IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS)
e-ISSN: 2319-2380, p-ISSN: 2319-2372. Volume 7, Issue 10 Ver. II (Oct. 2014), PP 72-81
www.iosrjournals.org

First report of infection of *Rhizomucor pusillus* PKBSG strain in *Channa punctatus* - its isolation, identification by sequencing of D1/D2 region of 28s rRNA-DNA genes and host-pathophysiology

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Abstract: *rRNA genes are attractive targets for developing PCR identification of fungal pathogens in cultured fishes. Infected fresh water Channa puntatus, were brought alive from Canning fisheries and aquatic resource, Canning, India to parasitology laboratory, Department of Zoology for pathogenic examination. Macroscopic production of wide ribbon-like, coenocytic hyphae on the head, skin and fins was observed. The fungal species was isolated and characterized on the basis of morphological and molecular analysis. The species was identified as Rizomucor pusillus. Fragment of D1/D2 region of 28S rDNA gene was amplified by PCR with a band size of 574bp and 564 bp respectively. The D2 partial sequence of 28S rDNA (HG313769) of this strain was submitted to EMBL. Dendrogram was constructed using reported nucleotide sequences of domains D1/D2 of the nuclear 28S rDNA from different related taxa. HG313769 was found most closely related to JN315054 and JN315053 submitted from Korea. The effect of this pathogenic fungus on host physiology was observed by monitoring changes in biochemical, hematological and histological parameters. In our present research, results showed significant decrease in concentration of RBC, Hb, PCV, glucose, total protein and albumin – globulin (A/G)ratio whereas significant increase in AST, ALT, cholesterol after 10 and 20 days of post- infection with increased doses of 10^6, 10^7 and 10^8 CFU. The cumulative mortalities of the intraperitoneal infection increased along with the sporangiospore concentrations; the highest mortality observed was 70% with10^8 CFU as compared to the control. This is the first report of R.pusillus infection in fresh water Channa fish.*

Keywords: *Channa punctatus, dendrogram, histopathophysiological study, Rhizomucor pusillus PKBSG, rRNA genes*

I. Introduction

Rhizomucor belongs to the family Mucoraceae under class Zygomycetes. They are reported to be pathogenic in birds, animals and humans [1-3]. The genus *Rhizomucor* is distinguished from *Mucor* by its thermophilic nature and presence of stolons, poorly developed rhizoids at the base of the sporangiophores.

Zygomycosis refers to infections caused by fungi of the zygomycota phylum. Till date, some 665 species have been described but the infections in humans and animals are generally caused by genera such as Mucorales, causing subcutaneous and systemic zygomycosis (Mucormycosis) - *Rhizopus, Rhizomucor* and *Mucor*. The infecting fungus invades vessels of arterial system, causing embolization and subsequent necrosis of surrounding tissue with high morbidity [4-6]

The characteristic features are greyish brown colored mycelium with typical sympodially branched yellow-brown sporangiophores always with a septum below the globular sporangium.

The infectivity involved with human zygomycosis is well documented. zygomycosis also occurs in fish, a *Mucor* sp. was isolated from *Takifugu obscures* in Jiangsu province, China was found to cause zygomycosis [7].Another Systemic zygomycosis from farmed tilapia fish in Virginia, USA was also reported [8]. However, the identification by phenotype only to genus level is inconclusive so it is difficult to associate a disease specifically with a species [9], and accurate and quick treatment for the fungal pathogens may therefore be delayed.

In the present study, integrated approaches including phenotype and molecular analysis of the D1/D2 region of LSU (Large SubUnit: 28S rDNA) have been done to identify the *Rhizomucor pusillus* isolated from diseased snake headed, *Channa puntatus* of India. The histopathological and electron microscopic studies of the affected organs of the host have been done together with haematology and serum biochemical analysis to observe the physiological changes. This is the first reported episode of zygomycosis in fish from India.

II. Materials and methods

2.1 Microscopic examination, fungus isolation and culture

In February 2013, a group of diseased snake headed *Channa puntatus* (17-22cm long) were captured from fish farms of Canning, Canning Soth 24 parganas**,** India, and brought alive to the parasitology laboratory for experiment. The most noticeable clinical symptoms of the diseased fish were wide ribbon-like coenocytic

hyphae, subsequent necrosis on the head, skin and fins area with lethargic movement. For the isolation of the fungus the affected organs were aseptically examined by 20% KOH, stained with Lactophenol cotton blue and after that necrotic tissue materials from the dorsal region were inoculated on Potato Dextrose Agar (PDA) (Hi Media) to culture the fungal strain. The fungal strain was isolated and purified following standard methodology [10]. The pure strain was sub cultured on PDA at 37°-55° C.

The morphological analysis was performed by slide culture technique [11].The scanning electron microscopy was done [12] using, Hitachi S -530 Scanning Electron Microscope at accelerating voltages of 15 and 20KV.

2.2 Genotypic characterization of fungal pathogen

DNA was isolated from the fungal culture and ITS-1, 5.8S rRNA, 28S rRNA and ITS-2 regions were amplified by PCR and its quality was checked on 1.2% agarose gel. A single band of 650 bp was observed (Fig.1). The PCR amplicon was purified and sequenced using DF (ITS1) and DR (ITS2) primers in BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.

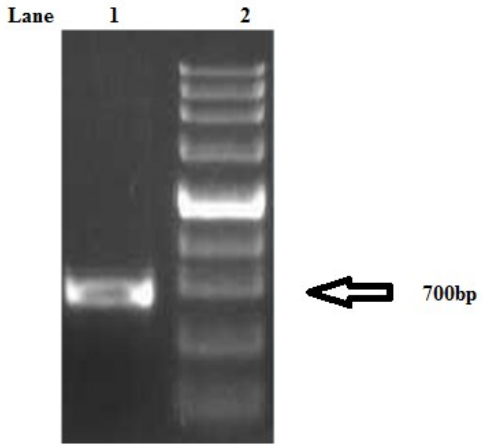

FIGURE 1 Gel image showing, Lane 1: D1/D2 region of LSU (Large subunit 28S rDNA) amplicon b and Lane 2: DNA marker.

2.3 Infectivity experiment

Approximately fifty healthy snake headed fresh water *Channa puntatus* (length 17–22 cm; weight 50-70grm) were obtained from a commercial fish farm. Among them forty fishes were divided into four groups (10 fish in each group) and kept in tanks under similar conditions (water volume 50 L; temperature 24–25 °C). There was no history of disease or abnormality and were acclimatized for half a month before infectivity experiment.

Inocula were prepared from cultures of the strains on potato dextrose agar (PDA) slants for seven days at 37-55° C to obtain sufficient sporulation. 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. 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- formining unit) were counted after 18 h.

Three groups of *Channa puntatus* were injected with 200 µL fungal suspension containing $10^{6,}$ 10^7 and 10^8 CFUmL/l doses respectively and one group was used as control and injected with 200 µL sterile 0.68% NaCl containing 0.05% Tween 80. *Channa puntatus* were exposed for ten and twenty days for the infectivity experiment.

2.4 Haematology and biochemical analysis

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. Blood parameters like RBC (Red blood cells), WBC (White blood cells), PCV (Packed cell volume), Haemoglobin concentration (Hb), MCV (Mean corpuscular volume), MCH (Mean corpuscular haemoglobin) and MCHC (Mean corpuscular haemoglobin concentration) were estimated. Total RBC and WBC count were determined by using an improved Neubauer haemocytometer [13] and the packed cell volume (PCV) was determined by using microhematocrit capillary tube [14] Haemoglobin content in blood

was determined by using Sahli's haemocytometer. Absolute values like MCV, MCHC and MCH were calculated following standard methodology [15].

The samples without anticoagulant were centrifuged at 2000 r. p. m. 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).Total protein concentration in serum was analyzed [16].Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was also determined along with Alkaline phosphatase(ALP) [17,18]. Albumin was determined by bromocresol green method [19]. The level of Serum cholesterol and glucose was also estimated [20,21].Blood serum globulin was calculated by subtracting the concentration of albumin from that of the total protein and albumin/globulin ratio (A/G ratio) was calculated by dividing albumin concentration over that of globulin[22].

2.5 Histopathological study

Samples of liver, spleen, gills, kidney and skin were collected from normal and artificially infected host fish and fixed in Bouin's fixative for 24 hours, dehydrated in ascending grades of alcohol and cleared in xylene. The fixed tissues were embedded in paraffin wax and sectioned into five micrometres thick, stained with hematoxylin and eosin [23] .Then the sections were examined on light microscope and photographed by using a Olympus make phase contrast microscope with attached camera.

2.6 Scanning electron microscopic study of tissues infected with fungal pathogen

The tissues of the host fish with artificial infection were excised and fixed in 2.5% glutaraldehyde solution for two hours at 4 ° C followed by dehydration with ethanol, and washing with absolute acetone and amyl acetate mixture in 3:1, 2:2 and 1:3 ratios respectively and finally with 100% amyl acetate. The tissues were then critical point dried using CO_2 in a HCP:2 Critical Point Dryer (Hitachi), coated with metallic gold in an IB-2 ion coater and examined in a Hitachi S-530 Scanning Electron Microscope at accelerating voltages of 15 and 20KV.

III. Statistical analysis

All the data were analyzed by Students't' test between control and infected groups. The mean values are compared at 1% level of significance ($P < 0.01$)

IV. Results

4.1Microscopic examination and morphological identification

The surface colony color of *Rhizomucor pusillus* PKBSG was initially white which in time turns to grey to yellowish brown. Microscopic appearance shows the presence of nonseptate broad hyphae, rudimentary rhizoids, sporangia and yellow-brown sporangiospores (Fig. 2a) with numerous spores (Fig.3f). Sporangiospores are smooth-walled and globose to subglobose shaped measuring 3-5 µm in diameter (Fig. 3a-d).The rudimentary rhizoids (Fig. 3a and Fig.2b) are located on stolons between the sporangiophores which are irregularly branched, in an umbel at the apex (Fig. 3e). Sporangia are brown in color, round shaped, with well developed columella (Fig. 2a and Fig. 3a) having diameter ranging from 40 – 80 µm. Temperature growth range: minimum 20-27°C; optimum 35-55°C; maximum.

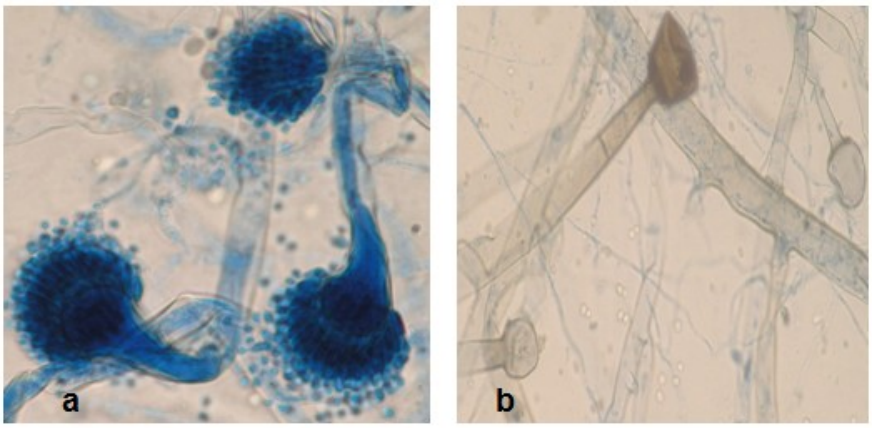

Figure 2a Lactophenol cotton blue stained light microscopic picture of nonseptate broad hypahe and Sporangiophore attached with oval shaped columella at 100x ;b: KOH stained image showing rudimentary rizoids between the sporangiophores at 100x

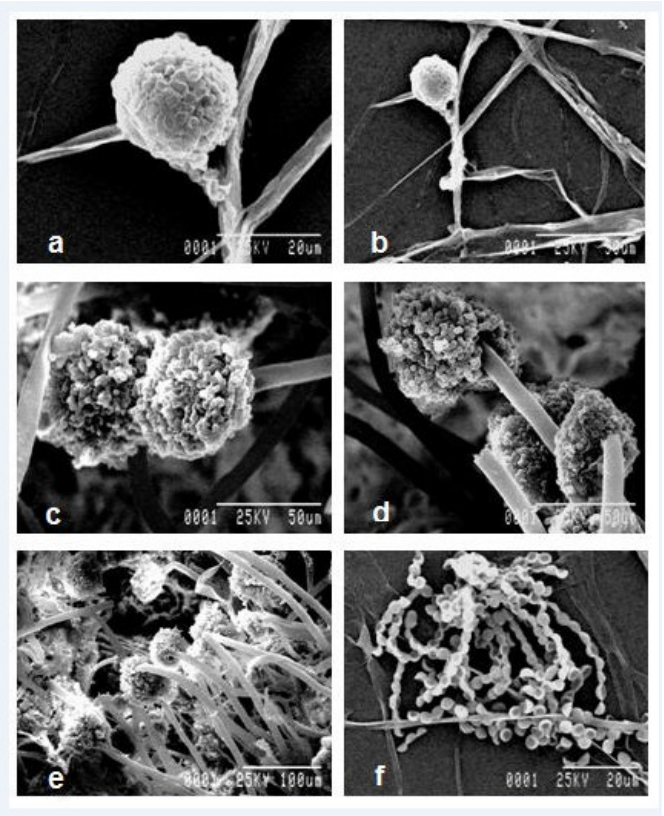

FIGURE 3(a-f) Scanning electron microscopic image of *Rhizomucor pusillus* at 100x
(a) Globose sporangia (3-5µm) at the tip of the hyphae;scale bar=50 ,(b) Rudimentary rhizoids between the irregularly branched sporangiophores; scale bar=25 µm , (c and d) Ovoid well developed columella along with unicellular small sporangiophores; scale bar-50µm, (e) Irregularly branched sporangiophores with branches arranged in an umbel at the apex; scale bar=25 µm, f)Ovovoidal sporangiophores with ornamented walls;scale bar=25 µm.

4.2Molecular identification

For molecular identification a D1 region (574-bp) and D2 region (564 bp) of 28s rDNA gene fragment was amplified using gene specific primers DF-1 (5'- acccgctgaacttaagc-3') and DR-2 (5'-ggtccgtgtttcaagacgg-3'). The reverse sequence (564 bp) was submitted in EMBL Gene Bank (accession number HG313769). The BLAST results revealed that HG313769 showed closest homology (100%) with HM849716, JX088738, JN315053, JN315054 and AF113475. Among these reported gene sequences JX088738 was submitted from Kaktiya University, Warangal, India[24] whereas genes with accession number HM849716, JN315054 and AF113475 from Netherlands, Korea and USA respectively. The fungal sequence from Kalyani, India was most distantly related to HM849717 from Fungal Biodiversity Centre, Netherlands [25]. They also found that ITS diversity was up to 20% in *Lichtheimia* and 35% in *Mucor*, while *Rhizopus arrhizus* and *Rhizopus microsporus* deviated by 29.8%.

The phylogenetic tree was inferred using the Neighbor-Joining method [26] and constructed using MEGA 5 [27] (Fig. 4). Based on maximum identity based on nucleotide sequences, BLAST results were compared to other taxa like *Rhizopus*, *Mucor*, *Rhizopus pussillus*, *Rhizopus michei* and *Lichtheimia*. The dendrogram reveals three major clusters; A, B and C. Cluster A bears a total of twenty reported nucleotide sequences, ten from *Rhizopus* species (HM849666, HM849667, HM849668, HM849669, HM849671, HM849670, HM849672, HM849659, HM849673 and HM849674), eight from Mucor (HM849688, HM849690, HM849685, HM849683, HM849684, HM849679, HM849676 and HM849677) and one each of *Rhizomucor pusillus* (HM849716) and *Rhizomucor michei* (HM849717). Five nucleotide sequences of *Lichtheimia* species (GQ342909, GQ342946, GQ342903, GQ342950 and GQ342933) and sequences (FJ345356, JX088738 and EU257379) showing 100% homology with the fungal sequence from India were included in cluster B. HG313769 was most closely related to JN315054 and JN315053, were kept in cluster in C. JN315054 and JN315053 (28S ribosomal RNA gene partial sequence of *Rhizomucor pusillus* strain) was submitted from National Agrobiodiversity Center, Korea (unpublished work)[28].

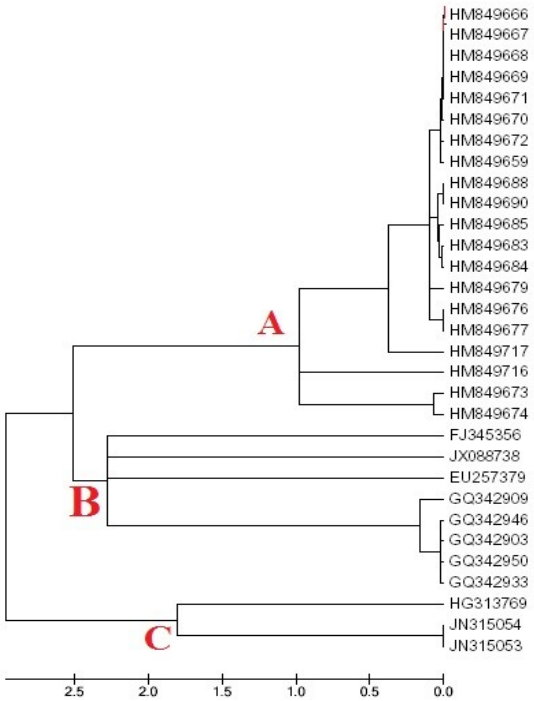

FIGURE 4 Phylogenetic tree of taxa with distances computed using the Maximum Composite Likelihood method.

4.3Infectivity experiment

Before fungal inoculation with intraperitoneal injection, no fish death was observed. Mortalities have been recorded during the experimental infections. Along with the increasing concentration 10^6, 10^7 and 10^8 CFU of sporangiospore suspension the cumulative mortality ascend correspondingly from 30% to 40% and with highest mortality (70%) within the given time of exposure and the time to death of fish was reduced.

All fishes were examined for gross pathological changes. The dead or moribund fish were checked for the presence of the fungal pathogen. The main clinical symptoms includes large swollen liver and conspicuous fungal colonies after 20 days of post infectivity experiment that appeared on the mouth, gills, eyes, and fins and localized areas of the body surface (Fig. 5). The fungal growth appeared white or grey thin threads resembling a tuft of cottony white patches on the body (Fig. 5) .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, irregular and sluggish movement.

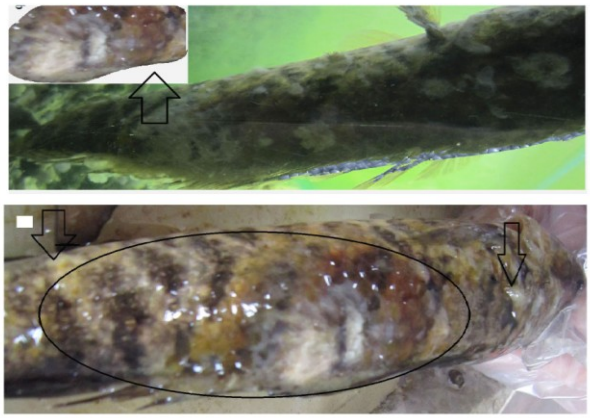

FIGURE 5(a-b) :Apperance of fungal infection on the mouth, gills, eyes, and fins and localized areas of the body surface as white or grey thin threads resembling a tuft of cottony white patches on the body of fish after exposure to the infectivity experiment.

Mortality in each group was recorded and dead fishes were removed immediately to prevent contamination. Doses, the cumulative mortality and time of first death have been shown in Table 1.

Infectivity trials showed that isolated fungal strain PKBSG was pathogenic for snake headed *Channa* fish.

Table 1Showng the percentage of mortality with the doses

Dose (CFUmL/l)	No. of fish examined	No. of dead fish	No. of fish survived	Percentage mortality (%)
10^6 CFUmL/l	10	3	7	30
10^7 CFUmL/l	10	4	6	40
10^8 CFUmL/l	10	7	3	70

4.4 Hematology and biochemical analysis

After one day of post infection, with Zygomycosis; no significant changes in the haematological and serum biochemical parameters have been observed. A significant decrease in RBC, Hb and PCV was observed in *Channa punctatus*, after ten and twenty days of post infection with the increased amount of doses. MCV and MCH showed a high significant increase at the same period of sampling in comparison to uninfected fish (Table 3).In ten and twenty days exposed fishes, the recorded values of plasma cholesterol, alkaline phosphatase, GPT/AST and GOT/ALT led to increase while the total protein, serum albumin and globulin and glucose concentration tends to decrease in respect to the control group (Table2).

Table 2 Effect of *Rhizomucor pusillus* on some serum biochemical parameters of *Channa puntatus* before and after treatment in comparison with control (Mean±SE)

SL. NO.	parameters	No of fish examined	Control		10^6 CFUmL/l		10^7 CFUmL/l		10^8 CFUmL/l	
			10 days	20 days	10 days	20 days	10 days	20 days	10 days	20 days
1.	Glucose (mg dl^{-1}) Normal range (49.6-87.07)	10	87.07	71.95 ± 4.29	66.10±21.5 423	62.1±12.7	52.42± 2.29	42.5 ±4.307	34.68 ±7.83	35.134±9. 85
2.	Cholesterol (mg dl^{-1}) Normal range (120.78-140)	10	130.93 ±1.97	131.9 ±1.35	144 ± 1.48	149.9±5.2 4	154.82 ±9.83	159.66± 6.3	162.04±4. 26	188.59±0. 287
3.	Total protein (g dl^{-1}) Normal range (2.5-6.0)	10	5.56±0.31	5.473 ±1.31	4.58 ±1.05	4.42±1.05	3.98±0 .32	3.15±0. 32	2.33±0.88 87	2.24±0.72
4.	Serum albumin (g dl^{-1}) Normal range (2.5-4.2)	10	3±0.357	2.95±0.09	2.65±0.08	2.24±0.35 7	1.61±0 .061	1.05±0. 5739	1.58±0.29 4	0.368±0.0 66
5.	A/G Normal range (1.4-1.9)	10	1.7±0.23	1.61±0.061	1.572±0.13 9	1.02±0.40	1.21±0 .8	0.86±0. 05	0.8161±0. 2946	0.61±0.03 5
6.	SGOT/AST (UL^{-1}) Normal range (140-315)	10	147.43±5.6 8	179.43±5.6 8	190.9±89.0 19	191.57±0. 199	203.59 ±0.287	243±0.6 40	345.76±1. 228	416±172. 55
7.	SGPT/ALT (UL^{-1}) Normal range (30.2-36.42)	10	34.167 ±1.067	37.93±0.26 5	73.35±1.65	76.78±0.3 45	82.733 ±2.34	84.89±0 .0789	90.86±0.0 905	103.59±0. 287
8.	Alkaline phosphates (mg dl^{-1}) Normal range (125.47-145.35)	10	130.60±2.9 1	133.16±9.0 3	142.13±8.9 3	149.58±4. 60	151.57 ±6.3	159.905 ±8.82	161.6±31. 362	189.12±0. 207

Table 3Effect of *Rhizomucor pusillus* on some haematological parameters of *Channa puntatus* before and after treatment in comparison with control (Mean±SE)

SL. NO.	parameters	No of fish examined	Control		10^6 CFUmL/l		10^7 CFUmL/l		10^8 CFUmL/l	
			10 days	20 days	10 days	20 days	10 days	20 days	10 days	20 days
1.	RBC(10^6 μL^{-1}) Normal range (2.8-4.8)	10	4.738±0.43	3.38±0.25	2.81 ± 0.11	2.63–3.26	2.35	2.13±1.4 1	2.076±0.5 85	1.75 ± 0.31
2.	WBC (10^3 μL^{-1}) Normal range (7.0-10.8)	10	7.33± 4.88	7.87±1.68	8.18±1.074	8.63±1.69	9.58±0. 54	10.36±0. 56	11.08±1.3 43	12.166±1. 98

3.	Hg (g dL^{-1}) Normal range (11.0-14.5)	10	13.7± 1.08	13.19 ±1.12	12.14±1.87	10.85±2.370	9.12 ± 1.64	8.9	7.08 ± 1.04	6.5± 1.84
4.	PCV (%) Normal range (41-46)	10	43.458±4.00	41.28 ± 8.22	36.613± 1.57	31.57 ± 9.16	28.836 ±6.2	27.93 ± 0.35	26.5±0.23	24.9±0.28
5.	MCV (Ft) Normal range (90.8-145)	10	94.28	101.83 ± 1.25	107.27±19.5	132.7 ± 27.08	150.6 ± 12.17	167±0.74	177.66 ±6.33	179±0.28
6.	MCH (pg) Normal range (28.5-43.15)	10	31.28 ± 0.89	32.35±6.708	43.025±6.10	53.45±5.06	56.992 ±21.36	62.53±4.52	65.31±6.56	71.6±4.52
7.	MCHC (%) Normal range (30.0-31.5)	10	31.9 ± 1.11	31.30±2.78	30.15 ± 3.88	30.46±0.5217	28.2±0.84	29.1±1.273	27.78 ± 0.84	25.25±1.879

4.5Scanning electron microscopical findings

SEM studies revealed the penetration of fungal hyphae in the tissues of liver, kidney, skin and gill lamellae of the host causing damage and haemorrhage of the liver cells which has been indicated by the increase in number of RBC cells in liver tissue infected with the strain (Fig.6b). Many of the nonseptate, broad and branched hyphae have been observed in ulcer granulated tissue of the liver leading to degeneration of cells near the hyphae (Fig. 6a). A fungal growth has been noticed inside the kidney leading to formation of outgrowth in the said tissue due to the accumulation of fungal hyphae (Fig. 6c-d). Furthermore the study showing the Skin and gill containing fungal hyphae and fungal spore leading to disintrigation of the skin and damage of gill lamellae (Fig. 6 e-h)

Besides, due to fungal infection spleen cell have been infiltrated by the cells of other organs which indicate the damage to the tissue (Fig.6i).

4.6 Histological findings

A series of histopathological changes have been observed in the tissues of liver, kidney, spleen, skin and gill. Inflammation of tissues, tissue necrosis and circulatory disturbances has been noticed. The regular arrangement of hepatocytic cells of liver was irregular with small vacuolar spaces. Nucleus was hardly visible in most of the cells. Many erythrocytes were observed in the hepatic tissue section (Fig.7B).Part of the hepatic tissue was necrotic and numerous melanomacrophage centres were observed in the sections of infected fishes.

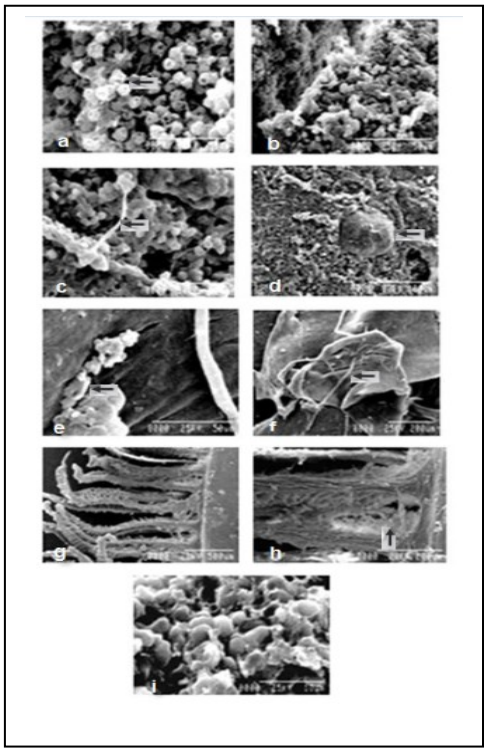

FIGURE 6

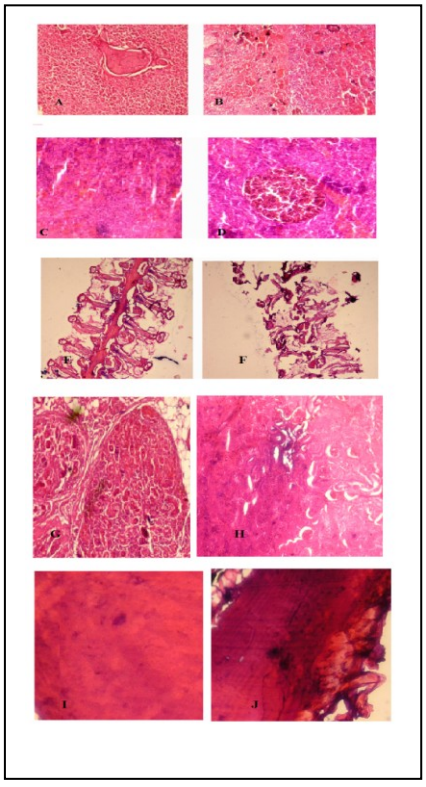

FIGURE 7

FIGURE 6 (a-i) Histological sections of different tissues from disea*sed Channa puntatus* under scanning electron microscopy. (a-b)Nonseptate ,broad hyphae invasion in liver tissue; cells near the hyphae degenerate and many erythrocytes are observed in the hepatic tissue section; 1500x, scale bar=50 µm and 25 µm; (c-d) part of the connective tissue in kidney was proliferated and an outgrowth was observed due to inflammatory cell infiltration; part of the renal tubule atrophied,1500x,400x scale bar=50 µm; (e-f)Skin section showing fungal invasion and fungal spore that cause disintrigation of skin cells,150x;scale bar=200 µm; (g-h)Gill containing messes of fungal infection caused fungal hyphae between the gill lamellae,1000x,Scale bae=200 µm ; (i)spleen showing damaged cells from other tissues indicating damage of liver and kidney due to infection,1000x;scale bar=10 µm.

FIGURE 7 (A-J) Histological sections of different tissues from diseased and healthy freshwater Channa puntatus.

Figure 7(A-B) Showing infection in liver in respect to control fish where arrangement of hepatocytic cells of liver was irregular with many erythrocytes and melanomacrophage centers in the infected hepatic tissue section;(C-D) showing the swelling in the endothelium of splenic ellipsoidal capillaries, thickened trabeculea , hemorrhage with melanomacrophage centersin respect to the controlsections;(E-F)showing disintegration and damage of gill lamellae in respect to intact gill lamellae in the control section;(G-H) showing proliferation of the connective tissue leading to incrassatation and atrophication of part of the renal tubule with glomerular expansion, resulting reduction of Bowman's space in respect to the control sections (I-J) showing degenerated tissue architecture of the infected skin in respect to the healthy longitudinal pattern in the control sections.

The spleen of infected fish mainly showed swelling in the endothelium of splenic ellipsoidal capillaries, thickened trabeculea and haemorrhage. Several melanomacrophage centres were observed (Fig.7D). The disintegration of gill lamellae in respect to normal due to fungal infection have been observed (Figure 7E-F).Gill sections revelled hypertrophy of the lamellar epithelium. In addition, fusion of adjacent lamellae, dilation and haemorrhage in gill filaments have also been also seen (Fig. 7F).

Fungal infection in kidney resulted in proliferation of the connective tissue leading to incrassatation and atrophication of part of the renal tubule .Furthermore, an extensive necrosis of hematopoietic cells, glomeruli and tubular epithelial cells as well as dilatation of peritubular capillaries have also been observed (Fig. 7H). The Bowman's space reduced due to expansion of Glomerulas whereas, in normal condition the glomerulus evenly distributed with proper Bowman's space (Fig. 7G).

Skin epithelium of the host fish were arranged in longitudinal pattern in normal condition (Fig.7I) but the tissue architecture was found to be degenerated due to fungal infection. (Fig.7J)

V. Discussion

The present study focuses on the morphological and molecular identification of the pathogenic fungal strain *Rhizomucor pusillus*PKBSG causing zygomicosis .This is the first report of the identification and molecular characterization of *Rhizomucor pusillus* isolated from snake headed *Channa puntatus* of India.

These results of biochemical haematological experiments also supported the facts that the strain PKBSG is found to be pathogenic to the fresh water live fish.

The fungal infection leads to abnormal decrease in glucose level, as the fungus consumes sufficient quantities of glucose of the host causing glycopyruvic intoxication leading to abnormal decrease in glucose level in serum which can be defined as a secondary response to stress as the level of the deviation from the normal range is a measurement of stress response which has been widely used in a variety of fish species [29,30].The mean values of ALT (alanine aminotransferase) and AST (aspartate aminotransferase) showed significant differences between control and infected group of host fish. As liver is the primary organ and the major site for detoxification reaction, therefore, a significant increase in liver enzymes due to stress, leads to higher metabolic activity and elevated concentrations of amminotransferase enzymes [31] The increase of blood enzymatic activity is due to leakage of these enzymes from damaged hepatic cells and thus raising levels in blood.

The levels of total protein and cholesterol are also considered to be major indices of the health status of fish. This study revealed significant differences in the values of these parameters between control and infected group of host fish. Since proteins are mainly involved in the architecture of the cell so its quantity is dependent on the rate of protein synthesis or on rate of its degradation. During stress due to fungal infection fishes need more energy to detoxify the toxicant to overcome stress; hence in order to meet the increased energy demand depletion of protein fraction in serum has been observed and due to their degradation the quality of protein also gets affected which results in impaired incorporation of amino acids in polypeptide chain. The reason for the loss of protein from serum may be attributed to the increased level of transaminase activity indicating the rapid utilization of reserve food like protein and carbohydrate under stress condition.

The above consideration coupled with the differences in the contents of serum total protein, albumin and globulin represent a variation in metabolism. The osmotic pressure is being regulated by albumin which participates in the ionic balance. It contributes to the elimination of toxicants, transport of organic molecules and

also serves for protein synthesis after degradation [32]. As compared with globulin, the relatively higher level of albumin in infected fish indicates that its concentration in serum is significantly influenced by stress. The change in serum cholesterol is related to the liver metabolism [33]. Elevated level of cholesterol indicates disorders of lipid and lipoprotein metabolism, especially liver-impaired physiology [34].Elevation in the ALP (Alkaline phosphatase) activity in serum may be indicative of renal and liver damage.

Blood is another good indicator to determine the health of an organism [35]. It also acts as a pathological reflector of the whole body. Hence, the haematological parameters are important in diagnosing the functional status of the host infested by fungal pathogen and also to evaluate the physiological condition and nutritional state of fish [36]

Since erythrocyte characteristics partly determine efficiency of oxygen transport from respiratory systems to tissues [37,38] changes in their number and volume could influence metabolic performance [39] Reduction in percentage of haemoglobin, PCV, RBC level was as a result of decrease in appetite in infected fish or more likely to be the direct of catabolic effect of cortisol on the fish tissues [40]. The changes in PCV is due to the release of catecholamine, which can mobilize red blood cells from spleen [41] and induce red blood cell swelling as a result of fluid shift into the intracellular compartment [42].

The increase in WBC count occurred as a pathological response since the WBC plays important role during infestation by stimulating the haemopoietic tissues and the immune system by producing antibodies and chemical substances working as defence against infection. This could also attribute to increase in the number of lymphocytes in the diseased fish.

The values of MCV and MCH have been significantly increased in infected fish in comparison to healthy one (Table 3) which confirms the occurrence of macrocytic (pernicious) anaemia in the infected fish.

Acknowledgement

One of the authors (SG) is thankful to the University Grants Commission, New Delhi, India for granting fellowship to carry out the research work. Thanks are also due to Prof. Alicia E. Toranzo, Professor Universidad de Santiago de Compostela, Spain, Spain 15782, for taking the pain in kind suggestion during the research work.

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