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Immune response and status of epizootic ulcerative syndrome (EUS) affected fishes *Channa punctatus* and *Clarias magur*

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Abstract: In Epizootic ulcerative syndrome (EUS) affected *Channa punctatus* and *Clarias magur,* the serum total proteins were decreased by 24.63% and 38.75% respectively along with A:G ratio. During immune response, the antibodies produced were found to be positively exponential with the amount of antigens (bacteria, fungus and viruses). The present changes may be because of the changing environmental conditions especially temperature fluctuations producing stress conditions in the fishes.

Keywords: *Channa punctatus*, *Clarias magur,* EUS, Immune response, Serum proteins

INTRODUCTION

Fishes are inevitably subjected to various kinds of stresses in farms that may lead to down regulation to immunity and as a result the outbreak of infectious diseases may occur (Prasad and Verma, 2004). The emergence and spread of aquatic freshwater diseases are a major concern (Johnson and Paull, 2011) and one such aquatic disease implicated in mass mortalities of cultured and wild fish in many countries is Epizootic Ulcerative Syndrome (EUS) Baldock *et al.* (2005).

Large-scale mortality occurs among the fresh water fishes often due to environmental stress followed by pathogenic attacks and parasitic afflictions causing a tremendous loss to the nation.

The course of events from stress to predisposition to infection, include physiological changes, the consequences of which are to enforce barriers, normally preventing entry of bacteria to fish inner systems and at the same time incapacitating fish defence responses and immune reactions (Mazeaud *et al.*, 1977; Barton and Iwama, 1991). Wounds are some common entrance points for some of the bacterial and viral infections, which in turn invite fungal secondary invaders such as *Saprolegnia* species. Lesions may have mixed infections. Naturally and artificially infected snakeheads have been shown to produce an antibody response against *Aphnomyces invadans* (Thompson *et al.*, 1997), and the cellular macrophage response is also considered to be important in enabling fish to resist infection (Wada *et al.*, 1996). Sampling studies in Thailand have yielded an increasing number of isolates showing morphological and electrophoretic similarities to SHRV. Two such isolates were obtained in 1992, nine in 1994, and nine in 1996 (Kanchanakhan, 1996). Further two virus isolates were obtained in 1997, (Lilley *et al.*, 1998). In addition to rhabdoviruses, several birnaviruses and a single reovirus have also been isolated from ulcerated fish.

At present it is difficult to say the exact causative agent of EUS and the economic loss of fishes due to EUS. The more work on immunological responses of fish towards invading pathogens is required. Hence, the present work was undertaken to study this severe problem of EUS and immunological response in the fishes like *Channa punctatus* and *Clarias magur*.

MATERIALS AND METHODS

The present study was carried out by collecting the suspected diseased fishes from near by EUS prone water bodies (Wadali and Malkhed lakes: Maharastra at Latitude 20.96'N and Longitude 77.75'E) with the help of fishermen and were brought to the laboratory and maintained in glass aquaria for a week-to study the type of infection.

The healthy fishes weighing about 25±2 gm and of approximately equal length (10±1cm) were kept in aquarium containing 75 litre of water and were acclimatized for 15 days. They were regularly fed on pellet feed and 25% water of the aquarium was also regularly changed at an interval of 7 days to maintain the water quality. 10 fishes were kept in one aquarium each.

The Experiment was carried out on six sets of 10 fishes in each group as below:

***C. punctatus*:**

Group I : Control (Healthy)

Group II: Naturally EUS affected

Group III: Artificially infected fishes injected with 0.2ml homogenate of ulcerated skin tissue from the diseased ones

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C. magur

Group IV: Control (Healthy)

Group V: Naturally EUS affected

Group VI: Artificially infected fishes injected with 0.2 ml homogenate of ulcerated skin tissue from the diseased ones.

The tissue suspension of ulcerated skin tissue was prepared in sterile fish saline (0.3%). A tissue (500 mg) homogenate was prepared by taking affected or ulcerated skin in fish saline (5ml).The tissue suspensions of naturally EUS affected fishes were inoculated to confirm the presence of bacteria and fungi. Then 0.2 ml of this positive suspension was injected intraperitonially immediately below the skin on lateral sides of the healthy fish (Group III) and they were again released in the aquaria. The experimental fishes were sacrificed at the interval of 7 days, 14 days and 21 days for various studies. No mortality was observed during these 21 days.

Blood was obtained by amputating the tail. Blood was collected in eppendorf tubes, allowed to clot at room temperature for 60 mins and then spun down at 2500 rpm and the serum was removed. Serum of 6 fishes from the same group was pooled and then estimations were carried out. The serum was stored at -20^{0}C until it was assayed for total protein, RID and SDS-PAGE.

Total protein (mg / 100 ml) in serum of fishes was estimated by Biuret method cited in Varley (1975). For immunological studies, electrophoresis and Radial Immunodiffusion Assays (RIA) were carried out. Estimation of immunoglobulins, before and after the inoculation was carried out by densitometer to evaluate the stress caused by the pathogens.

Serum proteins (albumin and immunoglobulin) from control, naturally EUS affected and artificially EUS infected fishes were separated by SDS-PAGE (Laemmli, 1970) and were run on the densitometer for finding out the fractions of immunological proteins.

Polyclonal antibodies of fungi, viruses and bacteria developed in naturally EUS affected fishes were used during the present experiment. The polyclonal antibodies are usually present in serum and hence serum from naturally EUS affected fish and artificially EUS infected fishes was used by mixing it in the agarose gel.

Student's 't' test was used, $p < 0.05$ was regarded as moderately significant and $p < 0.01$ as significant (Fischer, 1950).

RESULTS

In naturally EUS affected *C. punctatus* as well as after 21 days of challenge infection (Artificially EUS infected) the serum protein level was declined significantly ($p<0.05$) when compared with serum protein level in healthy *C. punctatus*. In *C.magur,* similar observations are made. However, the serum proteins were found to be decreased more (34.14 to 38.75%) when compared with the results in *C. punctatus*. The α_1, α_2, β as well as γ serum globulins were found to be elevated in all the naturally as well as artificially infected *C. punctatus* and *C. magur*. This has resulted into decreased A:G ratio (Table 1).

A:G ratio in naturally EUS affected *C. punctatus* was 1.14 and that in artificially EUS infected fishes was found to be 0.72. These results are highly significant ($p<0.01$). In *C. magur* the A:G ratio was 3.23, 0.94 and 1.49 in healthy, naturally EUS affected and artificially EUS infected fishes respectively.

Serum proteins in healthy and EUS affected *C. punctatus* and *C. magur* are shown in Table 2 and Fig. 1. The serum of healthy *C. punctatus* exhibited 8 proteins bands with R_f values 0.31, 0.36, 0.39, 0.48, 0.54, 0.59, 0.67, and 0.72. However, the serum of naturally EUS affected fishes showed 8 bands of proteins with R_f values 0.31, 0.36,

Table 1. Serum proteins in control and EUS infected fishes.

Serum proteins	*C. punctatus*			*C. magur*		
	Control	Naturally affected	Artificially infected (21 days)	Control	Naturally affected	Artificially infected (21 days)
Total Protein (mg/100g)	5.28±0.15	4.08*±0.08 (-22.73)	3.98*±0.22 (-24.63)	4.98±0.09	3.28**±0.11 (-34.14)	3.05**±0.08 (-38.75)
Albumin	74.64%	53.43%	42.09%	76.37%	50.97%	59.84%
α_1	8.98%	15.96%	9.31%	11.48%	19.97%	10.18%
α_2	5.55%	8.26%	14.37%	4.10%	9.15%	14.30%
β	5.54%	12.67%	17.78%	4.36%	8.36%	10.55%
γ	5.26%	11.66%	16.43%	3.67%	1.53%	5.10%
Globulin	25.33%	46.55%	57.89%	23.61%	48.01%	40.13%
A:G	2.94	1.14** (-61.23)	0.72** (-75.52)	3.23	0.94** (-70.9)	1.49** (-53.87)

Values are mean of 6 replicates ± SE; Percent change over control given in parenthesis; * P< 0.05 , ** P<0.01

Table 2. Electrophoretic seperation of proteins from healthy and EUS affected fishes.

R_f values of protein markers	Molecular weights of protein markers (KD)	Serum protein values			
		C. punctatus		*C. magur*	
		R_f Control	R_f Infected	R_f Control	R_f Infected
0.31	66	0.31	0.31	0.48	0.36
0.36	43	0.36	0.36	0.54	0.48
0.48	29	0.39	0.48	0.70	0.64
0.54	14	0.48	0.54	0.85	0.74
		0.54	0.59	0.82	0.85
		0.59	0.61	0.90	0.96
		0.67	0.67	0.93	
		0.72	0.72		

0.48, 0.54, 0.59, 0.61, 0.67 and 0.72. Thus one new band of R_f value 0.61 was seen with absence of band with R_f value 0.39.

Similarly serum of healthy and naturally EUS affected *C. magur* was analysed for different proteins (Table 2 and Fig.1). Serum of healthy *C. magur* exhibited 7 bands on the gel with R_f values 0.48, 0.54, 0.70, 0.82, 0.85, 0.90 and 0.93. Serum of naturally EUS affected fish showed presence of 6 different proteins and R_f values 0.36, 0.48, 0.64, 0.74, 0.85 and 0.96. Standard Protein mixture with known molecular weights were also run simultaneously with R_f values, 0.31, 0.36, 0.48 and 0.54.

2 µl of extracts in fish saline from the EUS affected tissue of *C.punctatus* (7,14 and 21 days post injection and naturally EUS affected fish) were poured separately in 4 wells already prepared in agrose gel having serum from naturally EUS affected fish and after 24 hours antigen-antibody reactions were visible as rings of different diameters. The well in which extract of EUS affected tissue (7 day post injection) was poured, showed antibody - antigen reaction with a diameter of 7 mm. The results are shown in Table 3. The antibodies developed in naturally affected fishes were almost equal to the antibodies developed in artificially EUS infected fishes after 21 days of post-injection.

DISCUSSION

Serum total protein level in EUS affected fishes was declined significantly with decreased A : G ratio. Antigen-antibody reactions in EUS infected fishes were confirmed by radial immunodiffusion test and it was found to be positively exponential. These EUS affected fishes showed immune response by developing corresponding antibodies. However, it appears that they compromised the immune status during EUS. Leibmann and Reidmuller (1960) and Reidmuller (1965) reported decreased quantities of serum proteins as well as albumin-globulin ratio in infected fishes. It was suggested that toxin produced by the pathogenic bacteria interfered with protein metabolism.

Ecological inputs on immune parameters of organisms can be manifold. Variable environmental conditions like temperature, availability of nutrient resources, and genetic diversity of pathogens favor variable immune traits, as this will increase the chance to survive and reproduce in this environment. For instance, ambient temperature modulate intensity as well as nature of fish immune response to pathogens (Koellner and Kotterba, 2002; Xu *et al.*, 2011).

Exposure to *Aphanomyces invadans* spores is a key factor causing EUS (Baldock *et al.*, 2005) and once a pathogen is in area, subsequent outbreaks of infectious diseases in fishes are closely linked to environmental conditions, particularly temperature and other water quality variables through their effects on stress and the immune system (Bly and Clem, 1992; Wedemeyer, 1996; Hrubec *et al.*, 1996). Richards and Pickering (1979) also reported severe hypoproteinaemia in *Saprolegnia* infection of brown trout from spawing streams. This loss of protein may be associated with loss of protein from the extra vascular fluid at sites of fungal damage (Hargens *et al.*, 1974) and may explain the rapidity with which the fungal-infected fishes die. Oomycete infections cause significant problems in aquaculture and have been implicated in the decline of some wild fish stocks (Van West, 2006).

Thus, it appears that certain environmental factors,

Table 3. Radial immuno-diffusion test for antigen-antibody reaction in EUS affected fishes.

Antigens	Antigen-antibody reaction
Antigens from 7 day post injection fish	7.0 mm
Antigens from 14 day post injection fish	9.5 mm
Antigens from 21 day post injection fish	11 mm
Antigens from Naturally EUS affected fish	10.5mm

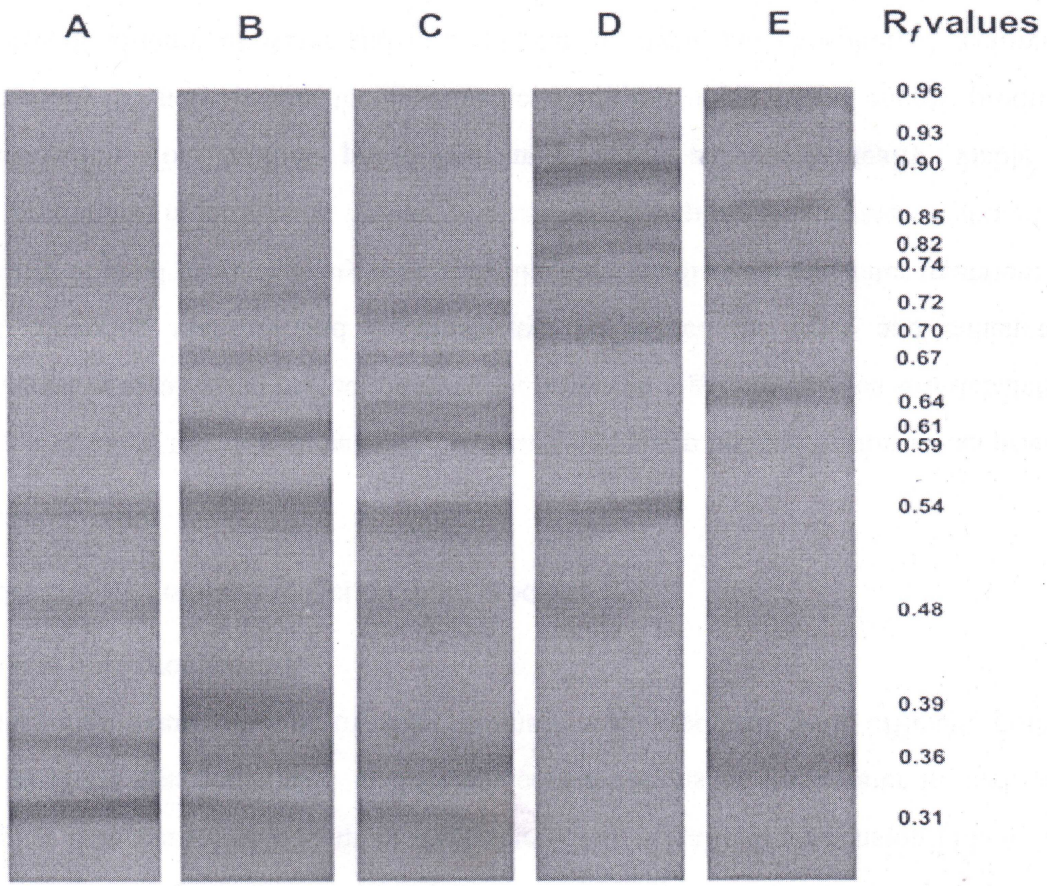

Fig. 1. *Separation of serum proteins from naturally and artificially infected fishes. A: Proteins with known molecular weight, B: Separation of serum proteins of healthy C. punctatus, C: Separation of serum proteins of naturally EUS affected C. punctatus., D: Separation of serum proteins of healthy C. magur, E: Separation of serum proteins of naturally EUS affected C. magur.*

pollutants, bacteria, fungi and viruses are responsible for outbreak of EUS in fishes. The immune response shown by the EUS affected fishes is evident from antigen – antibody reactions and hence there is a possibility of development of resistant strains of such fishes by manipulating their genome. Therefore, the immune-modulation of fish eggs or larvae is proposed as a potential method for improving larval survival by increasing the innate responses until their adaptive immune responses are sufficiently developed to mount an effective response to the pathogens like fungus, bacteria and viruses.

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