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NOTE

Immunofluorescence of the epizootic ulcerative syndrome pathogen, *Aphanomyces invadans*, using a monoclonal antibody

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ABSTRACT: A monoclonal antibody (MAb), designated 3gJC9, was raised against a protein antigen of *Aphanomyces invadans*, the oomycete pathogen that causes epizootic ulcerative syndrome (EUS). The antigen was expressed on the surface of hyphae and secreted extracellularly. MAb 3gJC9 did not cross-react with other oomycete or fungal pathogens of fish, although it did react to the crayfish plague pathogen *A. astaci*. The MAb was used for immunofluorescent staining on histological sections of fish infected with EUS, and was found to be more sensitive than conventional staining methods for detecting *A. invadans*. It thus has utility in confirming the case definition of EUS. It also revealed very small filamentous structures, the significance of which is unclear, but they may represent an early stage of infection, thus allowing earlier detection of the disease, since they are not detected using conventional staining methods.

KEY WORDS: *Aphanomyces invadans* · Monoclonal antibody · Immunohistochemistry · IFAT · Epizootic ulcerative syndrome · Extracellular product

Aphanomyces invadans (= *piscicida*) is the pathogen that causes epizootic ulcerative syndrome (EUS) (Hatai et al. 1977, Roberts et al. 1994), and is one of the most economically destructive diseases of fresh- and brackish-water fish in the Asia-Pacific region (ACIAR 1998, Chinabut 1998). It is an oomycete protist with filamentous hyphae, which infiltrates the muscle tissue of fish. EUS is characterised by large ulcers on the surface of the fish (Viswanath et al. 1997), and causes death by osmotic imbalance (Cruz-Lacierda & Shariff 1994), or through secondary infections that enter through the ulcers (Boonyaratpalin 1989).

Histopathologically, *Aphanomyces invadans* is characterised by massive necrosis of muscle around the invading hyphae, which subsequently become enclosed by granulomas (Wada et al. 1994, Viswanath et al. 1997). At present, the most commonly reported method for histopathological examination of EUS is Grocott's (1955) methenamine silver stain (GMS) for muco-polysaccharides (Wada et al. 1994, Viswanath et al. 1997), although it is unable to distinguish between *A. invadans* and the large number of opportunistic fungal and oomycete pathogens that may take advantage of the ulcers raised by EUS (Lilley & Roberts 1997). Identification of *A. invadans* is essential to confirm the case definition of EUS, which is 'the presence of invasive *Aphanomyces* infection and necrotizing ulcerative lesions typically leading to a granulomatous response' (Roberts et al. 1994).

Lilley et al. (1997) developed polyclonal antibodies against *Aphanomyces invadans* in an attempt to establish a more specific diagnostic test, but found that the antibodies cross-reacted with a wide range of pathogenic and non-pathogenic oomycetes. Cross-reactivity is a frequently reported problem with antibodies raised to oomycetes, and has severely restricted the usefulness of polyclonal antibodies produced in a number of different studies (Bullis et al. 1990, Peterson et al. 1996).

Monoclonal antibodies (MAbs) have been widely used as a diagnostic tool in the study of fish diseases (Adams et al. 1995), and also in the research of oomycetes (Hardham et al. 1985, Estrada-Garcia et al. 1989, Beakes et al. 1995). Following the problems of cross-reactivity associated with the polyclonal rabbit anti-*Aphanomyces invadans* serum encountered by Lilley et al. (1997), the intention of the present study was to develop MAbs that specifically detect *A. invadans*.

Materials and methods. A range of pathogenic and non-pathogenic oomycete isolates, listed in Table 1, was used in the study. They were all maintained on agar media based on glucose and peptone, and were induced to sporulate by washing and placing in filtered, autoclaved pond water (Miles et al. 2001). Germination was induced by collecting the propagules

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Table 1. Comparison of reactivity of monoclonal antibodies (MAbs) against *Aphanomyces invadans* isolate PA8 1 d germlings with 1 d germlings of other oomycetes, measured by the intensity of fluorescenece. Intensity was categorised as indistiguishable from negative control (– –), higher than negative control but less than that to PA8 (–), or indistinguishable from the reaction to PA8 (+). American Tissue Culture Collection numbers are given in parentheses where appropriate. EUS: epizootic ulcerative syndrome; UM: ulcerative mytosis

Isolate Species	Isolated from	Location	Supplied by	Description	Cross-reactivity of MAbs 1gA A11	1Ge e5	2gM F5	3gF E11	3gJ C9
PA8 *Aphanomyces invadans*	Striped snakehead *Channa striata*	Nonthaburi, Thailand	Dr. J. H. Lilley	EUS pathogen	+	+	+	+	+
B99C *Aphanomyces invadans*	Reba carp *Cirrhinus reba*	Mymensingh, Bangladesh	Dr. J. H. Lilley	EUS pathogen	+	+	+	+	+
T99G2 *Aphanomyces invadans*	Giant gourami *Osphronemus gouramy*	Bangkok Noi, Thailand	Miss. V. Panyawachira	EUS pathogen	+	+	+	+	–
UM3 *Aphanomyces invadans*	Atlantic menhaden *Brevoortia tyrannus*	Wicomoco River, Maryland, USA	Dr. V. Blazer	UM pathogen	+	+	+	+	–
FDL458 *Aphanomyces astaci*	White-clawed crayfish *Austropotamobius pallipes*	River Arrow, Hertfordshire, UK	Dr. D. J. Alderman	Crayfish plague pathogen	–	– –	– –	– –	–
ASEAN1 *Aphanomyces laevis*	Fish pond water	Kasetsart, Bangkok, Thailand	Dr. L. G. Willoughby	Plant pathogen	– –	– –	–	–	– –
SA11 *Aphanomyces* sp.	Striped snakehead *Channa striata*	Nonthaburi, Thailand	Dr. J. H. Lilley	Non-invasive wound pathogen	++	–	–	– –	– –
84-1240 (62427) *Aphanomyces* sp.	Atlantic menhaden *Brevoortia tyrannus*	North Carolina, USA	Dr. M. J. Dykstra	Non-invasive UM isolate	– –	– –	– –	– –	– –
99ExtAph *Aphanomyces* sp.	Striped snakehead *Channa striata*	Bangkok, Thailand	Dr. J. H. Lilley	Non-invasive wound pathogen	– –	– –	– –	–	– –
ACHLYA99 *Achlya* sp.	Climbing perch *Anabas testundineus*	Bangkok, Thailand	Dr. J. H. Lilley	Non-invasive wound pathogen	– –	– –	– –	– –	– –
TF23 *Saprolegnia* sp.	Striped snakehead *Channa striata*	Udon Thani, Thailand	Dr. L. G. Willoughby	Non-invasive wound pathogen	– –	– –	–	– –	– –
P32 *Saprolegnia ferax*	Lake water	Lake Windermere, UK	Dr. L. G. Willoughby	Saprophyte	– –	–	– –	– –	– –
795 (42060) *Saprolegnia australis*	Skelly *Coregonus lavarentus*	Lake Ullswater, UK	Dr. L. G. Willoughby	Saprophyte	–	–	– –	– –	– –
TP41 (42062) *Saprolegnia parasitica*	Brown trout *Salmo trutta*	Hatchery, Lake Windermere, UK	Dr. L. G. Willoughby	Saprolegniasis pathogen	–	–	– –	– –	– –
E3 (36144) *Saprolegnia diclina*	Lake water	Lake Windermere, UK	Dr. L. G. Willoughby	Saprophyte	+	+	–	– –	– –
3501d *Leptolegnia caudata*	Unknown	India	Prof. M. W. Dick	Insect pathogen	– –	– –	–	– –	– –

produced by sporulation, and diluting the pond water containing them in an equal volume of glucose-peptone broth media containing 100 mM calcium chloride to stimulate germination (Deacon & Saxena 1998). They were incubated overnight at 24°C and collected by centrifuging at 3000 × *g* for 20 min.

The germlings were prepared for experimental use by fixing in formaldehyde as described by Burr (1991), and after a final wash with phosphate buffered saline at pH 7.4 (PBS), the germlings were resuspended in PBS and the concentration of the suspension determined by counting the germlings in a haemocytometer.

Soluble antigens were collected after disruption of the germlings by shaking with 0.5 mm silicon beads for 160 s in a bead shaker (Biospec Products). The concentration of the soluble antigen was assessed spectrophotometrically.

Preparation of monoclonal antibodies: The MAbs were prepared after Adams et al. (1995). Three BALB/c mice received 3 × 100 µl intraperitoneal injections of soluble antigen prepared from germlings of *Aphanomyces invadans* isolate PA8 over a 60 d period. The antigen was prepared in autoclaved PBS and mixed 1:1 with the Titremax gold adjuvant (CytRx Corporation). Mice initially received soluble antigen prepared from a suspension of germlings at 2.8×10^5 ml^{-1}, then 32 d later they were boosted with soluble antigen obtained from 4.0×10^5 germlings ml^{-1}, and 19 d later with soluble antigen obtained from 2.5×10^5 germlings ml^{-1}. The last immunisation was given intravenously 28 d later, when 100 µl of soluble antigen from 2.5×10^5 germlings ml^{-1} prepared in PBS was injected into the caudal vein of the mice. Three days later, the B-cells were harvested from the spleen of the mice and fused with SP2 myeloma cells to make hybridomas.

Hybridomas producing antibodies against *Aphanomyces invadans* were identified by dot-blot and ELISA (Adams et al. 1995), and positive wells were expanded and recloned 3 times until monoclonal cell lines were obtained. The isotypes of the MAbs were determined by ELISA, using isotype-specific secondary antibodies (Sigma Chemical).

Characterisation of MAbs by Western blot: To determine the molecular weights of the antigens recognised by the MAbs, 2 samples of soluble PA8 germling extract, and 1 of extracellular products (ECP) from culture, were prepared. All of the protein was digested from 1 sample of soluble extract by digesting it with 1 mg ml^{-1} proteinase K (Sigma) for 60 min at 60°C.

The ECP was prepared by filtering a broth culture of isolate PA8 germlings through grade 541 filter paper (Whatman PLC), then through 0.45 and 0.22 µm sterile filters (Sartorius). The concentration of the filtrate was increased by centrifuging at 3000 × *g* for 30 min in a Vivascience concentrator with a molecular weight cut-off of 5 kDa (Sartorius), which increased the concentration of solutes larger than 5 kDa by approximately 18 times.

All samples were subjected to electrophoresis, transferred to nitrocellulose paper and analysed by Western blot after Lilley et al. (1997), with the following modifications: the nitrocellulose membrane was incubated in the hybridoma supernatant overnight at 22°C, and in the secondary antibody, 1% v/v biotin-labelled anti-mouse immunoglobulin (Ig) (Diagnostics Scotland) diluted in antibody buffer (1% w/v bovine serum albumin [Sigma] in PBS) for 90 min at 22°C. This was followed by a further 90 min incubation, in 1% v/v streptavidin peroxidase (Diagnostics Scotland) diluted in antibody buffer. The molecular weight of bands recognised by the MAbs were calculated using standards run at the same time as the gel.

Staining of fixed germlings by immunofluorescence antibody technique (IFAT): Initial screening of the MAbs was carried out using IFAT on germlings obtained from 16 different oomycete isolates affixed to microscope slides (Table 1). The intensity and specificity of the staining was recorded. In preliminary studies, antibodies labelled with fluorescin isothiocyanate (FITC) were found to be considerably more effective for staining the germlings than those labelled with enzymes.

Microscope slides (Surgipath Europe) were prepared for immunoassays by immersing them in 3% v/v 3-aminopropyltriethoxysilane (APES) (Sigma) in acetone (Fisher Scientific) for 5 min. The slides were washed in 100% acetone for 5 min followed by a rinse in distilled water for 5 min before air-drying. The slides were divided into a number of sections with a PAP-pen, and 40 µl suspensions containing 2.5×10^3 germlings were added to each section. The germlings were allowed to air-dry onto the slides at 40°C, before washing the slides by gentle sluicing with PBS followed by 3 × 5 min incubations in PBS.

The IFAT was carried out according to Anderson (1990), with modifications: tissue culture supernatant containing the MAbs were applied to the samples for 4 h at 22°C, after which anti-mouse Ig labelled with FITC (Diagnostics Scotland) was applied at 2% v/v in antibody buffer, and incubated for 2 h at 22°C. Each slide contained a positive control of germlings of isolate PA8, and a negative control with germlings of the test isolate incubated with culture medium rather than tissue culture supernatant. The slides were stored in the dark at 4°C for no more than 7 d, before viewing under an Olympus IMT-2 microscope with a reflected epi-fluorescent attachment and exciter and barrier filters for FITC. The intensity of the staining obtained

with the MAbs against the test germlings was compared to the controls in order to evaluate each of the MAbs.

Immunohistochemistry by IFAT: After a number of preliminary trials, a technique based on a modification of the method of Lilley et al. (1997) was used to stain *Aphanomyces invadans* in the tissues of EUS-infected fish. Tissue sections were treated as stated, but the sections were placed onto APES-treated slides. The tissue sections were incubated with neat hybridoma supernatant overnight at 4°C in a humidified chamber and then with FITC-labelled anti-mouse Ig for 1 h. The reaction was enhanced with a second cycle (Linsenmayer et al. 1988), by repeating the application of the goat serum, MAb supernatant and secondary antibody, and all wash steps described by Lilley et al. (1997). After a final wash step, 5% v/v methyl green in PBS was added for 5 min to reduce background staining (Lannan et al. 1991). The slides were washed in running water for 5 min, mounted with a cover slip and stored in the dark at 4°C for not more than 7 d before examining.

Determining the specificity of the MAbs against Aphanomyces invadans: To confirm the specificity of the MAbs, IFAT was performed on tissue sections collected from a range of fish tissues infected with various oomycete and fungal diseases (Table 2). In the case of the sections infected with crayfish plague, the effect of adding a third reaction cycle was also assessed. Any reaction with hyphae in the sections was recorded.

Comparison of IFAT with GMS: Tissue samples were collected from 48 striped snakehead *Channa striata* that had been experimentally infected with EUS by immersion challenge with *Aphanomyces invadans* (Miles 2001). Two sections were cut from each of 48 sections and mounted on APES-treated slides. One section was stained with GMS, following the protocol of Chinabut & Roberts (1999), and 1 by IFAT. The presence or absence of *A. invadans* was assessed and compared between the 2 sections.

Results and discussion. Five MAbs were identified that reacted with *Aphanomyces invadans* isolate PA8. All 5 were used in IFAT, and 4 were found to cross-react with several oomycetes other than *A. invadans* (Table 1). The fifth, an antibody of isotype IgM designated MAb 3gJC9, gave the strongest and clearest staining of *A. invadans* germlings and only cross-reacted with 1 other isolate, *A. astaci* (Table 1). This is a crustacean pathogen, which is taxonomically similar (Hart 1998), but does not infect fish (Lilley & Roberts 1997, Oidtmann et al. 1999). The fluorescent staining with MAb 3gJC9 was observed over the entire surface of all *A. invadans* germlings stained (Fig. 1), and a similar reaction was observed with the germlings of *A. astaci* (Table 1). Since MAb 3gJC9 did not cross-react with the germlings screened from other species of oomycte found in fish, this was the only MAb to be used in the remainder of the study.

In Western blot analysis with MAb 3gJC9, bands were observed with both undigested germling extracts and ECP, but no reaction was obtained with samples of germling extract (isolate PA8) digested with proteinase K, indicating that the MAb recognised a protein epitope present on both *Aphanomyces invadans* germlings and their ECP (Fig 2). A band was recognised at 33 kDa in both cases, while further bands were recog-

Table 2. Tissue sections of aquatic organisms with hyphal diseases other than epizootic ulcerative syndrome (EUS) that were used to assess cross-reactivity of monoclonal antibody (MAb) 3gJC9

Section reference	Host species	Pathogen	Disease	Supplied by
5950020U	Striped snakehead *Channa striata*	*Saprolegnia* sp. Isolate TF29	Injection challenge	Dr. J. H. Lilley
500-007SKIN	Channel catfish *Ictaurus punctatus*	*Saprolegnia* sp.	Saprolegniasis	Dr. L. Khoo & Dr. A. Grooters
R970036B	Atlantic salmon *Salmo salar*	*Exophiala* sp.	Exophialasis	Dr. R. Collins
599-1070-1	Channel catfish *Ictaurus punctatus*	*Branchiomyces* sp.	Branchiomycosis	Dr. L. Khoo & Dr. A. Grooters
82-1240	Atlantic menhaden *Brevoortia tyrannus*	Unknown	Ulcerative mycosis	Dr. E. J. Noga
1247/96/II	Noble crayfish *Astacus astacus*	*Aphanomyces astaci*	Crayfish plague	Dr. B. Oidtmann
629/96/c	Noble crayfish *Astacus astacus*	*Aphanomyces astaci*	Crayfish plague	Dr. B. Oidtmann

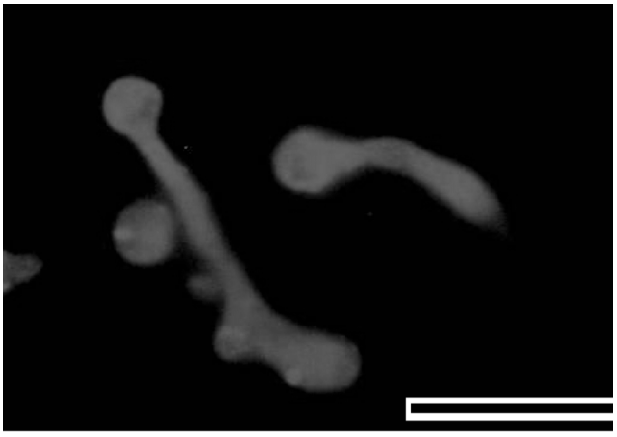

Fig. 1. *Aphanomyces invadans* isolate PA8 1 d germlings stained by immunofluorescence antibody technique (IFAT) with monoclonal antibody (Mab) 3gJC9. Photographed at 1000× magnification; scale bar indicates 10 µm

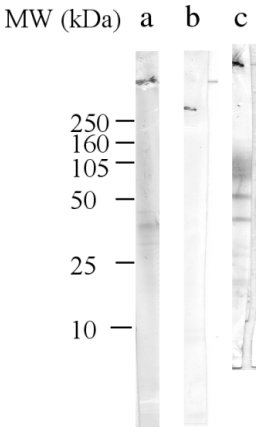

Fig. 2. Response monoclonal antibodies (MAbs) 3gJC9 in Western blots to lanes: (a) 1 d germling extract; (b) 1 d germling extract digested with proteinase K; and (c) extracellular products from 1 d germlings

nised at 35 and 38 kDa in the germling extract, and a doublet at 25 kDa and single bands at 23, 24, 46, 72 and 102 kDa in the ECP preparation.

When muscle sections of EUS-infected striped snakeheads were stained with MAb 3gJC9 in IFAT, strong fluorescence could be seen around the hyphae in infected muscle sections (Fig. 3). Most of the fluorescence was concentrated in a rim around the hyphae, although the surrounding tissue also fluoresced, particularly where it was heavily necrotic. Well-developed granulomata appeared to concentrate the fluorescence much closer to the hyphae. No fluorescence was observed in undamaged muscle tissue in uninfected fish, or away from the locus of infection in infected fish (data not shown). Sections treated with hybridoma culture medium as a negative control had lower levels of background fluorescence compared to tissue sections infected with the pathogen, although a weak non-specific reaction was observed around the cell walls of the hyphae. This was much weaker and easily distinguishable from sections stained with MAb 3gJC9, and was not strong enough to photograph clearly, so no figure is included here.

The antigen recognised by MAb 3gJC9 was located in necrotic and inflamed tissue surrounding *Aphanomyces invadans* hyphae, suggesting the tissue damage seen in the early stages of *A. invadans* infection is due to ECPs secreted by the hyphae. Where granulomatas had formed, the ECP antigen was confined to a small area immediately surrounding the hyphae, which implies that a major function of the granulomatous reaction characteristic of EUS may be to prevent the spread of ECPs.

At the periphery of the infected site, pinpoint areas of fluorescence were observed around the hyphae (Fig. 3), often extending into parts of the sections that still contained intact muscle fibres. In some cases, developed hyphae were absent or only found in the dermis, and only the sources of pinpoint fluorescence appeared in the musculature (Fig. 4). At higher magnifications, the pinpoints appeared filamentous, but no clear photographs were obtained at magnifications above ×40. The shape and immunogenicity of these, and the fact that they were nearly always found near more developed hyphae, suggests that they may be a hitherto unreported part of the *Aphanomyces invadans* mycelium. They were not stained by GMS, which is possibly why

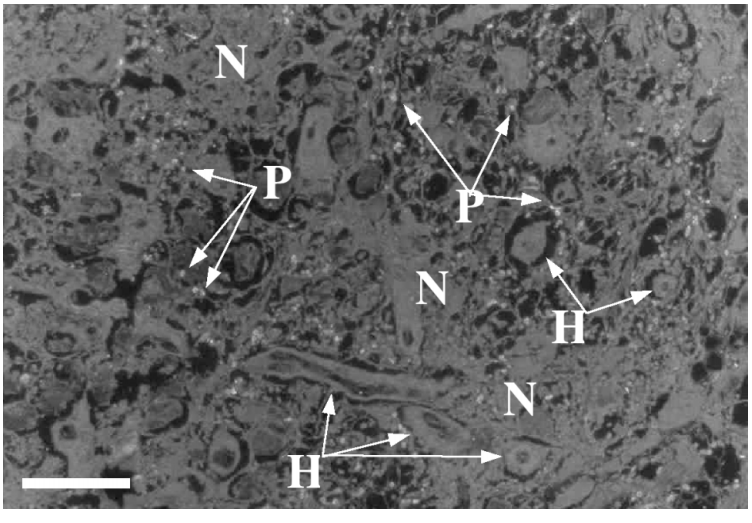

Fig. 3. *Aphanomyces invadans* in striped snakehead *Channa striata* muscle tissue stained with 2-cycle immunofluorescence antibody technique (IFAT) with monoclonal antibody (MAb) 3gJC9, showing developed hyphae (H) surrounded by myonecrosis (N) and pinpoint sources of fluorescence (P). Photographed at 100× magnification; scale bar indicates 10 µm

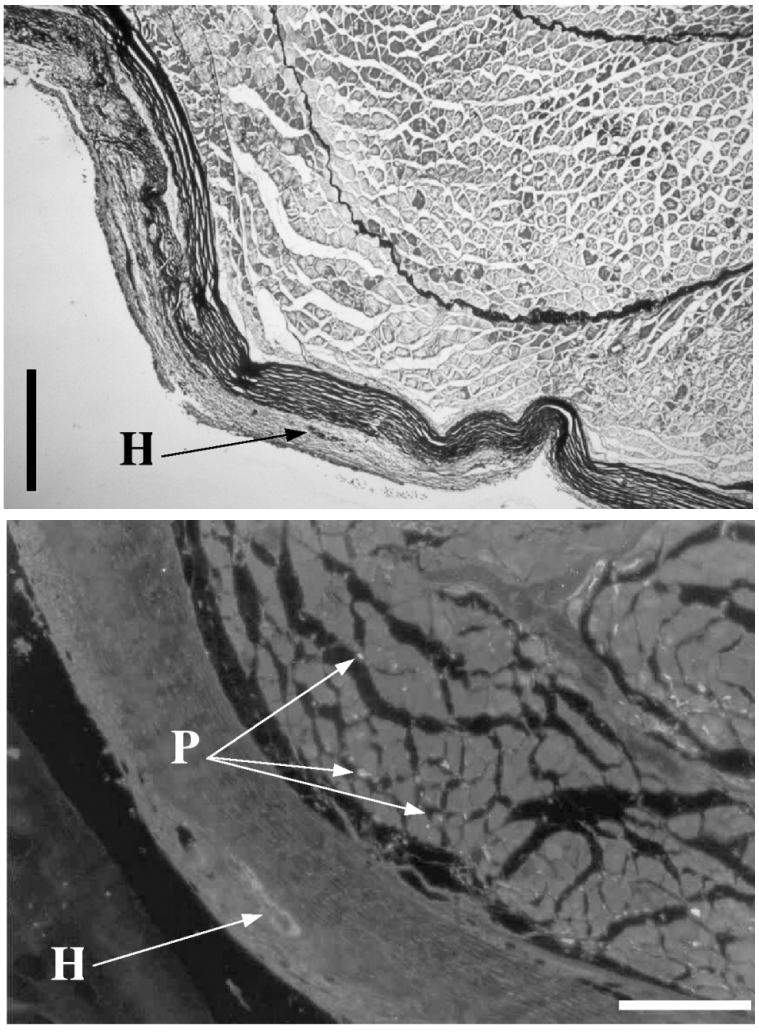

Fig. 4. Muscle section of striped snakehead *Channa striata* sampled 11 d after immersion challenge with *Aphanomyces invadans* and stained with (a) Grocott's (1955) methenamine silver stain (GMS) and (b) 2-cycle immunofluorescence antibody technique (IFAT) using monoclonal antibody (MAb) 3gJC9. Note the hyphae in the dermis (H) and pinpoint sources of fluorescence (P) that are only visible in the section stained by IFAT. Photographed at 40× magnification; scale bars indicate 50 µm

they have not been reported before, and they were nearly always found in apparently undamaged tissue, or necrotic tissue that had probably only recently become infected. In some cases, they were the only sources of fluorescence that had penetrated beyond the dermis, so they may be involved in the earliest stages of infection. If they are indeed an early stage of the mycelium, they may produce very little muco-polysaccharide which would be stained by GMS, but be rich in the ECP that contains the antigen that 3gJC9 recognises. They were never found in sections where the hyphae were enclosed by granulomata, which further supports such a possibility. However, the purpose of this study was not to investigate the nature of these filaments, and further investigation is necessary before any firm conclusions can be drawn as to their form and function.

Many pathogenic oomycetes use enzymes expressed extracellularly to invade their hosts, including *Aphanomyces astaci*, which uses them to penetrate the chitinous cuticle of crayfish (Söderhäll & Unestam 1975). The pathology of *A. invadans* infections often involves considerable tissue damage some distance away from the hyphae (Wada et al. 1994), implying that it secretes ECPs that are important to the process of infection. The nature of the ECPs from *A. invadans* remains obscure, although the haemagglutinating and haemolytic properties associated with the mycelium (Kurata et al. 2000) are likely to be caused by enzymes expressed extracellularly.

Of the 48 GMS-stained muscle sections sampled from striped snakeheads challenged with EUS, invasive hyphae could be seen in 23 of them, and were apparently absent in the other 25. Of all the samples, those which stained positive with GMS also appeared positive with IFAT. In addition, 2 sections where no hyphae had been observed following GMS were identified as positive by IFAT. This was because either the hyphae were confined to the dermis and therefore did not appear invasive, or the hyphae simply were not visible with the GMS stain. There were a further 3 cases in which hyphae could not be seen, but the filamentous structures described above were present. Equivalent structures were not observed in sections stained with GMS (Fig. 4). These results show that the immunohistochemical stain was as sensitive as GMS in detecting invasive *Aphanomyces invadans* hyphae, but was also able to detect *A. invadans* in cases where their presence could not be established by GMS.

In sections of fish infected with *Saprolegnia* spp., *Exophiala* sp. and *Branchimyces* sp., the fluorescence of the hyphae was no stronger than the background. However, Section 82-1240 from an Atlantic menhaden *Brevoortia tyrannus* infected with ulcerative mycosis showed strong fluorescence of the hyphae in a manner identical to the sections of EUS-infected fish, supporting the conclusion of Blazer et al. (1999, 2002) and Kiryu et al. (2002) that the large-scale fish mortalities that have been reported from the SE USA since 1984

(Noga & Dykstra 1986), dubbed ulcerative mycosis, are in fact caused by *Aphanomyces invadans*.

Sections of crayfish infected with *Aphanomyces astaci* did not induce as strong a reaction as sections of EUS-infected fish, but the hyphae fluoresced strongly after a third reaction cycle. Most IFAT and immunohistochemistry protocols used in fish diagnostics do not involve multiple reaction cycling (Adams et al. 1995), but the use of 2 cycles is not unprecedented, as Linsenmayer et al. (1988) reported the use of up to 4 cycles with some MAbs. In the present study, 3 cycles were necessary to elicit clear and repeatable fluorescence from *A. astaci* in crayfish muscle, although *A. astaci* germlings appeared to elicit a response as strong as that for *A. invadans*. The fact that the 2 species are genetically more similar to each other than to other *Aphanomyces* spp. (Hart 1998), and that they are the only invasive animal pathogens in the genus, implies that the ECP antigen detected by MAb 3gJC9 may be involved in the pathogenicity of both species. However, the nature of this antigen was not established in this study, and further research is necessary to establish whether this is indeed the case.

The principal application of MAb 3gJC9 is likely to be in the confirmation of the presence of *Aphanomyces invadans* in infected fish, which must be carried out histopathologically in order to confirm the case definition of the disease (Roberts et al. 1994). Although the IFAT procedure described here is more time-consuming than GMS, and requires an epifluorescence microscope, it is more sensitive and detected *A. invadans* in 5 sections where it was not detected by GMS. In particular, the use of IFAT to stain *A. invadans* hyphae in the dermis of fish may enable detection of early stages of the infection. The same hyphae in a section stained with GMS would not be identified as *A. invadans*, as they do not appear to be invasive and so would be considered to be a saprophyte or opportunist.

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