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Studies on Ulcerative Disease Caused by ProvidenciastuartiiBacteria in Indian Major Carp, Labeo rohita (Ham.)

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Abstract: Rohu (Labeo rohita) is a species of fish of the carp family cyprinidae. A survey was carried out from September, 2012 to August, 2013 to study the prevalence of bacterial ulcerative disease in L. rohita, cultured in a freshwater farm located at Moongilthuraipattu Village of Villupuram District, Tamil Nadu, India. To mimic the infection in healthy fish, organs such as liver, gill and ulcerative skin were used to prepare the inoculum in PBS buffer. The inoculum was injected into healthy animals through intramuscular injection, immersion challenge and oral route. The tissue supernatant from infected rohu fish was screened for isolation and screening of the causative organism responsible for ulcerative lesion in L. rohita. Based on the biochemical, morphological and molecular features the bacterial culture isolated from infected rohu was tentatively identified as Providencia species like bacterium which was further confirmed by the analysis of its 16S rRNA gene using PCR and DNA sequencer and it has been identified as P. stuartii. Further, its partial sequence was deposited in GenBank (KF155520.1).

Key words: Labeo rohita, Providencia stuartii, 16S rRNA gene, GenBank (KF155520.1)

I. INTRODUCTION

Providencia species are found in multiple animal reservoirs, including flies, birds, cats, dogs, cattle, sheep, guinea pigs, penguins, and are resident oral flora in reptiles such as pythons, vipers, and boa. Providencia species are also found commonly in soil, water and sewage. Examples of Providencia infections in animals include neonatal diarrhea due to P. stuartii infection in dairy cows and enteritis caused by P. alcalifaciens infection in dogs. P. rettgeri has been isolated in crocodiles with meningitis/septicemia and in chickens with enteritis [1]. P. heimbachae has been isolated in penguin feces and from an aborted bovine fetus [2]. The genus Providencia, belonging to the family Enterobacteriaceae, consists of 9 species, namely P. alcalifaciens, P. stuartii, P. rettgeri, P. rustigiani, P. heimbachae, P. vermicola, P. sneebia, P. burhodogranarieaand P. thailandensis [1], [3-6]. In human, Providencia species have been isolated from urine, stool, blood, sputum, skin and wound cultures. One case study has described P. stuartii as the etiology of infective endocarditis,[7]. Papadogiannakis[8] studied on the P. stuartii infection with severe skin ulceration and cellulitis isolated from a dog. Among Providencia species, P. rettgeri is the only species isolated from farmed fish (Hypophthalmichthysmolitrix) in Israel. Till date, it is the only report implicating this organism as a fish pathogen[9]. In view of acknowledging this, P. vermicola was the first species isolated from freshwater fish, L. rohita and its partial 16S rRNA sequence was deposited in GenBank by the authors of this study (accession no. KF155518.1.).

In the present study, moribund young L. rohita with clinical signs of ulcer on the abdomen and pectoral fin surfaces was observed in freshwater fish farms. A work was undertaken to isolate the causative organism responsible for mortality of Indian major carp, rohu. Experimental infection for high mortality of L. rohita was carried out to reproduce the infection in healthy fish using the bacterial cultures isolated from the infected fish. Llobrera and Gacutan[3] studied the Aeromonas hydrophila associated with ulcerative disease epizootic in Laguna de Bay, Philippines. John Thomas[11] studied the ulcerative disease caused by A. caviae-like bacterium in Indian catfish, Clariasbatrachus. The
gram negative, rod shaped bacterium isolated from Indian freshwater carp, L. rohita was tentatively identified as P. stuartii. In order to acquire more information on the taxonomic position, DNA from the Providencia sp. was isolated, polymerase chain reaction was performed and 16S rRNA gene of the sample was amplified and sequenced to confirm the species affecting the freshwater fish, L. rohita. The partial gene sequence of 16S rRNA belongs to P. stuartii was deposited in GenBank, with accession no. KF155520.1

II. MATERIALS AND METHODS

A. Collection and Maintenance of Naturally Infected L. rohita

The moribund freshwater fish L. rohita (56-69g) with the signs of red ulcers on the abdomen and base of pectoral fin were collected from a fish farm located at Moongilthuraipattu Village of Villupuram District, Tamil Nadu and transported within three hours at 30°C in live state to the laboratory in an aerated polythene bag. In laboratory, the infected animals were maintained in 75 L aquarium tanks with tap water at a temperature of 27-30°C. The animals were fed with commercial fish feed.

B. Physico-chemical Parameters of Water

The physico-chemical parameters of the pond water such as temperature, pH, dissolved oxygen (DO), biochemical oxygen demand (BOD) and chemical oxygen demand (COD) of the pond were determined following standard protocols [12].

C. Isolation and Characterization of Bacterial Strain from Infected L. rohita.

The organs liver, gill and ulcerative skin from naturally infected fish were dissected out and homogenized with sterile PBS buffer. The diluted samples were plated on nutrient agar, Aeromonas agar and tryptcase soy agar by spread plate technique and incubated at room temperature for 24-48 h. Overriding colonies (from tryptcase soy agar) were selected and again streaked on tryptcase soy agar for locating pure cultures. These pure cultures were maintained on tryptcase soy agar for further biochemical and molecular characterization studies. Biochemical characterization was done and the bacterial isolates were identified according to Bergey’s manual of Determinative Bacteriology[13].

D. Molecular Identification of Bacterial Strain

1) Bacterial Genomic DNA Isolation:

Genomic DNA was extracted and purified by following the standard techniques [14], [15] with slight alterations. Luria broth was inoculated with a loop full of pure culture and incubated overnight. From this, 1.5 mL of grown culture was centrifuged at 10000 xg for 30 minutes, the resulted pellet was suspended in 600 µL of TE buffer. To this, 45 µL of 10% SDS was added followed by addition of 5 µL lysozyme and mixed well. The mixture was incubated for 1 h at 37° C. After incubation, 500 µL of phenol: chloroform was added and the mixture was mixed well by inverting the tube until the formed two phases completely mix with each other. Then the sample was centrifuged at 10000 xg for 15 min. The aqueous phase was transferred to a new centrifuge tube, to this equal volume of phenol: chloroform was again added and centrifuged at 10000 xg for 15 min. The resulted aqueous phase was mixed with 50 µL of 3M sodium acetate in a new micro centrifuge tube and mixed well. To this, 300 µL of isopropanol was added and mixed gently to precipitate the DNA, then the mixture was centrifuged at 10000 xg for 10min. The resulted pellet was washed with 70% ethanol for 30 sec and centrifuged at 8000 xg for 1-2 min. The obtained pellet was resuspended in 100 µL of TE buffer and stored at 4° C for further analysis. Then the sample (5 µL) was allowed to run in 0.8% agarose gel to confirm the presence of bacterial genomic DNA under UV light.

2) Bacterial PCR and DNA Sequencing:

30-cycle amplification was performed in a DNA thermal cycler (Eppendorf, Germany). For a 30µL reaction: 15µL of Taq DNA polymerase PCR master mix, 3µL of amplified DNA, 3 µL of Universal bacterial forward primer
E. Collection and Maintenance of Experimental Animals

Disease-free, Labeo rohita were collected from the Department of Fisheries, Mettur Dam, Tamil Nadu with no record of ulcerative syndrome. The live fish were transported to laboratory in an aerated bag and maintained in 700 L FRB tank with continuous aeration at room temperature (27- 30°C) with tap water. The animals were fed twice a day with commercial fish feed.

F. Reproduction of Bacterial Infection

Ulcerative skin from the naturally infected fish was cut and homogenized with PBS buffer. The homogenized sample was centrifuged at 1000 xg for 10 min at 4°C. Healthy and active fish were injected intramuscularly with 5U of suspension and maintained for a period of 7 days. The control fish were injected intramuscularly with 5U of sterile PBS. Experimental fish were examined often for clinical signs of disease and mortality.

G. Experimental Infection of Bacterial Isolate in Healthy L. rohita

Infectivity of the new bacterial isolate from the naturally infected fish was studied in healthy individuals. Immersion, intramuscular and oral route of administrations were followed to determine the mode of infection and the pathogenicity of bacterial isolate in healthy carp, Labeo rohita based on the standard protocols [16], [17].

H. Preparation of Bacterial Inoculum

The bacterial cultures isolated from naturally infected fish was grown on nutrient agar for use in pathogenicity experiments. The pathogenicity of the bacterial isolate was tested by bath immersion, intramuscular injection and oral administration. The bacterial count was determined by standard dilution and plating methods [18].

I. Experimental Infection by Immersion Method

The experiment was carried out by following [11] with some modifications. Healthy fish (12 fish per tank) were reared in aquarium tanks of 75 L capacity containing sterilized freshwater with continuous aeration. Air stones and air tubes were sterilized by immersing them in 5% sodium hypochlorite and by washing them thoroughly with sterilized water before use. The tanks were covered to prevent contamination. Aseptic techniques were used throughout the experiment. Fish were fed with commercial fish feed. For the experimentally induced infection, the fish were exposed to different concentrations of bacterial cells (10^2, 10^4, 10^6, 10^8 and 10^10 CFU ml^-1). The control consisted of fish exposed to sterile freshwater alone.

J. Infection by Intramuscular Injection

The experiment was carried out by following [11] with some modifications. Fish (12 per dosage and tank) were maintained in 100 L FRB tank containing sterilized freshwater at room temperature. The bacterial isolate was inoculated into healthy fish through intramuscular injection, near the dorsal fin at doses of 10^8, 10^9, 10^10 and 10^12 CFU per animal. Control fish were inoculated only with sterile PBS buffer.

K. Oral Infection

Fish were individually secluded in the aquarium tanks and starved for 24 h. Each fish was fed with a piece of fish meat which was injected with 1 ml of bacterial suspension (10^{12} CFU). The fish were fed thrice, with an interval of 8 h. After the last feeding with infected meat, the animals were fed with non-infected meat for 7 days. In the control group, fish were fed only with non-infected meat. In all experiments, animals were examined once in 8 h a day for clinical signs of disease and mortality.
L. Confirmation of pathogenicity

The specific action of the bacterial sample isolated from naturally infected fish as a pathogen was confirmed by re-isolating the bacterium from the liver, gill and ulcerative skin of moribund fish to fulfil Koch's postulates. The samples were inoculated on tryptase soy agar plates by spread plate technique for the isolation of bacterial pathogen. The isolated bacteria were recognized using the earlier described procedure.

III. RESULTS AND DISCUSSION

The physico-chemical features of the fish farm water were determined. The ranges of temperature, pH, DO, BOD and COD were: 26-30 °C, 6.3-8.1, 5.7-7.1 mg/L, 2.36-2.89 mg/L and 183-209 mg/L, respectively. A high temperature of 30 °C was recorded during summer. Death of fish due to bacterial pathogen extended to 99-100% within 2-3 days after the presence of ulcer on the surface of infected fish samples. The clinical signs of the diseased fish contained ulcerative lesions on the abdomen/body surface and bases of the pectoral fin with reddish color on the surface of the infected portions. The bacterial inoculum prepared from ulcerative tissue samples of diseased fish alone produced clinical indication of ulcerative abrasion in the disease free, healthy L. rohita fish under experimental condition. Studies were carried out on different organs like ulcerative skin, liver and gill acquired from the infected fish samples. Single prevailing bacterial culture isolated from each of the media was tested for their infectivity in the healthy L. rohita, but the bacterial isolate from trypticase soy agar and nutrient agar alone resulted mortality and reproduced the signs of ulcerative lesions in healthy L. rohita. This specific bacterial isolate alone was selected and identified based on the colony morphology, biochemical, physiological and molecular identification. Colonies grown on TSA plates were Gram-negative rods, motile, circular, 2.1 – 2.2 mm in diameter, slimy and convex. Colonies are smooth with entire edges and an intense characteristic smell was produced with growth on trypticase soy agar. The biochemical characterization of the bacterial culture resulted in positive for citrate, inositol, glycerol, gelatin, sorbitol, lyssole, D-Mannose, D-Serine, catalase, indole, phenyl alanine, glucose, nitrate reduction, trehalose, and methyl red. Negative results for D-Mannitol, D-Xylose, cellobiose, esculin, sorbitol, pigmentation, oxidase, lactose, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, H₂S, Voges-Proskauer, gas from glucose, L-Arabinose, maltose, raffinose, salicin, D-Xylose, deoxyribonuclease and lipase. Based on the biology, biochemical characterization and morphology of the isolated bacteria from infected L. rohita was tentatively identified as P. stuartii like bacterium. Further confirmation of P. stuartii like bacterium by analyzing its 16S rRNA gene was performed using PCR. For this, bacterial universal primers 27F & 1525r were used for the amplification of 16S rRNA gene and the results were shown in Fig.1. The sequencing of the strain discovered a homology of 99% with P. stuartii. Hence it is a Providencia sp. with features of P. stuartii.

The exposure of L. rohita to Providencia like bacterium was tested by immersion, intramuscular injection and oral infection. The maximum concentration of Providencia sp. like bacterium (30 x 10⁶ CFU ml⁻¹) caused 33.33, 43.33, 56.6, 63.3 and 87.3% of mortalities through immersion method at 18, 30, 48, 84 and 108 h of post exposure, respectively. The LC₅₀ value of Providencia sp. like bacterium was determined. It was found to be 3.22 x 10³, 3.69 x 10⁴ and 5.85 x 10⁴ CFU ml⁻¹ at 18, 36 and 54 h of post injection respectively. The highest concentration, 54 x 10⁷ and 54 x 10⁶ possible cells of Providencia like bacterium per animal caused 100% death within 96 and 60 h of post infection respectively. During bath exposure, whereas the lower presentation of 54 x 10³ and 54 x 10⁴ feasible, study bacterial cells, per animal caused 43.33 and 73.3% death within 120 and 108 h of post infection. The LD₅₀ value of Providencia sp. like bacterium for intramuscular course was defined at different time intervals and was found to be 2.1 x 10² and 1.04 x 10³ per animal after 72 and 96 h of post injection, respectively. There was no mortality in the oral route administered L. rohita fish. The infection of Providencia sp. like bacterium was established by satisfying Koch’s postulate.

Rohu is considered to be the high number of fish cultivated than other major carps in India. It has high market value and are easily available for culture. The risk of diseases in rohu also increased in making the rohu susceptible to diseases in association to the exotic carps [18]. Diseases are the most serious limiting factors in aquaculture, because of increased density of fish in restricted water where the fish pathogens can transmit from one to another [19]. The rohu is
being mainly affected by bacterial pathogens. Among bacterial pathogen, ulcer disease is the major one, followed by columnaris and dropsy. Among bacterial pathogens, P. rettgeri was one of them which caused a mass mortality among farmed silver carp, Hypophthalmichthys molitrix in Israel during 1976. Till date, this has been the only report associating this organism as a fish pathogen [9]. This disease tempted severe epidemics of ulcerative diseases in farmed fish. From 2012, an ulcerative disease which is caused by a bacterium has been witnessed in nursery and grow-out ponds located in the Villupuram District of Tamil Nadu, India. The clinical signs of infected rohu fish include large red ulcer lesions on the abdomen and base of pectoral fin. The indications in the infected rohu fish agrees with the reports of [20], [11] who have reported a related clinical indication in other bacterial infections. Isolation and biochemical characterization were performed on organs such as liver, Gill and ulcerative skin from infected rohu using nutrient agar (NA), Aeromonas agar and trypticase soy agar (TSA). Single pure colony was isolated from TSA and NA plates which exactly revealed the presence of culture colony in the specific medium, which was again studied in healthy rohu fish to confirm that the bacterial sample from infected rohu fish was a pathogen in order to fulfil the Koch’s postulates. Many authors have reported that, Aeromonas and Pseudomonas spp. are causing major infections in L. rohita (FAO, 2007) which persuade with severe lesions of ulcerative diseases in fish from Southeast Asia especially in India. In afford with colony morphology, biochemical and molecular identifications the disease caused in L. rohita was identified as Providencia sp. like bacterium. This is the second report in L. rohita for the presence of Providencia sp. occurring naturally in cultured freshwater fish. Hence, only one report was available about the infection of P. rettgeri isolated and studied in silver carp, H. molitrix in 1976 reported by [9]. Similar ulcerative lesion induced by Aeromonas sp. in many fishes were stated in many reports [21], [22], [11]. Hence in the present study, the bacterial culture Providencia sp. like bacterium was confirmed as a pathogen to satisfy Koch’s postulates in normal rohu fish. DNA isolation and PCR were executed to detect the bacterial strain at molecular level using universal primers specific to 16S rRNA gene of prokaryotes. The application of 16S rRNA gene sequence is to study the bacterial phylogeny and taxonomy which include important reasons like (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the roles of the 16S rRNA gene over time has not changed, recommending that random sequence changes are a more precise degree of time (evolution); and (iii) the 16S rRNA gene (1500bp) is large enough for informatics purposes [23], [11]. The PCR band (~1500bp) confirmed the presence of Providencia sp. Species identification was done using 16S rRNA gene sequencing. Unfortunately, on no account definition for species identification using 16S rRNA gene sequencing exhibited. In not any studies does the definition of a species “match” ever exceed 99% similarity [11]. The species of Providencia was identical as P. stuartii and it (Providencia) is the second report for its infection in L. rohita, its 16S rRNA gene partial sequence was deposited in GenBank with accession no. KF155520.1. Whereas, the bacterium P. vermicola was first isolated and reported from India by [4] from an infected juvenile nematode, Steinernemathrophilum. The authors of this study have submitted the 16S rRNA partial sequence of P. vermicola bacteria from L. rohita in GenBank with accession no. KF155518.1. The responsibility of rohu fish, L. rohita to P. stuartii was tested by bath exposure (immersion), intramuscular injection and oral route in L. rohita. Finally, the significant gateway of the pathogen is by means of penetration of tissue at the site of wounds or injuries. The expiry by the above experiment showed that the pathogenicity of P. stuartii in L. rohita depends mainly on the doses and period of exposure.

IV. CONCLUSION

Bacterial diseases are responsible for high mortality in both wild and cultured farm fish. The real role of microorganisms vary from a major pathogen to that of an opportunistic pathogens which makes its host organisms (fish) moribund by commencing infection development. Bacterial flora of fish is directly proportional to its environment. In the present study, clear variations in physico-chemical parameters has been observed which may be considered as conducive condition for the outbreak of disease. Biochemical and molecular characterization of the species confirmed that it is the first report, species stuartii of genus Providencia causing infection and mortality in the freshwater fish, L. rohita infection. From the pathogenicity experiments it is evident that the main portal entry of the pathogen is by means of penetration of tissue at the site of rashes or wounds and the mortality of experimentally infected animals depends on the dosage and time period of exposure.
ACKNOWLEDGEMENT

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Description of Providencia vermicola isolated from diseased Indian major carp, Labeo rohita (Hamilton, 1822)

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a b s t r a c t
A strain of Gram-negative, urease-positive, motile, Providencia bacteria, with colony size of 1.8–2.2 mm was isolated from trypticease soya agar (TSA)-medium, originally isolated from diseased Indian major carp, Labeo rohita (rohu) during the survey period from August, 2012 to July, 2013 at a commercial fish farm in Nerijipettai-village, near Mettur Dam, Tamil Nadu, India. The diseased rohu showed ulcerative lesions on the abdomen surface and bases of the pelvic fin with reddish coloration. The present study investigated the species through biochemical reaction and PCR amplification of 16S rRNA locus. Experimental infection assays with reproduced isolate was conducted and pathogenicity (by immersion, intramuscular and oral route) was demonstrated in healthy rohu fish. Histopathological analyses of the pathogen exposed gill, liver and skin showed moderate structural variations. Since no Providencia was detected in fish feedstuffs and pond water as the source of this pathogen remains unknown, our study revealed that the Providencia-infection may be due to the handling methods and from the poultry feces aside from the human feces from nearby ponds. Hence, Providencia isolate is regarded as an opportunistic pathogen for rohu. This is the first report of Providencia which can cause disease in cultured fish, L. rohita.

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1. Introduction

The genus Providencia includes urease-producing Gram-negative bacilli that are responsible for a wide range of human infections. Providencia species are found in multiple animal pools including flies, cats, birds, dogs, cattle, sheep, penguins and guinea pigs and are resident oral flora in reptiles like pythons, vipers and boas. The genus Providencia, belonging to the family Enterobacteriaceae, consists of 9 species, viz. Providencia alcalifaciens, Providencia stuartii, Providencia rettgeri, Providencia rustigianii, Providencia heimbachae, Providencia vermicola, Providencia sneebia, Providencia burhodogranariea and Providencia thailandensis (Janda and Abbott, 2006; Jumeja and Lazaro, 2009; Khuntongpan et al., 2013; Somvanshi et al., 2006). Among those, P. rettgeri is the only species isolated from farmed fish (Hypophthalmichthys molitrix) in Israel. To date, it is the only report implicating this organism as a fish pathogen (Bejerano et al., 1979). Among bacterial pathogens, Vibrio species have been predicted as fish pathogens; particularly, Vibrio alginolyticus is known to be associated with diseases of many farmed and wild fish globally (Austin, 2010; Austin and Austin, 2007; Austin and Zhang, 2006; Frans et al., 2011). However, the role of Providencia sp. as a primary pathogen has been questioned in fish pathology insofar as its recovery from diseased animals has been erratic (Austin and Austin, 2007).

The present study pertains to the description of characteristics of a unique microbial species isolated from the diseased fish, Labeo rohita. Further, an infection experiment was conducted on L. rohita using the cultured Providencia sp. and the pathological changes of the diseased/infected fish have been described.

2. Materials and methods

2.1. Collection and maintenance of naturally infected L. rohita

A total of 29 moribund freshwater fish, L. rohita (~68-85 g) showing the symptoms of ulcers on the abdomen, base of pelvic fin and around the head were collected from a commercial fish farm in Nerijipettai-village, near Mettur Dam, Tamil Nadu, India during August, 2012–July, 2013 i.e., August, September, October and November, 2012 & July, 2013. The moribund fish samples were transported to the laboratory within one and a half hour under continuous aeration at a 30 °C temperature.

2.2. Physico-chemical characteristics of pond water

The temperature, pH, dissolved oxygen (DO), biochemical oxygen demand (BOD), and chemical oxygen demand (COD) of the pond water were determined by adopting standard procedures. All chemical analyses were performed according to standard procedures described by APHA (1998).
2.3. Isolation and characterization of bacterial strain from infected L. rohita

The organs (liver, gill and ulcerative skin) from naturally infected fish were dissected out and homogenized with sterile PBS buffer. The diluted samples were plated on nutrient agar, Aeromons agar and tryptase soy agar by spread plate technique and incubated at room temperature for 24–48 h. Dominant colonies from tryptase soy agar were selected and streaked again on tryptase soy agar plate towards re-isolating the pure cultures. These pure cultures were maintained on tryptase soy agar for further biochemical and molecular characterization studies. Biochemical characterization was done and the bacterial isolates were identified according to Bergey’s manual of determinative bacteriology (Buchanan and Gibbons, 1974).

2.4. Bacterial genomic DNA isolation, PCR & sequencing

Genomic DNA was extracted and purified by following the techniques of Claridge (2004) and Grimont and Grimont (1995) with some alterations. Then the resulted DNA sample (5 µl) was allowed to run in 0.8% agarose gel to confirm its presence under UV light. After confirmation, the DNA sample was PCR amplified using universal bacterial primers and screened at the 16S rRNA locus and the resulted PCR product was purified and sequenced. Then, the sequence was analyzed using Chromas Lite version 2.0 and aligned with reference genotypes from GenBank. The details of the primer sequences, amplified product sizes and annealing temperatures are given in Table 1. P. vermicola (DSM-17386) was procured from DSMZ, Germany for comparison.

2.5. Fish feedstuff and water sample analyses

In order to determine the source of the pathogen, feedstuff and pond water were analyzed using PROVIDENCIA selective medium (TSA medium) and using PCR by employing genus-specific primers. Feedstuff samples were dissolved with sterile saline, optimum supernatants and water samples were streaked on selective medium plates for isolation of PROVIDENCIA sp., 100 ml of pond water sample was concentrated to 1 ml after being spun at 2000 ×g for 15 min and the resultant sample was used for confirmation of PROVIDENCIA through PCR detection.

2.6. Collection and maintenance of experimental animals

Healthy, disease-free carp fishes, L. rohita were obtained from the Tamil Nadu-state Inland Fisheries Department, Mettur Dam. The live fish were transported to the laboratory in aerated bags and maintained in a 700 L FBB tank with continuous aeration at room temperature (27–30 °C). The health status of the fish was detected based on physical appearance and internal organs followed by swabs from body surface, gill and liver immediately upon arrival at the lab and at 14 day intervals later (Austin and Austin, 1989). All fish were acclimatized for 14 days prior to use and fed twice a day with commercial fish feed.

2.7. Reproduction of bacterial infection

Sections of abraded part of infected fish were cut and homogenized with PBS buffer. The homogenized sample was centrifuged at 1000 ×g for 10 min at 4 °C. Healthy and active fish were injected intramuscularly with 50 µl of suspension and maintained for a period of 7 days. The control fish were injected intramuscularly with 50 µl of sterile PBS. Experimental fish were examined twice a day for clinical signs of disease and mortality.

2.7.1. Experimental infection of bacterial isolate in healthy L. rohita

Infectedness of the new bacterial isolate from the naturally infected fish was studied in relation to healthy individuals. Immersion, intramuscular and oral route methods of administrations were followed to determine the mode of infection and the pathogenicity of bacterial isolate in healthy carp, L. rohita based on the protocols followed by Egidius (1987) and John et al. (2013). The bacterial count was determined by standard dilution and plating methods (Ducklow et al., 1980).

2.7.2. Experimental infection by immersion method

The experiment was carried out by following John et al. (2013) with slight modifications. Fish were exposed to different concentrations of bacterial cells (10^3, 10^4, 10^5, 10^6 and 10^7 CFU ml^-1). The control consisted of fish exposed to sterilized freshwater alone.

2.7.3. Infection by intramuscular injection

The experiment was carried out by following the method of John et al. (2013) with some modifications. The bacterial isolate was inoculated into healthy fish through intramuscular injection, near the dorsal fin at doses of 10^3, 10^4, 10^5, 10^6 and 10^7 CFU per animal. Control fish were inoculated only with sterile PBS buffer.

2.7.4. Oral infection

Fish were individually isolated from the aquarium tanks and starved for 24 h. Each of the fish was fed with a piece of fish meat which was already injected with 1 ml of bacterial suspension (10^12 CFU). The fish were fed thrice, with an interval of 8 h. After the last feeding with diseased meat, the animals were fed with non-infected meat for 7 days. In the control group, fish were fed only with non-infected meat. In all experiments, animals were examined once in every 8 h for their clinical signs of disease and mortality.

2.7.5. Confirmation of experimental infection by PCR

The pathogenicity in experimentally infected animals was confirmed by using nested primers in PCR. PROVIDENCIA specific 16S-forward and reverse primers were used (Shima et al., 2012). The details of the primer sequences, amplified product sizes and annealing temperatures are given in Table 1. For this, the bacterial DNA was isolated from the pure cultures which were obtained from experimentally infected fish skin samples by following the procedures as described in Sections 2.3 & 2.4.

2.7.6. Validation of pathogenicity

The precise action of the bacterial sample isolated from naturally infected fish as a pathogen was confirmed by re-isolating the bacterium from the liver, gill and ulcerative skin of moribund fish to fulfill Koch’s postulates. The samples were inoculated on tryptase soy agar plates by spread plate technique for isolation of bacterial pathogen.

2.8. Tissue preparations & histology

At the end of exposure time after intramuscular injection, the fish samples were sacrificed after 48 h of infection by decapitation. Gill, liver and skin along with control fish organs were dissected out, were fixed in 10% neutral buffered formalin solution for 24 h and then were processed for paraffin embedding. Paraffin blocks of gill, liver and skin were cut at a 6 µm thickness and stretched on decontaminated glass slides. After deparaffinization, sections were stained with hematoxylin-eosine and observed under light microscope. The histopathological changes in the organs were examined in the randomly selected sections.

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Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Annealing temp (°C)</th>
<th>PCR product size (bp)</th>
<th>Sequences</th>
<th>Primer orientation</th>
</tr>
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<tbody>
<tr>
<td>16S rRNA external</td>
<td>55</td>
<td>1500</td>
<td>AGGTTTGATCTGTCGTCGAG</td>
<td>Upstream</td>
</tr>
<tr>
<td>16S rRNA internal</td>
<td>50</td>
<td>515</td>
<td>ACCGATAATCTCTTAGG</td>
<td>Upstream</td>
</tr>
</tbody>
</table>

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Histopathological changes induced by treatments in the tissues were photographed.

3. Results

The physico-chemical features of the fish farm water were determined. The ranges of temperature, pH, DO, BOD and COD were: 26–32 °C, 6.8–7.9, 5.9–7.8 mg/L, 2.36–2.89 mg/L and 168–196 mg/L, respectively. A high temperature of 31–32 °C was recorded during summer. Death of fish due to bacterial pathogen extended to 99–100% within 2–3 days after the presence of infection on the surface of infected fish samples. The clinical signs of the diseased fish contained ulcerative lesions on the abdomen/body surface and bases of the pelvic fin with reddish coloration on the surface of the infected parts.

From the 29 moribund fishes investigated, the skin samples of only 16 fishes revealed the presence of Providencia. The bacterial inoculum prepared from ulcerative skin sample of diseased fish alone produced clinical indication of ulcerative abrasion in the disease free, healthy L. rohita fish under experimental condition.

Colonies grown on TSA plates were found to be Gram-negative rods, motile, circular, 1.8–2.2 mm in diameter, shining, slim, convex, and dense with a brownish centre and hyaline periphery. After 48 h of growth soluble brown pigment was observed, that colored the medium around the colonies. The colonies of entire edges were found to be smooth and characteristic, and intense smell was produced during their growth on trypticase soy agar. The biochemical characterization of the bacterial culture showed positive reactions for catalase, nitrate reduction, acid production from glucose & d-mannose, urease, inositol and L-arabinose, and negative reactions to amygdalin, sucrose, rhamnose and arginine dihydrolase. Based on the above characteristics the bacterial genus is tentatively identified as Providencia.

The genomic DNA was isolated and observed under UV light. Further confirmation of P. vermicola-like bacterium by analyzing its 16S rRNA gene was performed using PCR. For this, bacterial universal primers (27F & 1525r) were used for the amplification of 16S rRNA encoding gene and the results are shown in Fig. 1. The sequencing of the strain revealed a homology of 99% with P. vermicola. Hence, it is a Providencia sp. with features of P. vermicola. The partial gene sequence of 16S rRNA of P. vermicola was deposited in GenBank with accession no. KF155518.1. Based on the molecular analysis the bacterium has been confirmed as P. vermicola.

For the source determination of P. vermicola, the fish feedstuff and water samples were investigated using specific primers during PCR, but no positive results were acquired.

The immersion method administration of Providencia sp. like bacterium (31 × 10^6 CFU mL^-1) caused 33.3, 40, 56.6, 63.3 and 73.3% of mortalities at 18, 24, 48, 84 and 96 h of exposure, respectively. The LC50 value of Providencia sp. like bacterium was found to be 3.01 × 10^6, 3.38 × 10^6 and 5.1 × 10^6 CFU mL^-1 at 18, 30 and 48 h of postinjection respectively.

The intramuscular injection of the maximum concentrations of 52 × 10^6 and 52 × 10^6 feasible cells of Providencia like bacterium per animal caused 100% death within 72 and 48 h of booster respectively. But the application of the lowest concentrations of 52 × 10^3 and 52 × 10^3 feasible study-bacterial cells per animal caused 52.6 and 73.3% of deaths within 96 h of booster. The LD50 value of Providencia sp. like bacterium for intramuscular course was defined at altered time intervals and was found to be 1.98 × 10^6 and 1.01 × 10^6 per animal after 60 and 84 h of postinjection, respectively. There was no mortality in the oral route administered L. rohita fish.

The PCR study revealed the presence of a specific band at 515 bp (Fig. 2). The gill of L. rohita is normally composed of primary lamellae arranged in rows, bulged on the lateral sides of which there are alternately arranged secondary lamellae with periodic distributions as pillars. Mainly there was cartilaginous cores with traces of sinusoidal blood spaces and these structures are evenly distributed (Figs. 3A & 4A). The experimentally infected fish showed epithelial hyperplasia, lamellar fusion (shrinking), epithelial lifting, epithelial necrosis and desquamation. And the cartilaginous rod at the core of primary lamella was found to be disrupted (Figs. 3B & 4B). The hepatic parenchymatous appearance was observed with homogenous cytoplasm, increased cytoplasmatic vacuolation, blood sinusoids and central nucleus in liver (Figs. 3C & 4C). In experimental fish, hepatic necrosis and irregular cytoplasmatic vacuolation were seen with converging sinusoids (Figs. 3D & 4D). Normal skin of L. rohita showed the presence of mucus gland with architectural structures (Figs. 3E & 4E). In the experimental fish skin, the mucus gland was very much disrupted (Figs. 3F & 4F).

4. Discussion

Generally, outbreaks of bacterial diseases are largely responsible for the high mortality of wild and farm-cultured fish. The real role of microorganisms varies from a major pathogen to that of an opportunistic one which make their hosts (fish) become moribund by commencing infection development. Presently observed ulcerative disease breakout could be related to the recorded unusual variations in the concentrations of pH and BOD. Similar physico-chemical factors that induced disease outbreaks in rohu fishes were earlier reported by Roberts (1989) and Mastan and Osman Ahmed (2013).

P. vermicola sp. nov. was first reported by Somvanshi et al. (2006) which was mainly based on the results of 16S rRNA gene sequencing. However, reports on Providencia sp. associated with fish-sources are rare. P. rettgeri was one of those that caused mass mortality among farmed silver carp, H. molitrix, in Israel during 1976 (Bejerano et al., 1979). And to this date, that is the only report pertaining to the Providencia caused fish disease. Presently, the fish infection by Providencia like bacterium has been established by satisfying Koch’s postulate. Further, the recorded specific band at 515 bp confirmed the

![Fig. 1. Agarose gel showing the amplification of 16S rRNA gene of Providencia vermicola.](image)

![Fig. 2. Agarose gel showing the amplification of 16S rRNA gene of Providencia vermicola.](image)
Fig. 3. Gill, liver and skin histology of Labo rohita in control (A, C, E) and intramuscular exposed organs after 48 h (B, D, F). A: Micrograph of gill tissue from control showing the thickness of primary lamellar epithelium, appearance of secondary lamellar epithelium, and normal presence of central vein. B: Gill of exposed fish showing epithelial lifting, necrosis and desquamation. C: Control group showing hepatocytes with central nucleus, converging sinusoid RBC and vacuolation. D: Liver of exposed fish showing hepatic necrosis, irregular vacuolation and jamming of central vein. E: Control group of skin showing mucus gland with architectural structures. F: Skin of exposed fish showing the disrupted mucus gland and architecture.

Fig. 4. Gill, liver and skin histology of Labo rohita in control (A, C, E) and immersion exposed organs after 48 h (B, D, F). A: Micrograph of gill tissue from control showing the thickness of primary lamellar epithelium, thin and squamous appearance of secondary lamellar epithelium, and normal appearance of central vein. B: Gill of exposed fish showing epithelial lifting, necrosis, cartilaginous disruption and desquamation. C: Control group showing central vein in hepatocytes with central nucleus. D: Liver of exposed fish showing hepatic necrosis, irregular vacuolation and converging blood sinusoids. E: Control group of skin showing mucus gland with architectural structures. F: Skin of exposed fish showing the disrupted mucus gland and shrunken architecture.
presence of P. vermicola and its infection in fish through immersion and intramuscular mode of administration. Moreover, the molecular analysis showed that our strain is closely related to P. vermicola with a 99% similarity in 16S rRNA sequence analysis. The role of the 16S rRNA gene over time has not changed and, hence, the recommendation that random sequence changes are more precise over an evolutionary period besides large base pair presence is enough for informative purpose.

The observed marked degeneration of the gills and other organs in 48 h infected fish would be prone to other complications. As the gills of fish are responsible for respiratory and osmoregulatory processes, oxygen uptake and disruption of osmoregulatory function might have occurred. Further, toxicity induced liver-damage might prevent its detoxification role, as opined earlier by Figueiredo-Fernandes et al. (2007) and Mishra and Mohanty (2008). Importantly, the infection caused skin damages were largely facilitating the microbial invasion and further colonization (Mac Law, 2001).

The P. vermicola may be a member of normal flora of rohu fish but it becomes pathogenic under some specific conditions and therefore an opportunistic one to fish. The findings during our infection-experiment in rohu fish underlined the pathogenicity potential of P. vermicola.

This is the first case report of Providencia species associated with fish disease in India. During infection experiment, it was confirmed that bacterial transmission through intramuscular route caused fish mortality.

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