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Pathological and Pathobiology of Epizootic Ulcerative Syndrome (Eus) Causing *Aspergillus fumigatus* and its Immunological Response in Freshwater Fish of Channa Striatus

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Abstract

Aspergillus fumigates is an oomycyte fungi most frequently recognized as a causative agents of epizootic ulcerative syndrome (EUS) it is a seasonal and epidemic pathogens of great important in cultured fishes in both freshwater and estuarine environments. EUS is a complex infectious etiology which leads to necrosis ulcerative lesions and granulomatous response in freshwater fishes of Channa striatus. This is the cause of death of approximately 92 species that has been recorded in wild as well as in commercial culture systems worldwide. Different environmental and biological factors are responsible for the growth and establishment of *A. fumigatus*, which is further, attracts secondary pathogens to enter the lesions thus, increasing the severity of the fungal infection. The proper methods for identification of *A. fumigatus*, includes PCR detection and light microscopy. In order to discover new effective treatment to control the disease, a better understanding of the infection process is necessary. The studies on fungal infection in freshwater fish of Channa striatus indicate the immune response pattern in fish against the *A. fumigatus* that serves as an important key for the development of targeted therapeutics and vaccines to prevent the disease and to maintain EUS free aquaculture systems. The immune mechanisms that respond to stimulation, interaction between the immune system of host species and *A. fumigatus*, different factors of *A. fumigatus* and its pathological conditions as well as various approaches of treatment has been discussed.

Keywords: *Aspergillus fumigatus*; EUS; PCR; Granulomatous

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Introduction

The Epizootic ulcerative syndrome (EUS) is an invasive and aggressive disease of both freshwater and estuarine fisheries. Over the past 30 years, there has been a continuous increase in the prevalence of lesions or ulcerative mycosis associated issues. The EUS is known by distinct names in different geographic regions like red spot disease (RSD) in Australia [1] mycotic granulomatosis (MG) in Japan and ulcerative mycosis (UM) in USA [2] have given a detailed report on the association between EUS, RSD, MG and UM. This is confirmed by the aquaculture biologists worldwide, the above mentioned diseases are recognized and commonly known by the term of EUS. The disease was first identified in Japan in 1971 and similar conditions were identified in 1972 in South East Queensland (Australia). Later, these outbreaks had spread widely across the North America and Asia-Pacific region; and caused substantial economic loss to the aquaculture industries in various countries including Thailand, China, Vietnam, Lao PDR, Malaysia, Myanmar, Cambodia, Sri Lanka, Bangladesh, India, Philippines, Hong Kong, Nepal, Bhutan, Singapore and Pakistan. It was believed that a diverse group of organisms and environmental factors were responsible for these diseases since the symptoms differed with each outbreak. Multiple etiologies have been proposed for the

Diseases based on the organisms isolated from the infected fish in which primary cause for the disease was assumed to be from bacteria, viruses, fungus and parasites (Kamilya and Baruah 2014). There is no clear evidence whether the UM and EUS were caused by the similar pathogens. In relation to the EUS, lesions on the fish body may be caused due to multiple reasons which are not in line with the traditional opinion.

Different Factors influence the pathogenicity of *A. fumigatus*

An understanding of the role of environment and its impact on the growth of *A. fumigates* is important to improve the disease management system against EUS infection. EUS outbreaks it is a seasonal occurrence depending on a number of abiotic and biotic factors which are include behavioral changes. These changes influence the extent of fungus infection and lesion induction in an aquatic environment. There are several studies [3] that examined the possibility of an association between EUS outbreaks and the environmental factors that influence the disease.

Temperature

Water temperature is one of the main environmental factors in EUS infection. Generally, EUS outbreaks occur at moderately low temperatures or at the year's cold season. Several studies demonstrated the EUS induction by injecting the *A. fumigatus* zoospores in different fish population like roach Rutilus rutilus (Khan et al. 1998), Snakehead murrel Channa striatus at various temperature ranges between 11 and 310 C. These studies showed that the temperature range of 19 to 23 C produced a higher scale of mortality. Fungal infection proportionally increases the mortality rate of fish which was higher specifically during unstable low temperature. Such outbreaks have been reported in 1988 and 1999 in India and earlier in China and Bangladesh due to lower temperatures [4] Overall reports indicate that the fungus was able to induce the infection at a temperature range of 22–300C, where the highest growth was attained. More tests have demonstrated that they do not grow at 370C or survive above 370C. The low water temperature around 20–220C, and rapid decrease in water temperature were immunosuppressive and induced epidermal changes which led to loss of mucus that makes the fish more susceptible to the infection. Hence, the disease outbreak during the winter causes significant mortalities in many freshwater and brackish water fishes in both wild and culture conditions. This clearly indicates that the oomycete is an opportunistic pathogen that establishes the disease when fishes were under stress and immunosuppressant condition.

PH, dissolved O_2 and other associated factors

The combination of various environmental factors including irrelevant conditions in water pH, alkalinity, ambient temperature, region, etc., they were all associated with EUS outbreaks (Nsonga et al. 2013). Such overlap among these factors provide stressful condition to the fishes, which are finally suppress the host immunity and induces lesions on fishes skin that leads to EUS outbreaks. There was a decrease in pH in the water collected from the EUS infected zone which was around 4.5–6.0; and the water color varied from rusty-brown to reddish-brown. Minerals like iron and aluminum.

Materials and Method

Epizootic Ulcerative Syndrome (EUS)

The Epizootic ulcerative syndrome (EUS) is one of the major diseases affecting both fresh and brackish water fishes. EUS is also known by various names according to its outbreak like Mycotic granulomatosis (MG) and ulcerative mycosis (UM). The spread of this disease affects the source of revenue for fisherman and fish farmers, which threatens to the sustainable food supply for local population majorly depending on fish as an affordable source of animal protein. Till now, EUS had been reported in more than 10 fish species of both fresh and estuarine environment of the Asian continent alone. In India, the incidences of disease in different species were recorded in certain genera of fishes. The highly susceptible species are Channa sps., Puntius sps., Mastocembelus sps., *Anabas* sps., *Clarias* sps., *Mystus* sps., *Glossogobius* sps., and Heteropeneustes sps. It commonly begins as reddening in a small expanse over a single scale, which subsequently spreads to the adjacent scales, finally forming a characteristic ulcerative red spot. Further advancement leads to often scales falling and ulcer becoming deep hemorrhagic due to hyphal growth, which invades the muscular tissue and reaches the internal organs especially the abdominal cavity [5] .The severity of infection, is further increased when they get affected by secondary pathogens. EUS is an epidemic with increased susceptibility of infection in water environment. Spontaneous healing of EUS was observed in a few cases; however, much affected fish dies even at the juvenile stage, it is also vulnerable to young adults. EUS outbreak is a seasonal occurrence which gets manipulated by several biotic and abiotic factors including temperature, pH, salt concentration, rainfall, pesticide, fertilizers, minerals, organic and inorganic components and heavy metals in water [6]. These factors influenced by the availability of motile zoospore and enhanced the ability of spore to attach and germinate over the fishes.

Primary Agent

Multiple infectious agents were initially believed to have caused EUS, including bacteria, fungus, and viral agents caused by Aspergillus sp. in different organisms like skin, gills and liver etc., *A. fumigatus*, various secondary pathogens enter and colonize the lesions and the lesion gradually develops into the ulcer stage. In general, the infection was found when the motile zoospore attached in the fish skin, especially at the injured area. The spore germinates and hyphal growth occurred which invade broadly into the surrounding of the skin and deep into the underlying muscle tissue. Histopathology analysis revealed that the EUS is characterized by penetrating hyphae surrounded by granulomatous inflammation. The EUS infection is commonly in during the winter season, when the water temperature downfalls between 18 and 220C. At such low temperature, the host immunity gets suppressed that favors the growth of oomycete over the fish skin, which results in high mortality. Till date, no report existed where the primary zoospore has been isolated from the water. Further studies are required in this aspect to understand the mechanism of initiation and establishment of

EUS in the host and the virulence factors association in provoking the disease which helps to explore the potential targets against *A. fumigates* from further spreading. [7] Reported the microbial flora present in EUS infected *C. striatus* and identified different bacterial isolates such as *A. hydrophila*, *Flavobacterium* sp in higher numbers followed by other species including *A. salmonicida*, *Staphylococcus* sp., *Yersinia enterocolotica, Vibrio vulnificus* and *Shigella* sp. isolated *Streptococcus faecalis*, *A. hydrophilla, Shigella* sp., *Cellobiosococcus sciuri* and *Micrococcus luteus* from ulcer of EUS infected Cirrhinus mrigala.

Secondary Bacterial Agent

Several types of bacterial species have been isolated from the infected fish from various countries. Most often isolated bacteria are *Aeromonas hydrophila* and *A. sobria*, followed by other bacteria such as Vibrio anguillarum, V. vulnificus, Alteromonas putrefaciens, and Plesiomonas shigelloides. *Aeromonas* species were continually isolated as the main source of secondary infection. Twenty seven percentages of Aeromonas isolates of EUS infected fish collected from the Indo-Pakistan region are A. veroniibiovar sobria. Unique characteristic nature of this clonal group expressed certain virulent factor which is able to agglutinate the fish erythrocyte and cause of EUS infection [8].

Secondary viral agents

In all cases, a viral particle was routinely visualized during the EUS outbreak, subsequently identified as rhabdovirus and infected pancreatic necrosis virus in snakehead fish. An experimental study revealed the frequent isolation of rhabdovirus from the infected fish and its capability of inducing dermal lesions in cell culture. Challenging the snakeheads (Channa striatus) with rhabdovirus by intramuscular injection induced small hemorrhagic lesions at 20–250C but not above 280 C. Ranavirus is another group of virus belonging to iridoviridae family which were a long suspect of EUS causative due to the repeated isolation from aquatic vertebrate at various taxonomical level, including largemouth bass Micropterus salmoides ornate burrowing frog Limmodynastes ornatus, leopard tortoise Geochelone pardalis pardalis, green python Chondropython viridis and perch Perca fluviatilis [9]. Largemouth bass virus (LMBV), a type of ranavirus was first isolated and identified in Florida and later in several other locations of United States reported the presence of ranavirus by isolation, propagation and characterization also they confirmed the ability to form an ulcerative lesion in largemouth bass.

Host Response against *A. fumigatus*

The immune system is a complex network of cells, tissue, and organs that simultaneously communicate and work together to protect the host against the infectious agents. The system has the ability to distinguish self and non-self-molecules and responds to an antigen with significant specificity. The fish immune system mainly relies on innate immunity that is responsible for the recognition of foreign molecules and triggers an immune response by activating humoral factors as well as immune cells which further activates the specific adaptive immune system that together eliminates the invading pathogens.

Results and Discussion

Pathobiology of *A. fumigatus*

The presence of *A. fumigatus* in Epizootic Ulcerative Syndrome infected fish was detected and described by many studies, few considered the mechanism by which they reach the fish and destroy or surpass immune responses. A major study in India found that the pathology of the EUS in different species was remarkably similar. Initial clinical symptoms are generally small skin lesions in pinhead size, with very few noticeable disturbances. The Histopathology examination at this point indicates that necrosis and inflammation are associated with hyphae. The lesions then increase in size and generally granulomata develop around the hyphae, while lesions have a diameter of 2–3 cm. If unchecked, the lesions continue to expand in width and depth and expose the skeletons as necrotic inflammation and granulation destroying several muscle tissues [10]. The end cause of death is not clear, but blood hemoglobin and serum protein levels decrease dramatically during infection (Cruz-Lacierda and Shariff 1994). This indicates that the risk of fatal serodilution can occur via open lesions. Opportunistic bacterial infections often quickly develop in open lesions that can be the ultimate cause of death. The various diversity of oomycete saprophytes can infect the damaged tissues but are not the key primary pathogen. It implies that *A. fumigatus* express strong virulence factors; however, very little has been done to determine what they are hem agglutinating or their hemolytic properties. Fish may react in a similar manner to higher vertebrates by generating antibodies for these pathogens. Many studies investigate the reaction of fish to hyphal pathogens and antibody prodution on response to *A. fumigatus*. The stretch snake and rainbow trout from an endemic EUS area produced antibodies in respond to *A. fumigatus* as demonstrated by Both the species have developed unique antibodies against *A. fumigatus* and the antibody response provided defense in either case was not identified. In few experiments, the EUS-resistant species were focused in order to assess the reason for their resistance. The progressive histopathological study was noticed [11] carried out a similar study and discovered that tilapia responded which was observed by histopathology, although it showed granulation **(Figure 1- 3).**

Hematological changes

A. fumigatus infection in fish is characterized by hemorrhagic lesions which produce a granulomatous response. Experimental infection in fish gives a great insight into understanding the EUS infection and their morphological and behavioral changes at a specific time. During *A. fumigatus* infection, the hematological parameters such as Red Blood Cells (RBC), Hemoglobin (HB), Packed Cell Volume (PCV) was found to decrease compared to the healthy fish. The fungal infection induced extravasation of blood and reduction of haemosynthesis which in turn fails the hematopoietic tissue to release the blood cells (Malathi et al. 2012). Infected fish shows highly elevated level of White blood cells (WBC) and Mean Corpuscular Hemoglobin Concentration (MCHC) count. Hence, the increase in MCHC is due to macrocytic anemia. Similarly, Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Volume (MCV) were found to decrease in

Figure 1 Microbial images of Aspergillus Fumigatus.

Figure 2 Red spotted and infected Channa striatus.

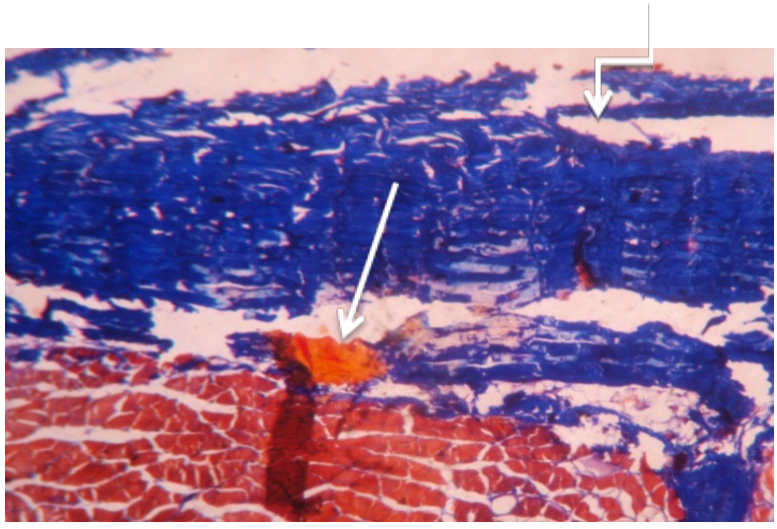

Figure 3 Histopathological distrusted skin.

the infected fishes over the control that indicates hypochromic anemia. A noticeable increase in eosinophil and lymphocytes was observed in EUS infected fishes [12] **(Table 1).**

Innate Immune Response

Initially, after the pathogen entry, various pattern recognition receptors (PRRs) such as lectins, dectins, and TLRs get activated and they further activate the downstream molecules including chemokines and other cytokines and signaling molecules which ends up in the activation of different immunological counters against *A. fumigates*. Oxidative stress plays a major role in fish pathogenicity and in disease progression. Vertebrate carries several antioxidant enzymes that play an important role in the elimination of excess reactive oxygen species (ROS) from the cell in order to protect the host from its toxic effects. The role of various antioxidant molecules such as superoxide dismutase and thioredoxin during *A. fumigates* infection has been reported in *C. striatus*. Antimicrobial proteins (AMP) are proposed to act as an immune defense by exerting broad spectrum microbicidal activity against the invading pathogenic microorganism. The involvement of various lectins and other antimicrobial proteins such as lysozymes have been reported during *A. fumigatus* infection which was directly involves in the cleavage of the membrane as well as act as signaling molecules in activating further downstream activities. Proteases and protease inhibitors are found to be potentially involved in defense mechanism against fungal pathogens. The activity of different cathepsins and other proteases in *C. striatus* has also been described that when the fish infected with fungus it showed an elevated level of cathepsin L in spleen and liver. This indicates that both hematopoietic and lymphoid organs are interlinked in producing specific immune response. The role of Kazal type serine protease inhibitor has also been reported against *A. fumigates* infection which indicates the involvement of inhibitory activity against fungal proteases. Apoptosis plays an important role in multicellular organism in removing the infected cells by activation of several apoptotic factors. During fungal infection, host cells get infected and the apoptotic process is initiated to avoid further development of diseases. Correspondingly, during *A. fumigatus* infections, key apoptotic factors such as caspase (Kumaresan, Ravichandran et al. 2016) and tumor necrosis factor and their receptors get activated and protect the normal cells from further infection.

Treatment strategies and perspectives

EUS infection has been reportedly transmitting horizontally from one fish to another with an alarming rate by developing repulsive lesions in susceptible fish. Once, after the outbreak in pond/lake, the movement of live EUS infected Channa punctatus carries the pathogen wherever the water flows. Flooding also causes the spread of EUS among inland fishes that are reported in several Asian countries. There is currently no effective EUS therapy available. However, there are certain useful preventive measures adopted to control the spreading of EUS. The key preventive measure is to ensure that the infected river water should not come in contact with the cultured fish farms. The most effective way to control the EUS is to follow the regulation of quarantine and health certification guidelines for the movement of live fish between the countries which prevent the disease from entering a particular country. Regular monitoring of cultured fish is important to identify the fish health and to prevent infection by parasitic skin pathogens due to the physio-chemical imbalance of the environment. In case of susceptible species like *C. striatus*, Catla catla, Labeo rohita, etc., they should be farmed in an endemic area with healthy husbandry, an absolute essential. Precautionary steps include selection of clean water source which should be Oomycete-free and obtaining seed stock from pathogen-free certified hatchery. Pond studies in Bangladesh suggested that drying out and liming of ponds once after the occurrence of EUS is a good practice to decrease the severity and further spreading of EUS. Quite a lot of studies have reported the usage of malachite green with other disinfectants like hydrogen peroxide and potassium permanganate ranging between 1 ppm and 10 ppm for the treatment of EUS as partially effective in preventing the induction and as a curative to initial ulcer sores (Das and Das 1993). Attempt in using turmeric at high concentration (5000 ppm) along with lime, neem leaves and seeds provide some encouraging results in controlling EUS and curing up ulcerated fishes. However, molecular level studies on the pathogen is required to address the exact virulence nature of the organism to control its growth, propagation and virulence by targeted therapeutic approach. As described earlier, there is no adequate treatment for EUS. Currently, most of the measures

Table 1. Haematological parameters of control and infected Channa striatus.

S.No	Blood Parameters	Control Fish	Infected Fish	Percentage change (%)	P values
1	HB (g/dl)	10.1±0.33	9.1±0.29	-90.9	0.0373
2	Haematocrit (PCV) %	32.7±0.68	28.3±0.47	-71.7	0.0001
3	MCV (M.gms)	56.7±0.94	65.7±1.32	-34.3	0.0001
4	MCH (M.gms)	21.4±0.61	18.9±0.31	-81.1	0.0015
5	MCHC (M.gms)	36.539±0.925	33.159±0.174	-66.841	0.0021
6	RBCs ($10^{6/ml}$)	4.2±0.27	1.89±0.13	-98.11	0.0001
7	WBCs ($10^{3/ml}$)	33505±674.13	39000±756.450	38900	0.0001
8	Lymphocytes %	58.2±0.341	61±0.614	-39	0.0009
9	Neutrophils %	34.8±0.64	29.3±1.67	-70.7	0.0059
10	Monocytes %	5.8±0.37	4.4±0.74	-95.6	0.0057
11	Eosinophils %	2.6±0.37	4.1±0.38	-95.9	0.0124
12	Basophils %	2.2±0.28	2.8±0.38	-97.2	NS

Results were statistically analyzed by student't'- test and values presented in the table are Mean± SD.

taken to control the infection are ineffective and have some disadvantages. Malachite green, which is used for the treatment against *A. fumigates* are considered as hazardous to humankind. Liming of the pond that shows some fair outcomes in controlling the infection in agricultural field fails to show such effectiveness at in vitro condition. Potassium permanganate and hydrogen peroxide at recommended concentrations (1-10 ppm) do not demonstrate any activity against fungal sporulation. Chitosan is partially considered for its inhibitory effect, though it is costlier. Hence, only low concentrations of commercially available fungicides can be used with acceptable LC50 against aquatic animals. But the irony is that there is no such drug available which could be effective in controlling EUS in such acceptable concentration. There is no available protective vaccine for use. Nevertheless, snakehead fish immunized with a crude extract from the *A. fumigatus* reacted with humor immune response as identified through the western blot analysis and Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Diagnosis

Wet mounts, smears, Electron microscopy or cytopathology are not appropriate for the diagnosis of *A. fumigates* infection. For any effort to regulate or control it, unambiguous EUS identification is crucial. Histopathology, oomycete isolation or amplification of the polymerase chain reaction can be accomplished for clinically affected fish diagnosis of infection with *A. fumigates*. The Aquatic Animal Health Research Institute (AAHRI), Thailand, has been appointed by the International des Epizooties (OIE) as the EUS reference laboratory. EUS case definition can only be evaluated histopathologically and attempts to define EUS on the bases of clinical indications created a great deal of confusion. A EUS outbreak in South Vietnam was outlined in 1973, six years before any other report in Asia, but there has been no histopathology, so it is uncertain whether the disease was effectively EUS or some other ulcerative infection (Pham 1994). It is possible to obtain a more favorable identification by isolating *A. fumigatus* from infected tissue and their development rate, pathogenicity and sporangia morphology. Isolation has a time-consuming disadvantage and is not always efficient and doesn't always succeed (Lilley et al. 1998). Development of *A. fumigatus* infection may differ among species. Three phases of EUS lesions that are prevalent to most incidents were identified by Viswanath et al. (1997), from hemorrhages of pinhead size with massive lesions that can damage skeletal muscle tissue and its structure. As clinical signs as red spots or slight to massive ulcerative lesions in fish usually develops in the body, the disease's early signs include loss of appetite and the fish are getting darker. Changed in behavior involved the infected fish floatig close to the water surface and becoming hyperactive by irregular pattern of movement in the water surface.

Conclusion

The severity of EUS outbreak and lack of effective methodology becomes the subject of interest in developing a new therapeutic strategy to combat the disease. Fish largely depends on innate immunity, though it is unsuccessful in claiming resistance against the pathogen. During the infection, certain key humoral innate immune molecules are triggered and further they activate various molecular systems including SOD, protease and protease inhibitors, caspase and antimicrobial peptide which are largely expressed effectively in killing the pathogen. These nonspecific immune mechanisms and their associated elements functioned as an immunomodulatory. Therefore, these molecules can be targeted for immune modulation or for the development of therapeutic agents, which can improve the immune response in the fish against EUS-like conditions.

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