* hybridisation

Standard methods for histopathology can be found in Chapter 2.3.0.

3.5.4. Samples for other tests

None

3.6. Pooling of samples

Pooling of samples from more than one individual animal for a given purpose should only be recommended where supporting data on diagnostic sensitivity and diagnostic specificity have been evaluated and found to be suitable. The effect of pooling on diagnostic sensitivity has not been thoroughly evaluated, therefore, larger animals should be processed and tested individually. However, smaller life stages (e.g. fry) can be pooled to provide a minimum amount of material for testing.

4. Diagnostic methods

The methods currently available for pathogen detection that can be used in i) surveillance of apparently healthy animals, ii) presumptive diagnosis in clinically affected animals and iii) confirmatory diagnostic purposes are listed in Table 4.1. by animal life stage.

Ratings for purposes of use. For each recommended assay a qualitative rating for the purpose of use is provided. The ratings are determined based on multiple performance and operational factors relevant to application of an assay for a defined purpose. These factors include appropriate diagnostic performance characteristics, level of assay validation, availability cost, timeliness, and sample throughput and operability. For a specific purpose of use, assays are rated as:

+++ =   Methods are most suitable with desirable performance and operational characteristics.
++ =   Methods are suitable with acceptable performance and operational characteristics under most circumstances.
+ =   Methods are suitable, but performance or operational characteristics may limit application under some circumstances.
Shaded boxes = Not appropriate for this purpose.

Validation stage. The validation stage corresponds to the assay development and validation pathway in chapter 1.1.2. The validation stage is specific to each purpose of use. Where available, information on the diagnostic performance of recommended assays is provided in Section 6.3.

WOAH Reference Laboratories welcome feedback on diagnostic performance of recommended assays, in particular PCR methods. Of particular interest are any factors affecting expected assay sensitivity (e.g. tissue components inhibiting amplification) or expected specificity (e.g. failure to detect particular genotypes, detection of homologous sequences within the host genome). These issues should be communicated to the WOAH Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

Table 4.1. WOAH recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals

Method	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis[1] of a suspect result from surveillance or presumptive diagnosis			
	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV
Clinical signs	+	+	+	NA	+	+	+	NA				
Squash mounts					+	+	+	1	+	+	+	1
Histopathology					++	++	++	1	++	++	++	1
Cytopathology												
Cell or artificial media culture					++	++	++	1	+	+	+	1
Real-time PCR												
Conventional PCR					++	++	++	1				
Conventional PCR followed by amplicon sequencing									+++	+++	+++	1
In-situ hybridisation									++	++	++	1
Bioassay												
LAMP												
Ab ELISA												
Ag ELISA												
Other antigen detection methods												
Other method												

LV = level of validation, refers to the stage of validation in the WOAH Pathway (Chapter 1.1.2); NA = not available; PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively.

[1]For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). [2]Susceptibility of early and juvenile life stages is described in Section 2.2.3.

Shading indicates the test is inappropriate or should not be used for this purpose.

4.1. Observation for clinical signs

Using observational data of clinical signs (see Section 2.3.2 Clinical signs, including behavioural changes) for targeted surveillance, a sample of the fish population should be examined live with a sample size sufficient to meet survey design assumptions as described in Chapter 1.4 of the Aquatic Code. Surveys should be conducted during seasons that favour clinical manifestation of infection with *A. invadans* or when water temperatures are in the range 18–25°C.

4.2. Squash mounts

Aphanomyces invadans can be detected using microscopic examination of squash preparations prepared 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. Histopathology and cytopathology

Aphanomyces invadans can be detected using microscopic examination of fixed sections, prepared as follows:

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.

4.3.1. Histological procedure

Standard methods for processing are provided in chapter 2.3.0. H&E and general fungus stains (e.g. Grocott's stain) will demonstrate typical granulomas and invasive hyphae.

4.3.2 Histopathological changes

Early lesions are caused by erythematous dermatitis with no obvious oomycete involvement. *Aphanomyces invadans* hyphae are observed growing in skeletal muscle as the lesions progress 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.

4.4. Cell culture for isolation

4.4.1. Isolation of *Aphanomyces invadans* from internal tissues

The following are two methods of isolation of *A. invadans* adapted from Lilley *et al*. (1998) and Willoughby & Roberts (1994).

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 medium 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 glucose/yeast extract (GY) agar (see Table 4.1) and transferred to a fresh GY agar tube once every 1–2 weeks (Hatai & Egusa, 1979).

4.4.2. 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 spp., as described in Lilley *et al.*, 1998. *Aphanomyces 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 Lilley & Roberts (1997). *A. invadans* can be identified by PCR amplification of the rDNA of *A. invadans*.

4.4.3. 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 glucose/peptone/yeast (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 4.4.3.1), 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.

Table 4.4.3.1. Media for isolation, growth and sporulation of Aphanomyces invadans cultures

GP (glucose/peptone) medium	GPY (glucose/peptone / yeast) broth	GPY agar	GY agar (glucose/ yeast)	Autoclaved pond water
3 g litre^{-1} glucose 1 g litre^{-1} peptone 0.128 g litre^{-1} MgSO$_4$.7H$_2$O 0.014 g litre^{-1} KH$_2$PO$_4$ 0.029 g litre^{-1} CaCl$_2$.2H$_2$O 2.4 mg litre^{-1} FeCl$_3$.6H$_2$O 1.8 mg litre^{-1} MnCl$_2$.4H$_2$O 3.9 mg litre^{-1} CuSO$_4$.5H$_2$O 0.4 mg litre^{-1} ZnSO$_4$.7H$_2$O	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.

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.

4.4. Nucleic acid amplification

PCR assays should always be run with the controls specified in Section 2.5 *Use of molecular techniques for surveillance testing, confirmatory testing and diagnosis* of Chapter 2.3.0 *General information* (diseases of fish). Each sample should be tested in duplicate.

Extraction of nucleic acids

Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and can be checked using a suitable method as appropriate to the circumstances.

4.4.1. Real-time PCR

No real-time PCR methods for detecting *A. invadans* in fish tissues are available.

4.4.2. Conventional PCR

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 (Phadee *et al.*, 2004b; Vandersea *et al.*, 2006).

DNA preparation from A. invadans-*infected tissue*

Small pieces of *A. invadans*-infected tissue (25–50 mg) are suitable for DNA extractions (Phadee *et al.*, 2004a).

Diagnostic PCR technique

Three published techniques are specific to *A. invadans*. Oidtmann *et al.* (2008) demonstrated cross reactivity of the Phadee *et al.* (2004b) assay with *A. frigidophilus* when more than 10 ng of template DNA of *A. frigidophilus* was used in the PCR.

Pathogen/ target gene	Primer (5'–3')	Concentration	Cycling parameters
Method 1: Vandersea *et al.*, 2006); GenBank Accession No.: AF396684; amplicon size: 234bp			
Aphanomyces invadans (ITS1)	Fwd Ainvad-2F: TCA-TTG-TGA-GTG-AAA-CGG-TG Rev Ainvad-ITSR1: GCT-AAG-GTT-TCA-GTA-TGT-AG	0.025 nM 0.025 nM	35 cycles: 95°C/30 sec, 56°C/45 sec, 72°C/2.5 min
Method 2: Phadee *et al.*, 2004b; GenBank Accession No.: AF396684; amplicon size: 550bp			
Aphanomyces invadans (ITS1-ITS2)	Fwd ITS11: GCC-GAA-GTT-TCG-CAA-GAA-AC Rev ITS23: CGT-ATA-GAC-ACA-AGC-ACA-CCA	500 nM 500 nM	35 cycles: 94°C/30 sec, 65°C/30 sec, 72°C/1 min
Method 3: Oidtmann *et al.*, 2008; GenBank Accession No.: EU422990; amplicon size: 564			
Aphanomyces invadans (ITS1-ITS2)	Fwd BO73: CTT-GTG-CTG-AGC-TCA-CAC-TC Rev BO639: ACA-CCA-GAT-TAC-ACT-ATC-TC	600 nM 600 nM	35 cycles: 96°C/1 min, 58°C/1 min, 72°C/1 min

4.4.3. Other nucleic acid amplification methods

None.

4.5. Amplicon sequencing

Nucleotide sequencing of all conventional PCR amplicons (Section 4.4.2) is recommended as one of the final steps for confirmatory diagnosis. *Aphanomyces invadans*-specific sequences will share a high degree of nucleotide similarity to one of the published reference sequences for *A. invadans* (Genbank accession AF396684).

The size of the PCR amplicon should be verified, for example by agarose gel electrophoresis. Both DNA strands of the PCR product must be sequenced and analysed in comparison with reference sequences.

4.6. *In-situ* hybridisation

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 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 fluorescent oomycete hyphae appear as green fluorescence against the dark tissue background. The detailed protocols were published by Vandersea *et al.* (2006). Using the FISH technique, *A. invadans* can be visualised very well in thinly sliced tissue.

4.7. Immunohistochemistry

None.

4.8. Bioassay

Fish can be experimentally infected by intramuscular injection of 0.1 ml suspension of 100+ motile zoospores into fish susceptible to infection with *A. invadans* at 20°C. Histological growth of aseptate hyphae, 12–25 μm in diameter, should be demonstrated in the muscle of fish sampled after 7 days, and typical mycotic granulomas should be demonstrated in the muscle of fish sampled after 10–14 days.

4.9. Antibody or 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. (Lilley *et al.*, 1997b). However, a specific monoclonal antibody against *A. invadans* was found to have high specificity and high sensitivity to *A. invadans* using immunofluorescence. This monoclonal antibody could detect *A. invadans* hyphae at the early stage of infection (Miles *et al.*, 2003).

A monoclonal antibody-based flow-through immunoassay was developed by Adil *et al.* (2013). This assay was found to have high analytical (0.007 mg ml^{-1}) and diagnostic specificity comparable to PCR.

4.10. Other methods

Serological methods for detection and identification of *A. invadans* in diseased 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. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

The test for targeted surveillance to declare freedom from infection with *A. invadans* is examination of target populations for gross signs of infection with *A. invadans* (as described in Section 4.1 Observation for clinical signs).

If fish show gross signs consistent with infection with *A. invadans*, they should be categorised as suspect 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 4.1.

6. Corroborative diagnostic criteria

This section only addresses the diagnostic test results for detection of infection in the absence (6.1) or presence of clinical signs (6.2) but does not evaluate whether the infectious agent is the cause of the clinical event.

The case definitions for suspect and confirmed cases have been developed to support decision-making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. If a Competent Authority does not have the capability to undertake the necessary diagnostic tests it should seek advice from the appropriate WOAH Reference Laboratory, and if necessary, refer samples to that laboratory for confirmatory testing of samples from the index case in a country, zone or compartment considered free. There are currently no WOAH Reference Laboratories designated for EUS.

6.1. Apparently healthy animals or animals of unknown health status[1]

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. Hydrographical proximity to, or movement of animals or animal products or equipment, etc., from a known infected population equate to an epidemiological link. Alternatively, healthy populations are sampled in surveys to demonstrate disease freedom.

6.1.1. Definition of suspect case in apparently healthy populations

The presence of infection with *A. invadans* shall be suspected if at least one of the following criteria is met:

i) Observation of clinical signs consistent with infection with *A. invadans*[2]

ii) A positive result obtained by any of the diagnostic techniques described in Section 4.

6.1.2. Definition of confirmed case in apparently healthy populations

The presence of infection with *A. invadans* is considered to be confirmed if one or more of the following criteria is met:

i) Histopathology consistent with infection with *A. invadans* and positive result by PCR and amplicon sequencing

ii) Histopathological changes consistent with infection with *A. invadans* and positive result for *in-situ* hybridisation

iii) Artificial media culture and positive result by PCR and sequencing of the amplicon

6.2 Clinically affected animals

6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with *A. invadans* shall be suspected if at least one of the following criteria is met:

i) Gross pathology or clinical signs associated with infection with *A. invadans* as described in this chapter, with or without elevated mortality

ii) Positive result by a recommended molecular detection test

---

1   For example transboundary commodities.

2   Note that surveillance of apparently healthy populations for EUS is based on examination of target populations for clinical signs of infection with *A. invadans* (see Section 5 Test[s] recommended for surveillance to demonstrate freedom in apparently healthy populations).

iii) Histological changes consistent with infection with *A. invadans*

iv) Visual observation of hyphae characteristic (direct or by microscopy) of *A. invadans*

v) Culture and isolation of *A. invadans*-type colonies

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with *A. invadans* is considered to be confirmed if one or more of the following criteria is met:

i) Visualisation of hyphae under squash mounts and a positive result by PCR and sequencing of the amplicon

ii) Histopathological changes consistent with infection with *A. invadans* and a positive result by PCR and sequencing of the amplicon

iii) Histopathological changes consistent with infection with *A. invadans* and positive result for *in-situ* hybridisation

iv) Artificial media culture and a positive result by PCR and sequencing of the amplicon

v) Positive result for *in-situ* hybridisation and a positive result by PCR and sequencing of the amplicon

6.3. Diagnostic sensitivity and specificity for diagnostic tests [under study]

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with *A. invadans* is provided in Table 6.3.1. and 6.3.2. (no data are currently available for either). This information can be used for the design of surveys for infection with *A. invadans*, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic performance may vary under different conditions. Data is only presented where tests are validated to at least level 2 of the validation pathway described in Chapter 1.1.2 and the information is available within published diagnostic accuracy studies.

6.3.1. For presumptive diagnosis of clinically affected animals

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation

DSe: = diagnostic sensitivity, DSp = diagnostic specificity.

6.3.2. For surveillance of apparently healthy animals

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation

DSe: = diagnostic sensitivity, DSp = diagnostic specificity.

7. References

ADIL B., SHANKAR K.M., NAVEEN KUMAR B.T., PATIL R., BALLYAYA A., RAMESH K.S., POOJARY S.R., BYADGI O.V. & SIRIYAPPAGOUDER P. (2013). Development and standardization of a monoclonal antibody-based rapid flow-through immunoassay for the detection of *Aphanomyces invadans* in the field. *J. Vet. Sci.*, **14**, 413–419.

AFZALI S.F., HASSAN M.D., ABDUL-RAHIM A.M., SHARIFPOUR I. & SABRI J. (2013). Isolation and identification of *Aphanomyces* species from natural water bodies and fish farms in Selangor, Malaysia. *Malaysian Appl. Biol.*, **42**, 21–31.

ANDREW T., HUCHZERMEYER K., MBEHA B. & NENGU S. (2008). Epizootic ulcerative syndrome affecting fish in the Zambezi river system in Southern Africa. *Vet. Rec.,* **163**, 629–632.

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., Lester R. & Bondad-Reantaso M.G., eds. Fish Health Section, Asian Fisheries Society, Manila, Philippines, 555–585.

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.

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.

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.

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.

CAVALIER-SMITH T. & CHAO E.E.Y. (2006). Phylogeny and Megasystematics of Phagotrophic Heterokonts (Kingdom Chromista). *J. Mol. Evol.,* **62**, 388–420.

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.

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.

CRUZ-LACIERDA E.R. & SHARIFF M. (1995). Experimental transmission of epizootic ulcerative syndrome (EUS) in snakehead, *Ophicephalus striatus. Dis. Asian Aquac.,* **II**., 327–336.DIEGUEZ-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.

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

EUROPEAN FOOD SAFETY AUTHORITY EFSA (2011a). Scientific Opinion on Epizootic Ulcerative Syndrome. *EFSA J.,* **9**, 2387.

EUROPEAN FOOD SAFETY AUTHORITY (EFSA) (2011b). Report of the technical hearing meeting on Epizootic Ulcerative Syndrome (EUS). *EFSA Support. Publ.,* **8**, 1–16.

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS (FAO) (1986). Report of the expert consutation on ulcerative fish diseases in the Asia-Pacific region (TCP/RAS/4508). Bangkok, August 1986. FAO, Regional Office for Asia and the Pacific, Bangkok, Thailand.

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS (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.

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.

GOMO C., HANYIRE T., MAKAYA P. & SIBANDA S. (2016). Outbreak of epizootic ulcerative syndrome (EUS) in Seranochromis robustus fish spesies in Darwendale dam, Zimbabwe. *African J. Fish. Sci.,* **4**, 204–205.

HANJAVANIT C. (1997). Mycotic granulomatosis found in two species of ornamental fishes imported from Singapore. *Mycoscience*, 38, 433–436.

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.

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.

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

HAWKE J.P., GROOTERS A.M. & CAMUS A.C. (2003). Ulcerative Mycosis Caused by *Aphanomyces invadans* in Channel Catfish, Black Bullhead, and Bluegill from Southeastern Louisiana. *J. Aquat. Anim. Health.*, **15**, 120–127.

HERBERT B., JONES J.B.B., MOHAN C.V. V. & PERERA R.P.P. (2019). Impacts of epizootic ulcerative syndrome on subsistence fisheries and wildlife. *Rev. Sci. Tech.,* **38**, 459–475.

HUCHZERMEYER C.F., HUCHZERMEYER K.D.A., CHRISTISON K.W., MACEY B.M., COLLY P.A., HANG'OMBE B.M. & SONGE M.M. (2018). First record of epizootic ulcerative syndrome from the Upper Congo catchment: An outbreak in the Bangweulu swamps, Zambia. *J. Fish Dis.*, **41**, 87–94.

HUCHZERMEYER K.D.A. & VAN DER WAAL B.C.W. (2012). Epizootic ulcerative syndrome: Exotic fish disease threatens Africa's aquatic ecosystems. *J. S. Afr. Vet. Assoc.*, **83**, 1–6.

IBERAHIM N.A., TRUSCH F. & VAN WEST P. (2018). *Aphanomyces invadans*, the causal agent of Epizootic Ulcerative Syndrome, is a global threat to wild and farmed fish. *Fungal Biol. Rev.,* **44**, 1–13.

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.

KIRYU Y., SHIELDS J.D., VOGELBEIN W.K., KATOR H. & BLAZER V.S. (2003). Infectivity and pathogenicity of the oomycete *Aphanomyces invadans* in Atlantic menhaden *Brevoortia tyrannus*. *Dis. Aquat. Org.*, **54**, 135–146.

KUMAR P., SARKAR P., STEFI RAJU V., MANIKANDAN V., GURU A., ARSHAD A., ELUMALAI P. & AROCKIARAJ J. (2020). Pathogenicity and Pathobiology of Epizootic Ulcerative Syndrome (EUS) Causing Fungus *Aphanomyces invadans* and Its Immunological Response in Fish. *Rev. Fish. Sci. Aquac.*, **28**, 358–375.

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

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.

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

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

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.

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

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.

MCHUGH K.J., CHRISTISON K.W., WEYL O.L.F. & SMIT N.J. (2014). Histological Confirmation of Epizootic Ulcerative Syndrome in Two Cyprinid Species from Lake Liambezi, Zambezi Region, Namibia. *African Zool.*, **49**, 311–316.

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

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.

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. Aquat. Org.,* **55**, 77–84.

NOGA E.J. & DYKSTRA M.J. (1986). Oomycete fungi assocaited with ulcerative mycosis in menhaden, *Brevoortia tyrannus* (Latrobe). *J. Fish Dis.,* **9**, 47–53.

OIDTMANN B. (2012). Review of biological factors relevant to import risk assessments for epizootic ulcerative syndrome (*Aphanomyces invadans*). *Transbound. Emerg. Dis.,* **59**, 26–39.

OIDTMANN B., STEINBAUER GEIGER S. & HOFFMANN R.W. (2008). Experimental infection and detection of *Aphanomyces invadans* in European catfish, rainbow trout and European eel. *Dis. Aquat. Org.,* **82**, 185–207.

PAGRUT N.K., GANGULY S., JAISWAL V. & SINGH C. (2017). An overview on epizootic ulcerative syndrome of fishes in India: A comprehensive report. *J. Entomol. Zool. Stud.,* **5**, 1941–1943.

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

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

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.

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.

TSUI C.K.M., MARSHALL W., YOKOYAMA R., HONDA D., LIPPMEIER J.C., CRAVEN K.D., PETERSON P.D. & BERBEE M.L. (2009). Labyrinthulomycetes phylogeny and its implications for the evolutionary loss of chloroplasts and gain of ectoplasmic gliding. *Mol. Phylogenet. Evol.,* **50**, 129–140.

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.

VISHWANATH T., MOHAN C. & SHANKAR K. (1998). Epizootic Ulcerative Syndrome (EUS), associated with a fungal pathogen, in Indian fishes: histopathology – 'a cause for invasiveness'. *Aquaculture,* **165**, 1–9.

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.

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: At the time of publication (2023) no WOAH Reference Laboratories for infection with *Aphanomyces invadans* (please consult the WOAH web site for the most up-to-date list: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: First adopted in 1995 as epizootic ulcerative syndrome. Most recent updates adopted in 2023.