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SHORT PAPER

Wohlfahrtiimonas chitiniclastica Fulminant Sepsis in Pangasius Sutchi-First Report

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Abstract

This communication provides an insight in to the emerging of new infection fulminant sepsis in *Pangasius sutchi* and aimed to screen prime pathogens involved in disease. The pathogen was isolated from diseased *P. sutchi* characterized by *morphological, biochemical and molecular* approach, which includes 16s r RNA gene sequencing. PCR amplified 16s RNA was separated using agarose gel electrophoresis, eluted product was sequenced and blast analysis was carried out to identify the pathogen. *Wohlfahrtiimonas chitiniclastica* with LD_{50} dose $10^{8.35}$ has been initiated re-infection in experimentally infected *Pangasius* fingerlings. This study provides the evidence of newly emerged *Wohlfahrtiimonas chitiniclastica* which was true causative agent of fulminant sepsis in *Pangasius.* There was no track record of *Wohlfahrtiimonas chitiniclastica* fulminant sepsis infection in *P. sutchi* till date around globe. To the best of knowledge, this is the first report of *Wohlfahrtiimonas chitiniclastica* infection in *Pangasius sutchi.*

Keywords: *Pangasius sutchi,* 16s r RNA gene sequencing, *Wohlfahrtiimonas chitiniclastica,* challenge test.

Introduction

Pangasius sutchi is a exotic fish introduced to 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 shows 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 to culture waters, these stress the fish in enormous rate and make them susceptible to different diseases, and 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 was reported by (Dung *et al*., 2008). These pollution problems may support the growth of *Wohlfahrtiimonas chitiniclastica* in culture waters. *Wohlfahrtia magnica* fly only develops in live vertebrates and livestock animals were reported earlier by (Nedelchev., 1998; Hall and Wall., 1995; Farkas *et al*., 1997; Toth *et al*., 1998). *Wohlfahrtiimonas* members show broad host range of humans and livestock animals like sheep, pig, cattle. The larvae of *Wohlfahrtia magnica* bearing *Gamma proteo bacterium* was reported by (Toth *et al*., 2008), it's very close to Ignatzschineria larvae, another bacterial member associated with this and it is responsible for wound myiasis in cattle was reported by (Toth *et al*., 1998). The bacterium was later discovered in swine waste by (Juteau *et al*., 2004). From fore gut of Romney breed sheep, strains E43 and $S5^T$ were isolated by (Toth *et al.,* 2008), closely belongs to *Gamma proteo bacterium. Wohlfahrtiimonas chitiniclastica bacterimia* infection in homeless women was reported by (Rebaudet *et al.*, 2009). First case of fulminant sepsis due to *Wohlfahrtiimonas chitiniclastica* was reported in homeless 70 year old patient by (Almuzara *et al*., 2011). The present study aimed to identify pathogens at molecular level from diseased *P. sutchi* suffering from fulminant sepsis and to prove them as primary agents in disease.

Materials and Methods

Collection of Water and Diseased Fish Samples

Sick fish samples were collected from above said locality ponds, moribund fish samples (10) were collected, brought to the laboratory, 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 color, shrunken gastro intestinal tract and spleen, and hemorrhages on internal body cavity. Three water samples were collected from sequential days 15 to 45 to check 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 liver, gastrointestinal tract and blood samples were collected with syringe, one transferred on to Rimler Shots agar medium along with visceral organ samples (Hi media, Bombay), and another blood sample processed by automatic cell counter (Procan, China). 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) according to manufacturer instructions. Later organisms were subjected to molecular characterization, to differentiate organisms up to species level.

DNA Extraction

Extraction of genomic DNA and PCR-mediated amplification of the 16s r RNA gene of bacterial strain was carried out as per the method described by (Stewart *et al.*, 1993). DNA from saturated bacteria liquid cultures was extracted by above said method, including collection of bacterial cell pellet by centrifugation; lysis of cell pellet was attained by suspending in TE buffer with 100Kg of proteinase K and 0.5% SDS final concentrations. After one hour of the incubation at 37ºC the lysate was treated with 80Kl of 5M NaCl and 100Kl of 10% CTAB solution. Cell lysate was incubated at 60ºC for 10min. 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 the salts. The DNA pellet was allowed by air drying and re-suspended in 50Kl of deionized water with 1Kl 10mg 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 50µl volume containing 2 µl of total DNA having 54ng per µl concentration, 200 M each of the four de oxy nucleotide tri phosphates, 1.5 µl $Mgcl_2$, 5µl 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 1min at 95 °C, annealing for 30 s at 56°C, and extension for 1min 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 according to 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 sequences were aligned with those of the reference microorganisms 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/**clustal**o/*). Neighbor joining phylogenetic tree (Figure 2) was constructed with the Molecular Evolutionary Genetic Analysis Package (MEGA VERSION 5.1) (Tamura *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

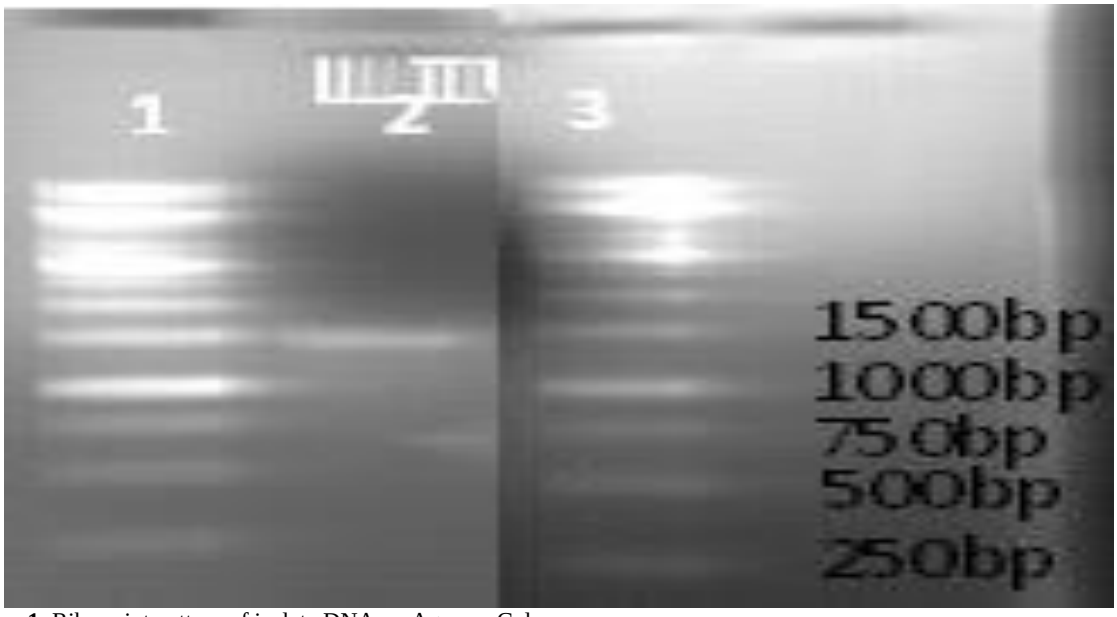

Figure 1. Ribo print pattern of isolate DNA on Agarose Gel.

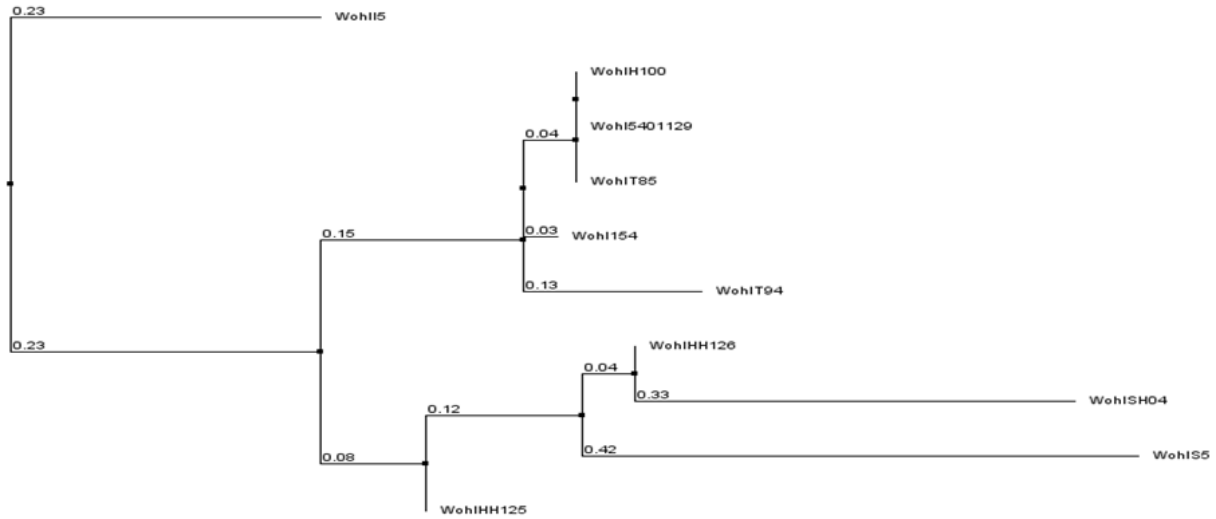

Figure 2. Neighbor- Joining tree constructed using Mega 5.1 showing phylogenetic relationships of 16s RNA sequences from diseases fish to closely related sequences from Gen Bank.

topologies.

Artificial Challenge Studies

Preparation of Bacterial Suspension

Bacterial suspension was prepared by culturing the isolates on TSA plates at 30 °C for 24 hours and harvested them with 50 ml of 0.85% physiological saline. Colony forming unit CFU per mL of this solution was determined by plating 10 fold dilution series. For this purpose, the solution was diluted with distilled water. Apparently active healthy, fingerlings of *Pangasius sutchi* (50±10 grams weight) were taken from the fish farm of Kaikalur, AP, India. They were stocked in 500L 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 0.1ml of different dilutions of bacteria. The final concentration of the bacteria injected to each was 10^5 - 10^8 CFU per mL. Control group fish was injected

with 0.1mL phosphate buffered saline without bacteria. Mortality was observed for 5 days, and pathogenicity was confirmed by re isolating the bacteria from experimentally infected fishes.

Results

The results of various physico chemical parameters of water were presented in (Table 1). Water chemistry results indicated that variations in ranges of pH, ammonia, nitrite, total dissolved solids, and alkalinity, show great impact on aquatic biota including fish. Inoculated fish isolate on RS medium plates were resulted in yellow color, small convex, entire, smooth glistening colonies. 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 isolate showed good growth on nutrient agar, brain heart infusion agar, and tryptone soya agar. The isolate grow best in the temperature range between 20 to 42ºC and resist up to 2 to 10% (w/v) salt concentration. Biochemical test results of yellow colored colonies are presented in the (Table 2). The yellow color colonies are positive for lysine, ornithine, citrate, H_2S, melonate, trehalose, esculin, and glucose tests. The yellow color colonies are negative for ONPG, urease, phenylalanine, vogues proskaver, methyl red, and indole. The haemotological parameters results are presented in (Table 3). The control group fishes showed normal ranges of hemoglobin, total leukocytes, and plate let count as 15.78g/dl, 59580/ µl, and 83,000 respectively, but diseased fishes show altered blood parameter ranges respective to above mentioned, their values as follows hemoglobin 4.7, total leukocyte count 13,300 and plate lets 53,000 respectively, it indicates that fishes were suffered from thrombocytopenia, leucopenia, and anemia.

Table 1. Various Physico- chemical parameters of water

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

Sequencing analysis revealed 100% identity with the sequence corresponding to the 16s r RNA gene of *Wohlfahrtiimonas chitiniclastica SH 04 strain* (Gen Bank accession number JQ796717.1). Experimental infection study confirmed the pathogenicity of *Wohlfahrtiimonas chitiniclastica* to *Pangasius sutchi*. The LD_{50} of *Wohlfahrtiimonas chitiniclastica* $10^{8.35}$ CFU per fish, which indicates the isolated strain, was highly virulent and capable of causing re- infection in *P. sutchi* and cause septic shock death in experimentally infected *Pangasius fingerlings* and showed similar sings to though collected from the tank outbreak (Table 4).

Discussion

For effective cultivation of the fish, good quality water is needed, due to lack of sustainable management practices in water quality, fishes prone to stress and susceptible to different diseases. All living organisms have optimum range of pH where the 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 oxygen. Nitrite results obtained? from feed can disrupt the oxygen transport in live fishes. Hardness of culture waters is depending 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*. After observing the gross symptoms of fish, we postulated emerging of new bacterial member's involvement in disease. Isolation and identification of pathogen were done in invitro by *cultural, biochemical, and molecular* methods. 16s r RNA gene sequence of *W. chitiniclastica SH 04* isolated from fulminant sepsis disease of *P. sutchi* (Gen Bank Accession number KC 463801) showed 100% identity with the sequence corresponding to the *W. chitiniclastica strain SH 04* (Gen Bank Accession number JQ 796717.1), which was isolated from *Chrysomya mega cephala* (Xiao- Meicao *et al.*, 2011). *W. chitiniclastica SH 04* with accession number KC 463801 showed 99% similarity with *W. chitiniclastica* (Gen bank accession number EU 484335), (Rebaudet *et al.*, 2009), and showed 99% similarity with *W. chitiniclastica* strain number 154 (Gen Bank Accession number JF 692205) (Marisa *et al.*, 2011). Artificial challenge studies determined

Table 2. Physical and biochemical characteristics of *W. chitiniclastica* SH 04

Characters	*W. chitiniclastica SH 04*
Colony colour	Yellow
Gram reaction	Negative
Shape (R/C)	Rod
Motility	Non motile
Growth at different temp ^{0}C	
20	Positive
25	Positive
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	Positive
4	Positive
6	Positive
8	Positive
10	Positive
Oxidative/Fermentative	Oxidative
Acid-fast test	Negative
Oxidase reaction	Positive
ONPG	Negative
Lysine	Positive
Ornithine Decarboxylase	Positive
Urease	Negative
Phenylalanine	Negative
Nitrate reduction	Variable
H_2S	Positive
Citrate	Positive
VP	Negative
MR	Negative
Indole	Negative
Production of acid from	
Melonate	Positive
Esculin	Positive
Arabinose	Variable
Xylose	Variable
Adonitol	Variable
Rhamnose	Variable
Cellobiose	Variable
Melibiose	Variable
Saccharose	Variable
Raffinose	Variable
Trehalose	Positive
Glucose	Positive
Lactose	Variable

Table 3. shows result of altered blood chemistry of diseased fish (vs) control group

Parameter	Control group Mean value± SD	Diseased fish
Hemoglobin	15.78±1.145	4.7g\ dL
TLC	59580±3723	13300 10^3µL
Neutrophils	15.02±1.613	15 µL
RBC	2.144±0.455	0.76 µL
Platelets	83,000±10393	53,000µL
PCV	23.32±4.352	9.5 µL
MCV	129.68±2.68	126.1fL
MCH	65.38±6.62	61.8pg
MCHC	50.12±4.796	49.4g\ dL

Table 4. shows lethal dose value CFU per ml of *Wohlfahrtiimonas chitiniclastica*

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	
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	10^8 35 cfu/ml
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	

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. CFU10^5 5.4 group shows 100% mortality of fishes within 48 hours. As per Reed and Muench formula LD_{50} 10^8 35 was determined for *P. sutchi*. All the experimentally infected fishes were died with septic shock. The identified organisms from moribund fishes are important members to cause infections in human and livestock animals like cattle, pig, sheep, not in fish. It is one of the emerging infection in *P. sutchi* around globe.

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