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Identification and Pathogenicity of *Pseudomonas aeruginosa* DJ1990 on Tail and Fin Rot Disease in Spotted Snakehead

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Abstract

Tail and fin rot disease (TFRD) is a big issue in the production of spotted snakehead, *Channa punctata* Bloch. The aims of the present study were to isolate and identify the bacterial pathogen causing TFRD, to detect histopathological changes in tissues (fin, tail, liver, and kidney), and to ascertain the antibiotic sensitivity pattern of the isolate. Out of six bacterial isolates, only the isolate DJ1990 was found to be the causal candidate of TFRD in *C. punctata*. Identical histopathological changes were detected in tail, fin, liver, and kidney under light and scanning electron microscope in both collected diseased fish and artificially infected fish. The isolate was identified as *Pseudomonas aeruginosa* strain DJ1990 (National Center for Biotechnology Information Ace. No. KX709967) based on the biochemical characterization tests and 16S rDNA sequence-based phylogeny analysis. Artificial challenge test demonstrated that the strain DJ1990 was highly virulent (100% mortality at 48 h of postinjection period at the concentration of 1.5×10^7 CFU/g of body weight) for *C. punctata*. The isolate exhibited sensitivity to the broad-spectrum antibiotics but was resistant against aztreonum. To the best of our knowledge, this is the first report of *P. aeruginosa* as a TFRD-causing candidate in *C. punctata*.

KEYWORDS

***Channa punctata*, histopathology, *Pseudomonas aeruginosa*, scanning electron microscope, tail and fin rot disease**

Aquaculture industries play an important role in maintaining economic growth in several countries such as China, India, Malaysia, Japan, Norway, and Brazil. According to the Food and Agriculture Organization (FAO 2016), India is the second-largest producer of fish after China. Disease in aquatic organisms, including fish, is a major threat to farmers and causes massive losses in production. Fish are susceptible to wide varieties of lethal diseases caused by different types of bacterial, fungal, viral, and parasitic agents (Taoka et al. 2006; Phani Kumar and Sree Ramulu 2013; Subharanjani et al. 2015; Banerjee et al. 2016; Nandi et al. 2017). According to FAO (2016), every year several countries, including India, face major losses in aquaculture production mainly due to bacterial diseases. Until now, several investigations have been carried out in the field of fish

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diseases and production; however, most of these studies have focused on major carps (*Catla catla*, *Labeo rohita*, and *Cirrhinus mrigala*) and catfish such as *Clarias batrachus* (Welker et al. 2005; Cruz et al. 2012; Banerjee et al. 2016; Nandi et al. 2017). The murrels, also known as *Channa punctata* (belonging to the family Channidae), constitute the dominant group of air-breathing freshwater fish in south and southeast Asian countries (Haniffa and Abdul 2011).

Channa punctata is an inexpensive source of animal protein and therefore is regarded as an important fish species. Hossain et al. (2009) reported that bacterial disease in *C. punctata* is a serious issue in Bangladesh, which causes about 7.5% mortality each year. The disease outbreak in *C. punctata* mainly occurs during the winter season. Among the bacterial diseases, the tail and fin rot disease (TFRD) is a major concern during the months of October to February in all North-East states of India. TFRD is a common disease in intensive fish culture systems, which is caused by several types of bacterial pathogens. Among them, Gram-negative bacteria, the genus *Aeromonas*, *Pseudomonas*, and *Vibrio*, are recognized as the most prevalent candidates (Faruk et al. 2004). Other genera reported to be associated with TFRD are Gram-positive bacteria such as *Flavobacterium* spp., *Acinetobacter* spp., and *Alcaligens* spp. (Rahman et al. 2010). In general, the symptoms of TFRD are slit, torn, or ragged tail fins, often with a white edge or signs of bleeding (Austin and Austin 1987). TFRD is also responsible for other symptoms such as skin ulcers, loss of color, and cloudy eyes. Austin and Austin (1987) have also reported petechial hemorrhages in internal organs such as liver and kidney as disease symptoms of TFRD. Therefore, the aim of the present investigation was to determine the etiology of TFRD of *C. punctata* that causes substantial mortality in the aquaculture sector. The present study was conducted to isolate and identify the bacterial pathogen responsible for TFRD. Along with effects on tails and other fins, we also have demonstrated the effect of TFRD in liver and kidney following the guidelines of Austin and Austin (1987).

Materials and Methods

Collection of Fish

During the winter months, diseased (clear symptom of TFRD) and healthy (no disease symptoms) *C. punctata* were collected from nearby fish firms in Guwahati, Assam, India. A total of 50 fish samples were collected and brought to the laboratory for investigation. The disease symptoms (ragged fins and blood hemorrhage on the surface of the skin and fins) were confirmed as per Austin and Austin (1987) guidelines.

Isolation of Bacterial Candidates

To isolate the causative agent, fish tissues (tail, fin, skin, kidney, and liver) were aseptically collected inside the laminar air flow. In brief, tissues were homogenized in 0.05 M phosphate buffer saline (PBS, pH 7.0), serially diluted, and plated on the tryptone soy agar medium (pH 7.0) (HiMedia, Mumbai, India). Plates were incubated at 30 C for 24 h. Separate colonies were picked and pure cultures were carried out by the repeated streaking method. Pure cultures were subjected to Koch's postulates. Isolate(s) that passed through Koch's postulates were the only ones considered for further studies.

In Vivo *Confirmation of Pathogenicity*

To check artificial infection, healthy (no external disease symptoms and normal swimming behavior) *C. punctata* with an average body weight ranging from 22 to 26 g were divided into five groups and acclimatized for 2 wk. Each group of fish (consisting of 10 individuals) were maintained in separate 25-L glass tanks in a flow-through system with dechlorinated, aerated, and temperature-controlled (28 ± 2 C) water before and during the experiments. The selected isolate(s) were grown in brain-heart infusion medium (HiMedia, Kolkata, India) at 28 C for 14 h, centrifuged at 5000 *g* for 10 min, and washed in 0.05 M sterile saline (pH 7.0), and the final pellet was suspended in sterile 0.05 M PBS solution (pH 7.0). The fish were injected intraperitoneally with 0.1 mL ($10^4 - 10^8$ CFU/mL) of pure cultured bacterial suspension. The control fish were injected with

0.1 mL of sterile 0.05 M PBS (pH 7.0). Clinical signs were recorded daily up to 7 d.

Histopathology

To detect histopathological symptoms, the liver and kidney tissue samples of control, naturally infected, and artificially infected fish (six fish in each group) were taken aseptically, fixed in 10% formaldehyde for 2 h, and embedded in paraffin wax. Sections of 5 µm each were cut with a microtome and stained with hematoxylin and eosin following the method of Ray et al. (2017) and examined under the light microscope (Leica DM 3000, Concord, Ontario, Canada).

Scanning Electron Microscopy (SEM)

Tail, dorsal fin, liver, and kidney tissue samples of control, naturally infected, and artificially infected fish (six fish in each group) were fixed in 2.5% glutaraldehyde (prepared in 0.1 M sodium cacodylate buffer, pH 7.2) for 3 h at 4 C following the method of Banerjee et al. (2015). The samples were then washed in 0.1 M sodium cacodylate buffer and postfixed in 1% buffered osmium tetroxide for 1 h. The samples were then washed first in the buffer and again washed with distilled water for half an hour. Dehydration was performed in ascending acetone gradation, followed by the tetramethylsilane (TMS) drying at 4 C for 10 min. The samples were then dried in TMS at 26 C and coated with gold using JFC-100 Ion sputter (JEOL USA, Inc., Peabody, MA, USA). The coated samples were observed under the JSM-6360 scanning electron microscope (JEOL USA, Inc., Peabody, MA, USA) with an accelerating voltage of 20 kV.

Biochemical Characterization of the Selected Isolate

The selected bacterial isolate was characterized by Gram's staining, standard biochemical assays, and carbohydrate fermentation tests (CFTs). Biochemical assays and CFTs were performed either manually or using the HiCarbo KB009-KT system (HiMedia, Mumbai, India) according to the manufacturer's instructions, except for the incubation temperature (28 C).

TABLE 1. *Colony morphology of the bacterial strains isolated from* Channa punctata *suffering from TFRD.*

	Colony morphology				
Isolates	Color	Margin	Diameter (mm)	Texture	Topology
DJ1987	Bright yellow	Rough	6.5	Slimy	Concave
DJ1988	Pale yellow	Entire	5	Slimy	Concave
DJ1989	White	Rough	2	Powdery	Flat
DJ1990	Transparent	Entire	3	Mucoid	Concave
DJ1991	Cream white	Entire	7	Mucoid	Flat
DJ1992	Orange	Rough	10	Slimy	Concave

TFRD = tail and fin rot disease.

The results of all biochemical tests along with CFT were interpreted following the guidelines provided by Bergey's Manual (Garrity et al. 2004) and Austin and Austin (2012). The bacterium was maintained in *Pseudomonas* selective agar media at 4 C.

16S rDNA Sequence Analysis

Genomic DNA was extracted using GSure Bacterial Genomic DNA Isolation Kit (GCC, Kolkata, India) following the manufacturer's instructions. The genomic DNA (40 ng) was used as the template in 50 µL reaction with polymerase chain reaction (PCR) master mix (Merk GeNei, Mumbai, India) containing 1X Taq Buffer, 2 mM $MgCl_2$, 0.5 mM dNTPs, and 1 U Taq polymerase along with 0.5 µM of Universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′) to amplify the 16S rRNA gene (Weisburg et al. 1991). The PCR conditions were: 5 min at 94 C, followed by 35 cycles (30 s at 94 C → 30 s at 50 C → 1 min 30 s at 72 C), and final extension at 72 C for 7 min using a thermo cycler (Applied Biosystems, Foster city, California, United States). The obtained sequence was submitted to the National Center for Biotechnology Information database. A phylogenetic tree was constructed by the neighbor-joining method using MEGA 6.0 software (Tamura et al. 2011; Hachioji, Tokyo, Japan). The distance matrix

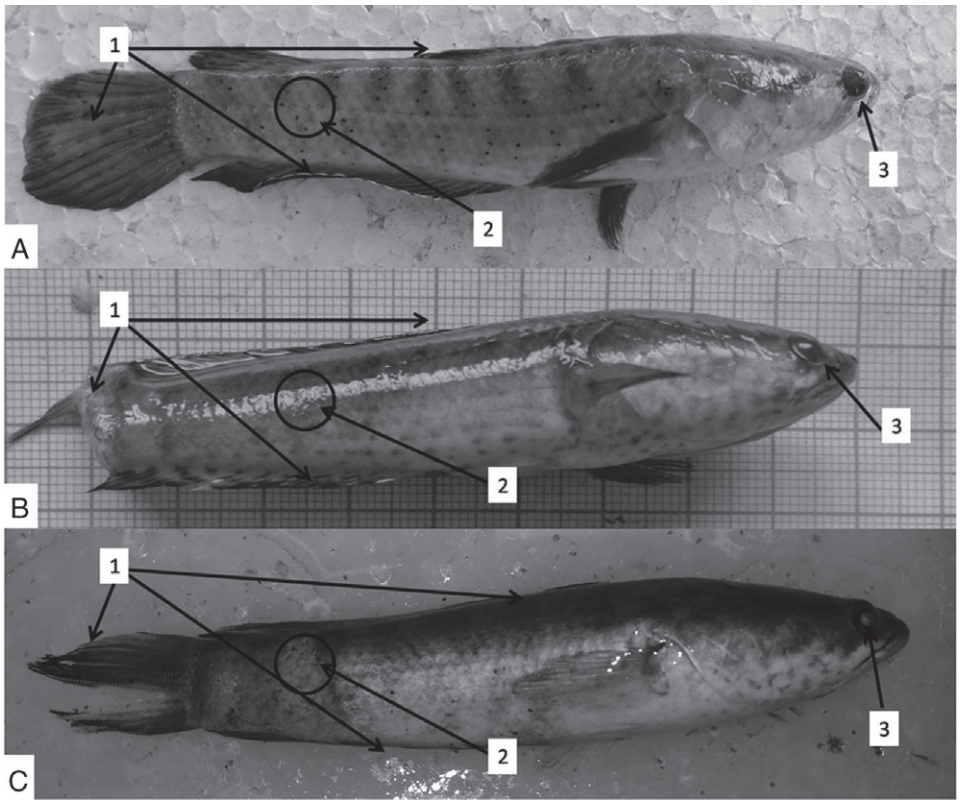

FIGURE 1. *Disease symptoms and the pathogenic effect of the bacterial strain DJ1990 on* Channa punctata*. (A) Control fish, (B) naturally infected fish showing symptoms of tail and fin rot disease (TFRD), and (C) artificially infected fish with strain DJ1990 showing symptoms of TFRD. TFRD symptoms are (1) torn or ragged tail and fins instead of entire tail and fins as evident in control fish, (2) loss of normal skin/scale color in comparison with control fish, and (3) cloudy eye in comparison with normal eye of control fish.*

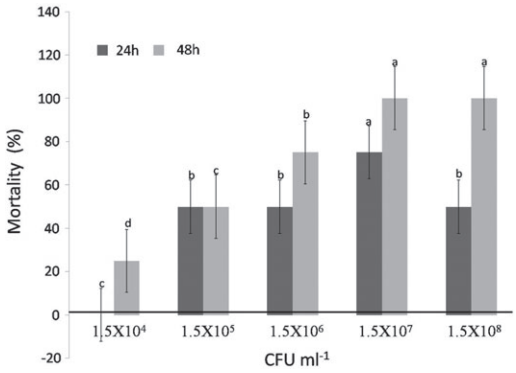

FIGURE 2. In vivo *mortality test of the bacterial strain DJ1990. Data are represented as mean* ± *SE.* n = *3 replicates. Different lowercase letters indicate significant* ($P < 0.05$) *difference.*

was calculated using Kimura's two-parameter correction.

Antibiotic Sensitivity Test

The antibiotic susceptibility of the isolate DJ1990 was studied following the Kirby–Bauer disk diffusion method (Bauer et al. 1966) in Mueller–Hinton agar Plates (pH 7.0) using commercial antibiotic disks (HiMedia, Mumbai, India). In brief, the entire agar surface was streaked horizontally, vertically, and around the edge of the plate to ensure a heavy growth. After allowing the plate to dry for 5 min, antibiotic disks were applied with the help of a Sensi-Disc dispenser. Each disk was gently pressed. All plates were kept in an inverted position at the same incubation temperature (37 C) for 48 h.

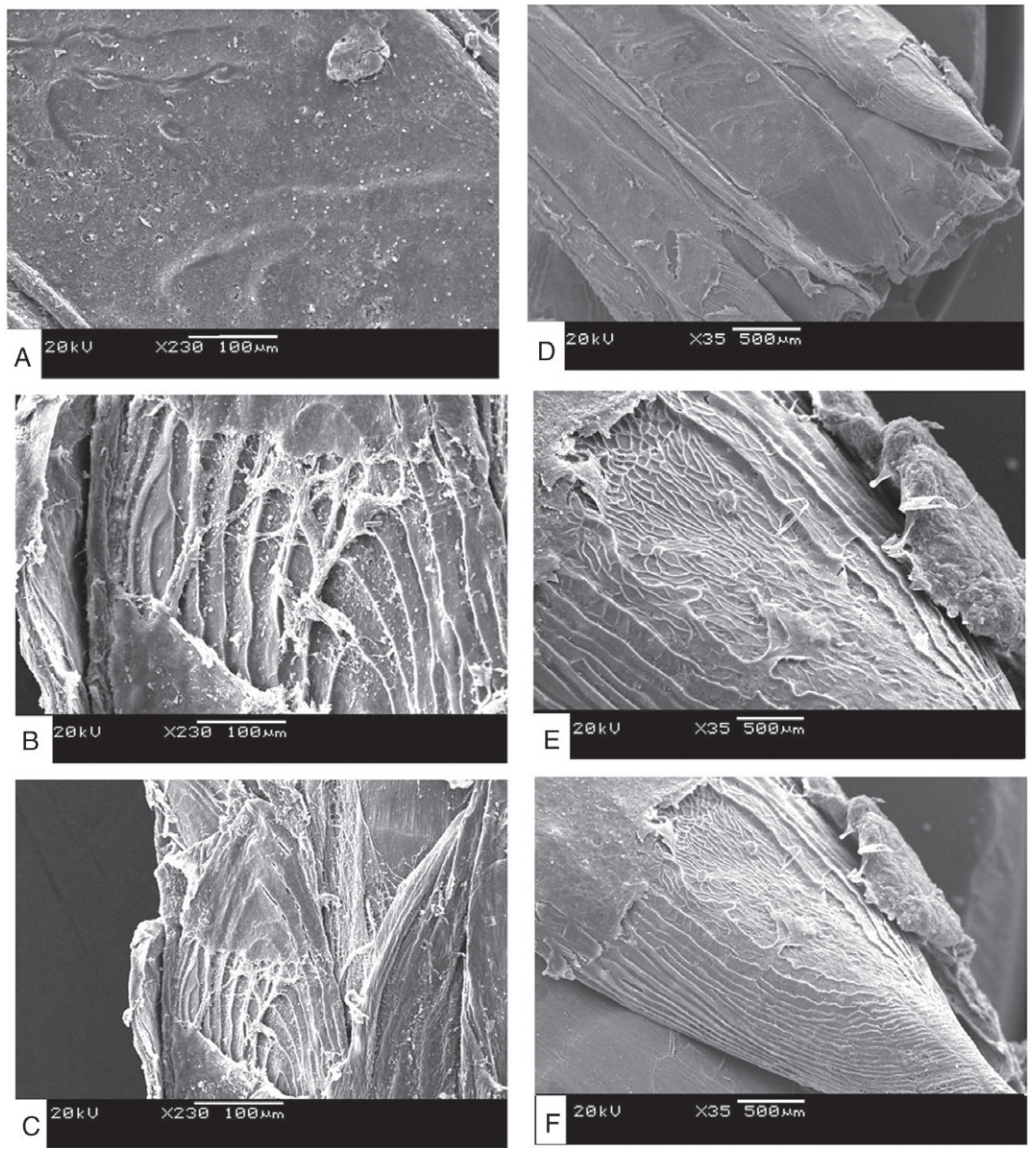

FIGURE 3. *Scanning electron micrograph of tail (A–C) and fin (D–F) from control (A, D), naturally infected (B, E), and artificially infected (C, F)* Channa punctata. *The erosion pattern of tail and fin is different but similar naturally infected (B, C) and artificially infected (E, F)* C. punctata.

The zone of inhibition was measured using a millimeter ruler.

Statistical Analysis

The ANOVA among different values was performed in Microsoft Excel 2007. The one-way ANOVA and Duncan's multiple range test were used with significance at the $P < 0.05$ level.

Results and Discussion

Bacterial Candidate

On the basis of distinctive colony morphology on nutrient agar plates, initially six isolates were chosen and pure cultures were performed by the repeated streaking method (Table 1). All strains were subjected to *in vivo* pathogenicity test. Only the strain DJ1990 was able to cause

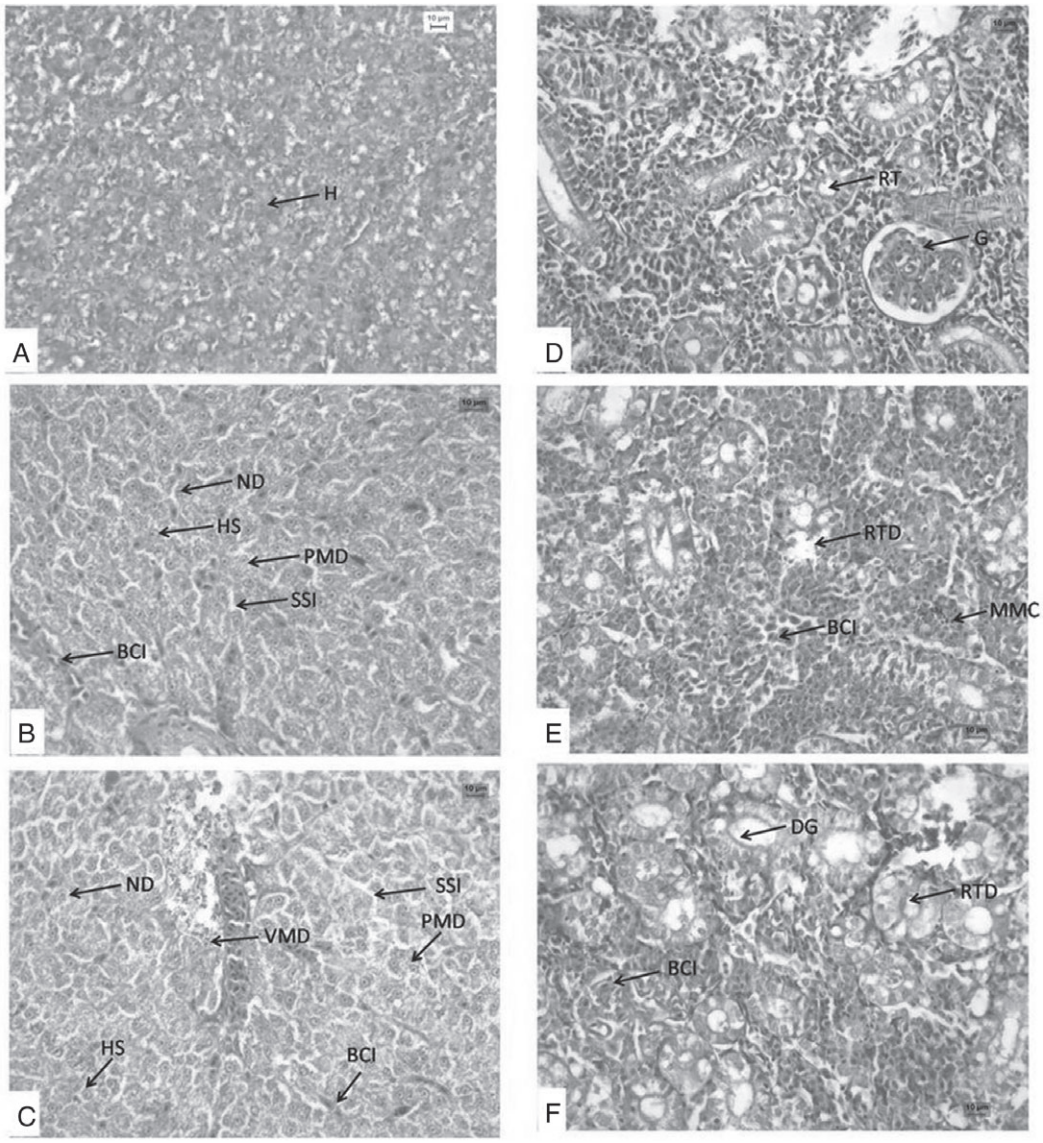

FIGURE 4. *Histopathology of liver (A–C) and kidney (D–F) from control (A, D), naturally infected (B, E), and artificially infected (C, F)* Channa punctata*. Different changes in liver (BCI, blood cell infiltration; HS, hepatocyte swelling; ND, nucleus degeneration; PMD, plasma membrane degeneration; SSI, sinusoidal space increase; VMD, vain membrane detachment) and kidney (BCI, blood cell infiltration; DG, deformation of glomerulas; MMC, melano-macrophage center; RTD, degeneration of renal tubules) were detected in both naturally and artificially infected fish when compared with control.*

TFRD with symptoms similar to those of the infection (Fig. 1) and was therefore selected for further investigation. The visible symptoms of TFRD detected in both naturally diseased fish and experimentally challenged fish were similar (Fig. 1).

Concentration-dependent Lethality

To check the disease-causing efficiency of the pathogenic strain, an *in vivo* experiment has been conducted with several doses of the selected bacterial strain (Fig. 2). It was observed that at lower concentration (1.5×10^4 CFU/mL) the

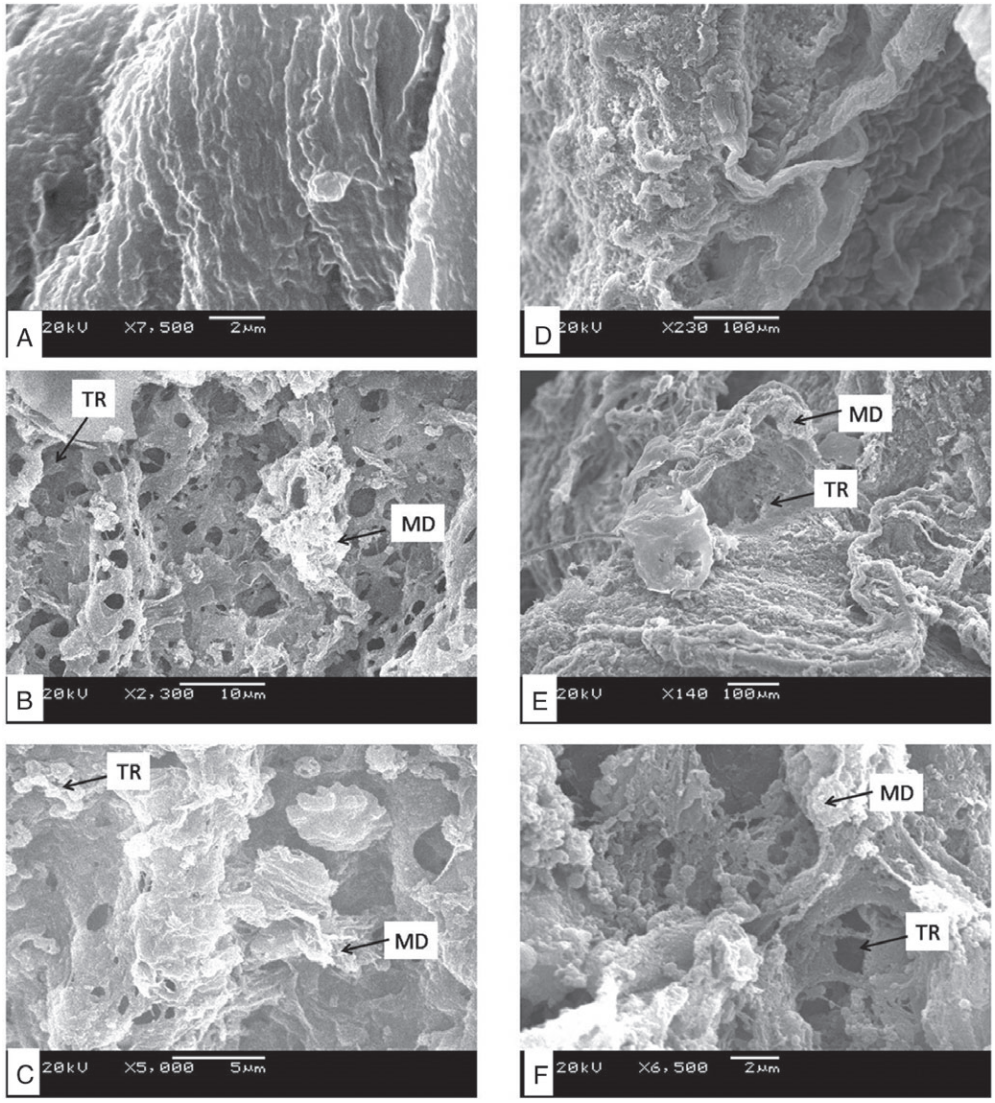

FIGURE 5. *Scanning electron micrographs of liver (A–C) and kidney (D–F) from control (A, D), naturally infected (B, E), and artificially infected (C, F)* Channa punctata*. Different changes in liver and kidney. MD and TR represent mucus deposition and tissue rupture, respectively.*

mortality was only 25% after 48 h of injection whereas at higher concentration, the mortality rate increased gradually and reached 100% (1.5×10^7 and 1.5×10^8 CFU/mL) at 48 h of postinfection period (Fig. 2). However, no such mortality was observed in the control fish, even after 13–14 d postinjection. The injected bacteria were reisolated from surviving fish (liver and kidney) and were confirmed by biochemical and 16S rDNA sequence analysis.

Aquatic animals, including fish, are always exposed to a wide range of pathogenic bacteria, which cause several types of diseases in fish such as ulcer, tail rot, fin rot, dropsy, and so on and create stress in fish. In the last few years, several reports have been published regarding bacterial diseases in fish and their mortality rate at different stages of the life cycle (Sakai et al. 1995; Chang and Liu 2002; Parthasarathy and Ravi 2011; Cruz et al. 2012; Dahiya et al. 2012;

TABLE 2. *Biochemical characterization of the strain DJ1990.*

Biochemical tests	Results
Catalase, oxidase, H_2S production	+
Indole, methyl red, Voges Proskauer	−
Starch hydrolysis	−
Carbohydrate fermentation tests	**Results**
Lactose, xylose, maltose, fructose, dextrose, raffinose, trehalose, melibiose	+
Galactose, sucrose	+/−
L-arabinose, mannose, inulin	+
Sodium gluconate	−
Glycerol	+
Salicin	+
Dulcitol	−
Inositol, sorbitol, adonitol, erythritol	+
Mannitol	−
α-Methyl-D-glucoside	+
Rhamnose, cellobiose, melezitose, α-methyl-D-mannooside, xylitol, O-Nitrophenol-β-D-galactosidase (ONPG), malonate utilization, sorbose	−
Esculin hydrolysis, D-arabinose, citrate utilization	+

+ = Positive reaction; − = negative reaction.

Ran et al. 2012; Saini et al. 2014; Koteshwar Rao and Benarjee 2015; Nandi et al. 2017). Hossain et al. (2009) examined the health status of *C. punctata* during the winter season and reported several types of disease signs in the mouth, gill, fin, and skin. Similarly, Marma et al. (2016) also reported disease symptoms (such as scale loss, red spot, and ulcer) in *C. punctata*. Several studies have reported the disease status of *C. punctata* during the winter season (Patwary et al. 2008; Koteshwar Rao and Benarjee 2015); however, none of them have identified the causative agent. In this study, we have reported the TFRD agent in *C. punctata*, which cause about 35% mortality in aquaculture sectors in Assam, India, during the winter months.

Histopathology

Furthermore, the pathogenicity of the selected bacterial strain was confirmed by histopathology and SEM examination of tail, fin, liver, and kidney tissues. In TFRD, tail and fins are the primary sites of infection. Scanning electron micrograph examination demonstrates similar types of erosion in tails and fins in both DJ1990-challenged fish and naturally TFRD-infected fish (Fig. 3). The results clearly indicate the potential of the isolate DJ1990 to cause TFRD in *C. punctata*.

The histopathology of liver of challenged and naturally infected fish exhibited a distinct plasma membrane degeneration and nucleus degeneration (Fig. 4). The hepatocyte swelling and blood cell infiltration (BCI) were prominent in infected fish (Fig. 4) compared with the control. Along with sinusoidal space increase, vain membrane detachment and abscess formations were also observed in the hepatic parenchyma (Fig. 4). In the histology of the kidney of control fish, normal structure and systematic arrangement of renal cells, glomerulas, and renal tubules were observed (Fig. 4D). However, degeneration of renal tubules, BCI, and deformation of glomerulas were detected in infected fish (Fig. 4). Hemorrhage and melano-macrophage center were also found in kidneys because of the release of blood cells. The above-mentioned observations clearly indicate disease symptoms in this fish species.

To further prove the pathogenicity of the selected bacterial isolate, SEM examination of liver and kidney tissues were also conducted. Marked tissue rupture and excessive mucus deposition in both liver and kidney were observed in both naturally and artificially infected fish (Fig. 5). It is already well established that kidney and liver are also infected during TFRD (Austin and Austin 1987; Loch and Faisal 2015). Our present observations are in accordance with the report of Austin and Austin (1987).

Characterization and Identification of the Selected Bacterial Strain

The isolate DJ1990 grew profusely on *Pseudomonas* agar base (SRL, Pune, India) medium supplemented with glycerol (2% v/v) and gave a characteristic blue pigment at 72 h of incubation. Cells were aerobic, motile, and Gram-negative rods. The biochemical reactions and CFT of the selected isolate DJ1990 corresponded with those of *Pseudomonas aeruginosa* (Table 2). Finally, the isolate DJ1990 was identified by

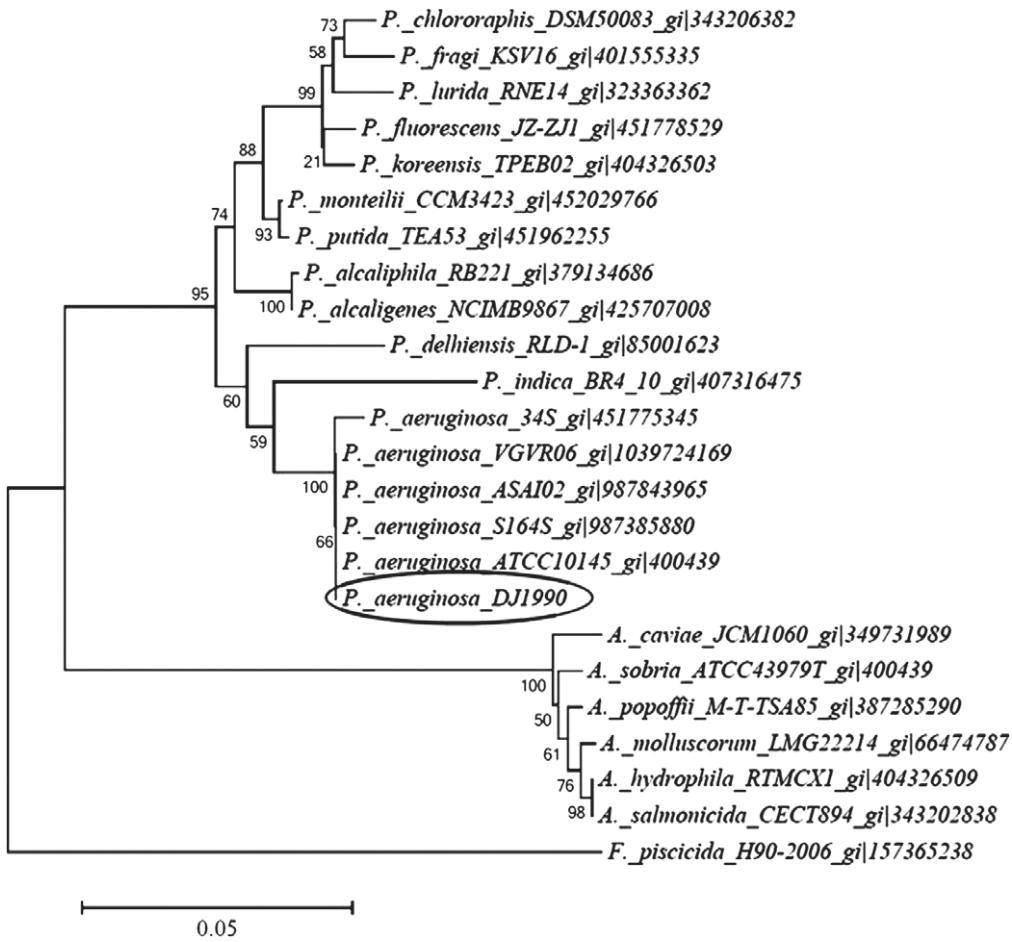

FIGURE 6. *Phylogenetic relationship of the strain DJ1990 with selected bacterial species available in the National Center for Biotechnology Information database based on 16S rDNA sequence.*

16S rDNA gene sequence analysis and the GenBank accession number was KX709967. The Basic Local Alignment Search Tool (BLAST) result revealed that the isolate DJ1990 was 99% identical with the *P. aeruginosa* strain ATCC10145. The phylogenetic tree of DJ1990 was constructed with other *P. aeruginosa* strains and other bacterial species using MEGA 6.0 (Hachioji, Tokyo, Japan) software (Fig. 6). The strain DJ1990 was clustered with different *P. aeruginosa* strain including ATCC10145 (accession number: gi636558415), and the clustering was supported by a boot strap value of 94. The bacterial strain *Flavobacterium columnare* strain IAM 14301 (accession number: gi2879938) was taken as an out-grouped member.

TABLE 3. *Susceptibility of the strain DJ1990 to various antibiotics.*

Antibiotics	Unit (μg)	Zone diameter (mm)
Piperacillin	100	23
Colistin	10	15
Piperacillin/tazobactam	10	28
Cefepine	30	27
Meropenem	10	27
Ciprofloxacin	10	27
Ceftazidime	30	25
Tobramycin	10	25
Aztreonum	30	R
Amikacin	30	26
Ticarcillin	7.5	23

R = resistance.

Different species of *Pseudomonas* are reported as potential aquatic pathogens. Toranzo et al. (1993) have reported that the Pseudomonad load in the kidney and livers of infected turbot was about 80% of the total microbial flora. Previously, Bullock et al. (1965) identified 19 strains of *Pseudomonas* from different infected freshwater fish species (Bullock et al. 1965). Similarly, Nabi et al. (2000) reported the infection of *Channa gachua* caused by *Pseudomonas fluorescens* and *Pseudomonas putida*. However, *P. fluorescens, P. aeruginosa, Pseudomonas anguilliseptica*, and *Pseudomonas* sp. were reported to be isolated from diseased *L. rohita* and *Cyprinus carpio* (Mastan 2013). Bacterial infection (deep ulceration and discoloration in the cephalic region and damaged caudal fins) in *C. punctata* from Dibrugarh, Assam, India, was also reported by Shah et al. (2012), although they have not identified the causative agent. It is now well established that *P. fluorescens* is a potent fish pathogen and has been reported by several researchers (Darak and Barde 2015; Banerjee et al. 2016). To the best of our knowledge, this is the first description of *P. aeruginosa* strain as a causative agent of TFRD in *C. punctata*.

Antibiotic Profiling

The antibiotic sensitivity test of the selected bacterium *P. aeruginosa* DJ1990 is tabulated in Table 3. The strain DJ1990 was found susceptible to the majority of tested antibiotics viz. piperacillin (100 µg), colistin (10 µg), piperacillin/tazobactam (10 µg), cefepine (30 µg), meropenem (10 µg), ciprofloxacin (10 µg), ceftazidime (30 µg), tobramycin (10 µg), amikacin (30 µg), and ticarcillin (7.5 µg). Surprisingly, the strain DJ1990 was found to be resistant against aztreonum (30 µg) (Kummerer 2009; Albuquerque Costa et al. 2015). Therefore, aztreonum (30 µg) might be used as a selective marker for this strain in future studies, including cloning. Several strains of *P. aeruginosa* are considered to be multidrug-resistant bacteria, which cause serious lung infection in humans (Banerjee and Ray 2016). However, the selected bacterial strain *P. aeruginosa* DJ1990 was detected to be sensitive to a wide spectrum of general antibiotics.

Conclusion

TFRD is a major economic problem that decreases the production of *C. punctata* and other major carps and ultimately affects the socioeconomical condition of the country. In this investigation, we identified a strain (DJ1990) of *P. aeruginosa* that causes TFRD in *C. punctata*. The pathogenicity of the isolated bacterial strain *P. aeruginosa* was confirmed by *in vivo* experiment, histopathological, and SEM examination. To the best of our knowledge, it is the first report of *P. aeruginosa* as a TFRD-causing candidate in *C. punctata*. Further investigations are required to find a solution to this problem.

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