CHAPTER 2.3.1.

**infection with *APHANOMYCES INVADANS* (Epizootic ulcerative syndrome)**

**1. Scope**

Infection with *Aphanomyces invadans* means all infections caused by the oomycete fungus *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) (Food and Agriculture Organization of the United Nations [FAO], 1986). 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 *Aphanomyces 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 hydrophila*), 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**

***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 |
| 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*  | *wells 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 archer fish |
| *Toxotes lorentzi* | primitive acher fish |

**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 sub-populations**

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 Indian major carp, catla, rohu and mrigal, revealed resistance to *A. invadans* (Pradhan *et al.,* 2007), even though they are naturally susceptible species. Experimental infections demonstrated that goldfish are susceptible (Hatai *et al.,* 1977; 1994), but common carp (Wada *et al.,* 1996), Nile tilapia (Khan *et al.*, 1998) and European eel, (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 *Aphanomyces 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**

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 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.

For recent information on distribution at the country level consult the OIE WAHIS interface (https://www.oie.int/wahis\_2/public/wahid.php/Wahidhome/Home/index/newlang/en).

**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**

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

**2.4.7. 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. PCR on tissue taken from the leading edge of the ulcer also should be attempted.

**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 since the fungus 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 are available. 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.

OIE 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 OIE Reference Laboratories so that advice can be provided to diagnostic laboratories and the standards amended if necessary.

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

| **Method** | 1. **Surveillance of apparently healthy animals**
 | 1. **Presumptive diagnosis of clinically affected animals**
 | 1. **Confirmatory diagnosis1 of a suspect result from surveillance or presumptive diagnosis**
 |
| --- | --- | --- | --- |
| **Early life stages2** | **Juveniles2** | **Adults** | **LV** | **Early life stages2** | **Juveniles2** | **Adults** | **LV** | **Early life stages2** | **Juveniles2** | **Adults** | **LV** |
| **Squash mounts** |  |  |  |  | + | + | + | 1 | + | + | + | 1 |
| **Histopathology** |  |  |  |  | ++ | ++ | ++ | 1 | ++ | ++ | ++ | 1 |
| **Cytopathology** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Cell or artificial media culture** |  |  |  |  | ++ | ++ | ++ | 1 | + | + | + | 1 |
| **Real-time PCR** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Conventional PCR** |  |  |  |  | ++ | ++ | ++ | 1 | ++ | ++ | ++ | 1 |
| **Amplicon sequencing4** |  |  |  |  |  |  |  |  | +++ | +++ | +++ | 1 |
| ***In-situ* hybridisation** |  |  |  |  |  |  |  |  | ++ | ++ | ++ | 1 |
| **Bioassay** |  |  |  |  |  |  |  |  |  |  |  |  |
| **LAMP** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Ab ELISA** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Ag ELISA** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Other antigen detection methods3** |  |  |  |  |  |  |  |  |  |  |  |  |
| **Other method3** |  |  |  |  |  |  |  |  |  |  |  |  |

LV = level of validation, refers to the stage of validation in the OIE Pathway (Chapter 1.1.2); PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification;
Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively.
1For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). 2Susceptibility of early and juvenile life stages is described in Section 2.2.3.
3Specify the test used. Shading indicates the test is inappropriate or should not be used for this purpose.

Diagnosis of infection with *A. invadans* in clinically affected fish may be achieved by histopathology, oomycete isolation or polymerase chain reaction amplification.

**4.1. 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).

**4.2. 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 cm3), 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.

**4.2.1. 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.

**4.2.2 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.

**4.3. Cell culture for isolation**

**4.3.1. 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 mm3, 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 mm3) 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 GY agar (see Table 4.1) and transferred to a fresh GY agar tube once every 1–2 weeks (Hatai & Egusa, 1979).

**4.3.2. 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 polymerase chain reaction (PCR) amplification of the rDNA of *A. invadans*.

**4.3.3. 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.

***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 glucose1 g litre–1 peptone0.128 g litre–1 MgSO4.7H2O0.014 g litre–1 KH2PO40.029 g litre–1 CaCl2.2H2O2.4 mg litre–1 FeCl3.6H2O1.8 mg litre–1 MnCl2.4H2O3.9 mg litre–1 CuSO4.5H2O0.4 mg litre–1 ZnSO4.7H2O | 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**

**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*.

*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 (Vandersea *et al.*, 2006).

*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 MgCl2, 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 (Phadee *et al.*, 2004b).

*Method 3*

The species-specific primer sites are located in the ITS1 and ITS2 regions. The forward primer is BO73 (5’-CTT-GTG-CTG-AGC-TCA-CAC-TC-3’) and the reverse is BO639 (5’-ACA-CCA-GAT-TAC-ACT-ATC-TC-3’). The PCR mixture contains 0.6 µM of each primer, 0.2 mM of each deoxynucleoside triphosphate, 1.5 mM MgCl2, 0.625 units of Taq DNA polymerase, and approximately 5 ng of genomic DNA (or 2.5 µl of DNA template extracted from 25 mg of infected tissue and suspended in 100 µl buffer) in a 50 µl reaction volume (Oidtmann *et al.*, 2008). The DNA is amplified under the following cycle conditions: 96°C for 5 minutes; 35 cycles of 1 minute at 96°C, 1 minute at 58°C and 1 minute at 72°C; followed by a final extension at 72°C for 5 minutes (Oidtmann, pers. comm.). The PCR product is analysed by agarose gel electrophoresis and the target product is 564 bp.

The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.

**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)

**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 *A. invadans*-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 H2O, 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 permeabilisation. 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*. (2006). Using the FISH technique, *A. invadans* can be visualised very well in thinly sliced tissue compared with freshly squashed 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* developed later 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 (0.007mg 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*. 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.

Using the gross sign test for targeted surveillance, a large 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.

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.

**6.1. Apparently healthy animals or animals of unknown health status**5F5F**[[1]](#footnote-1)**

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. Geographical 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*

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

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

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

v) Culture and isolation of *A. invadans*

**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. (**note**: no data are currently available). 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 two 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, qPCR: = real-time polymerase chain reaction.

**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, qPCR: = real-time polymerase chain reaction.

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**NB:** There is currently (2022) no OIE Reference Laboratories for infection with *Aphanomyces invadans*
(see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list: https://www.oie.int/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 2013.

1. For example transboundary commodities. [↑](#footnote-ref-1)