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CYTOGENETIC STUDY OF PATHOGENOMICS OF *ASPERGILLUS NIGER* ON THE FRESHWATER HOST FISH *ORIOCHROMIS NILOTICUS*

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ABSTRACT: Infected fresh water Tilapia *Oreochromis niloticus*, were brought alive from Canning fisheries and aquatic resource, Canning, India to parasitology laboratory, Department of Zoology for pathogenic examination. Macroscopic production of infection observed and the specimen was isolated and characterized on the basis of morphological and molecular analysis. The species was identified as *Aspergillus niger*. The effect of this pathogenic fungus on host physiology was observed by monitoring changes in biochemical, histological parameters. Native polyacrylamide gel electrophoresis have been used to analyze malate dehydrogenase (MDH), acid phosphatase (Acph) and peroxidase (Px) isoenzymes in different tissues (liver, kidney, muscle and heart) of the host fish in order to study the tissue specificity of these isoenzymes. The percentage amount of MDH in general varied significantly between muscle and different studied tissues. Peroxidase isoenzyme was recorded in liver and heart only with significant increase in liver. The cumulative mortalities of the intraperitoneal infection increased along with the sporangiospore concentrations; the highest mortality observed was 70% with108 CFU as compared to the control.

Key words: *Oriochromis niloticus*, histopathophysiological study, *Aspergillus niger*, Electron microscopy, Isoenzyme, Nucic acid

INTRODUCTION

The fishes are susceptible to diseases caused by a large number of infectious agents including true fungi, fungal-like microorganisms and other protists. In general, the number of reported fungal and fungal-like pathogens responsible for diseases in animals is on the increase globally (Fisher *et a*l., 2009,2012; Holdich *et al.*, 2009; 2009; Frick *et al.*, 2010; 2010). As such, they are truly emerging diseases with increasing incidence, geographic range, virulence and some of these fungal and fungal-like pathogens have recently been found in new hosts or are newly discovered (Berger *et al.*,

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1998; Brown 2000; Daszak *et al.*, 2000; Kim and Harvell 2004; Blehert *et al.*, 2009; Peeler *et al.*, 2010 and Cameron *et al.*, 2011). These pathogens are known to be opportunistic (Fisher *et al.,* 2012). In aquatic ecosystems these fungal species are presumably infectious through contamination of fish feed. Fungi and fungal-like pathogen detection in fish hosts is more complicated due to lack of direct observation of their hosts. This is particularly true in case of freshwater systems where, some diseases are chronic with no clear external symptoms (Gozlan *et al.*, 2005; Kocan and Hershberger, 2006 and Andreou *et al.*, 2011). The mortality rate of incubated eggs, due to such infection may raise upto 100%. Fish parasitic fungi generally belong to the phylum Eumycota. The subphylum mastigomycotina, zygomycotina and fungi imperfecti. The commonly encountered genera of this subphylum which are pathogenenic to fish are saprolegniasis, aspergilllosis and zygomycosis.

This study focuses on the current knowledge gaps on fungal microbes causing fish disease, their phathogencity along with the current methods of detection and a global ecological understanding of their impacts on fish host populations. During the period of the study pathogenic strain of fungus belonging to the phylum Ascomycota causing *Aspergillosis* has been isolated and identified from the common edible fish *Oriochromis niloticus* which is the most important and extensively cultured fish in West Bengal, India. Aspergillomycosis is principally a disease of tilapia *Oreochromis* sp. (Olufemi *et al.,* 1983). In this study investigations have been made to characterize the tissue-and organ-specific isoenzyme patterns (Fisher and Whitt, 1978; Leslie and Pontier, 1980; Berg and Buth, 1984; Holt and Leibel, 1987; Basaglia, 1991; Xia, *et al* 1992) among which little were concerned with fishes that eventually leaves a scope to see through the gap more intensely. Moreover, immune-cytotoxicity and cell pathogen interaction study was also done along with the study of genotoxic effects on fish DNA, RNA and proteins to understand the extent of damage caused by the pathogen in. Furthermore, histology-based monitoring by ultra structures of vital immunological organs to detect tissue damage and evaluation of blood biochemistry parameters were also performed as this simple approach can provide essential information on the physiological status of the host.

MATERIAL AND METHODS

Collection of fishes and sampling techniques: Fishes were collected weekly at regular interval. The infected fishes were identified by appearance of red spots on their body along with excess mucus secretion, damaged and infected gills and sluggishness. The diseased fish were brought to the laboratory and kept in medium aquaria (24x9x9) inches for further clinical examinations along with the isolation and morpho-taxonomy of the causative pathogen.

Methods for isolation and culture of fungal strains: Total length (TL) and body weight (BW) of each specimen fish was measured and health status was observed. The fish samples were disinfected by dipping the fish in 1% formaldehyde for 1 to 5 minutes to prevent secondary contamination by airborne spore. Samples were further transferred to 70% alcohol and finally several washings were given in sterilized distilled water by rinsing thoroughly. The fungi were then isolated from the infected fish organs with sterile needle and inoculated on Potato dextrose agar (PDA) (Hymedia). The process was performed in Laminar flow air cabinet to avoid any contamination. Further the agar plates were incubated at 28-30°C and fungal growth was observed after 4-7 days. The pathogenic colonies were isolated and microscopic analysis was done for the purpose of identification. The identified strain was maintained, preserved and stored in pure culture form for further study.

Phenotypic characterization using Light and electron microscopic study: Light microscopy was performed following the methodology of (Ke *et al,* 2009, Ghosh and Bandyo Padhya 2014**)**. Lactophenol cotton blue solution is a mounting medium and staining agent used in the preparation of slides for microscopic examination of the pathogen. Fungal elements are stained intensely blue.

SEM and TEM analysis: Scanning electron microscopy was performed (Afolayan et.al 1995) using ZEISS (CRNN, CU) Scanning Electron Microscope at accelerating voltages of 10 KV. Transmission electron microscopy was done following the protocol of AIIMS New Delhi (Electron Microscopy Department) with slight modifications (Robert *et al*, 1979)

Preparation of fungal inoculums: Inocula were prepared from the cultured strain of the isolated fungus on potato dextrose agar (PDA) slants for seven days at 37-55° C to obtain sufficient sporulation. Viability was determined by plating 10-fold dilutions prepared in 0.68% NaCl with 0.05% Tween 80. Plates were incubated at 37 ^{0}C, and CFU (Colony- forming unit) were counted after 18 hours. The OD of the solution was adjusted to 0.5 at 456nm which corresponded to 1×10^{7}CFU. Finally the spores were harvested by washing the agar surface with sterile 0.68% NaCl containing 0.05% Tween 80. Filtered suspensions of spores adjusted to the desired concentration for further set of experiments (LD50 challenge and infectivity experiment).

Determination of LD_{50} dose: For the LD50 challenge a total of 120 fish was taken. The fishes were divided into two major groups' viz. control group (20fish) that received no dose of fungal suspension and experimental group (100 fish) that received the fungal doses. The experimental group was divided into five subgroups to receive the five different doses which were prepared by serial dilution of the filtered fungal suspension. The five desired doses were of 10^{4} CFU/ml, 10^{5} CFU/ml, 10^{6} CFU/ml, 10^{7} CFU/ml and 10^{8} CFU/ml and were

given to the fishes by intraperitoneal injection. Each fish received 200µl of each of the dose mentioned above. Mortality in each group was recorded at every 12 hours daily and dead fishes were removed immediately to prevent contamination. The cumulative mortality for each dose, time of first death has also been recorded. All the data were analyzed using the Statistical Package for Social Sciences (SPSS) software Vs. 16 in a PC-compatible computer and the confidence level was 95%.

Infectivity Experiment: Infectivity experiment was conducted using healthy fishes obtained from pond (Diara Fish pond and hatchery (22°28′31″N 88°09′54″E) near railway station is a Kolkata Suburban Railway station on the Sheoraphuli-Tarakeswar branch line of Howrah railway division of the Eastern Railway zone) with no symptoms of being diseased. The body weight of the collected fishes ranges from 50-70gm with length ranging from17-22cm. prior to the experiment the fishes were disinfected with potassium permanganate (20 mg/ml) and were kept in glass aquaria with aeration, and water was maintained at a daily exchange rate of 5–10%. Water temperature was maintained at 22 – 26°C. All fishes were fed with artificial diet meal (ALG-OXY95) twice a day. The fishes were divided into two groups' viz. control group and infected group. Each group contained 10 fishes. The control group received no fungal suspension but 200 µL of sterile 0.68% NaCl containing 0.05% Tween 80 solution whereas the infected group received 200 µL of the 1/4th of the LD_{50} (0.685 CFU/ml) of the fungal suspension. The infectivity experiment was scheduled for 20 days and the fishes were then sacrificed to perform the assays to find out the pathogenic effect induced by the fungus.

In vivo Pathophysiological study via Light and Transmission electron microscopic study of tissue: Light microscopy was performed on liver, gills and muscle tissues. Oragans were aseptically dissected out from both normal and infected fishes. The tissues were fixed in 10% formaldehyde and then processed in the series of alcohol gradation For transmission electron microscopy study,the tissues were further fixed for transmission electron microscopy for ultrastructural study of the cells of the tissues. The samples were dehydrated through an acetone or ethanol series, passed through a "transition solvent" such as propylene oxide and then infiltrated and embedded in a liquid resin such as epoxy and LR White resin. After embedding the resin block was then thin sectioned by a process known as ultramicrotomy, sections of 50 - 70 nm thickness and collected on metal mesh 'grids' and stained with electron dense stains before observation in the TEM.

Biochemical analysis of blood: Blood samples were collected from caudal vein by using a sterile plastic syringe (2.5 mL) and immediately transferred into assay tubes, one containing EDTA (1.26 mg/ 0.6 mL) as an anticoagulant agent

and the other without EDTA. The samples without anticoagulant were centrifuged at 2000 r. p. m. (REMI C 24 Model, India) to collect the serum and stored at 4°C prior to analysis. Biochemical tests were performed for determination of serum Glucose, Cholesterol, Albumin, Aspartate aminotransferase (AST, E.C.2.6.1.1), Alanine aminotransferase (ALT, E.C.2.6.1.2) and Alkaline phosphatase (ALP, E.C.3.1.2.3.1) and total Protein. Total protein concentration in serum was analyzed (Dumas *et al*, 1971).Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was also determined along with Alkaline phosphatase(ALP) (Reitman and Frankel ,1957 Kind and King ,1954). Albumin was determined by bromocresol green method (Gustafsso,1976) . The level of Serum cholesterol and glucose was also estimated (Tietz *et al,* 1986, Mendel *et al*, 1954)

Isoenzymatic study of tissue: (Stegemann *et al.*,1985). Target organs like gill, heart, liver, spleen, kidney and muscle and were collected from the fish of both control and infected group. For sample preparation 100mg of tissue from each organ (gill, heart, liver, spleen, kidney and muscle) were minced in 500µl (1:5) of 0.1M Tris-HCl buffer (pH 7.4) and the lysate was centrifuged at 14000 rpm for 40 minutes at 4ºC and supernatant was preserved for enzyme analysis. Antioxidant enzymatic study for LDH, MDH and Peroxidase was performed. Native PAGE (10% native polyacrylamide gel) was performed for the qualitative study of the isoenzymes.

DNA, RNA and Protein profiling of tissue Isolation of DNA: 500 mg of tissue (gill, heart, liver, spleen, kidney and muscle) was taken and minced in lysis buffer and then DNA was isolated following the Phenol-chloroform method (Barnett *et al.*, 2012). The final pellet obtained was dried and dissolved in 100µl of water for the quantification of DNA.

Isolation of RNA: 500mg of fish tissue (gill, heart, liver, spleen, kidney and muscle) was homogenised on ice by adding Trizol reagent to it and RNA was isolated following the Trizol method. The final pellet obtained was washed in 75% ethanol and dissolved in RNAase free water and stored at -50ºC for further quantification.

Quantification of DNA and RNA: The quantification of DNA and RNA was done by UV spectrophotometric analysis (Aquamate, thermo scientific). The quantity of DNA and RNA was measured by obtaining the absorbance reading at 260 nm and 280 nm respectively.

Isolation of protein; 500 mg fish organs (gill, heart, liver, kidney, muscle and spleen) were thawed in urea-thiourea buffer (7 M urea, 2 M thiourea, 4% CHAPS, 45 mM Tris, 60 mM DTT and protease inhibitor- PMSF). Thawed samples were vortexed and kept at 4°C for 30 min. Mechanically disrupted and kept on ice. Samples were adjusted to 900 µl of lysis buffer (20 mM Tris, 100

mM NaCl, 1% Triton and Protease inhibitor - PMSF) and incubated for 15 min at 35°C. Reincubated in ice for 10 min. 100 µl of lysis buffer was added and incubated for 10 min along with DNAse I. The samples were centrifuged at 12000 rpm for 15 min at 4°C (middle aq phase bears protein). Extended delipidation was accompanied by tri-n-butylphophate-acetone-methanol precipitation. Precipitated proteins were estimated subjected to SDS-PAGE.

Characterization of protein: Characterization of protein was done by SDS-PAGE. 10% ploy acrylamide gel was casted and staining was processed using Coomasie Brilliant Blue. The gel was stained by incubating it overnight in staining solution bearing the dye followed by destaining. Finally the gel was photographed and bands were analyzed.

Protein Estimation: Protein concentration was calculated by Lowry's method (Lowry, *at al.*, 1951). Absorbance was measured at 750 nm by UV-VIS Elico spectrophotometer

RESULTS AND DISCUSSION

Identification by morphometric analysis: On Potatao dextrose agar media with a temperature growth range(20-27°C to 35-55°C) the surface colonies of the cultured strain found to consist of a compact white fungal colonies covered by a dense layer of dark-brown to black conidial heads. (Fig.1).

When studied under light microscopy the appearance of the strain shows the conidiophores with swollen end of hyphae. From hyphae numerous sterigmata radiates and ends in a short chains of conidia (Fig 2). Conidia are dark brown to black in colour, rough-walled, pigmented, one-celled, globose to subglobose structure having diameter ranges from 3.5-5.0 µm; conidial heads are large, globose, dark brown, radiated. Conidiophores are smooth-walled and become dark in colour towards the vesicle.

Scanning electron microscopy revealed two types of growth morphology; one without any support containing medium commonly known as pelleted section and another with solid support medium commonly called biofilm. Fungal pellets are a core of densely packed hairy regions containing radially growing hyphae containing conidia. Conidial heads were globose and later radiate into several conidial columns; measuring 3.0284 ×3.5003µm in diameter. The walls of the conidial heads were thick, smooth, pale brown and covers the entire surface of the vesicle, measuring vesicles 30–45 µm wide, phialides flask-shaped, 7–963–4 mm; conidia sub-globose, 3.5–4.8 mm in diameter (Fig 3).

LD_{50} challenge: LD_{50} challenge was performed with the fungal inoculation of three different concentrations of $2x10^3$, $4x10^6$ and $8x10^8$ conidia/ml CFU of sporangiospore suspension. Inoculation was injected intraperitoneally.

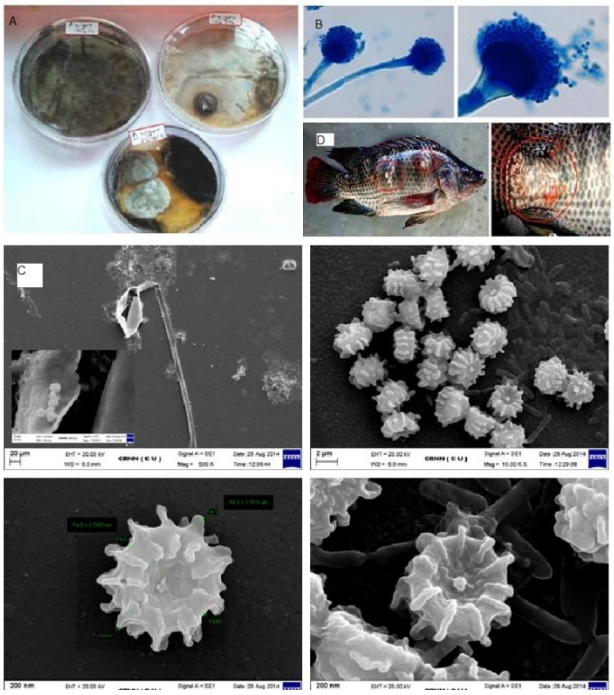

Fig. 1(A): Phtomicrograph showing morphology of *Aspergillus niger* at conidial stage in PDA (Hymedia) plate; 1(B) Lactophenol cotton blue mounted light microscopic appearance of the conidiophores of *Aspergillus niger*; 1(C) Phtomicrograph showing Scanning electron microscopic structure of *Aspergillus niger*; 1(D) Clinical symptoms in *Oriochromisniloticus*

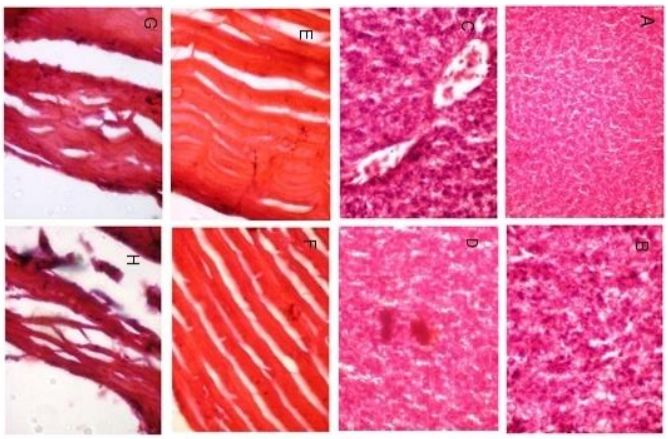

Fig. 2 Histological sections; (A-C) Normal liver; (B) infected liver with the presence of melanomacrophagic centers ;(D) infected liver with eosinophilic granules (E-F) normal muscle; (G-H) infected muscle with irregular striations.

Mortalities have been recorded during the experimental infections. The mortality was found to ascend from 30% to 40% and with highest mortality (70%) within the given time of exposure (Table-1). The $8x10^8$ cfu/ml was found to be the lethal

dose. 50% mortality was observed at the given dose of $4x10^6$ cfu/ml and $2x10^3$ cfu/ml of isolate did not give any significant variation. All the host fish were subjected for fungal isolation.

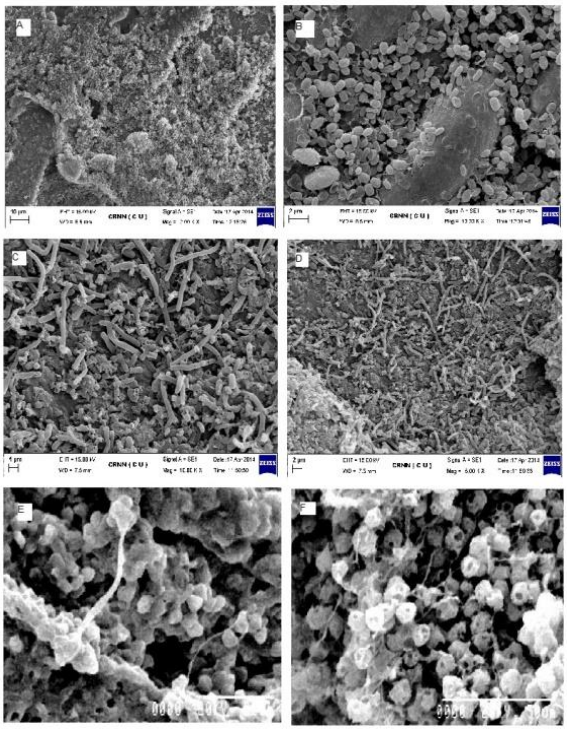

Fig. 3 (A-F) Scanning Electron Micrograph of histological section; (A and B) Scale; (C and D) Skin; (E and F) liver

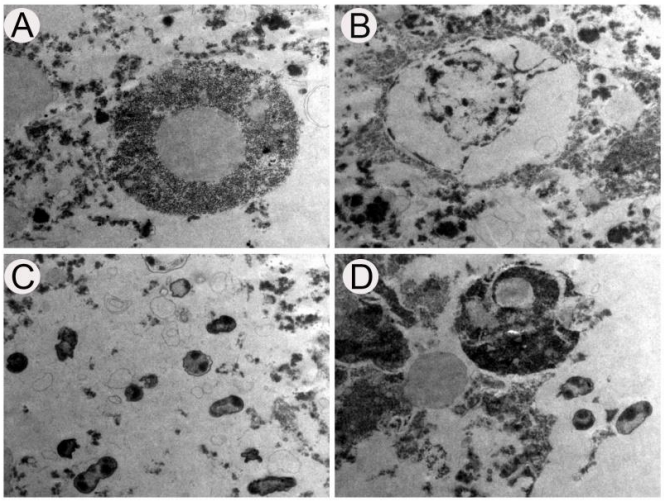

Fig. 4 (A-D) Transmission Electron Micrograph of cross section of hepatocyte cells of infected liver of host fish.

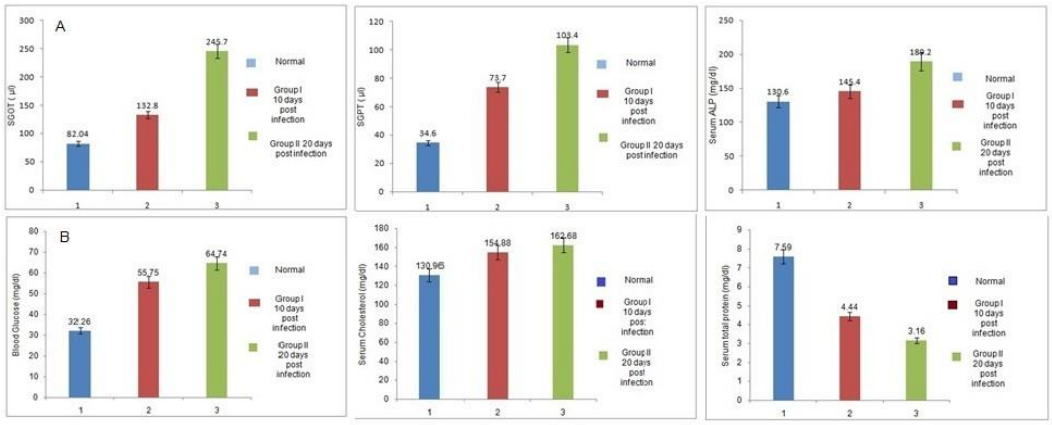

Fig. 5 (A): Different Enzyme concentrations (mean ± SD) namely GOT, GPT, ALP concentration of serum in host fish infected with *Aspergillus niger*; 5 (B) Different concentrations of serum glucose, cholesterol and total protein (mean ± SD) in host fish infected with *Aspergillus niger*

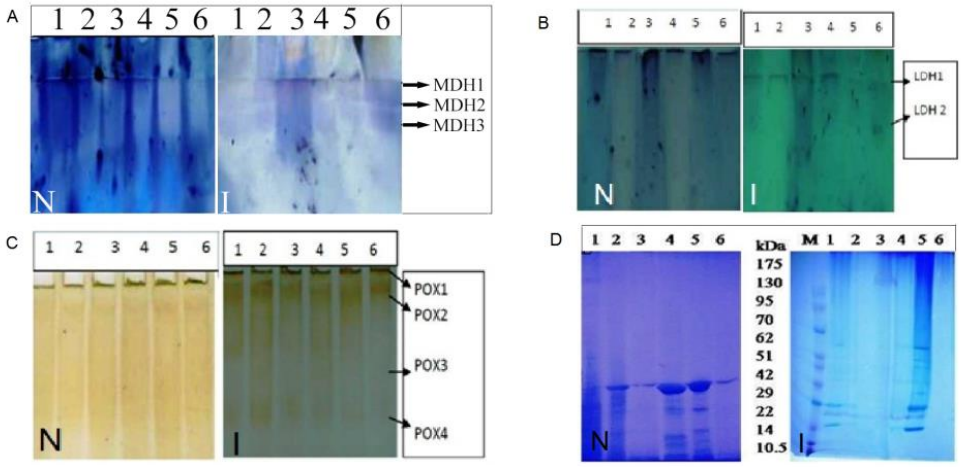

Fig. 6 Comparative study of isoenzymes of control and infected group of fish (A) MDH control; Lane 1-6 (1 liver, 2 Kidney, 3 spleen, 4 muscle, 5 heart, 6 gill ; MDH infected; Lane 1-6 (1 gill, 2 muscle, 3 kidney, 4 heart, 5 spleen,6 liver); (B) LDH control; Lane 1-6 (1 gill 2 heart, 3 liver, 4 muscle, 5 kidney, 6 spleen); LDH infected; Lane 1-6 (1 gill, 2 muscle, 3 liver, 4 kidney 5 heart,6 spleen); (C) Peroxidase control; Lane 1-6 (1 gill 2 heart, 3 liver, 4 muscle, 5 kidney, 6 spleen); Peroxidase infected; Lane 1-6 (1 gill, 2 liver, 3 heart, 4 muscle 5 kidney,6 spleen),6 (D) Protein profiling infected; Lane 1-6 (1 gill, 2 heart 3 liver, 4 muscle 5 kidney,6 spleen)

Clinical pathophysiology Clinical symptoms in host fish: After 20 days of post infectivity experiment, the main clinical symptoms observed were conspicuous fungal colonies on the skin, fins, scales and localized areas of the body surface (Fig.4).The fungal growth appeared as white thin threads resembling a tuft of cottony white patches on the body. The colour frequently changed to dark by accumulation of debris and the surface of the skin shows impressions of deep

lesions. The infected fish shows dissymmetrical movement with some changes in swimming pattern. The percent mortality of re-infected fish was found to increase with the increasing concentration of doses and days of treatment.

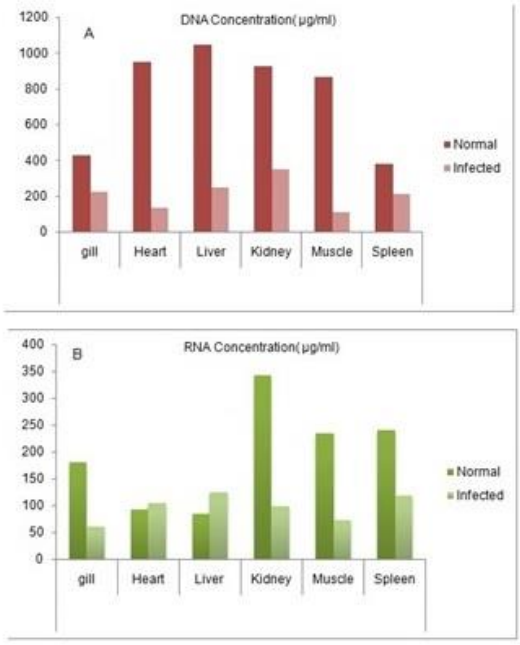

Fig. 7 (A) DNA Concentration in different tissues of host fish inoculated with *A. niger;* (B); RNA Concentration in different tissues of host fish inoculated with *A. niger*; (C) Protein profiling control; Lane 1-6 (1 muscle 2 gill, 3 heart, 4 liver, 5 kidney, 6 spleen)

Histological findings Light microscopic analysis: The main alterations found in the liver were irregular-shaped nuclei, nuclear hypertrophy and the presence of eosinophilic granules in the cytoplasm. melanomacrophages identified as rounded aggregates of cells containing dark-yellowish granules of various sizes, close to the vessels. (Fig. 5 a-d). Muscle fibers lack longitudinal pattern and compactness (Fig. 5 e-h).

Scanning Electron Microscopic analysis: The study showed depression on scales containing fungal hyphae and fungal spore (Fig. 6 a-b). Skins infected by hyphal penetration shows disintrigation of epidermal covering (fig 6 c-d). Hemorrhage observed in liver cells by increase in number of RBC cells in liver tissue (Fig.6 e-f). Many nonseptate, broad and branched hyphae observed in ulcer granulated tissue of the liver near the penetrating hyphae.

Transmission Electron Microscopic Study: Transmission electron micrograph of liver showed markedly vacuolated hepatocytes. Some were mildly degenerated, while others exhibited clumping of nucleoprotein at the periphery of the cytoplasm and central large vacuolation (Fig.7 a-b). Large vacuoles, containing fine-granular material with low electron density, accumulated in the cytoplasm.

Degeneration of the hepatocytic cell organelles has also been observed (Fig 7 c-d).

Biochemical changes in fish due to Aspergillus niger infection: Vital enzymes namely, GOT, GPT, ALP were also found to increase significantly (paired T- Test, $P < 0.01$) in the infected group. The activity of GOT enzyme in infected group was 380.5.±0.23 (UL^{-1}) whereas in normal fishes the enzyme activity was reported 263.43±0.24 (UL^{-1}). The GPT enzyme concentration was 109.59±0.09 (UL^{-1}) in infected group of fish, where as it was 36.04±0.11 (UL^{-1}) in control group of fish. The activity of ALP enzyme in normal fishes was 135.6±9.03 (UL^{-1}). In infected group, the activity of these the enzymes increased significantly and was observed as 185. .905±8.82 (UL^{-1}) than the control group of fish (Fig. 8 a-c). Blood glucose and total cholesterol concentration of serum in infected group has deviated significantly from the normal level (paired T- Test, $P < 0.01$). The blood glucose level in infected group was observed 34.907±0.95mg/dl which is much lesser than the normal level (79.51±1.38mg/dl). The total cholesterol concentration of serum in infected group was elevated to 188.5928±2.25mg/dl where as in normal fishes it was observed as 1034.25±2.17mg/dl (Fig.9 a and b). The total protein level in the serum of infected fish was found to be much lower in comparison to the control group. The protein concentration of the serum has significantly decreased (paired T- Test, $P < 0.01$) than the normal when exposed to increasing concentration of 2×10^3, 4×10^6 and 8×10^8conidia/ml CFU of sporangiospore suspension. The activity of protein decreased from normal 7.9 to 3.5 mg/dl (Fig. 9 c)

Isoenzymatic study: The isoenzyme malate dehydrogenase in infected sample showed three fractions in the electrophoretic pattern in the host fish (Fig. 10 b). The three fractions (MDH 1, MDH 2 and MDH 3) were recorded in all tissues only with the exception of kidney. The isoforms MDH 2 and MDH 3 were not observed in kidney. All the three isoforms which occurred in different tissues of host fish did not show much variation between heart and liver followed by muscle and gill. Malate dehydrogenase enzyme activity was recorded highest in spleen than any of the rest tissues. MDH isoforms were expressed significantly in spleen followed by kidney. The stress related enzyme shows an overall increase in activity in artificially infected host fish after 20 days as compared to control (Fig. 10 a). Figure 11 (a and b) revealed expression of lactate dehydrogenase with two isoforms LDH 1, LDH 2 and LDH 3 in host fish when exposed to determined dose (0.75×10^5 CFU/ml) of *Aspergillus* niger strains for 20 days. LDH 2 was present in gill, heart, liver, kidney and muscle whereas, LDH 3 was significantly observed in all tissues of the host fish. Only one isomorphic band, LDH 1 was expressed in muscle. Similar result was observed in case of heart and muscle too. The activity was recorded highest in liver

followed by spleen, kidney, gill, and heart and muscle .than any other tissue in control. There was no significant difference between spleen and kidney.

Regarding peroxidase isozyme, six isomorphic bands were recorded in different tissues (Fig. 12 a and b). It expressed as two fractions, Pox 4 only in heart and Pox 5 in liver and kidney. Pox 1 was expressed in all tissue without an exception whereas, isoform Pox 2 was absent in kidney and muscle. Similarly; Pox 3 and Pox 6 were not observed in case of gill and spleen respectively.

Cytotoxic analysis in tissues: DNA damage was observed in liver, muscle and spleen. Decreased concentration of DNA in the host tissues observed with higher doses (Fig-13). Maximum reduction of RNA concentration was found in gill, muscle and kidney (Fig 14).

Protein profiling analysis: The higher level of proteins in muscle tissue in groups of host fishes was seen and highly expressed lower molecular weight protein bands of 10.5,14, 22, 29 and 42 kDa was majorly expressed (Fig. 15 a) in respect to control groups protein fractions of 22 and 29 kDa (Fig 15b).

The present study focuses on the isolation, identification and morphometric analysis of the pathogenic fungus isolated from host fish *Oriochromis niloticus*. This study not only focuses on the fungal phenotype analysis but also its pathogenic effect on the host physiology. The infectivity experiment with the selected dose i.e. $1/4^{th}$ of the determined LD_{50} (Ghosh *et.al.* 2019) revealed that the isolated pathogen is capable of re-infecting the host fish, as the fish showed primary symptoms of infection after fungal inoculation. Different parameters when tested to confirm the pathogenic effect of the fungus on the host fish positive outcome was obtained.

To confirm the pathogenicity of the fungus, sign of acute infection in the liver and muscle were studied. The observations during this study like mild necrosis, rupture of portal vessels, vacuolizations of the hepatocytes is similar to the findings of Mohanty *et.al.*, 2008; El-Barbbary, 2010; Ghosh *et.al.*, 2014. The damage is caused due to the invasion of the fungal hyphae through the dermal layer and finally its adherence to the target organ and these observations were documented through the SEM and TEM studies.

The biochemical parameters it was observed that there was abnormal decrease in glucose level. The decrease was due to the fungal consumption of sufficient amount of glucose from the host causing glycopyruvic intoxication which ultimately led towards the abnormal decrease in serum glucose. This change can be defined as a secondary response to stress due to the pathogenicity of the fungus. The same parameter was tested for the measurement of stress response in a variety of fish species (Barton and Iwama, 1991 and Wendelaar Bonga, 1997).Liver enzymes like ALT, AST are the stress

indicator of body (Barton *et.al*, 1991; Van der Boon *et.al* 1991; Wendelaar Bonga, 1997). The quantitative analysis of these enzymes showed significant differences in between control and infected group of fishes and the probable reason was higher metabolic activity of the fish. As the liver is the primary organ and the major site for detoxification thus when the body is exposed to any sort of stress a significant increase in liver enzymes occurs leading to higher metabolic activity and elevated concentrations of amino-transferase enzymes *et.al*, 2005).

The major antioxidant enzyme system of fish constitute Lactate dehydrgenase (LDH), Malate dehydrogenase (MDH) and Peroxidase (Pox) and the study of this enzyme system was important during this work as the change in this enzyme system is an indicator of supply of oxygen in the living system (Chaudhuri *et al,*1998; Martinez *et al*, 2011). When the oxygen supply is inadequate, organisms switched from aerobic to anaerobic mode of metabolism in order to maintain their physiological function and in such conditions the antioxidant enzyme systems come into play. This enzyme system constitute the defense system of the organisms as well (Sharma and Jain ,2008; *et al,* 2009), and thus when pathogens enter into the fish body and toxicological response gets switched on, changes in this enzyme systems take place. In our study the electrophoretic behaviors of the isoenzyme system (fig. 10-12) was indicative towards the pathogen entry inside the fish body. LDH is a marker of common injuries, diseases and stress. Thus presence of LDH extensively in body tissues indicates its release during tissue damage by the fungal pathogen. LDH is involved in the respiratory process and there is increase in activity during the exposure of the host to the fungal pathogen. Pathogenic exposure causes an increase in the cellular metabolic processes in the organisms with higher energy availability (Tiwari and Singh, 2009). Thus ATPase activity also showed an increase in liver tissue of the infected group of fishes in comparison to the control group. This increase, which occurred with the LDH activity in muscle, may be the result of an increase in enzyme activity after being exposed to hypoxia. On the other hand the disruption of MDH was due to the induced alteration in carbohydrate metabolism leading to total glycogen depletion triggered by the fungal entry into the body (Mo *et.al,* 1975).

In animals, peroxidase enzymes are involved in functions like phagocytosis and immune cell response, cell adhesion, antioxidant reaction and the oxidative polymerization of hydroquinone to melanin. This enzyme is also considered as a good indicator for tissue specificity. Higher activity of this enzyme in liver after exposure to fungus was observed and this observation agrees with Mydlarz and Harvel (2007) who also suggested that the enzyme possesses an importance in the defenses of many different organisms and the level gets elevated upon

exposure to toxic environment. The hidden strategies of the organism to respond against artificial infection induced hypoxia lay in that its high affinity for lactate dehydrogenase, malate dehydrogenase and peroxidase, the stress enzymes in acute infection. This enzyme could also be considered as a good indicator for tissue specificity as. Twenty days of exposure to *Aspergillus* niger strain caused increased activity of peroxidase in gill and heart as compared to any other tissue.

Further the study on quantification of DNA and RNA was conducted and the result revealed a clear decrease in concentration of both the nucleic acids in the infected group of fish when compared with the control group. Fish growth showed an inverse correlation with nucleic acids content of the body. As discussed earlier that infection with the fungus Aspergillus leads to higher tissue damage and necrosis and thus cause degradation of the nucleic acids too which leads to the lesser synthesis of the structural and functional proteins and loss of body weight and thus the content of protein and DNA was found to be below the normal range in muscle, gill, kidney, spleen and liver during the infection (Ahmad, 2012).). From this, it clearly implies that fishes with *Aspergillus* PKBSG14 infection the fish must spend more energy to synthesize structural or functional proteins and bio transformer to resist the stressor's effects on homeostasis that is present in fungal toxins (David *et al,* 2004).

The level of total protein is considered to be one of the major indicators of the health status of fish as structural proteins mainly involved in the architecture of the cell and functional proteins determines the activity of the cell. The quantity of total protein is dependent on either the rate of synthesis or on rate of its degradation. During the stress induced by the fungal toxins the fishes need more energy to detoxify the toxicant just to overcome the stress (Ullah *et al.*, 2014); hence in order to that increased demand of energy depletion of protein fraction in serum is triggered and thus similar data was obtained. The primary mechanism of infection involves protein denaturation which results in molecular aggregation and mis-folding ((Karuppasamy, 2000). Synthesis of stress protein is also explained as a protective and repairing mechanism; synthesis of stress proteins therefore occurred to facilitate the repair of denatured proteins (Ahmad, 2012). The present investigations demonstrated that there were definite qualitative alterations in protein fractions and their intensity profiles in the muscle majorly and then other tissues such as kidney, liver and spleen due to infection. The reason for the loss of protein from serum may be attributed to the increased level of transaminase activity resulting rapid utilization of reserve food like protein and carbohydrate under stress condition (David, *et al* 2004, Parthasarathy and Joseph, 2011). The present study explains that host fish responded to the 1/4th of LD50 dose (.75×10^{5} CFU/ml) of *Aspergillus* niger by

altering the level of proteins and DNA in muscle, gill, kidney, liver and spleen. These alterations are sufficient indicators of their diseased condition. High molecular weight bands were observed in control which disappeared in infected fish.

The observations during the present investigation indicate definite physiological changes at the tissue, cellular and molecular level. The alterations in the tissue structure, changes in the quantitative and qualitative estimation of different biochemical and enzymatic parameters, and variations observed during nucleic and total protein quantification clearly indicate the pathogenic effect of the isolated strain of fungus Aspergillus. In the present study, pathogenic *Aspergillus* was proved to an effective inducer of stress causing modifications in the different physiological parameters in infected fish.

Ethical approval: The fishes were collected in fresh from different fish farms of West Bengal. Animal ethical care guidelines were followed as the fish were used in the study. It has been informed that as per CPCSEA instruction's protocol for experimentation on fishes does not require approval.

Conflict of interest statement: We declare that we have no conflict of interest.

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