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Antigen Characterization and Epitope Analysis of *Aphanomyces invadans* of Epizootic Ulcerative Syndrome Pathogen by Monoclonal Antibodies

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Abstract

A panel of three monoclonal antibodies (MAbs) C1 (IgG1), C13 (IgM) and C14 (IgM) was produced against *Aphanomyces invadans* for the first time in India. The MAbs C1 and C13 recognized four proteins, while MAb C14 recognized five proteins of the fungus in the Western blot. Specific reaction of the MAbs with the fungus was further confirmed by immunoperoxidase and immunofluorescence where reaction was clearly demonstrated along the hyphae and within granulomas. An epitope analysis of *A. invadans* in fish from different regions of India, employing present three MAbs by immunoperoxidase indicated antigen similarity of the said pathogen spread across and in India.

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Introduction

Epizootic ulcerative syndrome (EUS) has been one of the most destructive diseases affecting over 100 species of fresh and brackish water fish (cultured and wild) in the Asia-Pacific region since 1971, resulting in decrease in production and high economic loss. Although several isolates of virus and bacteria were recorded from EUS, none of these have been conclusively shown as primary etiological agents. Nevertheless, *Aphanomyces invadans*, an oomycete fungus is now considered as the cause of EUS due to its consistent presence in tissues of EUS affected fish (Lilley & Roberts 1997). Therefore, it would be pertinent to change the name Epizootic Ulcerative Syndrome to 'Epizootic Ulcerative Disease'.

Ulcerative diseases of fish such as red spot disease (RSD) in Australia and mycotic granulomatosis (MG) in Japan were also shown to be similar to EUS in protein profile analysis (Callinan et al. 1995) and reaction of rabbit antisera with proteins of *A. invadans* (Lilley et al. 1997a). Genome analysis of *A. invadans* from EUS, RSD and MG by rapid amplification of polymorphic DNA (RAPD)-PCR, showed that all the isolates were conspecific, and probably constitute a single clonal genotype. Furthermore, the isolates had identical nucleotide sequences within 18s nuclear rRNA gene region and hence were considered as similar (Lilley et al. 1997b). The PCR test (Panyawachira et al. 2000) and rabbit antisera based detection of the pathogen have been reported, but there were disadvantages as far as their specificity and cost concerned. Miles et al. (2003) used single monoclonal antibody (MAb) in immunofluorescence to detect *A. invadans*. However, studies on the fungus at the epitope level are lacking for comparison of isolates in India.

The present work describes the production of a panel of three monoclonal antibodies in order to characterize the antigen profile of *A. invadans* and compare it at the epitope level. This molecular approach is significant in forecasting the disease outbreak for other parts of the region enabling authorities to take suitable managerial steps and prevent further spread given its dreadful nature.

Materials and Methods

Fungus

Aphanomyces invadans B99C isolated from *Cirrihinus riba* during an EUS outbreak in Bangladesh was used in the present study. The isolate was injected into fingerlings of catla (*Catla catla*) and adult puntius (*Puntius* sp.) and re-isolated from them on glucose peptone penicillin-streptomycin (GP-penstrep) broth according to Willoughby et al. (1994).

Pathogenicity of the fungal isolate was confirmed by experimental infection to 10 each of catla and puntius with mycelial tips and zoospores (0.1 ml , $10^4\cdot\text{ml}^{-1}$). The fish were observed for clinical signs for a period of seven days and the tissues of fish showing typical EUS clinical signs were fixed in 10% buffered formalin for histopathology and immunohistochemistry.

Preparation of antigen

Antigen was prepared from the reisolated *A. invadans* B99C according to Lilley et al. (1997a) with modifications. Protein concentration of the fungal extract was estimated (Lowry et al. 1951), adjusted to $5\text{ mg}\cdot\text{ml}^{-1}$ with PBS (pH 7.2) and stored at -20°C until used.

Immunization of mice

Eight week old female Balb/C mice were immunized (i.p.) with 0.5 ml ($33\text{ }\mu\text{g}$ fungal protein) of 1:1 mixture of the purified mycelial extract of *A. invadans* and Freund's complete adjuvant. Mice were given a 0.3 ml ($20\text{ }\mu\text{g}$) booster (i.p.) with 1:1 mixture of the mycelial extract and Freund's incomplete adjuvant on the 15th day. On the 25th day another booster with 0.05 ml ($3.3\text{ }\mu\text{g}$) of extract in PBS was given (i.v.). Blood from mice was collected on the 29th day and antibody titre was estimated by enzyme linked immunosorbent assay (ELISA) as per manufacturer's specifications (Sigma, USA).

Rabbit antisera to *A. invadans* were maintained as positive control and PBS as negative control. Spleen cells from mice with serum ELISA titre more than 1:1000 were used for hybridoma production.

Hybridoma production

Hybridomas were produced according to Kohler & Milstein (1975) with modifications. Immunized mice were killed by cervical dislocation

and spleen cells were collected aseptically in serum free RPMI (Sigma, USA) medium. Spleen cells were then fused with SP2/O myeloma cells using polyethylene glycol (Sigma, USA) and seeded into fourteen 96-well tissue culture plates containing one day old spleen cells feeder layer prepared from un-immunized mouse. The plates were incubated in CO₂ incubator with humidified atmosphere (CO₂ 5 %) (Nuair, USA).

Immunodot assay for screening of hybridomas

Hybridomas were screened for antibody production from the 15th day onwards by an immunodot assay according to [Gayathri et al. \(2004\)](#). Development of clear purple blue dot on nitro-cellulose paper (NC) was considered positive. Hybridoma supernatant reacting with *A. invadans* but not with negative controls viz., saprophytic *Aphanomyces* and homogenate of healthy fish tissue was considered positive. Supernatants of positive hybridomas were also tested for their reaction with SDS-mercaptoethanol treated *A. invadans* antigen.

Positive hybridomas were further minicloned three times by serial ten fold dilution on 1 day old spleen cells feeder layer. Clones were screened for antibody production by immunodot assay and positive hybridomas cultured in 25 cm² tissue culture flasks for further characterization. Immunoglobulin isotype of the monoclonal antibodies (MAbs) was determined by capture ELISA (Sigma, USA) according to the manufacturer's instructions.

Characterization of MAbs by Western blot

Mycelial extract was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) ([Laemmli 1970](#)). The mycelial extract was mixed (1:4) with sample buffer [0.5 M Tris-HCl pH 6.8, glycerol 0.8 ml, 10 % (w/v) SDS 1.6 ml, 2-B mercaptoethanol 0.4 ml, 0.05 % (w/v) bromophenol blue] and boiled for 1'. The mycelial extract and molecular weight markers (Sigma, USA) were then electrophoresed in 15% gel at 200 V for 45-60' until the dye front reached the end of the separating gel. Two reference lanes (molecular weight markers and mycelial protein) were separated from the gel and stained with 0.1% (w/v) Coomassie blue-R for 5 h and destained with destaining solution. Proteins in the remaining lanes of the gel were transferred to NC at 100 V (250 mA) for 1 h using a mini-transblot apparatus (Bio-Rad, USA). Protein lanes on the NC were cut and reacted with blocking solution [3 % Bovine Serum Albumin (BSA)/Phosphate Buffer Saline (PBS)] followed by reaction with MAbs overnight at 4°C. After washing three times with PBS/Tween-20,

proteins were reacted with rabbit anti-mouse IgG horseradish peroxidase (Genei, India) (1:200) in 3% BSA/PBS for 1 h. After washing three times, substrate with 4-chloro 1-naphthol was added and development of purple blue bands was noted. The reaction was stopped by washing NC with PBS and dried.

Specificity of MAbs by immunohistochemistry

Immunoperoxidase

Muscle tissue of *A. invadans* (experimentally) infected catla was fixed in 10% buffered formalin. Sections were cut and washed with PBS and nonspecific binding sites were blocked with 500 μ l of 10% normal rabbit serum in 1% BSA in PBS for 30' in a humidified chamber. After washing with PBS, tissue sections were reacted with four day old cell culture supernatant of positive hybridomas for 1 h. After washing the slides with PBS, rabbit anti-mouse IgG horse radish peroxidase diluted 1 in 200 in 1% BSA in PBS was added and incubated for 30'. Followed by 3, 3' Diaminobenzidine (Sigma, USA) ($0.6 \text{ mg} \cdot \text{ml}^{-1}$ 0.05 mM Tris buffer pH 7.6 with 0.03 % H_2O_2) was added. After 5', the slides were immersed in tap water and then counterstained with 0.1% eosin for 3'. The sections were also stained with haematoxylin-eosin for comparison. Negative control consisted of test slides incubated with normal rabbit serum instead of MAb. Development of dark reddish brown colour within mycotic granuloma and along hyphae was considered as a specific reaction.

Immunofluorescence

Wax embedded muscle tissues of *A. invadans* infected fish were dewaxed, rehydrated, blocked with normal rabbit serum and incubated with supernatant of positive hybridomas as mentioned above under immunoperoxidase. Normal rabbit serum instead of MAb was used as negative control. The slides were washed with PBS followed by incubation with rabbit anti-mouse IgG FITC (Genei, India) diluted 1:40 with 1% BSA in PBS for 45'. The slides were washed twice with PBS and drained using tissue paper. The sections were mounted using glycerol with 1% n-propyl gallate (HiMedia, India) as antifading agent and observed under UV microscope (Olympus, BX-FLA).

Epitope analysis of *A. invadans*

Epitope analysis of *A. invadans* in six batches of EUS infected fish samples (catla, rohu (*Labeo rohita*), mrigal (*Cirrhinus mrigala*) and channa (*Channa striata*)) from various locations of India was carried out

using the panel of 3 MAbs by immunoperoxidase assay as explained earlier. All samples were confirmed to show EUS by histopathology.

Results

Pathogenecity of the isolate

Pathogenecity of *A. invadans* was confirmed by experimental infection using mycelial tips and zoospores. Typical ulcerative symptoms in catla and puntius were observed in both methods of infection, however, infection with zoospore was much severe than that by mycelial tips. *A. invadans* was re-isolated from the deeper part (skeletal muscle) of the ulcers of the experimentally infected fish. Tissue sections of experimentally infected fish showed presence of mycotic granuloma and the fungal hyphae consistently. The isolate showed aseptate, sparsely branching, slow growing hyphae, producing achlyoid form of zoospores typical of *A. invadans*.

Hybridoma production

A total of 60 hybridomas were obtained from two fusions. In immunodot assay, 23 hybridomas were positive against *A. invadans*, out of which nine showing strong reaction were selected. Out of these nine clones, supernatants of only three clones (C1, C13 and C14) which showed reaction with SDS-mercaptoethanol and heat treated *A. invadans* in immunodot test were selected (Fig. 1). There was no reaction of hybridoma supernatant with negative controls.

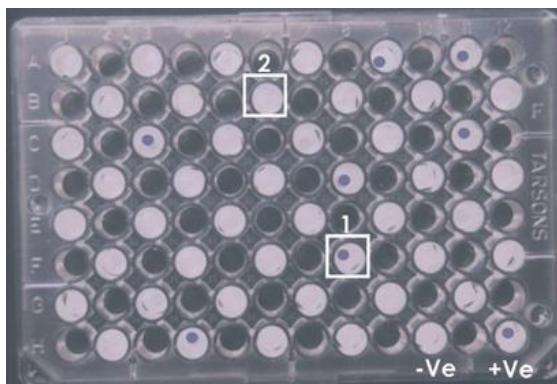
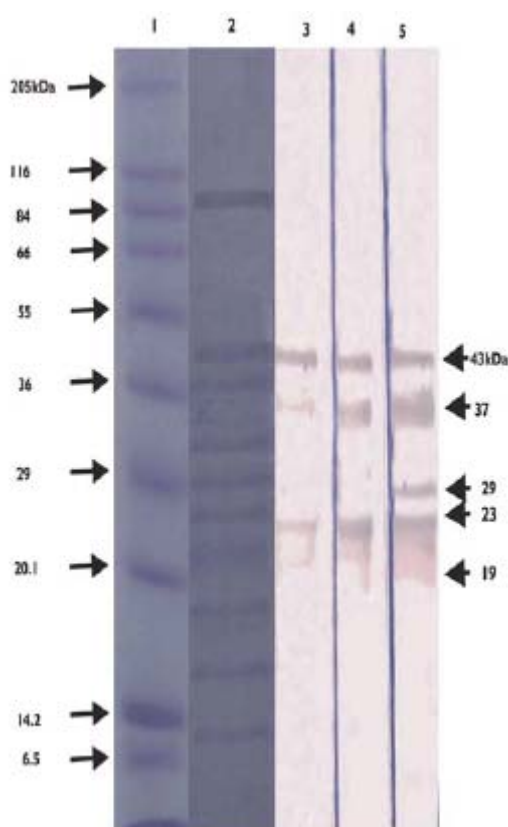


Figure 1. Screening of hybridomas by immunodot assay. Box 1: Clone reacting with *A. invadans* mycelial extract; Box 2: Clone negative to *A. invadans* mycelial extract; -ve: Negative control with saprophytic *Aphanomyces*/tissue homogenate from health fish/without mycelial extract of *A. invadans*; +ve: Positive control with mouse polyclonal antibody.

Characterization of MAbs by Western blot



In SDS-PAGE several protein bands of *A. invadans* varying from 6.5 to 100 KDa were recorded (Fig. 2). The MAbs C1 and C13 were similar in reaction recognizing four proteins viz., 43, 37, 23 and 19 KDa, while C14 in addition to four proteins, recognized another protein 29 KDa. The MAbs of C13 and C14 were of IgM type while C1 was of IgG1 type (Table 1).

Figure 2. Determination of *A. invadans* protein specificity of MAbs by Western blot. *A. invadans* mycelial extract subjected to SDS-PAGE, proteins transferred to NC paper and reacted with MAbs. Lane 1: Molecular weight marker; Lane 2: *A. invadans* mycelial extract; Lane 3: Reaction of MAb C1; Lane 4: Reaction of MAb C13; Lane 5: Reaction of MAb C14.

Table 1. Characterisation of monoclonal antibodies by Western Blot

MAbs	Antibody class	Intensity of reaction with <i>A. invadans</i> protein (kDa)				
		43	37	29	26	19
C1	IgG1	++++	+	--	+++	+
C13	IgM	++++	++	--	+++	++
C14	IgM	++++	+++	++	++	+++

Note: strong positive (++++); positive (+++); weak positive (++); slight reaction (+); no reaction (--)

Specificity of MAbs by immunohistochemistry

Immunoperoxidase with MAbs C1, C13 and C14 showed strong positive reactions (reddish dark brown color) selectively staining the hyphae inside the granuloma in EUS infected fish tissue (Figs. 3a-c). There was no reaction in negative controls. However, the color intensity of the reaction varied within a tissue section and between samples. There were no background reactions in any of the tests.

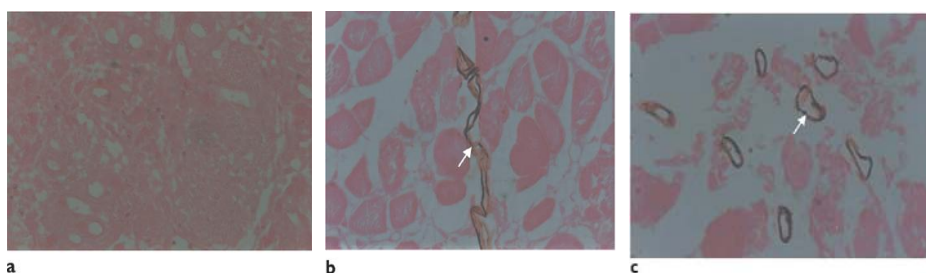


Figure 3. Determination of specificity of MAb by immunoperoxidase; a) Negative control (reaction with normal rabbit serum); b) LS; and, c) CS, reaction of MAb C1 selectivity with *A. invadans* hyphae

Immunofluorescence with MAbs C1, C13 and C14 showed a bright green yellow fluorescence staining the hyphae and centre of mycotic granuloma in muscle tissue sections of EUS infected fish (Fig. 4). There was no reaction with the negative controls.

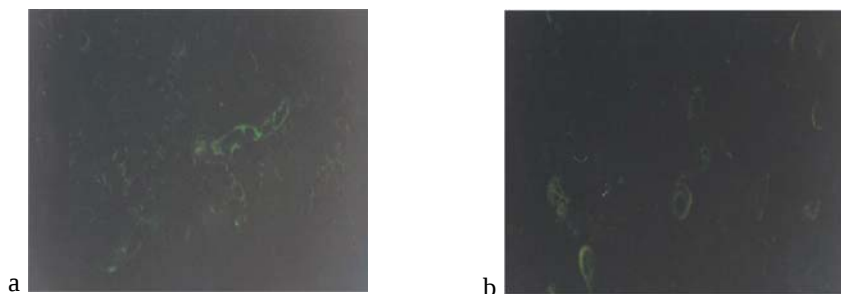


Figure 4. Determination of specificity of MAb by immunofluorescence. Reaction of MAb C1 with muscle tissue of rohu (*Labeo rohita*) with EUS; a) Reaction along the hyphae; b) Reaction with hyphae within mycotic granuloma

Epitope analysis of A. invadans

All 3 MAbs (C1, C13 and C14) reacted positively with six batches of EUS infected fish samples in immunoperoxidase (Table 2). The samples viz., R1, R2, MR1, MR2 and CM1 showed a strong reaction with all three MAbs. Tissue sections of experimentally infected catla (FC1) also showed positive reactions with all three MAbs. Fish samples obtained from Tripura showed intense reaction than that of the South Indian samples. However, the reaction pattern indicated that *A. invadans* from various regions of India are similar albeit subtle variations in intensity of color reactions. All six batches of samples showed mycotic granuloma with H and E staining.

Table 2. Epitope analysis of *A. invadans* from different species of EUS affected fish

Sample No.	Year of collection	Species	Place of collection	Type of water body	Clinical ulcers in the sample	Reaction of MAbs			Mycotic granuloma in H&E staining
						C1	C13	C14	
FC1	1999	<i>Catla catla</i>	Fish farm, College of Fisheries, Mangalore, Karnataka (13° 15' N, 74° 50' E)	Culture pond	P	++	+	++	P
R1	2002	<i>Labeo rohita</i>	Tripura (23° 50' N, 91° 30' E)	Culture pond	P	+++	+++	++	P
R2	2002	<i>Labeo rohita</i>	Tripura (23° 50' N, 91° 30' E)	Culture pond	P	+++	+++	++	P
MR1	2002	<i>Cirrhinus mrigal</i>	Tripura (23° 50' N, 91° 30' E)	Culture pond	P	+++	+++	++	P
MR2	2002	<i>Cirrhinus mrigal</i>	Tripura (23° 50' N, 91° 30' E)	Culture pond	P	+++	+++	+++	P
CM1	1997	<i>Channa striata</i>	Shimoga, Karnataka (13° 15' N, 74° 50' E)	Culture pond	P	++	++	+++	P

Note: Present (P); strong positive (+++); positive (++); relatively weak (+)

Discussion

Three MAbs C1, C13 and C14 which reacted with *A. invadans* antigen subjected to heat with SDS-mercaptoethanol were used in the present study. In Western blot, two MAbs C1 and C13 reacted with four proteins 43, 37, 23 and 19 kDa of *A. invadans*. The MAb C14, however, additionally reacted with 29 KDa protein. Further, the reaction of all three MAbs was clear and distinct with 43 KDa. The overall reaction pattern of MAbs with several proteins of *A. invadans* indicates sharing of similar epitopes by these proteins. Sharing of epitopes has also been observed in *Plasmodium halstedii*, the fungus causing sunflower downy mildew, in which two MAbs reacted with three proteins 68, 140 and 192 kDa in Western blot (Bouterige et al. 2000). A similar sharing of epitopes by two different proteins has also been reported in the case of white spot virus (WSSV) of penaeid shrimp employing MAbs (Poulos et al. 2001; Anil et al. 2002). Our hybridoma clones were considered truly monoclonal as the clones were minicloned thrice at a very high dilution. Further, different reaction patterns of three MAbs with five proteins and differences in their isotypes, C13 and C14 (IgM) and C1 (IgG1) support that these clones are different, reacting with three different epitopes.

Among the various proteins reported, 40 KDa was recognized using polyclonal antibodies in Western blot and was demonstrated to be immunogenic in both fish and rabbit (Thompson et al. 1997; Lilley et al. 1997a). Our MAbs recognized 43 KDa in Western blot. However, we observed that another low molecular weight protein 10 KDa which is immunogenic in fish (Thompson et al. 1997; Lilley et al. 1997a) was not found to be immunogenic in mice. This could be due to heat lability or susceptibility to the antigen preparation process of the above protein. Overall, there is an agreement in the protein profile of the isolate B99C compared with that of other isolates of *A. invadans*. These observations would clearly have an impact on the development of a vaccine against *A. invadans*.

Immunohistochemistry is significant in determining the specificity of MAbs when obtaining a pure culture of pathogen in question is difficult. Since immunohistochemistry makes use of tissue sections of infected samples (even preserved ones), there is a wider scope of its application in far off places. The specific nature of MAbs would be important for a retrospective analysis of infected samples where cause of mortality is yet to be ascertained. In the present study, the specificity of MAbs for the pathogenic fungus was confirmed by immunoperoxidase and immunofluores-

cence assays with the centre of mycotic granuloma and fungal hyphae reacting positive without any background reactions.

In the present study, MAbs against *A. invadans* did not react with saprophytic *Aphanomyces*, indicating the distinct antigen complex of the former with respect to the epitopes recognized by three MAbs in Western blot and immunohistochemistry. This fact further illustrated the specificity of MAbs to the pathogen. Bullis et al. (1996) demonstrated that MAbs against *Saprolegnia parasitica* did not react with *Aphanomyces* in indirect immunofluorescence suggesting that not all pathogenic oomycetes express the same antigen or epitopes.

The panel of three MAbs reacted with all six batches of fish samples from different parts of India in which EUS was confirmed by histopathology. The MAbs recognised *A. invadans* in all four batches of fish samples from Tripura, North Eastern India. This reaction was expected as Tripura being geographically adjacent to Bangladesh from where B99C isolate was collected. Specific reaction of MAbs with equal intensity for samples from Karnataka, Southern India had indicated the antigenic similarity. However, the reaction intensity of MAbs with CM1 sample from Karnataka was relatively weaker, probably due to formalin fixation and longer period of preservation which could have caused denaturation of some of the epitopes. Overall, the present study with three MAbs indicated that there was an antigenic similarity among the samples infected with *A. invadans* in the Indian subcontinent.

The isolates of EUS, RSD and MG were found to be similar by RAPD-PCR which suggested that these isolates are of a single clonal genotype (Lilley et al. 1997b). Therefore, it would be helpful to use our MAbs wherever natural EUS outbreak occurs to confirm the similarity and diagnosis across the world.

Conclusion

The present study, being the first of its kind in the country, has laid a molecular approach in using MAbs for disease diagnosis particularly of fungal origin providing enhanced control over preventive aquaculture management practices. Further, steps to develop field level application of this technology would help fish farmers in a greater way. Although, EUS has not been as severe as it used to be, there is no guarantee that the same

or similar disease would not surface again in the future. Therefore, the present research has established a very useful diagnostic method for such dreaded disease in aquaculture. The present effort resumes a greater significance in the face of increased demand for fish production from the aquaculture sector.

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