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ISOLATION AND IDENTIFICATION OF PATHOGENIC BACTERIA AND FUNGI ISOLATED FROM SKIN ULCERS OF *CIRRHINUS MRIGALA*

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ABSTRACT

Diseases like epizootic ulcerative syndrome (EUS), hemorrhagic septicemia, fin and tail rot, gill rot, dropsy etc. are caused in fish due to bacterial pathogens. The present investigation was carried out to isolate and characterize fish pathogens obtained from the diseased fish collected from different farms around Hisar, Haryana, India. Six types of pathogenic bacteria and a fungus were found to cause the epizootic ulcerative syndrome disease. A number of biochemical tests were carried out for identification of these causative agents. Six pathogenic bacteria viz. *Streptococcus* grp Q1, *Aeromonas hydrophilla, Shigella spp., Streptococcus faecalis, Cellobiosococcus sciuri, Micrococcus luteus* were identified. The fungus, *Aphanomyces invadens* was also identified and isolated from the diseased fish. Based on the programme PIBwin, ID scores were allotted to each bacterial isolate and matched/ compared with the standard scores of the reference bacterium and on this basis the bacterial isolates were tentatively identified. The pathogenicity of disease causing organisms was confirmed through both *in vitro* and *in vivo* experiments.

Key words: Aquatic, Bacteria, Disease, Fish, Fungi, Pathogen.

INTRODUCTION

Bacterial, viral and fungal diseases are very common in aquatic animals. As and when the environmental conditions are not stable, like sudden change in salinity, temperature, dissolved oxygen, pH or electrical conductivity etc. such changes become conducive for growth and proliferation of disease causing organisms on the host organism. Poor pond management practices and higher stocking rate often result into outbreaks of diseases which lead to mass mortality in fish (Kumar *et al.*, 1986; Dey, 1989). Innumerable diseases like epizootic ulcerative syndrome (EUS), hemorrhagic septicemia, fin and tail rot, gill rot, dropsy etc. are caused in fish due to bacterial pathogens and several of them are reported in scientific literature. Some of the important bacterial pathogens are *Flavobacterium sp., Photobacterium damsela* subspecies *pisicida* (Aoki *et al.*,1995; Aoki *et al.*, 1996) *Vibrio damsela, V. alginolyticus*, *V. cholerae, V. vulnificus, Pasturella piscida, Providencia rettgeri, Aeromonas hydrophila*, *A. salmonicida*, *Pseudomonas fluorescens*, *P. aeruginosa*, *Flexibacter columnaris*, *Edwarsiella tarda*, *Enterococcus*, *Staphylococcus aureus*, *Micrococcus sp.* (Dey, 1989; Kumar, 1989; Mukherjee *et al.*,1991) which have been identified as the most commonly occurring bacterial agents of fish diseases. In the present research work we have isolated and identified six types of bacteria and one fungi as causative agent of skin ulcers in Indian major carp (*Cirrhinus mrigala*).

MATERIALS AND METHODS

Culture and isolation of bacteria: For the culture and isolation of the pathogenic bacteria, methods suggested by OIE were followed. The specimens of diseased fish were collected and dissected; the affected tissue (skin lesions/muscle) was taken in a test tube, homogenized mannually and spread over the nutrient agar medium in Petri plates under aseptic conditions. These plates were incubated in B.O.D at 30 ± 1^{0}C for 24 h. Bacterial growth on the nutrient agar plate was observed after 24 h. Pure colonies of bacteria were isolated and obtained further by sub-culturing of the single colonies on nutrient agar by proper streaking method (OIE, 2006). These pure

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cultures were stored at -20^{0}C for further investigations/tests.

Identification of bacteria: Isolated pure cultures of bacteria were subjected to standard biochemical tests (primary and secondary) for identification as reported by Krieg and Holt (1984) and OIE (2006). The confirmation test of these bacteria was done with the help of selective media used for culturing that particular bacterium.

Culture of fungus: From the samples collected during survey of fish farms, fishes with pale, raised lesions and ulcerated skin were segregated for confirmation of fungal infection. The affected area of fish was scraped aseptically with the help of a sterile scalpel and homogenized in a manual homogenizer. The homogenized mixture was spread over the Czepacks medium in plates (Table 1), each containing about 25ml of this medium under aseptic conditions. The plates were sealed, incubated at 25^{0}C and examined daily. Emerging hyphal tips were repeatedly transferred on to fresh plates of Czepacks medium until cultures were free from contamination. The culture plate was labelled of the type specimen with date of inoculation.

Procedure for the characterization of fungus (OIE, 2006)

i) A drop of lecto-phenol was taken on the slide and fungal hyphae from pure culture were transferred aseptically on to it with the help of inoculation loop.

ii) The culture was spread to an even thin film on the slide.

iii) The fungal hyphae were stained with cotton blue and a permanent slide was prepared followed by observing the slide under the microscope. Identification and characterization of fungus was done by the pathology laboratory of the Department of Plant Pathology, College of Agriculture, CCS HAU, Hisar.

RESULTS AND DISCUSSION

Isolation and characterization of bacterial pathogens associated with EUS in Indian major carp: Isolation of bacterial flora from the surface lesions of EUS affected fish as well as from their muscle and gut revealed the occurance of *Areomonas hydroplhilla, Pseudomonas, Shigella, Streptococcus, Micrococcus, Cellobiosococcus, Acinetobacter, Streptococcus grp Q1.*

The results (Table 2) showed the bacteria obtained from Aquaculture Research and Training Institute, Hisar (ARTI) fish samples: three bacterial Isolates were obtained and isolate 1(a) revealed that this bacterium was gram negative, positive for catalase, anaerobic, orange colour and rod shape. The primary tests of isolate 2(a) revealed that this bacterium was gram positive cocci, catalase positive and had yellow colour colony. The primary tests of isolate 3(a) revealed that this bacterium was gram negative, aerobic, positive for catalase and had white colour colony. The results of primary and secondary tests are given in Table 2. Based on these tests an identification score of 0.99701, 0.99942, 0.99776 respectively were assigned to the isolates by the PIBWin Programme. On the basis of the identification scores, the bacterium species were identified as *Shigella* spp., *Streptococcus faecalis* and *Aeromonas hydrophilla.* The growth of these isolate on specific medias furthers confirms their identification (Table 6).

The bacteria (Table 3) obtained from Sandol farm fish samples: three bacterial isolates were obtained and isolate 1(b) revealed that this bacterium was aerobic, gram positive, non-fermenter, positive for catalase and had yellow orange colour colony. The primary tests of isolate 2(b) revealed that this bacterium was gram negative, aerobic, fermentive rods, positive for catalase and had white colour colony. The isolate 3(b) revealed that this bacterium was gram negative rods, aerobic, non fermenters and positive for catalase, oxidase. The results of primary and secondary tests are given in table. Based on these tests an identification score of 0.99631, 0.99776, 0.99595 respectively were assigned to the isolates by the PIBWin Programme. On the basis of the identification scores, the bacterium species were identified as *Micrococcus luteus*, *Aeromonas hydrophilla* and *Pseudomonas*

TABLE 1: Composition of Czpecks medium.

Ingredients*	Quantity
Sodium Nitrate	2 gm
Potassium phosphate	1 gm
Potassium chloride	0.5 gm
Ferrous sulphate	0.01 gm
Magnesium sulphate	0.5 gm
Sucrose	30 gm
Agar	20 gm
Distilled water	1 litre

TABLE 2: Physical characteristics and biochemical response of different bacterial isolates taken from diseased Mrigal (C. mrigala) from ARTI fish farm (Hisar).

Biochemical tests	Bacterial Isolate		
	1(a)	2(a)	3(a)
Gram reaction	-	+	-
Shape	Rod	Coccus	Rod
Colour of colony	Orange	White	White
Aerobic	-	Facultative	+
Anaerobic	+	-	-
Catalase	+		+
Oxidase	-	-	+
Glucose Acid	+	+	+
Urease	-	-	-
Simmon citrate	-	-	-
Starch hydrolysis	-	+	+
Ehrlich indole	+	-	
ONGP	+	-	
Nitrate-Nitrite	+	-	+
Adonitol	-	-	-
Cellobiose	-	+	-
Fructose		+	
Sorbitol	+	+	-
Sucrose	-	+	+
Tryptophan		-	
Arginine dihyrolase	-	+	+
Lactose	-	+	-
Maltose	-	+	+
Mannitol	+	+	+
Galactose		+	
Glycerol	-	+	+
Inositol	-	+	-
H/L (oxidative)	-		-
H/L (fermentive)	+		+
H/L (alkaline)	-		-
Methyl red 37^{0}C	+		+
Methyl red RT	+		-
Voges Proskauer 37^{0}C	-	+	-
Voges Proskauer RT	-	+	+
Growth at 37^{0}C	+	+	+
Growth at 5^{0}C	-	-	-
Growth at 42^{0}C	-	-	-
Motality at 37^{0}C	-		
Motality at RT	-		+
PIB win ID score	0.99701	0.99942	0.99776
Model ID score	0.00105	1.00000	1.00000
Bacterium identified	Shigella spp.	Streptococcus faecalis	Aeromonas hydrophila

flourescens. The growth of these isolate on specific medias furthers confirms their identification (Table 6).

The data in Table 4 showed bacteria obtained from Satrod fish farms: four isolates have been obtained namely, isolate no. 1(c), 2(c), 3(c), 4(c). Isolate 1(c) revealed that this bacterium was gram negative, aerobic, fermentive rods, positive for catalase and had white colour colony. The primary tests of isolate 2(c) revealed that this bacterium was gram negative, anaerobic, rod shape, positive for catalase and had orange colour colony. The primary tests of isolate 3(c) revealed that this bacterium was gram positive, aerobic and negative for catalase, oxidase. The primary tests of isolate 4(c) revealed that this bacterium was gram positive, non fermenters, aerobic, positive for catalase and had yellow orange colour colony. The results of primary and secondary tests are given in table. Based on

TABLE 3: Physical characteristics and biochemical response of different bacterial isolates taken from diseased Mrigal (C. mrigala) from Sandol fish farm (Sandol).

Biochemical tests	Bacterial Isolates		
	1(b)	2(b)	3(b)
Gram reaction	+	-	-
Shape	Coccus	Rod	Rod
Colour of colony	Yellow Orange	White	Green
Aerobic	+	+	+
Anaerobic	-	-	-
Catalase	+	+	+
Oxidase	-	+	+
Glucose Acid	+	+	-
Urease	+	-	-
Simmon citrate	+	-	+
Starch hydrolysis	-	+	-
Ehrlich indole			-
ONGP			
Nitrate-Nitrite	-	+	-
Adonitol	-	-	-
Cellobiose	-	-	-
Fructose	+		+
Sorbitol	-	-	-
Sucrose	-	+	-
Tryptophan	-		
Arginine dihyrolase	-	+	+
Lactose	-	-	-
Maltose	-	+	-
Mannitol	-	+	+
Galactose	-		
Glycerol	-	+	+
Inositol	-	-	+
H/L (oxidative)		-	+
H/L (fermentive)		+	-
H/L (alkaline)		-	-
Methyl red 370C		+	
Methyl red RT		-	
Voges Proskauer 37^0C	+	-	
Voges Proskauer RT	+	+	
Growth at 37^0C	+	+	+
Growth at 5^0C	-	-	-
Growth at 42^0C	-	-	
Motality at 37^0C			
Motality at RT		+	
PIb win ID score	0.99631	0.99776	0.99595
Model ID score	1.00000	1.00000	1.00000
Bacterium identified	Micrococcus luteus1	Aeromonas hydrophila	Pseudomonas flourescens

these tests an identification score of 0.99776, 0.99701, 0.99869, and 0.99631 respectively were assigned to the isolates by the PIBWin Programme. On the basis of the identification scores, the bacterium species were identified as *Aeromonas hydrophilla, Shigella* spp., *Streptococcus* gr Q1 and *Micrococcus luteus.* The growth of these isolate on specific medias furthers confirms their identification (Table 6).

The data of Table 5 were obtained from Charanjeet fish farm (Hansi): three isolates were obtained and the primary tests of the isolate 1(d) revealed that this bacterium was gram negative, aerobic, fermentive rods, positive for catalase and had white colour colony. Based on these tests, identification score assigned was 0.99776 and the species identified as *Aeromonas hydrophilla.* The growth of this isolate on *Aeromonas hydrophilla*

TABLE 4: Physical characteristics and biochemical response of different bacterial isolates taken from diseased Mrigal (*C. mrigala)* from Satrod fish farm (Satrod).

Biochemical tests	Bacterial Isolates			
	1(c)	2(C)	3(C)	4(C)
Gram reaction	-	-	+	+
Shape	Rod	Rod	Coccus	Coccus
Colour of colony	White	Orange	White Cream	Yellow Orange
Aerobic	+	-	+	+
Anaerobic	-	+	-	-
Catalase	+	+	-	+
Oxidase	+	-	-	-
Glucose Acid	+	+	+	+
Urease	-	-	-	+
Simmon citrate	-	-	-	+
Starch hydrolysis	+	-	+	-
Ehrlich indole		+	-	
ONGP		+		
Nitrate-Nitrite	+	+		-
Adonitol	-	-	+	-
Cellobiose	-	-	+	-
Fructose			+	+
Sorbitol	-	+	+	-
Sucrose	+	-	-	-
Tryptophan			-	
Arginine dihyrolase	+	-	-	-
Lactose	-	-	+	-
Maltose	+	-	+	-
Mannitol	+	+	+	-
Galactose			+	-
Glycerol	+	-	+	-
Inositol	-	-	-	-
H/L (oxidative)	-	-		
H/L (fermentive)	+	+		
H/L (alkaline)	-	-		
Methyl red 37^{0}C	+	+		
Methyl red RT	-	+		
Voges Proskauer 37^{0}C	-	-	+	+
Voges Proskauer RT	+	-	+	+
Growth at 37^{0}C	+	+	+	+
Growth at 5^{0}C	-	-		-
Growth at 42^{0}C	-	-		-
Motality at 37^{0}C		-		
Motality at RT	+	-		
PIB win ID score	0.99776	0.99701	0.99869	0.99631
Model ID score	1.00000	0.00105	1.00000	1.00000
Bacterium identified	*Aeromonas hydrophila*	*Shigella spp.*	*Streptococcus grp. Q1*	*Micrococcus luteus1*

specific Rimer-Sholts Medium Base confirmed that "isolate 1(d)" represented *Aeromonas hydrophilla* (Table 6). The results of primary tests of this isolate 2(d) revealed that this bacterium was gram positive, aerobic, non fermenter and positive for catalase and oxidase. Based on these tests, identification score assigned was 0.99973 and the species identified was *Cellobiosococcus sciuri.* The growth of this isolate on *Cellobiosococcus sciuri* specific Antibiotic Assay Medium-C confirmed that "isolate 2(d)" represented *Cellobiosococcus sciuri* (Table 6). The results of primary tests of the isolate 3(d) revealed that this bacterium was gram negative, anaerobic, rod shape and positive for catalase. The identification score was 0.99973 and the species was identified in isolate 3(d) as *Acinetobacter calcoaceticus.* The growth of this isolate on *Acinetobacter calcoaceticus* specific Eosin methylene blue medium further confirmed the "isolate 3(d)" (Table 6). The results of primary tests of this isolate 4(d) revealed that this bacterium was

TABLE 5: Physical characteristics and biochemical response of different bacterial isolates taken from diseased Mrigal (C. mrigala) from Charanjeet fish farm (Hansi).

Biochemical tests	Bacterial Isolates			
	1(d)	2(d)	3(d)	4(d)
Gram reaction	-	+	-	+
Shape	Rod	Coccus	Rod	Coccus
Colour of colony	White	White Cream	Cream	Yellow Orange
Aerobic	+	+	-	+
Anaerobic	-	-	+	-
Catalase	+	+	+	+
Oxidase	+	+	-	-
Glucose Acid	+	+	+	+
Urease	-	-	-	+
Simmon citrate	-	-	+	+
Starch hydrolysis	+	-	-	-
Ehrlich indole		-	-	
ONGP		-	-	
Nitrate-Nitrite	+		-	-
Adonitol	-	-	-	-
Cellobiose	-	+	-	-
Fructose		+		+
Sorbitol	-	+	-	-
Sucrose	+	+	-	-
Tryptophan		-		
Arginine dihyrolase	+	-	-	-
Lactose	-	+	-	-
Maltose	+	+	-	-
Mannitol	+	+	-	-
Galactose		+		-
Glycerol	+	+	-	-
Inositol	-	+	-	-
H/L (oxidative)	-		+	
H/L (fermentive)	+		-	
H/L (alkaline)	-		-	
Methyl red 370C	+		-	
Methyl red RT	-		-	
Voges Proskauer 37^0C	-	+	-	+
Voges Proskauer RT	+		-	+
Growth at 37^0C	+		+	+
Growth at 5^0C	-		-	-
Growth at 42^0C	-		+	-
Motality at 37^0C			-	
Motality at RT	+		-	
PIB win ID score	0.99776	0.99973	0.99999	0.99631
Model ID score	1.00000	1.00000	0.49254	1.00000
Bacteria identified	Aeromonas hydrophila	Cellobiococcus sciuri	Acinetobacter calcoacetius	Micrococcus luteus1

gram positive, non fermenters, aerobic, positive for catalase and had yellow orange colour colony. The identification score assigned was 0.99631 and the bacterium species was identified as *Micrococcus luteus.* The growth of this isolate on *Micrococcus luteus* specific Hugh Leifson glucose medium and Fermentation medium confirmed that "isolate 3(d)" represented *Micrococcus luteus* (Table 6).

These results, on the basis of confirmative tests, revealed that six bacteria inhabited the affected tissues of diseased samples of *Mrigal* fish in four fish farms surveyed for this study. The fish samples of ARTI were infected with *Shigella* spp., *Streptococcus faecalis,* and *Aeromonas hydrophilla* whereas samples of Sandol fish farm were infected with *Micrococcus luteus, Aeromonas hydrophilla and Pseudomonas flourescens.* Like wise, the fish of Satrod fish farm were infected with *Aeromonas hydrophilla, Shigella* spp., *Streptococcus* grp Q1 and *Micrococcus luteus* and the fish of Charanjeet fish

TABLE 6: Selective media used for the confirmation of presence of a particular bacterium in the lesions of diseased fish of Mrigal (*C. mrigala*).

Selective medium used	Isolates which developed the colonies	Characteristics of the colony of bacterium developed on the medium
Hugh Leifson glucose medium and Fermentation medium	1(b),4(c), 4(d)	Yellow colour pigment
Xylose deoxycholate agar	1(a),2(c)	Yellow-orange color colony
Azide Blood agar base	2(a)	Red color colony
Antibiotic Assay Medium. C	2(d)	Colorless colony
Pseudomonas agar F Base	3(b)	Colony show fluorescence
Rimler-Shotts Medium	3(a),2(b),1(c),1(d)	Green color colony
Eosin- Methylene Blue agar medium	3(d)	Dark blue color colony

farms were infected with *Aeromonas hydrophilla, Cellobiosococcus sciuri, Micrococcus luteus* and *Acinetobacter calcoacetius.* The identified fungus was *Aphanomyces invadens* which was present in the infected fish samples.

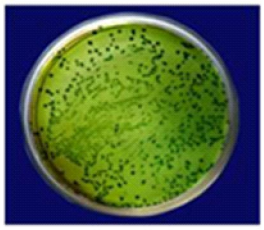
For the confirmation of the causative organism, three series of tests (primary, secondary and tertiary) were performed. Identification of bacteria was done with the help of Bergey's manual of microbiology (Krieg and Holt, 1984) in method. All the characteristics of obtained colony were matched with different colony characteristics presented in this manual. Under the primary test, the bacteria were differentiated with gram reaction into gram positive or gram negative. Under the secondary tests, pure colonies of bacteria were isolated and each bacterial isolate was subjected to many biochemical tests.

The results of these tests were then subjected to a computer software programme, 'PIBWin' (*http//www.soton.ac.in.uk*). Based on this programme PIB win ID scores were allotted to each bacterial isolate. These scores were then matched/compared with the standard scores of the reference bacterium and on the basis of similarity of the scores, the bacterial isolates were tentatively identified. The tertiary tests were done for the confirmation of these bacteria with the help of selective media used for culturing that particular bacterium. The growth of bacterium on the selective medium confirmed the presence of reference bacterium.

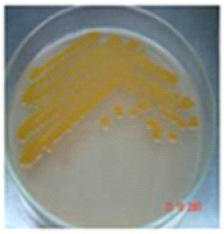
In the present study, in the recovered bacterial isolates from the diseased fish samples collected from different fish farms, *Aeromonas hydrophila* was present in all the fish samples collected from the four fish farms; other species like *Micrococcus luteus*, *Streptococcus faecalis, Cellobiococcus sciuri, Pseudomonas florescence, Streptococcus* grp Q1 and *Shigella* spp. was present selectively (Table 2- 6). (Chowdhury, 1998) reported the involvement of aeromonads and pseudomonads in the ulcer type disease of freshwater fishes. The involvement of *A. hydrophila*, *Micrococcus* spp., *Streptococcus* sp. and *Shigella* spp. might be the cause of highly significant secondary infection (ulcer formation) in the EUS in fish (Lilley, 1992). *A. hydrophilla* is also suspected to be the principal causative agent of ulcerative disease noticed in cultured fish in indo- pacific region (Tonguthai, 1985) and Thailand and Malaysia (Torres *et al.*, 1993). In the present study, *Aphanomyces invadans,* the only fungus was detected as very common fungal pathogen and this was present in all the diseased fish sampled. These results are in agreement with the findings as reported by Sarker (Sarker *et al.*, 1999) that the fungus (*Aphanomyces invadans)* was variably found in the EUS infected fishes. Among the investigated fish, only *C. mrigala* was found to be severely affected by this disease. Lilley *et al.* (1982) reported that in Indian major carps mrigal fish was more susceptible to EUS.

Bacterial pathogens cause heavy mortality in both cultured and wild fish species in different parts of the world. These are either obligate or facultative bacterial pathogens. Facultative bacterial pathogens become a potential threat when fish are under environmental and physiological stress (Wedemeyer, 1970). Six gram negative rods (*Aeromonas, Proteus, Citrobacter, Pseudomonas, Flavobacterium* and *Chromobacterium*) and three gram positive cocci (*Micrococcus, Streptococcus* and *Staphyllococcus*) which were potentially pathogenic were identified from *Aristichthys nobilis*

FIG. 1: Cultures of bacteria and fungus on specific media



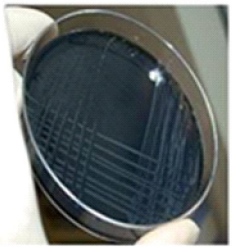
A. hydrophila on Rimler-Shotts Medium



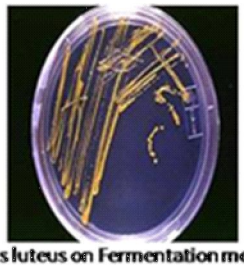
Shigella spp. on XLD Agar

Streptococcus faecalis on Blood agar base

Cellobiosococcus sciuri on Antibiotic assay medium C.



Acinetobacter calcoaceticus on EMB medium



Micrococcus luteus on Fermentation medium

Culture of fungus on Czpecks medium

and *I. idella* fingerlings (Shamsudin, 1986). Pathogens such as *Aeromonas hydrophila* and *Pseudomonas sp.* have been isolated from EUS-infected fishes (Boonyaratapalin, 1989; Torres, 1990; Fliermans *et al.*,1977; Pathiratne and Rajapakshe, 1998). Of these, the Gram-negative bacterium *A. hydrophila* is known to induce EUS-like lesions in *Carassius auratus* and *Cyprinus carpio* (Lio-Po *et al.*, 1998). Similarly *Aphanomyces invaden* is an invasive fungal pathogen isolated from beneath the ulcerative lesions of EUS-affected striped snakehead, *Channa striatus* (Lilley *et al.*, 1997). Transmission of EUS to snakehead (*Channa sp.*) without skin damage provides confirmatory evidence that *A. invadan*is associated with EUS as a primary pathogen (Kirk, 1974). Hence, EUS is a disease of mixed infections and *A. hydrophila* is commonly associated with skin ulcers and remains challenging to the fish farmer causing them high economic losses (Anbarasu *et al.*, 1998).

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