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Emerging *Acinetobacter schindleri* in red eye infection of *Pangasius sutchi*

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This communication provides an insight into the emerging of new infection "red eye" in *Pangasius sutchi* and aimed to screen the prime pathogens involved in disease. The pathogen was isolated from diseased *P. sutchi* and characterized by morphological, biochemical and molecular approach, which includes 16s r RNA gene sequencing. Polymerase chain reaction (PCR) amplified 16s RNA was separated using agarose gel electrophoresis, eluted product was sequenced and BLAST analysis was carried out to identify the pathogens. Identified virulent bacterial strain *Acinetobacter schindleri* with LD_{50} $10^{8.35}$ initiated re-infection in experimentally in infected *Pangasius* fingerlings. This study provided the evidence of *A. schindleri* which is true causative agents in red eye disease in *P. sutchi*. To the best of knowledge of this study, there was no track record of *A. schindleri* eye infection in fishes till date around the globe.

Key words: *Pangasius sutchi*, 16s r- RNA gene sequencing, *Acinetobacter schindleri,* LD_{50}.

INTRODUCTION

Pangasius sutchi is the exotic fish introduced in India from Thailand because of its high commercial value. The farmers of Janardhanapuram, Nandivada (Md), Krishna (Dist), Andhra Pradesh, culturing *Pangasius* in fresh water as intensive, monoculture with stock density of 50,000 per hectare, fed with floating feed having 20 to 23% protein, feeding rate up to 1.2 to 1.6% 10^3 kg body mass of fishes. *P. sutchi* is highly resistant species, and it is voracious feeder shown good food conversion rates (FCR) and give maximum sustainable yields (MSY) in short period. Nutrient rich feeds leave higher concentrations of ammonia and nitrite in culture waters and these stresses the fish in enormous rate and make them susceptible to different diseases. Culture waters with high organic matter pollute not only the tank, but also surroundings, and support the growth of many pathogens. For *Pangasius,* the most important bacterial diseases are bacillary necrosis; red spot have been reported by (Tu et al., 2008). These pollution problems may support the growth of *Acinetobacter* members in culture waters.

Acinetobacter members are found in water and act as common flora (José Américo, 2001; Marian, 1990). The alimentary tract of fresh water trout has *Acinetobacter* members (Trust, 1974). A significant increase in the microbial load of *Acinetobacter* members in ponds treated with different chemotherapeutics has been reported by Andreas Petersen (2002) and their entrance into culture waters

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Abbreviations: FCR, Food conversion rates; **MSY,** maximum sustainable yields; **PCR,** polymerase chain reaction; **SDS,** sodium dodecyl sulfate; **CTAB,** cetyl trimethyl ammonium bromide; **BOD,** biological oxygen demand; **COD,** chemical oxygen demand.

along with contaminated feed has been reported by Trevors et al. (1977). Although *Acinetobacter spp* acts as a severe human pathogen, there are only few studies to date that report it as a pathogen for fish. The genus *Acinetobacter* show wide range of distribution, recovered from soil, water, living organisms. The bacteria very quickly became important member in bacteria landscape in hospitals, responsible for number of nosocomial infections in humans like surgical wound, urinary tract, respiretory tract (Wolff et al*.,* 1997), pneumonia, secondary meningitis (Bukhary et al*.,* 2005), endocarditis (Levi and Rubinstein, 1996), peritonitis, skin and soft tissue infections (Fierobe et al., 2001). Kalidas Rit and Rajdeep (2012) have reported *Acinetobacter* sp and their member's cause nosocomial infections and susceptibility patterns for different antibiotics. The *Acinetobacter* members show resistance to wide range of antibiotics like ampicillin, carbapenems (Mussi et al*.,* 2005), carbenicillin, cephalosporin's (Heritier et al., 2006), amino glycosides, fluoroquinolones (Vila et al., 1993), carboxy pencillins (Joly and Guillov et al., 1995). They produce a wide range of amino glycoside inactivating enzymes (Buisson et al., 1990). Nemec (2001) reported *Acinetobacter schindleri* infections in human nosocomial infections, and Bouvet and Grimont (1986) firstly reported *A. haemolyticus* infections in humans. Emerging of new multi drug resistant bacterial pathogen, *Acinetobacter baumannii* associated with snake head *Channa striatus* eye infection has been reported by Rauta et al. (2011). The present study aimed to identify pathogens at molecular level from diseased *P. sutchi* suffering from red eye infection and to be proved as primary agents in disease.

MATERIALS AND METHODS

Collection of water and diseased fish samples

Diseased moribund fish samples (10) were collected from above said locality ponds, and brought to the laboratory. The fishes show different symptoms like gill impairment, erythro dermatitis, petechiae at lateral line, red mouth, redness at fin bases, swollen red colour anus, pop eye, red arched region around eye, swollen enlarged liver in light yellow colour, shrunken gastro intestinal tract and spleen, and hemorrhages on internal body cavity. Three water samples were collected from sequential days of 15 for 45 days to be checked the parameters like water temperature, pH, ammonia, nitrite, calcium, magnesium, alkalinity, hardness, chlorides, total dissolved solids, conductivity, and dissolved oxygen (APHA, 1988).

Isolation and identification of bacteria

A loop full of sample was collected with the help of inoculation loop from eye transferred on to Rimler Shots agar medium (Hi media, Mumbai). The plates were incubated at 37°C for 24 h. The nature of the cell wall of isolate was tested by gram staining method. For further differentiation, the culture was tested for biochemical characteristics with the Enterobacteriaceae kit (Hi media, Mumbai) as per manufacturer instructions. Later organisms were subjected for molecular characterization, to differentiate organisms up to species level.

DNA extraction

Extraction of genomic DNA and polymerase chain reaction (PCR) mediated amplification of the 16s r RNA gene of bacterial strain was carried out as per the method described by Neal Stewart et al*.* (1993). DNA from saturated bacteria liquid cultures was extracted by above said methodology, includes collection of bacterial cell pellet by centrifugation, lysis of cell pellet were attained by suspending in TE buffer with 100 µg of proteinase K and 0.5% sodium dodecyl sulfate (SDS) final concentrations. After 1 h of incubation at 37°C the lysate was treated with 80 µl of 5 M NaCl and 100 µl of 10% cetyl trimethyl ammonium bromide (CTAB) solution. Cell lysate was incubated at 60°C for 10 min. Degraded proteins from the cell lysate were removed by precipitation with phenol, phenol\ chloroform and chloroform treatment, respectively. Followed by protein precipitation, bacterial genomic DNA was recovered from the resulting supernatant by iso-propanol precipitation. Precipitated DNA pellet was washed with 70% alcohol for removal of salts. The DNA pellet was allowed for air drying and re suspended in 50 µl of deionized water with 1 µl 10 mg ml¯L RNA ase A enzyme for the removal of RNA. Quality of the isolated DNA was analyzed by resolving on 1% agarose gel electrophoresis with 1X TAE buffer.

PCR amplification

The variable V3 region of DNA coding for 16s RNA was amplified by PCR with primers F- 5'- AGAGTTTGATCCTGGCTCAG –3' and R-5'- GGTTACCTTGTTACGACTT–3'. All the PCR amplifications were conducted in 50 µl volume containing 2 µl of total DNA having 54 ng per µl concentration, 200 M each of the four de oxy nucleotide tri phosphates, 1.5 µl $MgCl_2$, 5 µl of individual primers and 1 IU of Taq polymerase. The PCR amplification, used for gene amplification was consisted of initial denaturation at 95°C for 3 min, followed by 39 cycles of denaturation for 1 min at 95°C, annealing for 30 s at 56°C, and extension for 1 min at 72°C and a final extension at 72°C for 10 min. Finally, the amplified PCR product was stored at 4°C. The samples were verified on 1% agarose gel (Lonza, USA) to know Ribo print pattern. The separated bands were excised from the gel (Figure 1) by using surgical blade for elution of DNA. The elution of DNA from agarose gel was carried out as per manufacturer instructions (Real Biotech DNA/PCR purification kit CAT NO 36105).

DNA sequence and phylogenetic analysis

For sequencing analysis, amplified PCR product was sent to EUROFIN Company. All the 16s r RNA partial sequence were aligned with those of the reference micro organisms in the same region of the closet relative strains available in the Gen Bank data base by using the BLAST N facility (http:// www.ncbi.nlm.nih.gov\BLAST) and were also tested for possible chimera formation with the CHECK CHIMERA program(http://www.35.8.164.52\cgis\chimera.cgi? Su: SSU). The sequences were further analyzed by using Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/). Neighbor joining phylogenetic tree (Figure 2) was constructed with the Molecular Evolutionary Genetic Analysis Package (MEGA VERSION 5.1) (Tamura K et al*.,* 2011). A boot strap analysis with 500 replicates was carried out to check the robustness of the tree. Boot strap re-sampling analysis, for the replicates was performed to estimate the confidence of the tree topologies.

Artificial challenge studies

Bacterial suspension was prepared by culturing the isolates on trypticase soy agar (TSA) plates at 30°C for 24 h and harvesting them with 50 ml of 0.85% physiological saline. Colony forming unit (CFU) per mL of this solution was determined by plating 10 fold dilu-

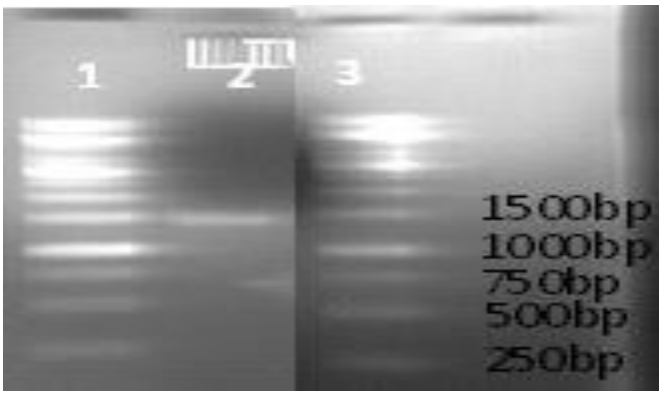

Figure 1. Ribo print pattern of isolate DNA on agarose gel.

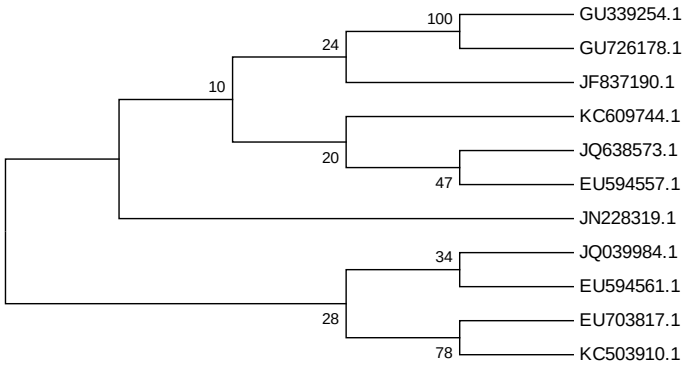

Figure 2. Neighbor-joining tree constructed using Mega 5.1 showing phylogenetic relationships of 16s RNA sequences from diseases fish to closely related sequences from Gen Bank.

Table 1. Physico- chemical parameters of water.

Parameter	Mean value± Sd
Water temperature	28.5±0.5°C
pH	8.2±0.458
Ammonia	1±0.416 mg\L
Nitrite	0.8±0.2 mg\L
Calcium	54.6±7.02 mg\L
Magnesium	91.3±6.42 mg\L
Alkalinity	473.3±30.55 mg\L
Hardness	155±5.56 mg\L
Chlorides	175.6±4.5 mg\L
TDS	1710±36 mg\L
Conductivity	1.1457±0.024 ms\cm^2
DO	1.76±0.25 mg\L

dilution series. For this purpose, the solution was diluted with distilled water. Apparently active healthy, fingerlings of *P. sutchi* (50± 10 g) were taken from the fish farm of Kaikalur, AP, India. They were stocked in 500 L cement tanks filled with fresh water and acclimatized in the laboratory condition for two weeks before starting the experiment. They were fed with standard diet in 2 divided doses daily during the experiment. Water was exchanged partially to remove left out feed and fecal matter. The lethal dose LD_{50} of the isolate was estimated according to Reed and Muench (1938). Five groups (Group1- 5) with 6 fish in each group were challenged with a series of dilutions of bacteria. The bacterial suspension prepared in phosphate buffered saline (0.15 M, pH 7.4) was injected to each fish intraperitoneally with 0.1 ml of different dilutions of bacteria. The final concentration of the bacteria injected to each was 10^5 - 10^8 CFU/mL. Control fish was injected with 0.1 mL phosphate buffered saline without bacteria. Mortality was observed till 5 days, and pathogenicity was confirmed by re-isolating the bacteria from experimentally infected fishes.

RESULTS AND DISCUSSION

The result of physico-chemical parameters of waters are presented in Table 1. Water chemistry results indicated that variation in ranges of pH, ammonia, nitrite and total dissolved solids, alkalinity, biological oxygen demand (BOD) and chemical oxygen demand (COD) show great impact on aquatic biota including fish. Inoculated fish isolates on RS medium resulted in green colour colonies are 1.5 to 2.5 mm in diameter, circular, convex, smooth and slightly opaque with entire margins. The nature of the cell wall composition of isolate was tested by gram staining method; the results confirmed the organisms as gram negative rods. The green colour colonies are positive for ONPG, lysine, ornithine, citrate, H_2S, voges proskaver, melonate, trehalose and negative for urease, phenyl alanine, nitrate reduction, methyl red, indole, esculin, melibiose, and glucose, respectively. The isolate showed good growth on nutrient agar, brain heart infusion agar, and tryptone soya agar. The isolate grew best in the temperature range of 30 to 37°C and pH 6 to 8. Biochemical results of green colonies are given in the Table 2. Sequencing analysis revealed a 100% identity with the sequence corresponding to the 16s r RNA gene of *A. schindleri YNB 103 strain* (Gen Bank accession number JQ 039984.1). Experimental infection study confirmed the pathogenicity of *A. schindleri* to *P. sutchi*. The LD_{50} of *A. schindleri* $10^{8.35}$ CFU per fish, which indicates the isolated strain, was highly virulent and capable of causing re-infection in *P. sutchi* and cause death in experimentally infected *Pangasius* fingerlings and showed similar signs even in the collected fishes from the tank outbreak (Table 3).

For effective cultivation of the fish, good quality water is needed; due to lack of sustainable management practices in water quality, fishes are prone to stress and susceptible to different diseases. All living organisms have optimum range of pH where growth is best. Water with high alkalinity not more buffered and the degree of pH fluctuation is high. Alkalinity changes can affect the primary productivity in cultured ponds. Dissolved oxygen is not at all problem to *P. sutchi* because it is air breathing fish. Elevated levels of ammonia causes gill damage and reduce the growth of fishes. Water temperature show direct impact on metabolism, feeding rates, respiratory rates of aquatic biota, and influence the solubility of oxy-

Table 2. Physical and biochemical characteristics of *A. schindleri* YNB 103.

Character	*A. schindleri*
Colony colour	Green
Gram reaction	Negative
Shape (R/C)	Rod
Motility	Non motile
Growth at different temp (°C)	
20	Negative
25	Negative
30	Positive
35	Positive
42	Positive
Growth on different media	
Nutrient agar	Positive
BHIA	Positive
Rimler-Shots agar medium	Positive
Tryptone soy agar	Positive
Growth in NaCl (w/v)	
2	Negative
4	Negative
6	Positive
8	Positive
10	Negative
Oxidative/Fermentative	Oxidative
Acid-fast test	Negative
Oxidase reaction	Negative
ONPG	Positive
Lysine	Positive
Ornithine Decarboxylase	Positive
Urease	Negative
Phenylalanine	Negative
Nitrate reduction	Negative
H_2S	Positive
Citrate	Positive
VP	Positive
MR	Negative
Indole	Negative
Production of acid from	
Melonate	Positive
Esculin	Negative
Arabinose	Variable
Xylose	Variable
Adonitol	Variable
Rhamnose	Variable
Cellobiose	Variable
Melibiose	Variable
Saccharose	Variable
Raffinose	Variable
Trehalose	Positive
Glucose	Negative
Lactose	Variable

Table 3. Lethal dose value CFU per mL of *A. schindleri*.

Group	Log dose	Death	Survived	Death	Cumulative survival	Total	Mortality ratio	Mortality (%)	LD 50
Control PBS 0.1ml	0	0	6	0	14	14	0/14	0	10^8 35 cfu/ml
CFU 10^8 2.1	0.322	2	4	2	8	10	2/10	20	
CFU10^7 3.4	0.531	3	3	3	4	7	3/7	42	
CFU$10^6$4.2	0.623	5	1	5	1	6	5/6	83	
CFU10^5 5.4	0.732	6	0	6	0	6	6/6	100	

gen. Nitrite results from feed can disrupt the oxygen transport in live fishes. Hardness of culture waters depends on levels of calcium and magnesium.

High total dissolved solids value directly indicates the presence of organic matter in culture waters. Culture water with high organic matter not only pollutes the tank, but also surrounding areas and support growth of different pathogens like causative agents of fulminant sepsis of *P. sutchi*. Some feed companies using animal meets, in place of soya while making feed pellets, it may be the indirect reason for entry of hospital landscape organisms in to aqua culture settings. Ponds treated with variety of chemotherapeutics to control different diseases, also affect the normal flora of pond bottom, it is also another reason to develop multi drug resistant bugs in to culture waters. After observing the gross symptoms of fish we postulated that emerging of new bacterial member's involvement in disease. Artificial challenge studies determined that *the* isolate can become pathogenic to *P. sutchi*. Out of 5 groups, control group fishes were injected with phosphate buffered saline, no mortality was observed, up to the end of the experiment. CFU 10^5 5.4 group shows 100% mortality of fishes within 48 h. As per Reed and Muench formula LD_{50} $10^{8.35}$ was determined for *P. sutchi*. To the best of knowledge, there was no track record of *A. schindleri* eye infection in fishes till date around the globe.

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