Induction of Skin Ulcers in Moon Light Gourami (*Trichogaster microlepis*) with *Aphanomyces invadans* Zoospores

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ABSTRACT

Epizootic ulcerative syndrome (EUS) is one of the seasonal and economically devastating diseases in the wild and farmed fresh water and estuarine fish. Thus, an experimental study was conducted by the Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM) from February to April 2012, to examine the susceptibility of Malaysia's indigenous fish to EUS infection. In this experiment, forty apparently healthy moonlight gourami (*Trichogaster microlepis*) (10 ± 2 g body weight and 7.5 ± 1 cm in body length) were kept at 20 °C and challenged by intramuscular injection of zoospores (0.1 ml of 10,000 spores ml^{-1} suspension) of *Aphanomyces invadans* (isolate NJM9701). Fish were observed daily for characteristic EUS clinical signs during the 14-day trial and sampled at 1, 2, 4, 6, 7, 8, 9, 10, 11, 13, 12, 14 days post-injection. The infected skin and muscle were then sampled for histopathological examination. The results demonstrated that injected fish started to develop lesions that were histopathologically and grossly identical to those found in naturally EUS-infected fish and they died within two weeks after the infection. The profoundly penetrating ulcers had characteristics such as severe dermatitis, myofibrillar degeneration, and deep necrotizing granulomatous myositis. Therefore, the result of this study proved that moonlight gourami was vulnerable to the EUS agent.

Keywords: Epizootic ulcerative syndrome (EUS), Gourami, *Aphanomyces invadans*, Infection, Histopathology

INTRODUCTION

Epizootic ulcerative syndrome (EUS) is an economically destructive disease that destroys a broad range of wild and farmed freshwater and estuarine fish particularly in Asia, Australia, some parts of USA and recently affected Africa as well (OIE 2009). Lilley and Roberts (1997) confirmed that

the oomycete, *Aphanomyces invadans*, also known as *A. piscicida* was the causative agent of EUS (Lilley and Roberts 1997); where else Chinabut (1998) stated that the clinical signs of EUS include petechial hemorrhages, profound necrotic ulcers of the skin and reformed in swimming behaviors. Microscopically the lesions were distinguished by signs such as mycotic granulomas associated with penetrating hyphae of *A. invadans* and cellular feedback amongst muscles and fibers (Noga *et al.* 1988). EUS diagnosis's procedure is based on clinical signs or demonstration of the EUS-characteristic mycotic granulomas in histopathological test and molecular diagnostic methods namely, PCR (OIE 2006).

Bruno and Wood (1999) stated that highly vulnerable fish to EUS were *Channa* spp. and significantly *Channa striata*, barbs, major carps, gourami and mullets. So far, there hasn't been any in-depth research conducted in Malaysia regarding to *A. invadans* pathogenicity to Malaysian indigenous freshwater fish species despite the fact that Malaysia has been through severe EUS outbreaks in the 1990's. In the present study research, an anabantid fish i.e. Moonlight gourami (*T.microlepis*) was selected for the experimental infection due to its importance in the Malaysian aquarium industry and its popularity in Malaysia's market at affordable prices. Moonlight gourami belongs to the suborder Anabantoidei with over 100 various colored species that are being traded in the tropical fish industry currently. This would include about the numerous species of the genus *Trichogaster* containing many of the more popular gouramis traded in the industry (Cole *et al.* 1999).

This study is concentrated on moonlight gouramis to determine its vulnerability to the EUS disease agent for import risk analysis in the future because this fish is being widely exported to the other countries in the recent years; therefore it is crucial to obtain a comprehensive knowledge regarding the risks of disease introduction via international trades. The significance of this task is based on reducing the potential risk of pathogen's penetration into Malaysia's wild and farmed fish populations and also controlling and programming preventive measures in this regards.

MATERIALS AND METHODS

Oomycete Culture and Sporulation

Zoospores for this study were obtained from *A.invadans* fungal isolate NJM9701 (courtesy of Dr. Oitdmann, U.K.), cultured on GP-Penstrep agar (glucose-peptone agar added with penicillin-streptomycin) (OIE 2007). The modified method as described by Johnson *et al.* (2004) was utilized for zoospores production. *A. invadans* mycelia were allowed to grow on sterile hemp seeds planted on the GP agar. Later 6-mm growing hyphae on the hemp seeds were transferred into GPY broth (glucose-peptone-yeast broth added with penicillin-streptomycin), incubated at 25°C for five days and eventually washed three times in autoclaved pond water (APW).

The mycelium mats from tip of colonies with seeds were loaded into 1.5 ml micro tubes containing APW for 24 hours. The number of zoospores in the suspension was quantified by using a Neubauer counting chamber and adjusted to the required concentration. Briefly, an aliquot of culture was preserved in 10% neutral-buffered formalin (1 ml culture: 5 ml formalin), centrifuged for 10 min at 150 rcf. The pellet obtained was resuspended in 1.8 mL APW and a 10 µL aliquot was quantified with the Neubauer (Johnson *et al*. 2004).

Challenge experiments and histopathology

Fish used in this challenge experiments were local Moonlight gourami (*T. microlepis*) weighing an average of 10 ± 2 g body weight and 7.5 ± 1 cm in total body length, which were purchased from a local fish wholesale trader. 60 apparently healthy fish were acclimatized in 75-liter aquarium equipped with air stones and aquarium heaters and maintained at 20℃, for one month before the experiment to observe for any disease signs. Two replicate challenge groups and one control w ere set-up utilizing 20 fish per tank. Fish were anaesthetized using MS-222 (tricane methanesulphonate) prior to injection with 0.1 ml of a 10,000 spores ml^{-1} suspension (i.e. 1000 spores/fish) intramuscularly into the left side of the body below the dorsal fin using a 27-gauge needle. The control fish were inoculated with an equal volume of APW and all fish were observed daily for characteristic EUS clinical signs during the 14-day trial. Two fish were sampled at 1, 2, 4, 6, 7, 8, 9, 10, 11, 13, 12, 14 days post-injection (pi) and killed with an overdose of MS-222.The infected tissues were sampled for histopathological examination. The excised tissues were fixed in 10% phosphate buffered formalin solution for at least 24 hours, processed through automatic tissue processor and embedded in paraffin. The paraffin blocks were then sectioned at 4-5 µm thickness with a rotary microtome and mounted on clean glass slides. Slides were then stained with routine Hematoxylin and Eosin (H&E) (Prophet 1992), before mounted with coverslip prior for viewing under light microscope.

RESULTS AND DISCUSSION

Within 24 hours after injection, zoospore-injected and control fish both showed reddening and scale loss at the injection site which seemed to be due to traumatic injury caused by needle penetration. From day 4 to 7 pi, cotton-like, whitish colonies were observed at the site of inoculation just in zoospore-injected fish, and the presence of fungal mycelium was confirmed by wet mount preparation which showed thin aseptate branching hyphae (Fig.1a, Fig.1b).

Changes in swimming behavior, manifested by forward and backward teetering movements, started to happen in injected group from day 7 pi, while control fish appeared normal (Fig.2). Deep penetrating focal ulcers that exposed the underlying musculature started to appear from day 5 pi (Fig.3). Histopathology results showed characteristic EUS mycotic granulomas in injected fish from day 8 pi. Extensive mycotic and myonecrotic lesions

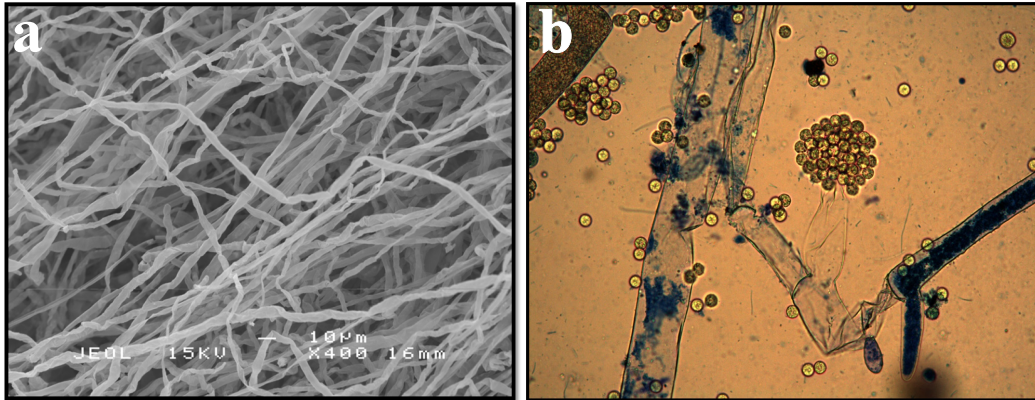Pertanika J. Trop. Agric. Sci. 37 (1): 133 - 140 (2014)

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Fig.1: Showing (a) Electron microscopy of *Aphanomyces invadans* reisolated from injected gourami tissues showing non-septate hyphae. SEM, X400. (b) Light microscopy of *Aphanomyces invadans* reisolated from injected gourami tissues showing primary zoospores (65µm).
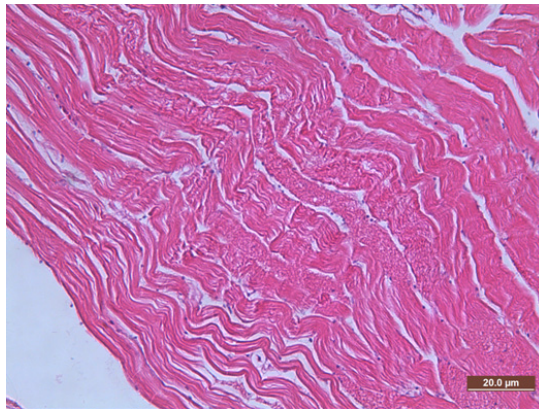

Fig.2: Longitudinal muscle section of APW injected gourami sampled at 13 days post injection. No microscopic lesion was observed as those seen in zoospore injected fish (H&E, X200).
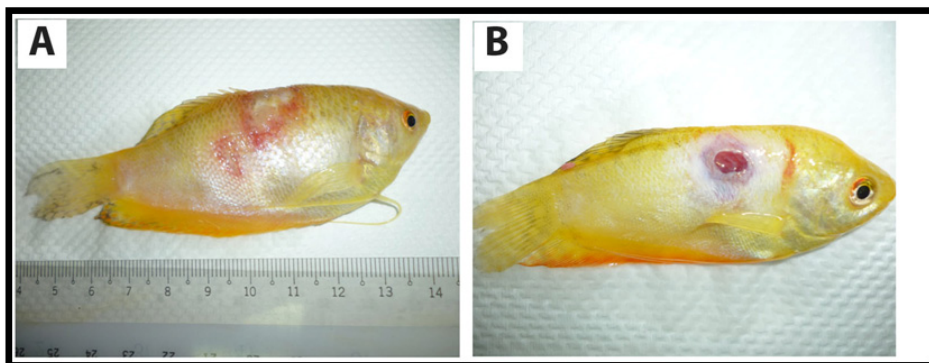

Fig.3: Experimentally infected gourami with *A. invadans* NJM 9701 at 100 spores/ml showed (A) necrotic ulcer surrounded with hyperaemic area, at day 6 post injection and (B) deep necrotizing ulcer at injection site seen at day 13, bordered with swollen hyperaemic ulcer and blanched margin.

were observed in muscles from day 6 pi. The injected muscle tissue area showed degenerating myofibrils infiltrated with inflammatory cells from day 8 (Fig. 4). Hyphae appearance could be detected in dermal and muscular layers. Large vacuoles were formed in degenerated muscles and granulomatous reactions were observed from day 9 pi (Fig. 5). Mortalities started to occur in injected fish from day 11 pi and by day 14 all injected fish were dead. All the dead fish had severe swollen hemorrhagic lesions with massive proliferation of hyphae in the lesion area of injected site similar to those caused by *A. invadans* in natural EUS infected fish.

This is the first research conducted in Malaysia on experimental infection of *Aphanomyces spp.* as an EUS aetiologic agent in Malaysian freshwater aquarium fish.
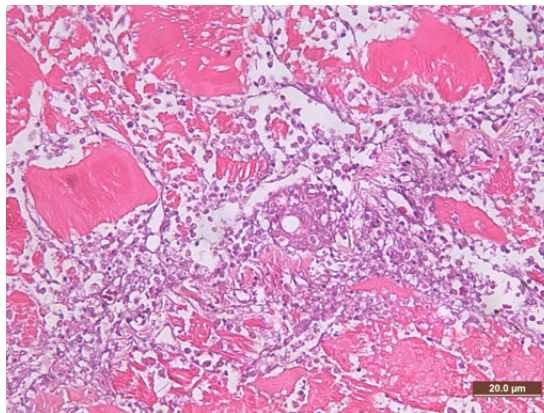

Fig.4: Histopathological section of zoospores-injected gourami sampled at 8 days post injection, showing degeneration of muscles fibres, interspersed with severe necrotic area and infiltrated with mononucleated inflammatory cells in lacunae-like spaces . Also seen mycotic granulomas (arrow).in the necrotic areas. Note the loss of muscles fibre architecture (H&E, X200).
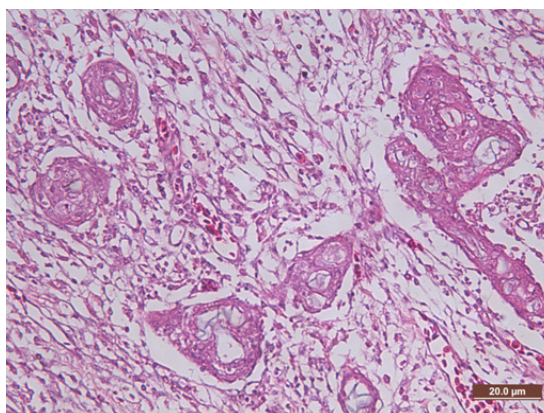

Fig.5: Histopathological characteristics of zoospores injected gourami sampled at 13 days post injection. Note the presence of mycotic granulomas (arrows), interstitial oedema and inflammatory cells infiltration in epidermis and dermis (H&E, X200).

This current study had successfully induced dermal ulceration in Moonlight gourami similar to natural EUS condition, via intramuscular injection of *A. invadans* strain NJM9701 live zoospores. Current findings indicated that the injected zoospores of *A. invadans* were able to grow in the muscles of Moonlight gourami. The zoospores were able to germinate into hyphae and proliferate extensively causing necrotizing granulomatous dermatitis with inflammatory response within very short period after injection. Lilley *et al*. (1998) stated that even though more than 100 fish species were reported to be affected by EUS, only a few of them had been validated by demonstrating the presence of mycotic granulomas in histological sections or by isolation of the pathogenic *Aphanomyces* fungus from tissues underlying the ulcers. Catap and Munday (2002) had also demonstrated EUS lesions in gourami (*T. trichopterus*), and sand whiting (*Sillago ciliate*) with injection of 0.05 ml of 1.7×1000 zoospores ml^{-1} suspension (85 spores/fish or 22 spores/g body weight. Both species of fish exhibited chronic granulomatous response and inflammatory cells, predominantly macrophages and lymphocytes, infiltrating the muscle and skin tissues at day 6–8 post-inoculation. This number of spores seems to be so much less in comparison with to our experiment but the research conducted by Khan *et al*. (1998) also reported dose regimes which varied by up to 7000–10,000 spores injected in various fish species and still obtained acceptable data. Mycotic granolumas were also observed in artificially infected ayu and carp (Wada *et al*. 1996). Experimental infection done in USA with *A. invadans* which were carried out in four species of estuarine fish lead to similar lesions, and severe pathologies in Atlantic menhaden (*Brevoortia tyrannus*) and Killifish were seen (Johnson *et al*. 2004). These results supported the results of current study which proved that some species of fish were sensitive than others, and fish having soft epidermal skin layers such as gourami was more vulnerable to be affected by *A. invadans*.

A limited number of researches have been conducted in utilizing bath challenge to expose fish to *A. invadans* spores. Atlantic menhaden which is one of the most vulnerable species to EUS was tested by aqueous exposure and confirmed that *A. invadans* was pathogenic via this route and also demonstrated that skin damage prior to pathogen exposure increased the prevalence of fish with ulcerous lesions and mortality (Kiryu *et al*. 2002; 2003). In addition, Sosa *et al*. (2007) demonstrated that more than 80% mortalities occurred in striped mullets (*Mugil cephalus*) injected with five spores per fish.

Oidtmann *et al*. (2008) had also conducted an experimental infection using similar *A. invadans* strain NJM9701 and showed that European catfish (*Silurus glanis*) produced typical EUS ulcerative skin lesions. In their study also, gouramis were used as positive control and showed to be more vulnerable to *A. invadans* infection as compared to European catfish. On the other hand, injected gouramies were indicated

changing in swimming behavior (forward and backward teetering movements) on Day 6 p.i similar to those were seen in moon light gourami on day 7 pi in present study. Saylor *et al*. (2010) described a mass mortality event of snakehead *Channa marulius* collected from freshwater bodies in Florida which clinical signs appeared within the first 2 days of captivity included petechiae, ulceration, erratic swimming, and inappetence which are similar to those are observed in this study.

Pradhan *et al*. (2008) discovered that, Indian major carp (*Catla catla)* greater than 1 year old seem to be resistant to infection by *A. invadans*, however, histopathological examinations were done by Baruah *et al*. (2012) revealed hyphae and granulomatous reactions in muscle tissue sections of Catla similar to those described in present study.

Since our knowledge on the vulnerability of fish species to EUS is still very limited, future studies should investigate the susceptibility of other local species especially those most likely to be affected in their natural environment. This will assist in assessing the most probable impact of an introduction of the pathogen into Malaysia and also provide useful information for import risk analysis of moonlight gourami to the other countries.

CONCLUSION

In conclusion, we have shown that moonlight gourami is vulnerable to an EUS pathogen, *A. invadans* via intramuscular injection. Therefore, it is recommended that EUS vulnerable species should not be cultured in high potential outbreak areas to avoid huge economic losses. However, since gourami is highly vulnerable to infection via inoculation; these species are ideal to be utilized as a laboratory model for further studies with *A. invadans* in Malaysia .

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