Transboundary and Emerging Diseases

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REVIEW

Review of Biological Factors Relevant to Import Risk Assessments for Epizootic Ulcerative Syndrome (*Aphanomyces invadans*)

B. Oidtmann

Epidemiology and Risk Team, Centre for Environment, Fisheries and Aquaculture Science (Cefas), Weymouth, Dorset, UK

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Correspondence:
B. Oidtmann. Cefas Weymouth Laboratory, Barrack Road, Weymouth, Dorset DT4 8UB, UK.
Tel.: +44 1305 206661;
Fax: +44 1305 206601;
E-mail: birgit.oidtmann@cefas.co.uk

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Summary

Epizootic ulcerative syndrome (EUS) is a disease affecting both wild and farmed fish in freshwater and estuarine environments. After it was first described in Japan in 1971, the disease has spread widely across Asia and to some regions of Australia, North America and Africa. In Asia and Africa, the spread of the disease has substantially affected livelihoods of fish farmers and fishermen. No reports are yet published showing the presence of the disease in Europe or South America. Given its epizootic nature and its broad susceptible fish species range, it would appear that the disease has the potential for further spread. This study provides a review of the scientific literature on several biological factors of the pathogen, *Aphanomyces invadans*, associated with the disease EUS and aspects of the disease that are relevant to undertaking import risk assessments (IRA) covering (i) Life cycle and routes of transmission; (ii) Minimum infectious dose; (iii) Tissue localization and pathogen load; (iv) Predisposing factors for infection and factors influencing expression of disease; (v) Carrier state in fish; (vi) Diagnostic methods; (vii) Survival in the environment; (viii) Permissive temperature range; (ix) Stability of the agent in aquatic animal products; (x) Prevalence of infection; and (xi) Affected life stages. Much of the biological information presented is relevant to a broad range of risk questions. Areas where data are lacking were identified, and the information provided is put into context with other aspects that need to be addressed in an IRA.

Introduction

Epizootic ulcerative syndrome (EUS) is a disease affecting wild and farmed fish. It was first described in Japan in 1971 (Egusa and Masuda, 1971) and has since globally lead to major epidemics in freshwater and estuarine fish. The disease has been described from Asia, Australia, North America and Africa. In the various geographical regions affected, the disease had initially been given different names (red spot disease in Australia, mycotic granulomatosis in Japan and ulcerative mycosis in the USA). It is now generally accepted that these are the same diseases as EUS (McKenzie and Hall, 1976; Noga and Dykstra, 1986; Callinan et al., 1989, 1995a; Dykstra et al., 1989; Roberts et al., 1993, 1994, Lilley et al., 1998; Baldock et al., 2005; Andrew et al., 2008, FAO, 2009). In Asia and Africa, the spread of the disease has substantially affected livelihoods of fish farmers and fishermen and in some cases threatened the sustainable food supply for local populations depending on fish as a relatively affordable source of animal protein (Lilley et al., 2001b, 2002; Choongo et al., 2009; FAO, 2009). No reports are yet published showing the presence of the disease in Europe or South America. Owing to the importance of the disease, it is listed by the World Organisation for Animal Health (OIE), and thus, OIE member countries are obliged to make an official notification to OIE in the event of an occurrence or an outbreak. *Aphanomyces invadans* is recognized to be the primary cause for EUS (Baldock et al., 2005); *A. invadans* belongs to the

oomycetes. Although long regarded as fungi because of their characteristic filamentous growth, the Oomycetida are not a member of the Eumycota but are classified with diatoms and brown algae in a group called the Stramenopiles or Chromista. A single clone of *A. invadans* is broadly distributed worldwide (Lilley et al., 1997).

Currently, there are several geographical regions, which remain unaffected by the disease. However, given its epizootic nature and its broad susceptible fish species range, it would appear that the disease has the potential to spread further.

Countries that are currently considered free from EUS may identify the introduction of *A. invadans* and the establishment of the disease in their territory as a potential hazard and may wish to investigate the likelihood of this to occur.

Risk analysis is a method investigating the likelihood and consequences of undesirable events. In the field of animal health, the method is often used to assess the risks of disease introduction via international trade, known as import risk analysis. An import risk analysis is divided into four steps: hazard identification, risk assessment, risk management and risk communication. The risk assessment step is further divided into release, exposure, consequence assessment and risk estimation. Guidelines stipulate that a risk assessment is transparent and evidence based. Data required to underpin the risk assessment step of an import risk analysis are biological, commodity and country factors (Murray, 2002; Murray et al., 2004; World Organisation for Animal Health OIE, 2010).

At the outset of an import risk assessment, a specific risk question is usually defined (e.g. what is the risk of introduction and establishment of pathogen x via the import of live fish of species y from country z), and information relevant to assess the risk of introducing the pathogen via this route will be collated (Murray, 2002; Murray et al., 2004). The information relevant to a specific risk question may vary. However, much of the biological information required is relevant to a broad range of risk questions for a specific pathogen or disease. The OIE Aquatic Animal Health Code (World Organisation for Animal Health OIE, 2010) provides guidance for the preparation of import risk assessments (IRAs) and provides examples for aspects to be covered in the release and exposure assessment of an IRA. To support IRAs for EUS, the scientific literature on several biological aspects of the pathogen associated with EUS, *A. invadans*, was reviewed: e.g. the life cycle, permissive temperature range and survival of the pathogen outside of the host. Apart from the preparation of IRAs, the information can be used to support risk-based surveillance for EUS.

Methodology

A database of EUS-related literature was established through searches of the Aquatic Sciences and Fisheries Abstracts (ASFA) and Scopus databases. These databases were searched using the search terms '*Aphanomyces invadans*', '*Aphanomyces invaderis*', '*Aphanomyces piscicida*' and 'Epizootic ulcerative syndrome'. In addition, a substantial number of references were identified through references in other published papers, email alerts (e.g. aquavetmed) or were in the collection of publications of the reviewer, which had been built up over several years. More than 300 publications were captured in the database when the review was undertaken. The following biological factors are covered by the review: (i) Life cycle of *A. invadans* and routes of transmission; (ii) Minimum infectious dose (MID); (iii) Host infection – tissue localization and information on pathogen load; (iv) Predisposing factors for infection/factors influencing expression of disease; (v) Carrier state in fish; (vi) Diagnostic methods and difficulties in distinguishing EUS from other conditions; (vii) Survival outside of the host (in the environment); (viii) Permissive temperature range; (ix) Stability of the agent (in aquatic animal products); (x) Prevalence of infection; and (xi) Affected life stages. To identify publications relevant to the respective subject, the database was searched for the presence of certain keywords in title or abstract. However, this method failed to identify several publications with relevant information. Therefore, to a large extent, publications referred to in the review were selected based on whether title or abstract suggested that information relevant to a respective subject might be contained.

Results

Life cycle of *Aphanomyces invadans* and routes of transmission

The infective life stage of *A. invadans* is the free-swimming zoospore that attaches to a fish host, encysts and germinates to develop vegetative aseptate hyphae invading and ramifying through host tissues (Lilley et al., 1998; Kiryu et al., 2003). The hyphae will invade the fish skin, muscular tissue and may reach the internal organs. The likely pathway of transmission of *A. invadans* from infected to naïve fish is through the formation of a sporangium developing from the mycelium in fish tissue at the fish surface, followed by the formation of primary spores in the sporangium. The primary zoospores are released at the tip of the sporangium where they form a spore cluster (Willoughby et al., 1995). The primary spores quickly transform into the secondary zoospores, which are reniform and biflagellate and can actively swim in water. The secondary zoospore remains motile for a

period that depends on the environmental conditions and presence of the fish host or substratum (World Organisation for Animal Health OIE, 2009). When the secondary zoospore finds a new fish host, the life cycle is closed. If a zoospore does not succeed in finding a new host or if triggered by environmental factors, it may encyst. The phenomenon, whereby several successive tertiary generations of zoospores may be produced from the secondary cyst, has been described in a number of oomycetes. *Aphanomyces invadans* isolates have been shown to produce one additional generation of motile zoospores from artificially encysted secondary zoospores, but no further generations could be induced (Lilley et al., 1999). This is in contrast to *Aphanomyces astaci*, where up to three successive generations can be induced (Cerenius and Söderhäll, 1984, 1985).

In most susceptible fish species, it appears that some form of skin injury is required to enable *A. invadans* to successfully penetrate the skin (Lilley et al., 2001b; also see predisposing factors). One of the few species so far shown to not require prior injury to the skin is Atlantic menhaden (Kiryu et al., 2003). There are no data on vertical transmission of *A. invadans*.

Although the assumed route of transmission is through the release of zoospores from the surface of infected live or dead fish, there are little data providing direct evidence for this. Similarly, there are no data on the number of spores released from infected fish and no data as to whether spore release would be limited to fish displaying clinical signs or whether also clinically healthy appearing fish may be a source.

Minimum infectious dose

Challenge experiments with *A. invadans* have only been undertaken in a small proportion of the species believed to be susceptible. Most challenge experiments were undertaken using intramuscular or subcutaneous injection of the pathogen into fish. In such studies, the authors usually provide the spore dose administered. Examples of species in which such experiments have been undertaken include rainbow trout, eels, European catfish, three spot gouramis (Oidtmann et al., 2008), roach, stickleback, tilapia, rosy barb (Khan et al., 1998), Atlantic menhaden (Kiryu et al., 2002, 2003), striped mullet (Sosa et al., 2007a) and sand whiting (Catap and Munday, 2002).

The doses administered by injection range from 1 to 15 000 spores per fish. Atlantic menhaden is believed to succumb to disease when exposed to a single spore; the LD50 in this species was determined as 9.7 spores i.m. (Kiryu et al., 2003). Relatively low numbers of spores have also been used in striped mullets, where in a group of fish injected with a calculated number of five spores per fish, high mortalities (≥80%) occurred (Sosa et al., 2007a).

Other species such as rainbow trout are susceptible to injection of ≥1000 spores per fish (Khan et al., 1998; Oidtmann et al., 2008). However, susceptibility has not been tested against fewer spores per fish, so the MID is not known.

Even when high numbers of spores were injected, in some species, clinical signs were relatively mild and mortalities not always observed [e.g. common carp, European eel, rainbow trout and Tilapia (Oidtmann et al., 2008; Khan et al., 1998; Wada et al., 1996)]. This suggests that there is a substantial range in the level of susceptibility between species. Whereas a single spore may be sufficient to induce disease in Atlantic menhaden, this is less likely to be the case in rainbow trout, where clinical signs were relatively mild compared with highly susceptible species such as three spot gourami.

A very limited number of studies have used bath challenge to expose fish to *A. invadans* spores. Kiryu et al. (2003) challenged Atlantic menhaden, a species highly susceptible to EUS, by aqueous exposure and confirmed that *A. invadans* was pathogenic by this route. Salinity of the rearing water for the fish had been decreased from 20‰ to 1‰ over a 30-day period prior to challenge. The authors bath challenged Atlantic menhaden with *A. invadans* spores at 100 spores ml^{-1} for 5.5 h. Over the 27-day observation period, 9 of 14 net stressed fish (64%) developed focal ulcers, whereas amongst the fish presumed free of mechanical skin damage at the beginning of the challenge, only 5 of 36 fish (14%) developed ulcerous lesions. Mortality was 64% in the net stressed group and 11% in the group without skin damage. The drop of salinity levels prior to challenge is likely to have introduced an additional stressor that may have affected fish susceptibility.

The minimal infectious dose of *A. invadans* is likely to vary depending on predisposing factors. Kiryu et al. (2003) demonstrated that skin damage prior to pathogen exposure increased the prevalence of fish with ulcerous lesions and mortality. Similarly, water temperature appears to have an important impact, as was observed in snakehead and attributed to the functionality of the immune system to respond to infection (Chinabut et al., 1995). The findings described in these studies are likely to be valid for other fish species. Conditions likely to have led to skin damage and stress in affected fish have been described in many natural outbreaks of EUS. Therefore, any studies reporting a MID will need to be considered in the context of skin damage present at the time of exposure to the pathogen, environmental conditions and stressors that may have influenced the MID.

Host infection – tissue localization and information on pathogen load

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 (Lilley et al., 1998; Kiryu et al., 2003). All researchers who described the histopathology of EUS have observed hyphae in the skeletal muscle tissue (e.g. Miyazaki and Egusa, 1972, 1973; Noga et al., 1988; Callinan et al., 1989; Chinabut et al., 1995; Das and Mukherjee, 1998; Ahmed et al., 1999; Chinabut and Roberts, 1999; Oidtmann et al., 2008). Apart from muscle tissue and skin, other tissue locations where hyphae have been observed in fish assumed or confirmed to be infected with *A. invadans* include

1 Kidney (Wada et al., 1996; Ahmed and Hoque, 1999; Chinabut and Roberts, 1999),
2 Liver (Vishwanath et al., 1998; Ahmed and Hoque, 1999),
3 Spleen (Vishwanath et al., 1998),
4 Pancreatic tissue (Vishwanath et al., 1998; Chinabut and Roberts, 1999),
5 Gut, including the gizzard (Vishwanath et al., 1998).
6 Parietal peritoneum (Wada et al., 1996; Chinabut and Roberts, 1999).
7 Swim bladder (Chinabut and Roberts, 1999),
8 Gonads (Vishwanath et al., 1998),
9 Spinal cord (Wada et al., 1996; Vishwanath et al., 1998; Chinabut and Roberts, 1999),
10 Meninges (Chinabut and Roberts, 1999),
11 Vertebrae (Wada et al., 1996; Chinabut and Roberts, 1999),
12 Inter-muscular bones (Vishwanath et al., 1998),
13 The mouth region (Chinabut and Roberts, 1999),
14 Jaw (Chinabut and Roberts, 1999) and
15 Orbit (Chinabut and Roberts, 1999).

Reports on hyphae in tissues other than muscle tissue are sporadic though. However, some studies only sampled skin or muscle lesions (e.g. Wada et al., 1996), and therefore, lesions in other tissues would not have been reported; where other tissues were tested and histopathology of these tissues is not reported, one may conclude that no lesions were observed in those tissues, although there is some uncertainty. Furthermore, in a number of studies, histopathological lesions are reported from other tissues, without an associated presence of fungal hyphae (e.g. Noga et al., 1988). A few studies do report the absence of fungal hyphae in other sampled tissues (e.g. Vishwanath et al., 1997; Ahmed and Hoque, 1999; Sosa et al., 2007a).

Given that the skin is the known portal of entry, surprisingly few studies describe the histopathology of the skin (Vishwanath et al., 1997; Sosa et al., 2007b). Skin lesions are always associated with infection. The degree of macroscopically visible skin damage can range substantially from tiny red spots to open dermal ulcers (Vishwanath et al., 1997; Chinabut and Roberts, 1999). The paucity of reports describing fungal hyphae in the skin is possibly a result of the inflammatory and degenerative processes in the skin, which may lead to the hyphae that were initially penetrating through the skin being dissolved.

Whether hyphae remain largely restricted to the muscle tissue may depend on whether the affected fish survive long enough for the pathogen to reach other tissues. An example of a particularly resilient fish species is snakehead (*Channa striata*), where lesions may occur in a large range of tissues and lesions reach a degree, where many other species would have already succumbed (Chinabut and Roberts, 1999). Another factor that could play a role is the level of susceptibility of the species. Fish species shown to control and recover from infection are more likely to restrict infection to skin and skeletal muscle (author's conclusion).

There are methodological problems in trying to quantify the amount of viable *A. invadans* per defined amount of tissue. Hyphae may be present at high density in affected tissues; however, histological methods have their limitations in quantifying fungal pathogens, as one would not be certain whether the hyphae were indeed *A. invadans* or secondary contaminants.

Cultivation methods, as are used to quantify viral load, cannot be applied in the same way. Isolation of *A. invadans* provides information on the absence or presence of the pathogen but has little value for quantification. If tissues were homogenized (as is carried out for virus titration), this is likely to damage the mycelium and the result may be no growth at all, wrongly suggesting absence of the pathogen. The most useful method to quantify would therefore be a method such as real-time PCR. However, such a method is currently not available.

Furthermore, the value of quantitative data on the density of hyphae in infected tissues may be limited. It is currently unknown how many spores are released from infected fish and at which stage during infection this happens. The amount of hyphae present in muscle tissue may have relatively little association with the amount of zoospores that may be released from an infected fish.

It is unclear whether *A. invadans* may continue to grow for some time in dead fish. Studies to support or refute this hypothesis are however not available. If the pathogen continued to grow, this would be in contrast to viral infections, where virus replication would be expected to cease as the host dies and a continuous decline in pathogen levels would be expected. The decline of *A. invadans*

would largely depend on exposure to enzymes released from host cells because of autolysis and from secondary contaminating micro-organisms.

The likelihood of *A. invadans* being released from aquatic animal products is likely to depend on a range of factors, including whether the infected tissue is placed in a moist or aquatic environment and the storage temperature.

Predisposing factors for infection/factors influencing expression of disease

Skin damage might be required to facilitate infection in some species. Experimental induction of EUS by bath challenge in some known EUS-susceptible species has proven difficult (Lilley et al., 2001b). Challenge experiments with strains of *A. invadans* from South-east Asia have suggested that zoospores attach to the dermis and not the epidermis before invasion (Lilley et al., 1998). One of the few fish species, in which experimental bath challenge was successful to date, is Atlantic menhaden. However, the incidence of ulcer formation was much higher in Atlantic menhaden to which skin damage had been inflicted prior to bath challenge, compared to untraumatized fish (Kiryu et al., 2002). In epidemics in the wild, Atlantic menhaden has stood out as particularly susceptible to the disease.

Several studies have reported an association of low pH values with EUS both in wild and in experimentally challenged fish (Bondad-Reantaso et al., 1992; Callinan et al., 1996; Fairweather, 1999; Choongo et al., 2009). Some authors hypothesized that low pH leads to skin damage and through this providing a portal for *A. invadans* infection (Fairweather, 1999; Callinan et al., 2005).

Outbreaks of EUS have been repeatedly reported in snakehead. In this species, skin lesions as a result of intraspecies aggression (biting) are considered a predisposing factor (Fairweather, 1999; Saylor et al., 2010). Another example where skin lesions may provide a portal for infection is infection of fish with dinoflagellates (Dykstra and Kane, 2000). In most species, skin injury is, therefore, considered to be required to provide a portal for infection. Causes of mechanical skin damage in the farming environment could be grading or on-farm handling for other reasons.

Another relevant factor influencing the occurrence of the disease and outcome of infection is temperature. Epizootic ulcerative syndrome has been associated with comparatively low water temperatures – either because of a sudden drop in temperature associated with massive rainfalls or in the cold season of the year (Bondad-Reantaso et al., 1992; Chinabut et al., 1995; Asoka et al., 1999; Catap and Munday, 1999; Lilley et al., 2002). When fish are injected with *A. invadans* spores and kept at (for the respective species and their normal habitat) low temperatures (e.g. 17 or 19°C in sand whiting and snakeheads), the host response is delayed compared to fish held at higher temperatures (in the preferred temperature range of the species) (Chinabut et al., 1995; Catap and Munday, 1998). Given the likely association of a delayed host response with a compromised immune system, other factors that impact on the host immune response, such as stress, may also enhance development of the disease.

Further predisposing factors that have been suggested to be involved in EUS outbreaks include water quality–associated factors [i.e. drop in water salinity, low alkalinity and high ammonia, generally poor water quality, low oxygen levels (Khan et al., 2001; Virgona, 1992; Mohan et al., 1999; Asoka et al., 1999; Callinan et al., 1989)], exposure to toxins (Dykstra and Kane, 2000) and infection with ectoparasites, bacteria or viruses (Lilley et al., 1998). Most of these factors may lead to or be associated with skin injury, which may be the real predisposing factor.

Some of the above-described factors may not only act as predisposing factors for infection, but also influence the outcome of infection. In this context, any factors influencing the capability of the host to respond to infection would need to be considered as potentially relevant.

Carrier state in fish

A carrier is defined as an animal that sheds infectious agent without demonstrating clinical signs (Thrusfield, 2005). Whether or not fish may develop a carrier state when infected with *A. invadans* is currently unknown. However, another Aphanomyces species, *A. astaci*, does induce a carrier state in North American crayfish (Unestam, 1969; Unestam and Weiss, 1970; Oidtmann et al., 2006). Invading hyphae are encapsulated by melanin deposited by the host. This contains the further growth of hyphae in the crayfish. When the carrier crayfish host moults or dies, *A. astaci* may be reactivated and form zoosporangia growing out of the crayfish cuticle. Similar to the life cycle of *A. invadans*, zoospores are released from zoosporangia, which can then find a new crayfish host (Cerenius and Söderhäll, 1984, 1985; Cerenius et al., 1988).

Epizootic ulcerative syndrome may not be reported from an area for a number of months or even years between outbreaks (Bondad-Reantaso et al., 1992; Chinabut, 1998). There are currently no published data, showing where the pathogen survives during periods with no outbreaks. Pond management procedures, such as liming, removal of the bottom mud, draining and drying of the pond, have been identified as protective measures against EUS (Lilley et al., 2001b). This may initially suggest that

the methods used destroy the pathogen in the environment and therefore the reservoir is the environment (if not treated). However, given that these activities are also associated with removal of fish from the pond, this conclusion could be wrong. There are usually fish surviving an EUS outbreak. Therefore, the question of the reservoir remains unresolved. If fish did develop a carrier state, it could be very difficult to detect. Pathogen numbers present in the host may be below the sensitivity of available detection methods, which would cause a problem for disease eradication.

Many other oomycetes are saprophytic and can survive outside of a host for extended periods of time. Saprophytic organisms can survive on many substrates. However, some oomycetes pathogenic to aquatic animals are not saprophytic, e.g. the causative agent of crayfish plague, *A. astaci*. *Aphanomyces astaci* depends largely on the presence of a crayfish host for its survival. Zoospores and cysts only have a limited lifespan outside of a host (Unestam, 1969; Oidtmann et al., 2002). It is currently unknown whether *A. invadans* depends on a live host for long-term survival.

Incubation period

In outbreaks in the wild, it is notoriously difficult to determine the incubation period, because the point of introduction of the pathogen is often unknown. Given the unresolved question of a latent or subclinical infection that may evolve into a clinical infection when conditions are right, it is difficult to put a figure towards the upper end of the range of the incubation period, which could possibly be several months, if not years.

One of the shortest incubation periods are described from young Puntius sp., and Indian major and minor carps. Mass mortalities may occur within 48 h of fingerlings of these species being introduced from the hatchery and – because of the rapidity of the event – is often mistaken as fish poisoning (Chinabut and Roberts, 1999).

The results from bath challenges undertaken with Atlantic menhaden suggest an incubation period of at least 5-day post-aqueous exposure in net stressed fish. Lesions occurred also at earlier stages post-exposure but were thought not to be related to EUS (Kiryu et al., 2002).

Diagnostic methods and difficulties in distinguishing EUS from other conditions

Clinical signs

During natural outbreaks, fish develop a wide range of skin lesions, ranging from mild reddening to severe ulcers. Such skin lesions are non-specific and have been associated with a large range of conditions ranging from mechanical damage, over various bacterial infections (e.g. Vibrio spp., Aeromonas spp.), viral infections, ectoparasites, toxic dinoflagellates to diseases of yet unclear aetiology (e.g. Red Mark Syndrome, cold water strawberry disease) (Burke and Rodgers, 1981; Stoskopf, 1992; Noga, 1996; Roberts, 2001; Vogelbein et al., 2001; Wildgoose, 2001; Woo et al., 2002; Lloyd et al., 2008; Verner-Jeffreys et al., 2008). Therefore, clinical signs are of little utility in deciding whether a fish might be infected with *A. invadans*.

Histopathology

In several of the EUS-susceptible fish species, infection of the muscle tissue leads to the formation of mycotic epitheloid granulomas (e.g. goldfish, gourami and snakehead; Miyazaki and Egusa, 1972; Hatai et al., 1994; Chinabut et al., 1995). However, such granulomas are not always associated with infection: *A. invadans* was isolated from Bluegills *Lepomis macrochirus*, largemouth bass *Micropterus salmoides* and American shad *Alosa sapidissim*, but granulomas were not observed despite fungal hyphae in histological sections of dermis and skeletal muscle (Sosa et al., 2007b). Similar observations were made in Channel catfish *Ictalurus punctatus*, black bullhead *Ameiurus melas* (Hawke et al., 2003) and European catfish *Silurus glanis* (Oidtmann et al., 2008). Instead of epitheloid granulomas, other inflammatory responses included multinucleated giant cells. Furthermore, highly susceptible life stages of highly susceptible species [e.g. fingerlings of Puntius sp. (barb), Indian major or minor carp] may die before a pronounced immune response can develop (Chinabut and Roberts, 1999). Several other factors may decrease the likelihood that mycotic granulomas are observed, including where (i.e. how far from the skin lesion) the tissue samples are taken from and the degree of necrosis in the lesion. In summary, absence of mycotic granulomas should not be considered as evidence that the investigated condition is not EUS.

PCR/molecular methods

A number of PCR methods have recently been published, with primers targeting the internal transcribed spacer (ITS) region or nearby located DNA regions of the pathogen (Lilley et al., 2003; Phadee et al., 2004a,b; Vandersea et al., 2006; Oidtmann et al., 2008). The ability to confirm the identity of isolates and detect the pathogen in clinical samples has greatly improved the capability of diagnosing the disease and detecting infection.

The ITS region is a target sequence frequently used for design of primers for specific diagnostic PCR assays. The ITS region has been shown to be prone to mutation, making it a suitable area to discriminate between closely related species. A potential disadvantage of targeting this

area is that mutation may also occur over time within a given species and therefore carrying the risk that primers do not detect strains with mutations in the target region. Sequences from about 20 *A. invadans* strains are publicly available for the ITS region or parts thereof. One problem is that some of the differences in these sequences occur in the target areas of primers for *A. invadans* PCR assays. The potential consequence of such sequence differences is a potential loss in test sensitivity or even failure to detect. As *A. invadans* has not always been isolated when outbreaks in new geographical regions occurred, new strains may have evolved but have not yet been identified. A comparison of three published PCR assays is provided in Oidtmann et al. (2008).

Another molecular method described is fluorescent peptide nucleic acid *in-situ* hybridization (FISH) that has the advantage of directly staining *A. invadans* hyphae (Vandersea et al., 2006).

Other methods for identification include isolation of the protist in pure culture, followed by an assessment of the morphology of hyphae, zoosporangia, type of zoospore release, macroscopical appearance in pure culture and growth characteristics at certain temperatures. However, because of the absence of sexual stages in its reproductive cycle, an important criterion for species determination is not available, allowing characterization based on morphological criteria only to family level, and not species level.

Survival in the environment and disinfection

How *A. invadans* survives outside the host is still unclear. Experiments undertaken by Lilley et al. (2001a) showed that zoospores of *A. invadans* isolate B99C can survive for at least 19 days at 20°C *in vitro*. Willoughby (1999, cited by Lilley et al., 2001a) reported that attempts to isolate *A. invadans* from pond water or soil have not been successful. Furthermore, *A. invadans* does not survive in water temperatures over 31°C for any length of time (Willoughby, 1993; cited by Chinabut et al., 1995). Given that water temperatures in tropical Asia regularly go up as high as 33–36°C, Chinabut et al. (1995) suggest that some form of resistant stage must exist either in the water, sediment or within some other carrier.

In sporulation medium supplemented with various levels of NaCl, *A. invadans* sporulated at 2‰, but not at 4‰ NaCl and not at any higher NaCl concentrations tested. *Aphanomyces invadans* grew on agar media containing 8‰ NaCl, but not on those containing 12‰ NaCl (Fraser et al., 1992).

Sun-drying and liming of fish ponds appear to be effective disinfection methods for EUS. However, studies on the survival of *A. invadans* in dry or moist environment are missing (Khan and Lilley, 2002). Similar to other oomycetes or water molds, general disinfection chemicals effectively destroy any *A. invadans* that might contaminate farms, fish ponds or fishing gear (Lilley and Inglis, 1997).

Permissive temperature range

Water temperature is an important factor in the development of EUS. Generally, EUS occurs when the water temperatures are comparatively low – either because of a sudden drop in temperature associated with massive rainfalls or in the cold season of the year (Chinabut et al., 1995; Lilley et al., 2002). So far, the majority of fish species affected by EUS are tropical species and in those climates, temperatures considered 'low' can be as high as 25°C (Pathiratne and Jayasinghe, 2001).

However, EUS has been observed in a wide temperature range: the disease occurred in freshwater ponds in Louisiana in the winter months at water temperatures from 10 to 15°C (Hawke et al., 2003) and in a rice fish pond in the Philippines at temperatures as high as 33°C (Bondad-Reantaso et al., 1992).

In experimental studies, EUS has been induced in roach (*Rutilus rutilus*) when fish were reared in water temperatures ranging from 11 to 16°C (Khan et al., 1998), in ayu (*Plecoglossus altivelis*) at temperatures as low as 15–16°C (Wada et al., 1996) and in snakehead at temperatures as high as 31°C (Chinabut et al., 1995). The studies at 11–16 and 15–16°C are the lowest temperatures reported to date. It remains to be shown whether clinical disease would also occur at even lower temperatures.

Comparing sand whiting held at 17 or 26°C and injected with *A. invadans* zoospores, fish at 17°C had delayed onset of granuloma formation, fewer granulomas and decreased healing (Catap and Munday, 1998). When snakeheads held at 19, 26 and 31°C were injected with *A. invadans* zoospores, more fish succumbed to disease at 19°C than at 26 or 31°C, possibly due to a reduced immune response (Chinabut et al., 1995). In summary, if fish are kept below their optimal temperature range, the outcome of infection is more likely to be fatal compared to fish at their optimal temperature range. However, infection also takes place in the optimal temperature range of the respective species, with the immune response likely to be more pronounced and effective in limiting the infection.

Given that no experimental study has yet investigated the susceptibility of fish at temperatures below 11°C, it might be useful to take into consideration the temperature range at which *A. invadans* grows *in vitro*. In a study investigating the growth of several *A. invadans* isolates between 6 and 42°C, all *A. invadans* isolates grew at 6°C,

but not at 37°C. The highest temperature tested, where growth was still observed was 34°C. Growth rates were highest between 22 and 30°C, and good growth still occurred at 14 and 34°C (Lilley and Roberts, 1997). Therefore, the permissive temperature range may be broader than the range described in live fish to date.

Stability of the agent (in aquatic animal products)

There are no published studies on the survival of *A. invadans* after being exposed to low temperatures as are used for chilling or freezing. The lowest temperature at which *A. invadans* is reported to grow on agar is at 6°C (Lilley and Roberts, 1997; several *A. invadans* isolates were tested), whereas three Australian *A. invadans* isolates did not grow at 3 or 37°C, but at all the other temperatures tested (13, 22, 25 and 31°C, Fraser et al., 1992). No studies are available that investigated whether *A. invadans* was still viable after exposure to cold storage temperatures, such as 4 or 0°C or freezing for defined periods of time.

Studies undertaken with the closely related *A. astaci* may provide some indication for the survival of *A. invadans* when exposed to low temperatures: *A. astaci* mycelia or spores kept at 0, 5 or 10°C were still viable after 2 weeks. Mycelium survived temperatures of −5°C for 7 days and at −15°C for 10 min but was no longer viable after 20 min at −15°C (Alderman, 2000). In infected crayfish tissue, one study reports that viable mycelium was not detected 3 h after exposure to −20°C (Alderman, 2000), whereas according to another study, mycelium was still viable after 48 h at −20°C, but not 72 h (Oidtmann et al., 2002).

There are no studies to show the level of decline of viable pathogen in infected tissues. Experimental studies undertaken with *A. astaci* were simply reporting presence or absence of the pathogen. Given that *A. invadans* is generally adapted to a higher temperature range, it is likely that *A. invadans* will be more affected by cold temperatures compared to *A. astaci*.

The results from the cultivation studies would suggest that *A. invadans* is likely to survive exposure to 4°C for some time – likely to be in the order of days. The effect of storage on ice (around 0°C) is unknown, and storage at −20°C would be expected to inactivate the pathogen within 72 h.

Prevalence of infection

Prevalence data from outbreaks of EUS in wild or farmed fish populations are mainly available from studies undertaken in Asia and Australia. Many of these studies investigated the association of environmental conditions with the occurrence of EUS.

A number of studies provide prevalence levels in estuarine fish populations: Two studies from Australia reported on EUS mainly in sea mullet (*Mugil cephalus*) sampled from estuarine areas of two rivers and found pronounced seasonal fluctuations of EUS, with prevalence ranging between 0% and 40%. EUS was present on almost all sampling occasions over the 16- and 37-month observation period in the studies, albeit in most of the months at levels below 15% (Rodgers and Burke, 1981; Virgona, 1992).

Studies on fish from brackish water areas are also reported from India and the Philippines. In the Philippines, Callinan et al. (1995b) sampled mullet (Mugil sp.) from a lagoon over a 4-month period. An outbreak of EUS occurred 3 months into the study. Whereas prevalence of EUS-affected fish was reported as 0 for the first 3 months of the study, it reached 40% within 20 days of the onset of the outbreak. Prevalence remained above 30% for about 10 days and then quickly declined. Mohan et al. (1999) reported prevalence data in mullets from two large brackish water ponds along the west coast of Karnataka, India, over a 6-month period. EUS-positive mullets were recorded during 2.5 months of the study with prevalence ranging from 3.8% to 55.5%.

Studies reporting prevalence in freshwater environments are available mainly from Asia. A cross-sectional study from Bangladesh provides prevalence data by districts and species, in both farmed and wild fish populations. Epizootic ulcerative syndrome was confirmed (by histology) in fish farms in 30 of 64 districts (47%). From wild fisheries, 49 districts (77%) were confirmed EUS positive. Across the total of 6433 wild fish and 6401 farmed fish examined for lesions, prevalence was 16.0% and 15.5%, respectively. The prevalence within the affected fish populations was low on some sites. Within species affected by EUS, the proportion of fish with lesions ranged from 0.3% to 53% (Lilley et al., 2001b). According to Sarker and Chowdhury (2001), prevalence of EUS during outbreaks in Bangladesh was 23–45% in farmed and 27–90% in wild fish. Examples of wild fish with high within-species prevalence include *Channa striatus* (49%), *Nandus nandus* (47%) and *Puntius ticto* (45%). Farmed fish species prevalence was comparatively lower.

Further data for Bangladesh are reported by Lilley et al. (2001b). Prevalence amongst 54 species, in which EUS was observed, ranged from 0.4% to 66.7% (35 000 wild fish of 69 species were investigated in total; Lilley et al., 2001b, unpublished survey data).

Further studies that investigated natural outbreaks report within-species or within mixed species population prevalence, which fall within the range described previously (Bondad-Reantaso et al., 1992; Pathiratne and Jayasinghe, 2001).

Cumulative incidence and mortality data for EUS following experimental bath challenge are reported by Kiryu et al. (2003). The authors bath challenged Atlantic menhaden (*Brevoortia tyrannus*) with *A. invadans* spores at 100 spores ml^{-1}. Over the 27-day observation period, 9 of 14 net stressed fish (64%) developed focal ulcerous lesions. In the groups of fish free of skin damage at the beginning of the challenge, only 5 of 36 fish (14%) developed ulcerous lesions. Mortality was 64% in the net stressed group and 11% in the group without skin damage.

The prevalence values reported in the publications mentioned earlier were sometimes based on different criteria as to what was considered a fish with EUS lesions. For example, whereas some studies considered fish as affected by EUS when they had ulcers (Lilley et al., 2001b; Pathiratne and Jayasinghe, 2001), other studies counted a broader range of clinical signs as EUS lesions (Rodgers and Burke, 1981; Virgona, 1992; Mohan et al., 1999). Again in other studies, criteria for counting a fish as EUS affected were not clearly described (Bondad-Reantaso et al., 1992; Callinan et al., 1995b; Sarker and Chowdhury, 2001). Furthermore, data were often presented as average prevalences across several sampling occasions (including times when EUS lesions were not observed), making it difficult to extract prevalence data during outbreaks across species and within species (e.g. Lilley et al., 2001b; Khan and Lilley, 2002).

Another level of uncertainty is introduced by the difficulty in discriminating EUS from other skin conditions. Skin lesions may be caused by a variety of factors, and the presence of skin lesion is not pathognomonic for *A. invadans* infection. Furthermore, sampling of fish populations during epidemics may be biased towards moribund fish, as these are less likely to be able to escape or avoid being caught, leading to a possible overestimation of prevalence levels.

To summarize, when interpreting the prevalence data summarized previously, it is important to remember that predisposing factors are usually required for EUS to express clinically and the extent to which such predisposing factors are present will influence the level of prevalence. Ample evidence also exists for varying levels of susceptibility between species, which has an impact on prevalence levels (depending on species present in an affected area) and degree of skin lesions to be expected for a specific species. In addition to these, prevalence data based on skin lesions may include fish that had skin lesions induced by other factors.

Affected life stages

Natural outbreaks of EUS are mostly described in subadult to adult fish (e.g. Lilley et al., 2001b, FAO, 2009). However, at the same time, this could be a result of the sampling methods usually applied (e.g. seine or gill netting). Similarly, experimental studies have usually been undertaken using intramuscular injection and are therefore biased towards larger fish sizes. The few studies reporting EUS in juvenile fish are of fingerlings of Puntius sp. as well as Indian major and minor carps (peracute infection associated with to mass mortalities, Chinabut and Roberts, 1999) of Indian major carp fry and fingerlings (unpublished observation by C.V. Mohan, referred to by Baldock et al., 2005) and of juvenile giant gouramis (size not provided, Lilley et al., 2002). Interestingly, Indian major carp yearlings appear to be resistant (unpublished observation by C. V. Mohan, referred to by Baldock et al., 2005) suggesting differences in susceptibility by life stage depending on species. In summary, although the majority of natural outbreaks and experimental challenges are reported from adult or subadult fish, depending on species, juvenile fish, including fry, may be affected.

Discussion

This study reviews the published peer-reviewed information available on *A. invadans* and the disease associated with the pathogen, EUS, with focus on biological factors relevant to IRAs. The information relevant to the susceptible species range and geographical spread will be presented elsewhere (B. Oidtmann and F. Berthe, in preparation).

The two main commodities internationally traded are live fish and aquatic animal products (for example, fish fillets imported for human consumption). The data from the literature review suggest that the risks associated with import of aquatic animal products are going to be limited, especially if the product was subjected to some sort of temperature treatment (e.g. cooking or freezing). The risk assessment could be kept quite simple by assessing whether the pathogen is likely to be viable in the imported commodity as a result of the treatments it has been subjected to.

However, the assessment will be more complex if live fish are imported from potentially infected sources. Other aspects relevant when undertaking an IRA (apart from biological factors) are country and commodity factors. Country factors in the context of the release assessment include, for example, information on incidence/prevalence of the disease, the quality of Aquatic Animal Health Services, as well as surveillance and control programmes in the exporting country. Obtaining country factor information is a complex task; the information is subject to change and was therefore not covered here. Nevertheless, information provided in this review on prevalence, factors relevant to expression of the disease and diagnostic

methods can be used to assess the likelihood that disease would be detected if present.

Examples for country factors in the context of the exposure and consequence assessment are aquatic animal demographics (e.g. presence and spread of known susceptible species) and geographical and environmental characteristics (e.g. hydrographic data, temperature ranges, water courses); examples for commodity factors are intended use of the imported aquatic animals or products (e.g. domestic consumption, restocking or use as bait) and waste disposal practices. These aspects are highly specific to the country for which the IRA is undertaken and were therefore not covered here.

For some of the biological factors, very little data were available. This is particularly relevant to the amount of zoospores released from infected fish, drop in infectivity of spores released into the aquatic environment over time, reservoirs for the pathogen during periods where no noticeable mortalities are observed, expression of the disease in a new geographical area and the MID. The issue of the MID is further complicated by the fact that it is likely to vary with water temperature, fish species, and presence, absence and degree of predisposing factors. In affected areas (which so far have largely been in tropical or subtropical climates), outbreaks were often associated with changes in water quality, leading to skin damage and/or stress of the exposed fish. Whether suitable conditions may occur in so far unaffected areas is less clear. Water temperature may also have an impact on the level of expression of clinical disease. It is therefore not clear whether EUS, if introduced into areas with temperate climate, will express in the form of dramatic outbreaks as have been described from tropical areas. Insufficient data are also available on the length of survival of spores, cysts or mycelium in the aquatic environment. Studies are recommended to address these knowledge gaps.

Aspects of uncertainty surrounding the susceptible species range are discussed in detail elsewhere (B. Oidtmann and F. Berthe, in preparation). In short, the authors show that susceptible species range for EUS is very broad and likely to be much broader than currently known. This poses especially a problem for countries that are believed to be free from the pathogen, but where information on susceptibility of resident fish species is limited, as the range of species occurring in the receiving country is very different to the species known to occur in areas where EUS has previously been observed. Similarly, aspects of different degrees of susceptibility between species are relevant to IRA, for release, exposure and consequence assessment (B. Oidtmann and F. Berthe, in preparation). As a result, several steps of the IRA will be associated with a high level of uncertainty.

Epizootic ulcerative syndrome has spread widely across Asia and was also identified in Australia, the USA and Southern Africa. Countries currently free from the disease may wish to undertake an IRA because they consider EUS a potential threat to their wild or aquaculture animals. Geographical areas at particular risk will be freshwater and estuarine areas in tropical or subtropical climates, such as Central America. For temperate climates, such as central Europe, the potential risk is more difficult to assess. Within Europe, conditions for establishment may be suitable all year-round in Southern Europe; in other parts of Europe, conditions may also be suitable seasonally (i.e. from spring to autumn). Climate change–associated temperature rises and weather events may further increase the risk (Marcos-López et al., 2010). The information provided through an IRA will assist a country to decide whether biosecurity measures should to be put into place to mitigate the risk of introduction, should it exist. It also provides information towards areas of particular risk of exposure or for establishment and can therefore assist with the design of risk-based surveillance for this disease.

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