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Dissemination, identification and treatment of *Aphanomyces invadans*, the causative agent of epizootic ulcerative syndrome in fish

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Summary

Epizootic Ulcerative Syndrome (EUS) is an emerging infectious disease of fish caused by the oomycete *Aphanomyces invadans*, which belongs to the class *Oomyceta* and the order *Saprolegniales*. It was first reported in the Indo-Pacific region and has been confirmed to affect 125 fish species. Clinical signs include red spots on the skin alongside necrotizing granulomatous dermatitis and myositis in association with *A. invadans* hyphae. In Europe, *A. invadans* has been detected in ornamental fish but no clinical cases have been reported. The presence of various bacterial and viral species isolated from *A. invadans*-infected fish indicates their possible role as opportunistic or predisposing factors. Several diagnostic approaches to detecting the presence of *A. invadans* have been described, such as monoclonal antibody (MAb)-based detection, flow through immunoassay (FTA) and pyrolysis mass spectrometry (PyMS). Techniques to discriminate strains of *Aphanomyces* species include Random Amplification

Zusammenfassung

Verbreitung, Nachweis und Bekämpfung von *Aphanomyces invadans*, dem Erreger des *Epizootic Ulcerative Syndrome* der Fische

Das Epizootic Ulcerative Syndrome (EUS), verursacht durch *Aphanomyces invadans*, ist eine relative neu auftretende infektiöse Fischkrankheit, die im Indo-Pazifischen Raum entstanden ist und gegenwärtig bei 125 Fischspezies nachgewiesen wurde. Das Auffinden einer nekrotisierend-granulomatösen Dermatitis und Myositis in Verbindung mit *A. invadans*-Hyphen bei der histopathologischen Untersuchung bestätigt den Verdacht des EUS. Klinisch präsentiert sich diese Erkrankung mit der Ausbildung von rötlichen Flecken auf der Fischhaut. In Europa fand man *A. invadans* auch bei Zierfischen, es gibt jedoch keine Berichte über klinische Fälle. *A. invadans* wird sowohl eine opportunistische, als auch prädisponierende Rolle zugeschrieben, da bei allen mit *A. invadens* infizierten Fischen auch Bakterien, Viren oder Parasiten gefunden wurden. Verschiedene diagnostische Methoden zum Nachweis von *A. invadans* wurden beschrieben, wie der Nachweis mittels monoklonaler Antikörper (MAb), Durchfluss-Immunoassays (FTA) und die Pyrolyse-Massenspektrometrie (PyMS). Molekularbiologische Verfahren zur Identifizierung von *Aphanomyces*-Spezies sind zufällig vervielfältigte polymorphe DNA (RAPD), PCR Vervielfältigung ribosomaler DNA (rDNA), rDNA Sequenzierung, Restriktionsfragmentlängenpolymorphismus (RFLP) *und* Peptid-Nukleinsäure *In-situ*-Hybridisierung Assay (FISH). Zahlreiche Behandlungsmöglichkeiten für das EUS sind aufgelistet, jedoch gibt es keine für Speisefische zugelassenen Chemotherapeutika oder Impfstoffe. Strikte Maßnahmen zur Biosicherheit sind daher notwendig, um die Ausbreitung der Erkrankung zu verhindern und auch das Risiko für Ausbrüche in Aquakulturen zu reduzieren.

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of Polymorphic DNA (RAPD), PCR Amplification of ribosomal DNA (rDNA), rDNA Sequencing, Restriction Fragment Length Polymorphism (RFLP) and Fluorescent peptide nucleic acid In-Situ Hybridization (FISH) assays. No approved chemotherapeutics or vaccines are available, so strict biosecurity measures are necessary to prevent the spread of disease and to reduce the risk of outbreaks in aquaculture facilities.

Abbreviations: AAHRI = Aquatic Animal Health Research Institute; CVA = Canonical Variate Analysis; EUS = Epizootic Ulcerative Syndrome; FTA = Flow-through Immunoassay; FISH = Fluorescent In-Situ Hybridization; GP = Glucose-Peptone; GY = Glucose-Yeast; AuNP = Gold Nanoparticle; GMS = Gomori Methenamine Silver; HSPs = Heat Shock Proteins; HCA = Hierarchal Cluster Analysis; MIC = Minimal Inhibitory Concentration; MAb = Monoclonal Antibody; OIE = Office International des Epizooties; psu = Practical Salinity Unit; PAS = Periodic Acid-Schiff; PyMS = Pyrolysis Mass Spectrometry; RAPD = Random Amplification of Polymorphic DNA; RFLP = Restriction Fragment Length Polymorphism

Introduction

Epizootic Ulcerative Syndrome (EUS) was first described in Japan (EGUSA and MASUDA, 1971). This waterborne disease is officially recognized as a "reportable disease" by the Office International des Epizooties (OIE) (BOYS et al., 2012; OIDTMANN, 2012). The causative agent is the oomycete *Aphanomyces invadans* and outbreaks have been reported from fresh and estuarine waters in multiple countries, often associated with high morbidity and mortality (above 50 %). The disease is particularly prevalent at colder temperatures (18 to 22 °C) during the winter and rainy seasons (VIJAYAKUMAR et al., 2013; OIE, 2015). No fewer than 125 fish species have been shown to be susceptible to the pathogen (KAMILYA and BARUAH, 2014) and juveniles are most often more susceptible than older fish. Conversely, there has been no report of infection of fish larvae and fish fry (OIE, 2015). Fish species with soft epidermal skin, such as Gourami and Catfish, are more susceptible to *A. invadans* than species with a scaled epidermis, such as Nile Tilapia (AFZALI et al., 2015).

Geographical distribution

Outbreaks of EUS have been reported from fresh and brackish waters in a number of countries (BARUAH et al., 2014). The first recorded outbreak occurred in Japan (EGUSA and MASUDA, 1971) and was followed by a report from Queensland, Australia the following year. Rapid spread occurred through the 1980's with reports of EUS outbreaks from many countries in the Asia-Pacific region, including Indonesia and Malaysia in 1980, from the USA in 1984 and from the Philippines in 1985. The disease was reported from Sri Lanka in 1987, Bangladesh and India in 1988 (PRADHAN et al., 2014) and Pakistan in 1995 (MINFAL, 1998). During 1997–98 skin ulcers observed in a variety of fish in Chesapeake Bay tributaries in North America were subsequently attributed to EUS (BLAZER et al., 1999). More recently, the disease has spread to Africa and the Middle East with reports from Botswana in 2006 and Iraq, Namibia and Zambia in 2007. Furthermore, fish losses in Canada, the East Coast of the USA and South Africa have also been attributed to EUS (ANDREW et al., 2008; SAYLOR et al., 2010; OIE, 2015; EFSA, 2011). EUS has recently been reported in various fish species in the Assam district in India by KAR et al. (2015) and in African catfish in Egypt by YOUSSUF et al. (2017).

There have been no reports of outbreaks of EUS in Europe, although several species, including *Siluris glanis* and *Oncorhynchus mykiss*, are susceptible to various degrees and *A. invadans* has been isolated from ornamental fish in several EU countries (OIDTMANN et al., 2008; EL-MATBOULI et al., 2014).

Clinical signs and histopathological findings

The behaviour of infected fish is characterized by a reduction in appetite. Fish become lethargic and float slightly beneath the surface or with the head out of the water and small red spots develop over the body. As the name of the disease suggests, the main clinical finding is generally the presence of dark-red circular or oval spots (2–4 cm) on the skin, with ulceration extending up to 0.5cm down to the muscles (CHINABUT and ROBERTS, 1999). Small erythematous foci are seen as early lesions on the skin and may develop into a necrotizing dermatitis and result in a deep dermal ulcer in the chronic stage (KHOO, 2000). Loss of scales and development of haemorrhages can be observed in early affected fish, while in more advanced stages the lesions can be so complete that the internal organs become apparent through the lesion (Fig. 1). Most fish species die at this stage but some species such as the Striped snakehead (*Channa striata*) can survive and the disease can progress to the complete destruction of the caudal peduncle or the erosion of the cranium or abdominal cavity (BARMAN et al., 2011).

According to the Aquatic Animal Health Research Institute (AAHRI, Thailand), only the necrotizing granulomatous dermatitis and myositis associated with *A. invadans* hyphae in fish should be regarded as an EUS positive case. Histological preparations from suspected cases are therefore required for confirmatory diagnosis (OIE, 2015). Alongside gross clinical signs, a histopathological examina-

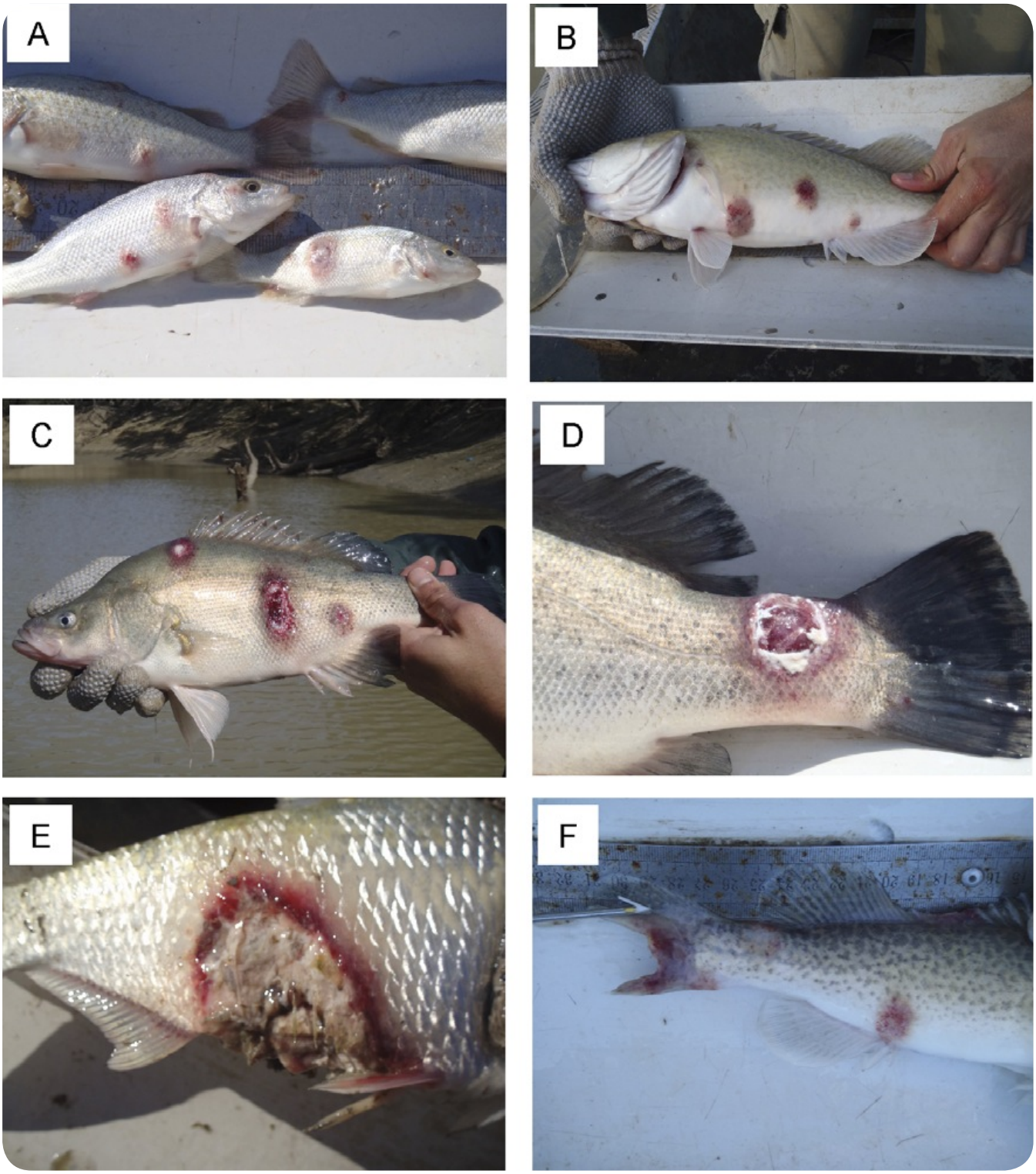

Fig. 1: Diseased fish collected from the Barwon-Darling River between Bourke and Brewarrina weirs in June 2010. Panels show A) *Leiopotherapon unicolor*, B) *Maccullochella peelii* with raised lesions, C,D) *Macquaria ambigua* and F) *Maccullochella peelii* with deep ulceration and muscle or fin necrosis and E) *Nematalosa erebi* showing severe ulceration and tissue necrosis exposing the peritoneal cavity and internal organs (from BOYS et al., 2012) / Kranke Fische aus dem Barwon-Darling-Fluss zwischen Bourke- und Brewarrina-Wehre im Juni 2010. Tafeln zeigen A) *Leiopotherapon unicolor*, B) *Maccullochella peelii* mit erhabenen Läsionen, C, D) *Macquaria ambigua* und F) *Maccullochella peelii* mit tiefer Ulzeration und Muskel- oder Flossennekrose und E) *Nematalosa erebi* mit schweren Ulzerationen und Gewebenekrosen unter Freilegung der Peritonealhöhle und der inneren Organe (aus BOYS et al., 2012)

tion can reveal the presence of branched, non-septate fungal hyphae surrounded by macrophages in the muscles (Fig. 2) (MCKENZIE and HALL, 1976) and skin scrapes (KHOO, 2000). Fungal granulomas are present underneath the epithelium on the margins of the ulcer (VISHWANATH et al., 1997; 1998).

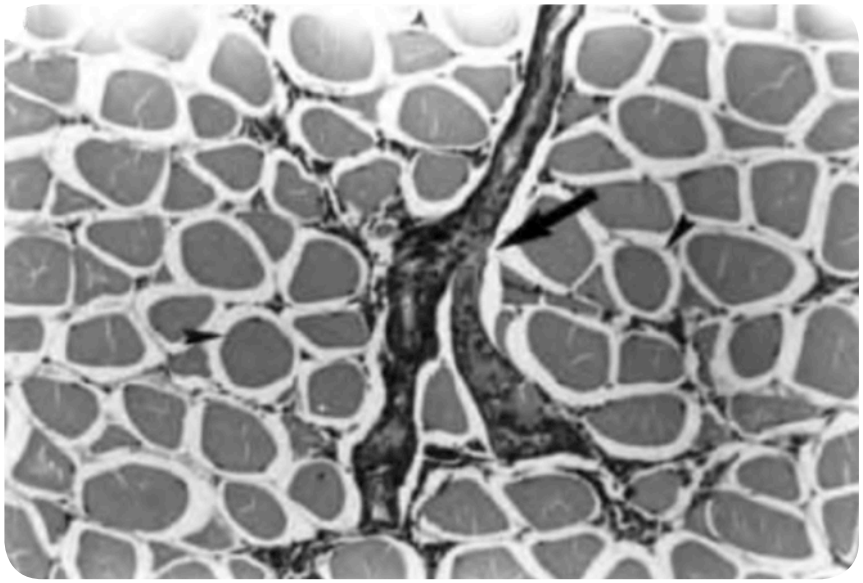

Fig. 2: Lesion area showing fungal hyphae (arrow) and adjacent normal muscle fibres (arrowheads) in mrigal carp at 12 dpi (H&E, x200) (from PRADHAN et al., 2007) / Läsionsbereich mit Pilzhyphen (Pfeil) und angrenzenden normalen Muskelfasern (Pfeilspitzen) in Muralkarpfen bei 12 dpi (H & E, x200) (aus PRADHAN et al., 2007)

The fungus is very invasive and spreads in all directions from the centre of the ulcer while muscle necrosis, macrophage infiltration, mycotic granulomas and fibrosis can also be observed at the site. Skeletal muscle degeneration and necrosis have also been reported, with invasion of fungal hyphae, presence of haemorrhages, infiltration of leukocytes at the site of fungal invasion and granulomatous inflammation (KIRYU et al., 2002). Penetrating hyphae have been observed on sites far away from the ulcer (PRADHAN et al., 2007) and on the contralateral site of the infected fish as well as in internal organs (VISHWANATH et al., 1998). The presence of mycotic granulomas in histological sections of affected tissues and organs is recognized as a confirmatory sign of EUS (MCHUGH et al., 2014; OIE, 2015). Degeneration of the gill in EUS-infected *Channa striatus* has recently been reported (ADINARAYANA et al., 2017).

In a few cases, some infected fish may not present the characteristic clinical signs, in particular the skin lesions, and may act as asymptomatic carriers for the infection (AGUIRRE-AYALA and VIDAL-MARTINEZ, 2015).

Isolation and culture

The fungus can generally be isolated from ulcers or muscle tissues and, because of the pathogen's fastidiousness, pure culture sometime requires repeated transfer of the hyphae on culture plates supplemented with antibiotics (AFZALI et al., 2013). Isolation and culture is most often performed on Glucose Yeast (GY) broth (AFZALI et al., 2013) or Glucose Peptone (GP) broth or agar (LILLEY et al., 1998).

The optimal culture temperature for WIC or PA7 strains is between 20 and 30 °C. No sporulation occurs below 5 °C or above 35 °C (KIRYU et al., 2005) and zoospores are only produced in a narrow range of room temperature. Some strains, for example the WIC strain, require 0, 1, and 2 practical salinity units (psu) but do not grow at a psu of 4 or higher.

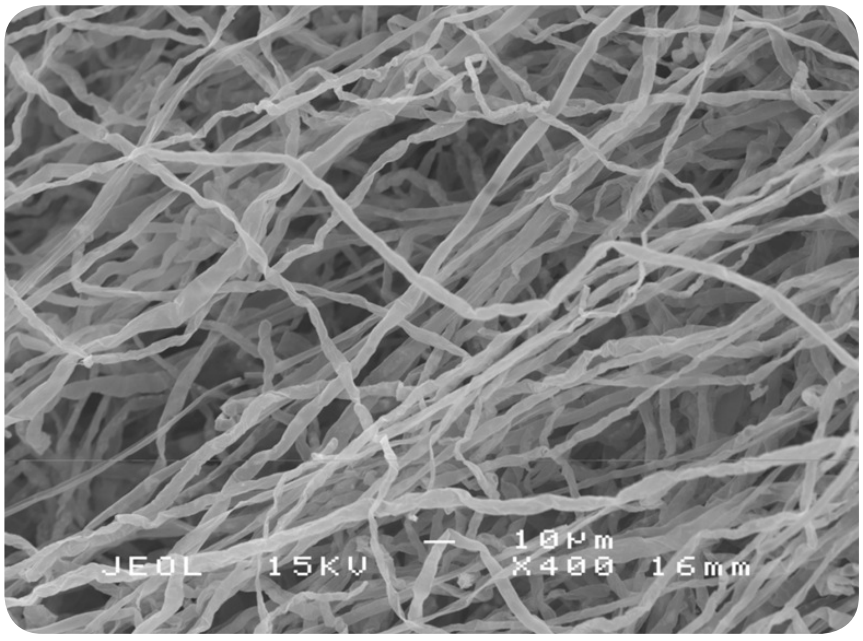

Fig. 3: Electron microscopy of *Aphanomyces invadans* reisolated from injected gourami tissues showing non-septate hyphae. SEM, x400 (from AFZALI et al., 2014) / Rasterelektronenmikroskopie von *Aphanomyces invadans*, reisoliert aus injiziertem Gourami-Gewebe, die nicht-septierte Hyphen zeigt. SEM, x400 (aus AFZALI et al., 2014)

Appearance and life cycle of *A. invadans*

A. invadans is a slow growing, non-septate microorganism (Fig. 3) that lacks reproductive structures (LILLEY et al., 1998; KIRYU et al., 2002, 2005). The diameter of the fungus ranges from 12 to 30 µm *in vivo* and from 5 to 20 µm in culture. On GP agar, the hyphae's branches are usually seen at right angles while the branching pattern is more vari-

Fig. 4: Growth of *Aphanomyces invadans* on Glucose-Peptone-Agar in a Petri plate. Hyphae are visible, especially on the peripheries of the mycelial mat. (author's own photograph) / Wachstum von *Aphanomyces invadans* auf Glukose-Pepton-Agar in einer Petrischale. Hyphen sind besonders an den Rändern der Myzelmatte sichtbar.

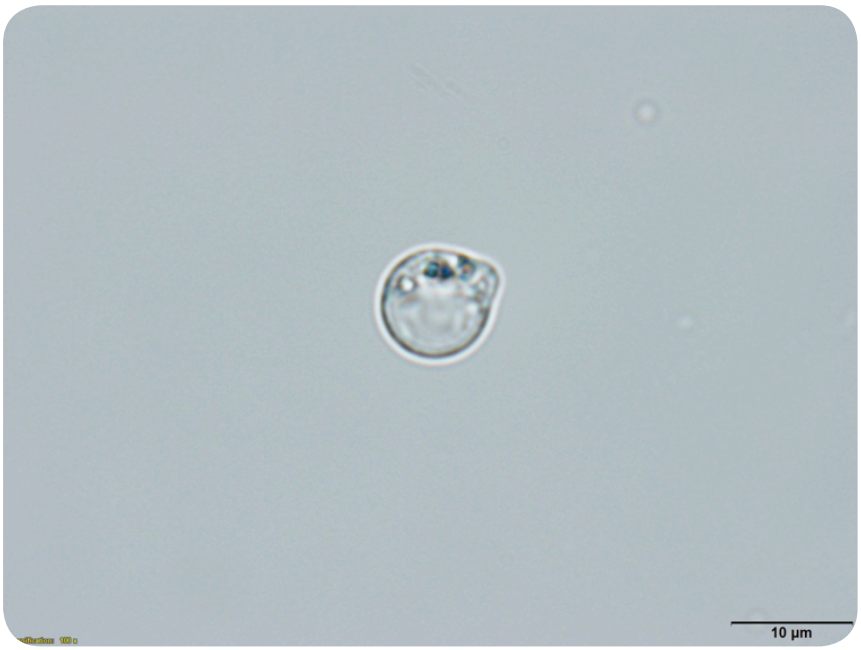

Fig. 5: Light microscopic image of the zoospore of *Aphanomyces invadans*. A small bud-like structure shows the start of the germination/hyphae development (x100) (author's own photograph) / Lichtmikroskopische Aufnahme der Zoospore von *Aphanomyces invadans*. Kleine Knospenstruktur zeigt den Beginn der Keimung / Hyphen-Entwicklung (x100)

able and different branching angles can be observed *in vivo*. *A. invadans* colonies on GP agar are slightly opaque and have a white velvety growth (Fig. 4) that covers the entire surface of the culture dish in about two weeks (ABBASS et al., 2004).

No oogonia are present in *A. invadans* but the fungus produces two forms of zoospores, primary and secondary. Primary zoospores develop in a sac-like structure called zoosporangia, which is of equal diameter to that of mycelia and is located at the terminal of the hyphae or between the upper and lower branches. The primary zoospores are circular in shape and encysted in cysts normally 6.5 µm in diameter (Fig. 5). Very rarely, the zoospores can form 'giant cysts' with a diameter of about 27 µm. The zoospore cysts may emerge and produce new zoospores, which turn into cysts again (LILLEY et al., 1998).

Upon release, primary zoospores quickly transform into secondary zoospores, which are biflagellate and motile. This form can survive in the water for a varying period of time, depending on environmental conditions (OIE, 2016). If the zoospores find a host/substrate they typically encyst and germinate to form fungal hyphae. Very rarely, a tertiary zoospore may be produced and released from the cyst (LILLEY et al., 1998; OIE, 2015).

Diagnosis

Diagnosis of the disease is still largely based on gross examination for the presence of external ulcers as well as on histopathological findings. According to the OIE, EUS-positive cases should be defined based on the presence of necrotizing granulomatous dermatitis and myositis in association with *A. invadans* hyphae (OIE, 2015). Skin scraping from ulcers of fish suspected to have EUS can be wet-mounted on slides and examined under the microscope to confirm the presence of fungal hyphae (KHOO, 2000). Gomori Methenamine Silver (GMS) or Periodic Acid-Schiff (PAS) stains have been used to visualize fungal hyphae (KHOO, 2000) but GMS is expensive and is not specific for *A. invadans*. Uvitex 2B, a fluorescent dye that binds chitin and is highly selective for fungi and algae, has been used to detect aquatic fungi and has proven very well able to detect oomycetes in tissues of aquatic animals (WADA et al., 2003), including *Aphanomyces* sp. in turtles (TAKUMA et al., 2011).

Antibody-based methods for the diagnosis of *A. invadans* have also been developed. For example, GANAPATHI et al. (2008) used monoclonal antibodies (MAb) to detect antigens on blots of tissue homogenates from ulcerated muscles; immunoblotting allows the early detection of the infection (NAIK et al., 2012). Similarly, MILES et al. (2003) used Mab in conjunction with fluorescent secondary antibodies on histological sections of infected striped snakehead. A flow-through immunoassay against the fungus has also been developed and has been reported to be both more sensitive (detection limit of 7 µg/ml and dilution of 10^{-11} vs. 56 µg/ml and 10^{-8}), as well as faster (10 vs. 90 min) than immunoblotting (ADIL et al., 2013).

LILLEY et al. (2001) applied Pyrolysis mass spectrometry (PyMS), a rapid and high resolution method, to discriminate between 45 closely related fungal isolates including *A. invadans*, *A. astaci, Achlya* and *Saprolegnia* species from Asia, UK and USA. They were able to distinguish *Aphanomyces* species from *Achlya* and *Saprolegnia* species. Canonical variate analysis (CVA) and hierarchal cluster analysis (HCA) of the pyrolysis mass spectra allowed the further discrimination between *A. invadans* and saprophytic strains.

LILLEY et al. (2003) used different molecular techniques to characterize fungal isolates: Random Amplification of Polymorphic DNA (RAPD), PCR Amplification of ribosomal DNA (rDNA), rDNA Sequencing and Restriction Fragment Length Polymorphism (RFLP) analysis. RAPD analysis in particular proved very sensitive in differentiating the genotypes of related fungi. An *A. invadans* species-specific peptide nucleic acid (PNA) probe has been developed for Fluorescent In-Situ Hybridization (FISH) assays and allows the detection of *A. invadans* in ulcerative mycotic fish lesions with a detection limit of 500 fg (VANDERSEA et al., 2006). KUAN et al. (2013) have developed an electrochemical genosensor using gold nanoparticles (AuNP) for the rapid detection of *A. invadans* and reported detecting *A. invadans* DNA at concentrations as low as 1.35 fg. QI et al. (2017) have recently developed an immunosensor to detect *A. invadans* using graphene-gold nanoparticles and reported a detection limit of 309 ng/ml. A real-time automated system for the detection of EUS has been reported by MALIK et al. (2017), who analysed images of EUS-infected fish with various algorithms and were able to detect the disease with 86 % accuracy.

Role of co-infections

Co-infection can have an important role in the development of aquatic diseases (KOTOB et al., 2017). Since 1983 several viruses, including members of the rhabdo, birna, reo- and ranavirus families and genera, have been identified and isolated from fish suffering from EUS (JOHN and GEORGE, 2012). Conversely, no virus could be isolated from several EUS outbreaks, suggesting that no viruses are required for EUS to develop (FRERICHS, 1995).

Aeromonas hydrophila and, more rarely, *Pseudomonas* species have been reported in association with EUS outbreaks (BOONYARATPALIN, 1989; PATHIRATNE et al., 1994). RAHMAN et al. (1999) isolated different bacteria from nine fish species suffering from EUS, including *A. hydrophila* and *Pseudomonas aeruginosa* as well as *Klebsiella aerogenes* and *Styphylococcus epidermidis*. However, BOONYARATPALIN (1989) found no bacteria in internal organs of the fish affected with EUS and it is therefore unlikely that bacteria are involved in causing EUS. In contrast to bacteria and viruses, parasites have not been consistently reported in association with EUS. It is nevertheless plausible that parasites play a part in some cases in weakening the fish and predisposing it to subsequent EUS infection, or that they cause superficial skin irritation and minor lesions that allow the fungal pathogen to enter the skin (CALLINAN and KEEP, 1989).

Although EUS has been suggested to be a multi-factorial disease, the variety of other agents isolated from clinical cases is more consistent with the interpretation that *A. invadans* is the only causative agent. It is likely that infection is facilitated when the immune system of the fish is compromised, for example because of another infection, or that EUS predisposes fish to secondary and opportunistic infections. However, the innate immune response seems to play a crucial part in keeping the common carp (*Cyprinus carpio*) resistant to infection with *A. invadans* (YADAV et al., 2016). Recent investigations on heat shock proteins (HSPs), which are produced in stressful conditions, and analysis of tissue-specific mRNA levels in *C. striatus* have revealed higher levels of HSPs when fish are under infection stress than when they are in an unstressed state. This suggests that HSPs are involved in the fish's immune response and defense mechanism. More studies in this area might open new avenues to explore (SATHYAMOORTHY et al., 2017).

Prevention and treatment

Although there have been attempts to develop and test vaccines against EUS, no commercial vaccine is available (THOMPSON et al., 1999; SAIKIA and KAMILYA, 2012). Similarly, while *in vitro* studies by CAMPBELL et al. (2001) have suggested that malachite green is effective against the fungus, this substance is banned in many countries and no available chemotherapeutic agent is known to be efficacious against the disease. WARRILOW et al. (2014) showed that clotrimazole is efficacious against *Saprolegnia parasitica*, which suggests that it might also be efficacious against other oomycetes such as *A. invadans*. JAHAN

et al. (2014) reported that the alkali-soluble fraction of paddy husk is able to cure *A. invadans* infections completely and to reverse clinical signs of the disease but the results still require confirmation.

Under these circumstances, the best control methods rely on sound biosecurity principles and husbandry practices (NSONGA et al., 2013; PALIĆ et al., 2015), including quarantine; removal of infected fish from the pond and improving water quality; and increased acidity, especially after a flood. Low chloride and alkaline levels in the water may increase the chances of EUS outbreak, as suggested by reports from Zambia (CHOONGO et al., 2009) and Bangladesh (SANAULLAH et al., 2001). Liming water with agricultural lime or by the addition of sodium chloride to the pond is thus likely to be beneficial. Because of the pathogen's limited tolerance of salinity, increasing the concentration of salt in the water could also result in clearing the disease (OIE, 2015).

SHAALAN et al. (2017) tested silver and zinc oxide nanoparticles on *A. invadans* and found that the *in vitro* growth of *A. invadans* was inhibited at MIC 17 µg/ml silver and 3.15 µg/ml zinc oxide nanoparticles. The approach to combat this oomycete is novel and *in vivo* experiments are required to establish control methods using these nanoparticles. Extracellular proteases, particularly serine proteases, secreted by *A. invadans* have been proposed to have a role in EUS pathogenesis (MAJEED et al., 2017) but further work is needed to reveal the pathogenic mechanisms. A better understanding of pathogenesis might help in identifying potential targets for therapeutic measures or a vaccine against EUS.

Suggestions and Conclusion

EUS is a relatively newly described fish disease and there is still a lack of effective strategies for its prevention and control. The risk of an EUS outbreak in an aquaculture operation may be mitigated by maintaining stable water quality and keeping the pH, temperature and alkalinity of the water within normal ranges. Physical injuries or trauma to the fish are thought to predispose fish to an outbreak of EUS (NSONGA et al., 2013).

In view of the high potential of *A. invadans* to spread through different ecosystems, of the susceptibility of several European fish species (EL-MATBOULI et al., 2014) and of the trend towards increasing average temperatures in the temperate regions of Europe, more attention should be paid to the risk of introducing EUS into European waters. The establishment of an EU surveillance programme for EUS, as suggested by EL-MATBOULI et al. (2014) and supported by the Aquatic Animal Health Code (OIE, 2015), should be seriously discussed.

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Fazit für die Praxis:
Bis zum heutigen Tage existiert kein Bericht über einen klinischen Ausbruch des EUS aus Europa. Eine Überprüfung der Importpolitik für lebende Zierfische sollte in Erwägung gezogen werden, um eine Einfuhr von EUS nach Europa weiterhin zu verhindern. Auch die Einführung von Überwachungsprogrammen scheint sinnvoll, um den aktuellen Status von EUS in Europa zu kontrollieren.

References

ABBASS, A.A., SHAHEEN, A.A., ABDEL-LATIF, A.M. (2004): Aphanomycosis in African Catfish "*Clarias gariepinus*". 1st Annual Conference, 1.-2. Sept. 2011, Faculty of Veterinary Medicine, Moshtohor, Benha branch, Zagazig University, Egypt , 479–492.

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

ADINARAYANA, P., KOTESWARA, R.D., SRINIASA, P.C., SRINIVASA, R.T., HYDER, I., SHARMA, S.V. (2017): Histopathological changes in the gills of fresh water fish *Channa striatus* (Bloch) infected with Epizootic Ulcerative Syndrome (EUS). Int J Fish Aquat Stud **5**, 515–518.

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

AFZALI, S.F., HASSAN, M.D., MUTALIB, A.R. (2014): Induction of skin ulcers in moon light gourami (*Trichogaster microlepis*) with *Aphanomyces invadans* zoospores. Pertanika J Trop Agric Sci **37**,133–140.

AFZALI, S.F., DAUD, H.H.M., SHARIFPOUR, I., AFSHARNASAB, M., SHANKAR, SHIV. (2015): Experimental infection of *Aphanomyces invadans* and susceptibility in seven species of tropical fish. Vet World **8**, 1038–1044.

AGUIRRE-AYALA, D., VIDAL-MARTINEZ, V. (2015): Experimental infection of Mayan Cichlid *Cichlasoma urophthalmus* with the oomycete *Aphanomyces invadans*. J Parasitol **101**, 485–487.

ANDREW, T.G., HUCHZERMEYER, K.D.A., MBEHA, B.C., NENGU, S.M. (2008): Epizootic ulcerative syndrome affecting fish in the Zambezi river system in Southern Africa. Vet Rec **163**, 629–631.

BARMAN, D., KUMAR, V., MANDAL, S.C. (2011): Epizootic Ulcerative Syndrome (EUS) – A great concern in modern aquaculture. World Aquaculture **43**, 63–65.

BARUAH, A., KAMILYA, D., SAHA, R.K. (2014): Epizootic ulcerative syndrome (EUS) in bata, *Labeo bata* (Hamilton 1822) from Tripura, India. Indian J Fish **61**, 141–144.

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

BOONYARATPALIN, S. (1989): Bacterial pathogens involved in the Epizootic Ulcerative Syndrome of fish in Southeast Asia. J Aquat Anim Health **1**, 272–276.

BOYS, C.A., ROWLAND, S.J., GABOR, M., GABOR, L., MARSH, I.B., HUM, S., CALLINAN, R.B. (2012): Emergence of epizootic ulcerative syndrome in native fish of the Murray–Darling river system, Australia: hosts, distribution and possible vectors. PLoS One **7(4)**, e35568.

CALLINAN, R.B., KEEP, R.J. (1989): Bacteriology and parasitology of Red Spot Disease in Sea Mullet, *Mugil cephalus* L., from Eastern Australia. J Fish Dis **12**, 349–356.

CAMPBELL, R.E., LILLEY, J.H., PANYAWACHIRA, V., KANCHANAKHAN, S. (2001): *In vitro* screening of novel treatments for *Aphanomyces invadans*. Aquacult Res **32**, 223–233.

CHINABUT, S., ROBERTS, R.J. (1999): Pathology and Histopathology of Epizootic Ulcerative Syndrome (EUS). Aquatic Animal Health Research Institute, Department of Fisheries, Royal Thai Government, Bangkok, Thailand, 33.

CHOONGO, K., HANG'OMBE, B., SAMUI, K.L., SYACHABA, M., PHIRI, H., MAGUSWI, C., MUYANGAAALI, K., BWALYA, G., MATAA, L. (2009): Environmental and climatic factors associated with epizootic ulcerative syndrome (EUS) in fish from the Zambezi floodplains, Zambia. Bull Environ Contam Toxicol **83**, 474–478.

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

EUROPEAN FOOD SAFETY AUTHORITY (EFSA) (2011): Panel on Animal Health and Welfare (AHAW): Scientific opinion on epizootic ulcerative syndrome. EFSA J **9**, 2387.

EL-MATBOULI, M., SALEH, M., SOLIMAN, H. (2014): Biosecurity risks associated with epizootic ulcerative syndrome and iridovirus in ornamental fish imported into the European Union. Vet Rec **174**, 1–2.

FRERICHS, G.N. (1995): Viruses associated with the epizootic ulcerative syndrome (EUS) of fish in South-East Asia. Vet Res **26**, 449–454.

GANAPATHI, M.N., RAJESH, K.M., SAHOO, A.K., SHANKAR, K.M. (2008): Monoclonal antibody-based detection of *Aphanomyces invadans* for surveillance and prediction of Epizootic Ulcerative Syndrome (EUS) outbreak in fish. In: BONDAD-REANTASO, M.G., MOHAN, C.V., CRUMLISH, M., SUBASINGHE, R.P (eds): Diseases in Asian Aquaculture VI. Fish Health Section, Asian Fisheries Society, Manila, Philippines, 157–168.

JAHAN, R., RAHMAN, S., REHANA, F., ANWAR, M.M., KALPANA, M.A., ANWARUL BASHAR, A.B.M., RAHMATULLAH, M. (2014): Treatment of Epizootic Ulcerative Syndrome (EUS) in *Anabas testudineus* with an alkali-soluble fraction from paddy husk. Adv in Nat Appl Sci **8**, 7–12.

JOHN, K.R., GEORGE, M.R. (2012): Viruses associated with epizootic ulcerative syndrome: an update. Indian J Virol **23**, 106–113.

KAMILYA, D., BARUAH, A. (2014): Epizootic ulcerative syndrome (EUS) in fish: history and current status of understanding. Rev Fish Biol Fisheries **24**, 369–380.

KAR, D., SINGHA, R., DAS, B.K., JOHN, R., GEORGE, R., MANSOOR, M., SELVA, M. (2015): Preliminary epidemiological studies on Epizootic Ulcerative Syndrome (EUS) in freshwater fishes of Assam. IJSRSET **1**, 256–263.

KHOO, L. (2000): Fungal diseases in fish. J Exot Pet Med **9**, 102–111.

KIRYU, Y., BLAZER, V.S., VOGELBEIN, W.K., KATOR, H., SHIELDS, J.D. (2005): Factors influencing the sporulation and cyst formation of *Aphanomyces invadans*, etiological agent of ulcerative mycosis in Atlantic menhaden, *Brevoortia tyrannus*. Mycologia **97**, 569–575.

KIRYU, Y., SHIELDS, J.D., VOGELBEIN, W.K., ZWERNER, D.E., KATOR, H. (2002): Induction of skin ulcers in Atlantic Menhaden by injection and aqueous exposure to the zoospores of *Aphanomyces invadans*. J Aquat Anim Health **14**, 11–24.

KOTOB, M.,H., MENANTEAU-LEDOUBLE, S., KUMAR, G., ABDELZAHER, M., EL-MATBOULI, M. (2016): The impact of co-infections on fish: a review. Vet Res **47**, 98.

KUAN, G.C., SHENG, L.P., RIJIRAVANICH, P., MARIMUTHU, K., RAVICHANDRAN, M., YIN, L.S., LERTANANTAWONG, B., SURAREUNGCHAI, W. (2013): Gold-nanoparticle based electrochemical DNA sensor for the detection of fish pathogen *Aphanomyces invadans*. Talanta **117**, 312–317.

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

LILLEY, J.H., BEAKES, G.W., HETHERINGTON, C.S. (2001): Characterization of *Aphanomyces invadans* isolates using pyrolysis mass spectrometry (PyMS). Mycoses **44**, 383–389.

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

MAJEED, M., KUMAR, G., SCHLOSSER, S., EL-MATBOULI, M., SALEH, M. (2017): *In vitro* investigations on extracellular proteins secreted by *Aphanomyces invadans*, the causative agent of epizootic ulcerative syndrome. Acta Vet Scand **59**, 78 doi: 10.1186/s13028-017-0347-3.

MALIK, S., KUMAR, T., SAHOO, A.K. (2017): A novel approach to fish disease diagnostic system based on machine learning. AIVP **5**, 49–57.

McHUGH, K.J., CHRISTISON, K.W., WEYL, O.L.F., SMIT, N.J. (2014): Histological confirmation of epizootic ulcerative syndrome

in two cyprinid species from Lake Liambezi, Zambezi Region, Namibia. Afr Zool **49**, 311–316.

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

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

MINFAL, (1998): Epizootic Ulcerative Syndrome (EUS) in fishes of Pakistan. Department for International Development, UK.

NAIK, M.G., SHANKAR, K.M. (2012): Epizootic Ulcerative Syndrome (EUS) in fishes: a review. Environ and Ecol **30**, 1045–1051.

NSONGA, A., MFITILODZE, W., SAMUI, K.L., SIKAWA, D. (2013): Epidemiology of Epizootic Ulcerative Syndrome in the Zambezi river system. A case study for Zambia. HVM Bioflux **5**, 1–8.

OFFICE INTERNATIONAL DES EPIZOOTIES, (OIE) (2015): Infection with *Aphanomyces invadans* (Epizootic Ulcerative Syndrome). Chapter 10.2. In: Aquatic Animal Health Code, 1-6.

OFFICE INTERNATIONAL DES EPIZOOTIES, (OIE) (2016): Infection with *Aphanomyces invadans* (Epizootic Ulcerative Syndrome). Chapter 2.3.2. In: OIE – manual of diagnostic tests for aquatic animals aquatic animal health code, 1–14.

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

OIDTMANN, B., STEINBAUER, P., GEIGER, S., HOFFMANN, R.W. (2008): Experimental infection and detection of *Aphanomyces invadans* in European catfish, rainbow trout and European eel. Dis Aquat Organ **82**, 195–207.

PALIĆ, D., SCARFE, A.D., WALSTER, C.I. (2015): A standardized approach for meeting national and international aquaculture biosecurity requirements for preventing, controlling, and eradicating infectious diseases. J Appl Aquaculture **27**, 1–35.

PATHIRATNE, A., WIDANAPATHIRANA, G.S., CHANDRAKANTHI, W.H.S. (1994): Association of *Aeromonas hydrophila* with epizootic ulcerative syndrome (EUS) of freshwater fish in Sri Lanka. J Appl Ichthyol **10**, 204–208.

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

PRADHAN, P.K., RATHORE, G., SOOD, N., SWAMINATHAN, T.R., YADAV, M.K., VERMA, D.K., CHAUDHARY, D.K., ABIDI, R., PUNIA, P., JENA, J.K. (2014): Emergence of epizootic ulcerative syndrome: large-scale mortalities of cultured and wild fish species in Uttar Pradesh, India. Curr Sci **106**, 1711–1718.

QI, X., CHEN, T., LU, D., CHEN, B. (2017): Graphene-Au nanoparticle based electrochemical immunosensor for fish pathogen *Aphanomyces invadans* detection. Fullerenes, Nanotubes, Carbon Nanostruct **25**, 12–16.

RAHMAN, H., KAR, D., BARMAN, N.N. (1999): Bacterial pathogen associated with Epizootic Ulcerative Syndrome in fishes in Assam. Indian J Anim Sci **69**, 1083–1085.

SAIKIA, D., KAMILYA, D. (2012): Immune responses and protection in catla (*Catla catla*) vaccinated against epizootic ulcerative syndrome. Fish Shellfish Immun **32**, 353–359.

SANAULLAH, M., HJELTNES, B., AHMED, A.T.A. (2001): The relationship of some environmental factors and the Epizootic Ulcerative Syndrome outbreaks in Beel Mahmoodpur, Faridpur, Bangladesh. Asian Fish Sci **14**, 301–315.

SATHYAMOORTHY, A., CHAURASIA, M.K., ARASU, M.V., AL-DHABI, N.A., HARIKRISHNAN, R., AROCKIARAJ, J. (2017): Differences in structure and changes in gene regulation of murrel molecular chaperone HSP family during epizootic ulcerative syndrome (EUS) infection. Fish Shellfish Immun **60**,129–140.

SAYLOR, R.K., MILLER, D.L., VANDERSEA, M.W., BEVELHIMER, M.S., SCHOFIELD, P.J., BENNETT, W.A. (2010): Epizootic ulcerative syndrome caused by *Aphanomyces invadans* in captive bullseye snakehead *Channa marulius* collected from south Florida, USA. Dis Aquat Organ **88**, 169–175.

SHAALAN, M.I., EL-MAHDY, M.M., THEINER, S., EL-MATBOULI, M., SALEH, M. (2017): *In vitro* assessment of the antimicrobial activity of silver and zinc oxide nanoparticles against fish pathogens. Acta Vet Scand **59**, 49. doi: 10.1186/s13028-017-0317-9.

TAKUMA, D., SANO, A., WADA, S., KURATA, O., HATAI, K. (2011): *Aphanomyces sinensis* sp. nov., isolated from juvenile soft-shelled turtle, *Pelodiscus sinensis*, in Japan. Mycoscience **52**, 119–131.

THOMPSON, K.D., LILLEY, J.H., CHEN, S-C., ADAMS, A., RICHARDS, R.H. (1999): The immune response of rainbow trout (*Onchorhynchus mykiss*) against *Aphanomyces invadans*. Fish Shellfish Immun **9**, 195–210.

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

VIJAYAKUMAR, R., RAJA, K., SINDUJA, K., GOPALAKRISHNAN, A. (2013): Epizootic Ulcerative Syndrome in fresh water and brackish water fishes. African J Basic & Appl Sci **5**, 179–183.

VISHWANATH, T.S., MOHAN, C.V., SHANKAR, K.M. (1997): Clinical and histopathological characterization of different types of lesions associated with Epizootic Ulcerative Syndrome (EUS). J Aqua Trop **12**, 35–42.

VISHWANATH, T.S., MOHAN, C.V., SHANKAR, K.M. (1998): Epizootic ulcerative syndrome (EUS), associated with a fungal pathogen, in Indian fishes: histopathology—'a cause for invasiveness'. Aquaculture **165**, 1–9.

WADA, S., YORISADA, Y., KURATA, O., HATAI, K. (2003): Histological detection of aquatic fungi by Uvitex 2B, a fluorescent dye. Fish Pathol **38**, 49–52.

WARRILOW, A.G.S., HULL, C.M., ROLLEY, N.J., PARKER, J.E., NES, W.D., SMITH, S.N., KELLY, D.E., KELLY, S.L. (2014): Clotrimazole as a potent agent for treating the oomycete fish pathogen *Saprolegnia parasitica* through Inhibition of Sterol 14α-Demethylase (CYP51). Appl Environ Microbiol **80**, 6154–6166.

YADAV, M.K., PRADHAN, P.K., SOOD, N., CHAUDHARY, D.K., VERMA, D.K., CHAUHAN, U.K., PUNIA, P., JENA, J.K. (2016): Innate immune response against an oomycete pathogen *Aphanomyces invadans* in common carp (*Cyprinus carpio*), a fish resistant toepizootic ulcerative syndrome. Acta Trop **155**, 71–76.

YOUSSUF, H., GWAD, E.A., EL-ASLEY, A., ELABD, H., SHAHEEN, A.A., ABBASS, A. (2017): Isolation of *Aphanomyces invadans* associated with skin lesions in African catfish "*Clarias gariepinus*". BVMJ **32**,193–197.