Monoclonal Antibody-based Detection of *Aphanomyces invadans* for Surveillance and Prediction of Epizootic Ulcerative Syndrome (EUS) Outbreak in Fish

M. GANAPATHI NAIK, K.M. RAJESH, A.K SAHOO and K.M. SHANKAR

Fish Pathology and Biotechnology Laboratory
Department of Aquaculture, College of Fisheries
Mangalore – 575 002, India

ABSTRACT

Epizootic Ulcerative Syndrome (EUS) has caused large scale mortality of fresh and brackish water fishes in several parts of South-East Asia including India since 1980s. EUS susceptible fishes such as *Mugil* spp., *Glossogobius* sp. *Sillago* sp., and *Platycephalus* sp. were collected from previously EUS affected brackish waters of South Canara and Udupi districts of Karnataka, India from June 2003 to June 2005. Tissues collected from grossly healthy and ulcerated fishes were screened for the presence of *Aphanomyces invadans* using a monoclonal antibody (MAb) based immunodot. Ulcers were only observed in fishes during the months of August and September each year, coinciding with low water temperature and low salinity. All the ulcerated fishes were found to be positive for *A. invandans* by immunodot. The immunodot could detect the fungus in grossly healthy fish in June two months before appearance of the ulcers in August. The immunodot could also detect the fungus in fishes with healed ulcers during late October and early November. Overall, among the grossly healthy fishes collected from June to November 20% of *Mugil* spp, 2% of *Glossogobius* sp, 10% of *Sillago* sp., and 4% of *Platycephalus* sp. were positive by immunodot. The study indicates that the immunodot could be used for early detection of *A. invadans* and to predict EUS outbreaks at least two months in advance of a disease episode.

Ganapathi, M. N., Rajesh, K.M., Sahoo, A.K. and Shankar, K.M. 2008. Monoclonal antibody-based detection of *Aphanomyces invadans* for surveillance and prediction of epizootic ulcerative syndrome (EUS) outbreak in fish, pp. 157-168. *In* Bondad-Reantaso, M.G., Mohan, C.V., Crumlish, M. and Subasinghe, R.P. (eds.). Diseases in Asian Aquaculture VI. Fish Health Section, Asian Fisheries Society, Manila, Philippines. 505 pp.

Corresponding author: M. Shankar, kalkulishankar@rediffmail.com

INTRODUCTION

Ever since its first report in 1972 in Australia, epizootic ulcerative syndrome (EUS), a destructive ulcerative disease caused by *Aphanomyces invadans,* has resulted in severe mortalities both in freshwater and in brackishwater fishes throughout the South East Asia (Kenzie and Hall., 1976; Hatai., 1994; Robert *et al.,* 1994b). In India, the first outbreak of EUS occurred in May 1988 in the northeast states and later the disease spread to Arunachal Pradesh in late 1988, Orissa and Bihar in 1989, Maharashtra, Uttar Pradesh, Andra Pradesh and Madhya Pradesh in 1990 and finally to Haryana, Kerala and Karnataka in 1991 (Kumar *et al*., 1991; Das and Das, 1993; Mohan and Shankar, 1994). Most recently EUS was confirmed in fish from Punjab Province in April 1996 and from Sindh Province in January 1998 (DFID, 1998). It has been reported that more than 100 fish species are affected by the disease (Frerichs., 1988; Lilley *et al.,* 1999). Susceptibility of *Mugil* spp. (mullets)*, Platycephalus* sp. (flatheads)*, Sillago* sp. (sillago)*, Glossogobius* sp. (goby) and *Terapon* sp. (therapon) to EUS in brackish water systems in India is well documented (Vishwanath *et al.,* 1997b, 1998). Severe chronic granulomatous mycosis has been consistently seen as the diagnostic pathology in all the EUS affected fishes from different parts of the world (Roberts., 1993, 1994a, b; AAHRI/IOA/NACA, 1997; Mohan and Shankar., 1994; Khan *et al.,* 1999). It is now well established that the surface ulcers and the fish mortality are because of the invasive activity of the fungus *A. invandans* (Vishwanath *et al.,* 1998). In addition to the presence of the causative agent *A. invadans*, several abiotic factors such as low water temperature, rapid change in salinity and dissolved oxygen have been reported to promote the disease (Phillips and Keddie, 1990; Virgona, 1992; Fraser *et al*., 1992). An ulcer is a non-specific clinical lesion, which may be caused by many different agents (Robert *et al.,* 1986). Not all ulcers are EUS related, because they do not occur in epizootic proportions or are not seasonal in nature (Shankar and Mohan, 2002). In light of this, the development of specific diagnosis for EUS is important to avoid confusion with other occasional and serious ulcerative conditions. Histopathology which can detect the fungus in advanced stages of the disease condition is tedious and time consuming. Our laboratory has developed a monoclonal antibody (MAb) based immunodot for specific and rapid detection of *A. invadans* (Gayathri *et al.,* 2004). In the present investigation, the immunodot was used to detect *A. invadans* for surveillance and prediction of EUS outbreaks in fishes from three brackish water bodies of Karnataka, India.

MATERIALS AND METHODS

Sample Collection

Three brackishwater bodies, Nethravati and Mulky estuaries in South Canara and Kundapur estuary in Udupi districts of Karnataka, India were selected for the study. EUS outbreaks were reported earlier from these water bodies. Ten EUS susceptible fish each belonging to *Mugil* sp.*, Platycephalus* sp.*, Sillago* sp. and *Glossogobius* sp. were collected once a month from the above water bodies from June 2003 to June 2005. However, due to the abundance of ulcerated fish during the month of August and September of each year, samples were collected twice a month for close monitoring. The sample consisting of both apparently

healthy and ulcerated fish was brought to the laboratory on ice and muscle tissue extracted for use in the immunodot. Water temperature and salinity were recorded to correlate the relation between incidence of EUS and environmental factors.

Preparation of fish tissue for the immunodot

Muscle tissue was taken from around the ulcerated site on the fishes, while the muscle tissue was randomly taken from 4 to 5 places of the body of apparently healthy fish. The muscle tissue was macerated in TNE buffer (0.02M Tris, 0.2M NaCl, HCl and 1mM EDTA, pH 7.4) at 1:10 w/v by using a pestle and mortar. The homogenate was allowed to settle for 5 min and the supernatant used in the immunodot assay.

Immunodot assay

The immunodot was carried out according to Gayathri *et al.* (2004) with slight modifications. Three µl of tissue homogenate prepared as above from the ulcerated and grossly healthy fish was dotted onto the nitrocellulose paper (pore size 0.2µm, Bio Rad). Semi-purified *A. invadans* was used as a positive control and tissue homogenate from a healthy fish, previously confirmed as negative for the fungus by histopathology and immunodot, used as negative control. The dots were air dried for 5 min. The dotted paper was blocked with 2 ml of 3% BSA (Bovine Serum Albumin, Merck, Mumbai) in PBS (Phosphate Buffer Saline) for 30 min followed by washing with PBS Tween-20. Later the paper was treated with 2ml of MAb C14 (IgM isotype, recognising epitopes on 43, 37, 29, 23, and 19 KD proteins of *A. invadans*) for 90 min. Pooled culture supernatant of the C14 clone from several batches of culture was used to maintain uniform level of the MAb throughout the study. The MAb was poured off and the paper was washed with PBS Tween-20. Then rabbit-anti-mouse IgG horseradish peroxidase (Genei, Bangalore) in 3% BSA (1:200) in PBS was added and incubated for 30 min. After washing 3 times with PBS Tween-20, substrate (4- Chloro-1-napthol/ H_2O_2) was added and a purple/ blue colored dot developed which was recorded as positive.

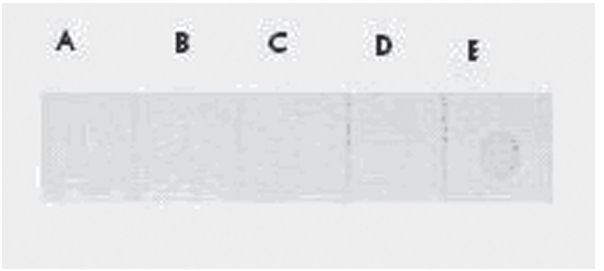
Reaction of the MAb in the immunodot with other ulcer causing aquatic pathogens such as *Aeromonas hydrophila*, *Bacillus* sp., *Saprolegnia* sp. and *Achlya* sp. was checked. These organisms were grown in the laboratory using suitable culture media for each organism. The colonies of the fungi were collected and homogenized with liquid nitrogen and suspended in PBS. Bacterial colonies were suspended in PBS. Three µl (2.5µg) antigen was dotted onto the nitrocellulose paper and examined for the MAb reaction by immunodot as above.

RESULTS

Specific reaction of the MAb with *A. invadans* in the immunodot is depicted in Figure 1. The MAb did not react with other ulcer- causing organisms such as *A. hydrophila*, *Bacillus* sp., *Saprolegnia* sp. and *Achyla* sp. In all the three brackish water bodies, EUS- outbreaks in fish, with visible ulcers were noticed during August and September each year coinciding with low water temperature and low salinity (Table 1). The immunodot could detect

Table 1. Detection and surveillance of *A. invadans* in fish by immunodot in Netravathi, Mulky and Kundapura estuary from June 2003 to June 2005.

Months	Netravathi estuary				Mulky estuary				Kundapura estuary			
	Water temperature (^{0}C)	Salinity (ppt)	Occurrence of EUS ulcer	Result of immunodot	Water temperature (^{0}C)	Salinity (ppt)	Occurrence of EUS ulcer	Result of immunodot	Water temperature (^{0}C)	Salinity (ppt)	Occurrence of EUS ulcer	Result of immunodot
June 2003	28.00	2.26	-	+	28.90	2. 76	-	+	29.20	3.54	-	+
July	28.80	0.00	-	+	29.80	0.96	-	+	28.70	0.78	-	+
Aug.-I	28.50	0.00	+	+	28.30	014	+	+	28.20	0.22	+	+
Aug. II	28.30	0.72	+	+	28.70	0.00	+	+	28.50	0.00	+	+
Sep. -I	28.90	1.62	+	+	28.90	0.70	+	+	28.80	0.85	+	+
Sep. - II	29.00	1.78	+	+	29.00	0.92	+	+	29.10	0.80	+	+
Oct.	28.90	2.18	-	+	30.20	2.46	-	+	29.60	2.37	-	+
Nov.	29.60	4.45	-	+	29.30	4.92	-	+	29.90	4.12	-	+
Dec.	30.30	12.17	-	-	29.80	12.64	-	-	29.50	13.86	-	-
Jan. 2004	30.70	17.86	-	-	30.20	15.24	-	-	30.30	18.13	-	-
Feb.	30.50	22.26	-	-	30.50	20.63	-	-	30.80	21.86	-	-
Mar.	31.20	25.18	-	-	31.30	28.60	-	-	31.00	26.74	-	-
April	31.80	27.68	-	-	31.40	29.34	-	-	31.20	29.65	-	-
May	32.20	30.20	-	-	31.90	30.40	-	-	31.70	30.92	-	-
June	28.30	2.26	-	+	28.70	4.32	-	+	28.90	4.10	-	+
July	28.40	0.00	-	+	28.90	0.45	-	+	28.20	0.58	-	+
Aug -I	28.60	0.00	+	+	28.50	0.00	+	+	28.00	0.00	+	+
Aug. II	28.20	0.00	+	+	28.20	0.00	+	+	28.30	0.00	+	+
Sep. I	29.00	1.30	+	+	28.80	0.52	+	+	28.50	0.93	+	+
Sep. II	28.70	1.25	+	+	28.60	0.72	+	+	28.20	0.65	+	+
Oct	29.80	2.20	-	+	29.60	2.86	-	+	28.80	3.12	-	+
Nov.	29.60	4.60	-	+	30.10	3.93	-	+	29.30	5.54	-	+
Dec.	30.50	13.00	-	-	30.80	14.17	-	-	29.90	15.14	-	-
Jan 2005	30.60	18.30	-	-	30.40	16.92	-	-	30.30	19.24	-	-
Feb	31.00	22.90	-	-	31.20	23.10	-	-	30.90	24.52	-	-
Mar.	31.50	26.00	-	-	31.70	27.30	-	-	31.00	27.36	-	-
April	31.80	28.00	-	-	32.00	29.23	-	-	32.00	30.20	-	-
May	32.40	30.60	-	-	32.20	30.80	-	-	32.60	30.80	-	-
June	28.80	3.20	-	+	28.70	3.93	-	+	29.20	5.10	-	+



A: *A. hydrophila*, B: *Bacillus* sp., C: *Achyla* sp., D: *Saprolegnia* sp., E: *A. invadans*

Figure 1. Immunodot of *A.invadans* with other ulcer causing agents, *A. hydrophila*, *Bacillus* sp., *Achyla* sp., and *Saprolegnia* sp.

A. invadans in all the ulcerated fishes in the three water bodies (Figure 2a). However, interestingly fishes collected during June and July of each year were apparently healthy, and immunodot could consistently detect the fungus in a good number of them (Figure 2b). EUS outbreaks were over by September in all the three water bodies, but immunodot could detect the fungus in the apparently healthy fishes during October and early November (Figure 2b). However, fishes collected from December to May of each year were consistently negative by immunodot (Figure 2c). Overall, among the grossly healthy fish tested from June to November by the dot, 20% of *Mugil* spp., 2% of *Glossogobius* sp, 10% of *Sillago* sp., and 4% of *Platycephalus* sp. were positive for the fungus (Figures 3a,b,c;, Table 2).

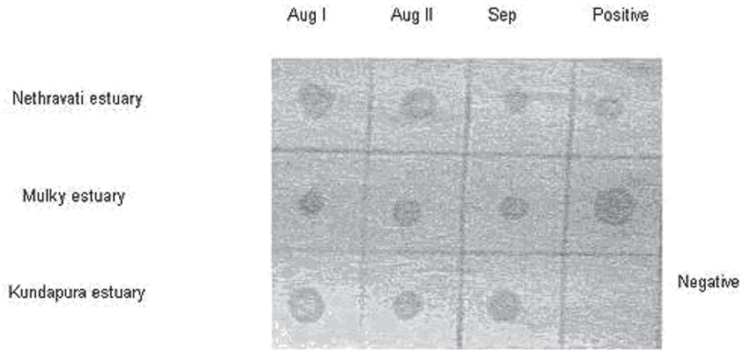


Figure 2a. Immunodot of fishes collected during August and September.

Figure 2b. Immunodot of fish sample without ulcer during June to November.

Figure 2c. Immunodot of fish sample (without ulcer) collected from December to May.

Table 2. Immunodot detection of *A. invadans* in individual fish species during June to November of 2003, 2004 and June 2005.

				Netravati estuary		Mulky estuary		Kundapur estuary	
Year	**Months**	**Fish type**	**No. of fish sampled**	**No. of fish + without ulcer**	**No. of fish + with ulcer**	**No. of fish + without ulcer**	**No. of fish + with ulcer**	**No. of fish + without ulcer**	**No. of fish + with ulcer**
2003	June	Mullets	10	2	-	3	-	2	-
		Goby	10	0	-	0	-	0	-
		Sillago	10	2	-	1	-	2	-
		Flat heads	10	1	-	2	-	1	-
	July	Mullets	10	3	-	2	-	4	-
		Goby	10	1	-	0	-	0	-
		Sillago	10	3	-	3	-	2	-
		Flat heads	10	2	-	2	-	0	-
	Aug.- I	Mullets	10	1	5	1	6	2	5
		Goby	10	0	2	1	1	0	2
		Sillago	10	2	3	1	3	1	3
		Flat heads	10	1	2	0	2	0	3
	Aug.- II	Mullets	10	0	7	1	6	1	5
		Goby	10	0	2	0	2	0	2
		Sillago	10	1	2	0	3	1	3
		Flat heads	10	0	3	1	3	0	2
	Sep. I	Mullets	10	1	5	1	4	0	4
		Goby	10	0	1	0	1	0	2
		Sillago	10	0	3	0	4	1	3
		Flat heads	10	0	3	1	3	0	2
	Sep - II	Mullets	10	2	3	1	4	1	4
		Goby	10	0	1	0	1	0	1
		Sillago	10	1	3	0	3	2	2
		Flat heads	10	0	2	1	2	1	2
	Oct.	Mullets	10	4	-	3	-	3	-
		Goby	10	1	-	1	-	1	-
		Sillago	10	3	-	3	-	4	-
		Flat heads	10	2	-	2	-	1	-
	Nov.	Mullets	10	3	-	2	-	3	-
		Goby	10	0	-	0	-	0	-
		Sillago	10	2	-	1	-	2	-
		Flat heads	10	0	-	0	-	1	-

Table 2. (continued)

2004	June	Mullets	10	3	-	3	-	2	-
		Goby	10	0	-	0	-	0	-
		Sillago	10	2	-	2	-	2	-
		Flat heads	10	1	-	2	-	1	-
	July	Mullets	10	4	-	4	-	4	-
		Goby	10	1	-	0	-	0	-
		Sillago	10	3	-	2	-	2	-
		Flat heads	10	2	-	1	-	0	-
	Aug.- I	Mullets	10	2	6	3	6	2	6
		Goby	10	0	2	1	1	0	2
		Sillago	10	2	3	1	3	1	3
		Flat heads	10	1	2	1	2	0	3
	Aug.- II	Mullets	10	0	6	1	7	1	7
		Goby	10	0	2	0	2	0	2
		Sillago	10	2	3	0	3	2	3
		Flat heads	10	1	3	1	3	0	2
	Sep. I	Mullets	10	0	5	2	6	3	4
		Goby	10	0	1	0	1	0	2
		Sillago	10	1	3	0	4	1	3
		Flat heads	10	1	3	1	3	0	2
	Sep - II	Mullets	10	3	3	1	4	1	4
		Goby	10	0	1	0	1	0	1
		Sillago	10	2	3	2	3	1	2
		Flat heads	10	0	2	1	2	2	2
	Oct.	Mullets	10	4	-	3	-	4	-
		Goby	10	1	-	1	-	1	-
		Sillago	10	3	-	3	-	2	-
		Flat heads	10	2	-	2	-	1	-
	Nov.	Mullets	10	3	-	3	-	3	-
		Goby	10	0	-	0	-	0	-
		Sillago	10	2	-	3	-	2	-
		Flat heads	10	0	-	0	-	1	-
2005	June	Mullets	10	2	-	4	-	3	-
		Goby	10	0	-	0	-	0	-
		Sillago	10	3	-	2	-	2	-
		Flat heads	10	1	-	1	-	1	-

(-): No ulcers found on fish

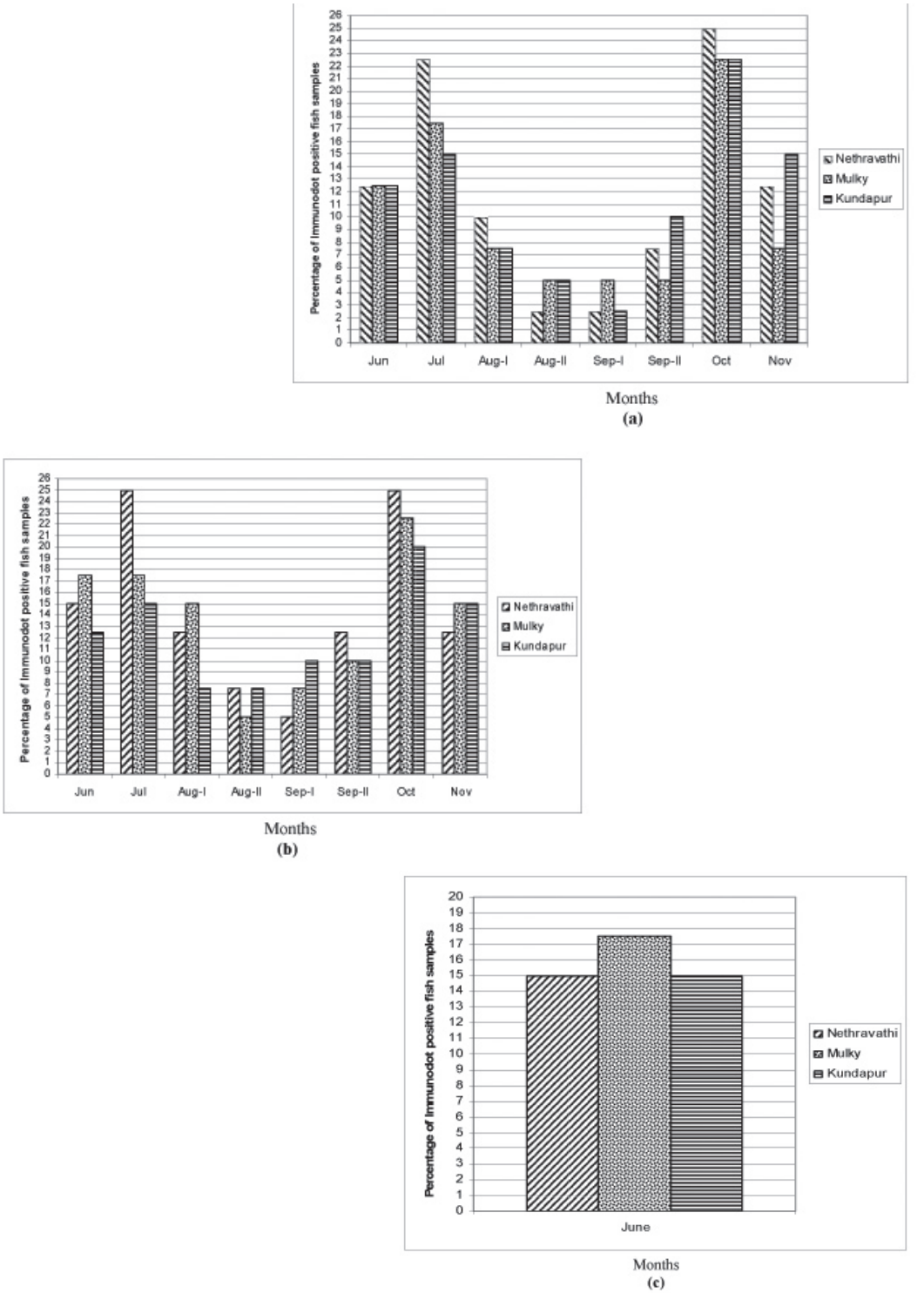


Figure 3. Immunodot detection of *A. invadans* in grossly healthy fish (pooled result of individual species) collected during June to November of 2003 (Figure 3a, 2004; Figure 3b) and in June 2005 (Figure 3c).

DISCUSSION

EUS outbreaks in fish, with typical clinical signs were recorded during August and September of each year in all the three brackish water bodies. During these months salinity and water temperature were low due to monsoon rains which favored development of EUS ulcers, which has been very well documented earlier in India (Mohan and Shankar, 1994; Vishwanath *et al.,* 1997a) and elsewhere (Lilley *et al.,* 1998). The dot detected the EUS fungus, *A. invadans,* in all the ulcerated fishes found during August and September. However, the dot could detect the fungus two months in advance in June in apparently healthy fish, without visible ulcers. It is well known that the fungus penetration into fish takes place at very early stage of the disease, where there may not be significant external clinical signs (Vishwanath *et al.,* 1997a). It indicates that the fungus after infection of fish in early June persisted for two months until favorable conditions such as low water temperature and low salinity are available for its rapid growth which causes ulcer. Lowering of water temperature and salinity may lower the innate defense competence of the fish to EUS ((Vishwanath *et al.,* 1997b; Chinabut and Roberts, 1999) and thus aggravate the ulcer condition. Furthermore, immunodot could also be used to detect the fungus in healing or healed fish during October and November. This shows the persistence of the fungal hyphae in fish tissue for at least two months after the disease outbreak. Conventionally, the fungus infection in EUS outbreak has been detected and confirmed by histopathology, characterized by presence of invasive fungal hyphae with granulomas. However, the fungus could not be detected in grossly healthy fish tissue by histopathology. Specific and sensitive detection of *A. invadans* in fish by DNA hybridization (Blazer *et al.,* 2002) and PCR (Panywachira *et al*., 1999) have been standardized, which however are sophisticated and time consuming. The MAb based immunodot is specific, simple and rapid requiring only 3 h to perform. The dot could detect the fungus in grossly healthy fish in June, two months before disease outbreaks in August, and in November, two months after EUS outbreaks. The MAb based immunodot has a detection sensitivity of 45-90 ng of the fungus protein. Therefore, the immunodot could be very useful for early and low level detection of the fungus ideal for prediction of EUS outbreak. Further, sensitivity of the immunodot is being evaluated with histopathology from the samples taken from experimental infections.

ACKNOWLEDGEMENT

The authors are thankful to Indian Council of Agriculture and Research (ICAR), New Delhi for funding the project. Research Associateship to Dr. K. M. Rajesh from CSIR, New Delhi is greatly acknowledged.

REFERENCES

AAHRI/ACIAR/IOA/NACA. 1997. Epizootic Ulcerative Syndrome (EUS) of fishes in Pakistan. A report of the findings of an ACIAR/DFID - funded mission to Pakistan, 9-19 March 1997.

Blazer V.S., Lilley, J.H., Schill, W.B., Kiryu, Y., Densmore, C.L., Panyawachira, V. and Chinabut, S. 2002. *Aphanomyces invadans* in Atlantic menhaden from Chesapeake Bay tributaries. *J. Aquat. Anim. Health* 11:340-349.

Chinabut, S and Roberts, R.J. 1999. Pathology and histopathology of Epizootic Ulcerative Syndrome (EUS). AAHRI, Bangkok. 33 p.

Das M.K and Das, R.K. 1993. A review of the fish disease, Epizootic Ulcerative Syndrome (EUS) in India. *Environ. Ecol*. 11: 134-145.

DFID. 1998. A report of the second mission to investigate Epizootic Ulcerative Syndrome in Pakistan. 19-30 April 1998.

Fraser, G. C., Callinan, R.B. and Calder, L.M. 1992. *Aphanomyces* species associated with red spot disease: an ulcerative disease of estuarine fish from eastern Australia. *J. Fish Dis.* 15: 173-181.

Frerichs, G. N. 1988. Ulcerative fish diseases in Srilanka. *Aqua. Cul. News* 6:1.

Gayathri, D., Shankar, K.M. and Mohan, C.V. 2004. Monoclonal antibody based immunodot test for epizootic ulcerative syndrome pathogen, *Aphanomyces invadans*. *Curr. Sci*. 87:289-291.

Hatai, K. 1994. Mycotic granulomatosis in ayu (*Plecoglossus altivelis*) due to *Aphanomyces piscicida*. *In* Roberts, R. J., Campbell, B. and MacRae, I.H. (eds.). Proceedings of the ODA Regional Seminar on Epizootic Ulcerative Syndrome, 25-27 January, Aquatic Animal Health Research Institute, Bangkok.

Khan, M.H., Lilley, J.H., Majumder, B., Sarker, M.G.A., Alaauddin, M., Hoque, A., Ahmed, G.U. and Choudhury, M.A. 1999. Cross sectional survey of Epizootic Ulcerative Syndrome (EUS) cases in Bangladesh. Paper presented at Fourth Symposium on Diseases in Asian Aquaculture. Cebu City, Philipines, 22-26, Asian Fisheries Society.

Kohler, G and Milstein, C. 1975. Continuous culture of fused cell secreting antibody of predefined specificity. *Nature* 256:495-497.

Kumar, D., Dey, R.K. and Sinha, A. 1991. Outbreak of Epizootic Ulcerative Syndrome (EUS) of fish in India, pp. 345-356 *In* Sinha, V.R.P and Srinivastava, H.C. (eds.) Aquaculture Productivity of Oxford and IBH Publishing Company, New Delhi.

Lilley, J., Bangyeekhan, E., Panyawachira, V. and Cerenius, L. 1999. Zoospore physiology of *Aphanomyces invadans.* Polyplanetism. *The AAHRI Newsletter* 8(2):6-8.

Lilley, J.H., Callinan, R.B., Chinabut, S., Kanchanakhan, S., Macrae, I.H. and Phillips, M.J. 1998. Epizootic Ulcerative Syndrome (EUS). Technical Handbook, AAHRI. Bangkok, Thailand.

Mckenzie, R.A and Hall, W.T.K. 1976. Dermal ulceration of mullet (*Mugil cephalus*). *Aust. Vet. J.* 52:230-231.

Mohan, C. V. and Shankar, K.M. 1994. Epidemiological analysis of Epizootic Ulcerative Syndrome of fresh and brackishwater fishes of Karnataka, India. *Curr. Sci.* 66:656-658.

Panyawachira, V., Lilley, J.H., Hart, D. and Kanchanakhan, S. 1999. A PCR-based technique for the identification of *Aphanomyces invadans*. *In* Fourth Symposium on Diseases in Asian Aquaculture, Cebu City, Phillipines, 22-26 November 1999. Asian Fisheries Society.

Phillips, M. J and Keddie, H.G. 1990. Regional Research Programme on relationships between Epizootic Ulcerative Syndrome in fish and the environment. *In* Report on the Second Technical Workshop,13-26 August 1990, NACA, Bangkok 133pp.

Roberts, R.J., Campbell, B. and Macrae, I.H. 1994a. Proceedings of Regional Seminar on Epizootic Ulcerative Syndrome (EUS). 25-27 January, Aquatic Animal Health Research Institute, Bangkok.

Roberts, R.J., Frerichs, G.N., Tonguthai, K. and Chinabut, S. 1994b. Epizootic Ulcerative Syndrome (EUS) of farmed and wild fishes, pp. 207-239. *In* Muir, J.F. and Roberts, R.J. (eds.). Recent Advances in Aquaculture. Vol. V. Blackwell Science.

Roberts, R.J., Macintosh, D.J., Tonguthai, K., Boonyaratpalin, S., Tayaputch, N., Phillips, M.J. and Millar, S.D. 1986. Field and laboratory investigations into ulcerative fish diseases in the Asia – Pacific region. Technical Report of FAO Project TCP/RAS/4508, Bangkok. 214 p.

Roberts, R.J., Willoughby, L.G. and Chinabut, S. 1993. Mycotic aspects of Epizootic Ulcerative Syndrome (EUS) of Asian fishes. *J. Fish Dis.* 16:169-183.

Shankar, K. M. and Mohan, C.V. 2002. Fish and Shellfish health management UNESCO, New Delhi. 104 p.

Virgona, J. L. 1992. Environmental factors influencing the prevalence of a cutaneous ulcerative disease (red spot) in the sea mullet *Mugil cephalus* L., in the Clarence River, New South Wales, Australia *J. Fish Dis*. 15:363-378.

Viswanath, T.S., Mohan, C.V. and Shankar, K.M. 1997a. Clinical and histopathological characterization of different types of lesions associated with Epizootic Ulcerative Syndrome (EUS). *J. Aqua.Trop.* 12(1):35-42.

Viswanath, T.S., Mohan, C.V. and Shankar, K.M. 1997. Mycotic granulomatosis and seasonality are the consistent features of Epizootic Ulcerative Syndrome of fresh and brackishwater fishes of Karnataka, India. *Asian Fish. Sci*. 10: 155-160.

Viswanath, T.S., Mohan, C.V. and 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.