Isolation and characterisation of virulent *Serratia marcescens* associated with a disease outbreak in farmed ornamental fish, *Poecilia reticulata* in Kerala, India

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ABSTRACT

Pathogenic strain of *Serratia marcescens* (NPSM-1) with multiple drug resistance was isolated from guppy *Poecilia reticulata* with clinical signs of fin rot and was confirmed by biochemical tests and 16S rRNA gene sequencing. The extra cellular proteins (ECP) of the bacteria exhibited marked cytotoxic activity in vitro on *Cyprinus carpio* koi fin (CCKF) cell line. The in vivo challenge studies confirmed that the isolate was highly pathogenic to fish when the fishes were injected with 1 x 10^4 CFU/fish and the same bacterium was re-isolated from infected fish, post-challenge. *S. marcescens* produced large zones of haemolysis on 10% sheep blood agar. The bacteria was found to carry virulence genes; extracellular metallo-protease gene (Pr596) and AHL synthase gene (SpnI). The bacterial isolate was tested to determine sensitivity against 16 antibiotics and was sensitive to only 5 viz., cefixime, chloramphenicol, ciprofloxacin, gentamycin and erythromycin. The study indicates that *S. marcescens* can cause disease in ornamental fish and the bacterium being a known human pathogen, may also cause infections in humans having direct contact with infected fishes. This is the first report describing *S. marcescens* as a pathogen of freshwater ornamental fish in India.

Keywords: Bacterial fish diseases, Guppy, Ornamental fish, *Poecilia reticulata*, *Serratia marcescens*

Introduction

Ornamental fish culture is one of the important and promising areas of aquaculture worldwide. In India, ornamental fish industry is expanding and is supported by government agencies such as the Marine Products Export Development Authority (MPEDA) (Silas et al., 2011). Diseases are inevitable with the rapid expansion of the aquaculture industry and pathogens have become one of the major bottlenecks to production. Variation in microbial community of water in the aquaculture systems is considered to be the major factor causing diseases and mortalities in fishes (Gomes, 1996). Austin et al. (1999) reported that higher quantities of organic material, changes in pH values and enhanced microbial populations resulted in infectious diseases in aquaculture.

The members of the family Enterobacteriaceae viz., *Yersinia ruckeri*, *Edwardsiella tarda* and *E. ictaluri* are recognised as fish pathogens (Sanders and Fryer, 1988). However, other enterobacteria such as *Proteus* sp., *Citrobacter* sp., *Hafnia* sp. and *Serratia* sp. have been associated with fish disease outbreaks (McIntosh and Austin, 1990). The genus *Serratia* comprising *Serratia liquefaciens*, *S. marcescens* and *S. plymuthica* have been associated with bacterial septicemia and mortalities in salmonids (Austin and Gibb, 1993), though these species are considered opportunistic pathogens.

*S. marcescens* is a well known cause of hospital acquired infections, including nosocomial pneumonia, wound infections, urinary tract infections and septicemia (Yu, 1979) and is a common microorganism present in soil and freshwater (Hejazi and Falkiner, 1997). Until now, there have been only a few publications concerning the fish infections caused by this microorganism. Baya et al. (1992) isolated *S. marcescens* from natural populations of white perch. *Morone americanus*, during the course of a bacteriological survey in USA. However, the emergence of multidrug resistant *Serratia* has been alarming not only in the medical field but also in aquaculture and agriculture sectors (Morohoshi et al., 2007). Recently *S. marcescens* has been isolated in an endemic disease outbreak from...
tilapia fish farms in Malaysia and its whole genome was sequenced (Chan et al., 2013). In the course of routine monitoring for diseases in ornamental fishes under the National Programme of Surveillance of Aquatic Animal Diseases (NSPAAD) in India, in June 2015, a farmer reported high mortality and morbidity in guppy, Poecilia reticulata with skin and fin rot lesions. The specimens were examined for important bacterial, viral and parasitic infections. We isolated a red pigmented bacterium which resembled S. marcescens from the affected fishes sampled from the farm. Because of the possible public health implications due to these bacteria, we aimed to characterise this microorganism; to determine whether S. marcescens is really the causative agent of such health disorders in guppy fish; and to select the most suitable antibiotic agent for the treatment of fishes affected by this bacterium.

Materials and methods

Fish sampling

The ornamental fish farmer from Kozhikode, Kerala reported several incidences of mortality in guppies (body weight range: 0.45 to 0.80 g) with fin and tail rot and mortality up to 40% during the rainy season (June-August) in 2015, with a history of not responding to antibacterial, antiparasitic and antiprotozoan treatments. Diseased (n = 25) fish were collected from the farm for detailed investigations. All fish were transported to the laboratory on ice within 6 h. The tissues viz., fin, gills, spleen and kidney from the affected fish were stored in 95% ethanol and Leibovitz’s L-15 tissue culture medium for screening of viruses viz., Koi Herpes virus (KHV), Iridovirus and spring viraemia of carp virus (SVCV) as described by Kumar et al. (2015) and isolation of viral pathogen, if any, respectively. A tissue homogenate was prepared from the pooled samples of fin, gills, spleen, heart and kidney from the affected fish and inoculated on different fish cell lines viz., pearl spot fin (PSF) (Swaminathan et al., 2010), catopra fish fin (CFF) (Swaminathan et al., 2013), Horabagrus brachysoma fin (HBF) (Swaminathan et al., 2014), Cyprinus carpio koi fin (CCKF) (Swaminathan et al., 2015), angelfish fin (AFF) (Swaminathan et al., 2016) and goldfish fin (GFF) (unpublished) maintained in our laboratory to screen for viral infections. The scrapings from skin, fin and gills of the affected fish were collected and examined under microscope for external parasitic infestation.

Bacterial isolation

For bacterial isolation, whole fish was homogenised in sterile phosphate buffered saline (PBS) in the ratio of 1:1 (w/v) and inoculated into trypticase soy agar (TSA; Himedia, India). The cultures were incubated at 28°C for 48-72 h and the number and diversity of colonies were determined. Preliminary tests allowed us to identify the dominant red pigmented colonies isolated from guppy as *Serrata* sp. and biochemical tests were conducted following procedures described by Barrow and Feltham (2004). Pure cultures were kept frozen at -80°C in double strength tryptic soy broth supplemented with 15% glycerol for further examination. For the taxonomic analysis, the reference strains of *S. marcescens* ATCC 1800 and *S. marcescens* isolated from natural populations of white perch, *Morone americana* (Baya et al., 1992) were included for comparison.

PCR amplification of 16S rRNA, metalloprotease and quorum sensing genes of *S. marcescens*

*S. marcescens* isolate was further confirmed by sequence analysis of 16S rRNA gene, extracellular metalloprotease gene (*Pr596*) and AHL synthase gene (*SpnI*) (Tao et al., 2007; Tariq, 2010). Total genomic DNA was isolated from pure bacterial cultures using the DNeasy blood and tissue kit (Qiagen) following the manufacturer’s instructions. The DNA concentration was quantified with a bio-spectrophotometer (Eppendorf, Germany) and adjusted to a concentration of 100 ng µl⁻¹. Universal primers 27F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5-TACG GCTATCTTGGTACGACTT-3), were used to amplify the 16S rRNA gene (Weisburg et al., 1991). The PCR products were sequenced at an automated sequencing facility (Scigenom Pvt. Ltd, India). The raw DNA sequences were edited using BioEdit sequence alignment editor version 7.0.5.2 (Hall, 1999). For molecular identification, homology comparison of 16S rRNA sequences of bacterial strains was performed using Basic Local Alignment Search Tool (BLAST) and Ribosomal Database Project (RDP). The sequences were compared for similarity between the sequences of collected bacterial isolates and the sequences available at GenBank and a phylogenetic tree was constructed by neighbour-joining method. Distance matrices were calculated using Kimura’s 2-parameter correction and stability of groupings and bootstrap analysis (1000 replicates) was conducted using MEGA 5.05 software (Tamura et al., 2011). *Vibrio cholerae* (GenBank Accession No. LC011458) was used as an out group. Additionally, two sequences of *S. marcescens* previously submitted to GenBank (Accession nos. AY498856 and EF194094) and other *Serrata* sp. viz., *S. liquefaciens*, *S. plymuthica*, *S. odorifera*, *S. glossinae* and *S. ficania* were included in phylogenetic analysis. The partial 16S rRNA, *Pr596* and *SpnI* gene sequences of *S. marcescens* isolate were deposited in the GenBank database.
**Virulent Serratia marcescens** from guppy fish

*Heamolytic activity*

*S. marcescens* produces a variety of virulence proteins, including haemolysin, which was reported to be the dominant virulence factor of the bacterium. The strain was tested for haemolysis activity on blood agar containing 10% sheep erythrocytes. Tryptone-yeast extract (TY) base (HiMedia, India) was used to prepare blood agar plates. The bacterial suspensions were streaked on to the plates and plates were evaluated initially after incubation for 24 h at 28°C and after further incubation for 12 h at 4°C. A clear and colourless zone around the colony indicates α-haemolytic activity.

*Challenge tests*

The bacteria, *S. marcescens* was grown in trypticase soya broth for 24 h at 28°C and then centrifuged at 2000 g for 10 min and cell pellets were suspended in sterile PBS to the final concentration of 1 x 10⁶ cells ml⁻¹. Healthy guppies weighing 0.50 - 0.75 g were used in challenge experiments. Before infection, the fish were anaesthetised with MS-222 (Sigma). For each bacterial strain, 10 guppy fish were injected intraperitoneally (i/p) with 100 µl of the bacterial suspension for testing Koch’s postulates and the same number of fishes were injected with sterile PBS, which were treated as the controls. Fishes were maintained in 100 l capacity glass aquaria with 50 l water with continuous aeration, daily 50% exchange of water and *ad libitum* feeding. The challenged fish were observed daily for infection after inoculation with *S. marcescens* bacterial suspension. Infected fish were examined, bacteria reisolated and reconfirmed by biochemical tests, PCR and sequencing as mentioned above.

*Antibiotic sensitivity tests*

*S. marcescens* isolates were tested for antibiotic susceptibility using disc diffusion method on Mueller Hinton Agar (HiMedia, India) (Bauer et al., 1966). A total of sixteen antibiotics (HiMedia, India) were tested: ampicillin (25 µg disc⁻¹), gentamycin (120 µg disc⁻¹), oxytetracycline (30 µg disc⁻¹), cefalexin (30 µg disc⁻¹), chloramphenicol (25 µg disc⁻¹), ciprofloxacin (30 µg disc⁻¹), cefixime (5 µg disc⁻¹), kanamycin (30 µg disc⁻¹), nitrofurantoin (100 µg disc⁻¹), erythromycin (10 µg disc⁻¹), amoxicillin (25 µg disc⁻¹), furazolidone (100 µg disc⁻¹), bacitracin (10 µg disc⁻¹), azithromycin (30 µg disc⁻¹), enrofloxacin (10 µg disc⁻¹) and cefixime/ clavulanic acid (5/10 µg disc⁻¹). Antibiotic sensitivity was assayed from the diameter of zone of inhibition formed around the discs. Manufacturer’s instructions were used to determine the sensitivity as sensitive, intermediate and resistant to antibiotics.

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pigmented bacteria revealed that it is an enterobacteria belonging to the species *S. marcescens* (NPSM-1).

According to Austin and Austin (2007), bacteria such as *Aeromonas* sp., *Bacillus* sp., *Citrobacter* sp., *Edwardsiella* sp., *Flavobacterium* sp., *Klebsiella* sp., *Proteus* sp., *Providencia* sp. and *Serratia* sp., are associated with fish disease and several bacterial pathogens have been isolated from freshwater fish by various workers (Kumar and Dey, 1988; Das et al., 1999; Novotny et al., 2004; Mohanty and Sahoo, 2007) in India. Recently Kumar et al. (2015) isolated a zoonotically important bacterial pathogen; *Proteus hauseri* causing mass mortality of ornamental koi carp in India. *S. marcescens* was isolated and confirmed only by biochemical characteristics from marine ornamental fish with ulcerative disease in India (Pramila, 2002). In the present study *S. marcescens* was isolated and identified from diseased guppy fish *P. reticulata*.

**Sequence analysis of 16S rRNA, metalloprotease and quorum sensing genes of *S. marcescens***

Approximately 1470 bp gene sequence of 16S rRNA was amplified after assembling the forward and reverse sequences. The 1408 bp and 1486 bp of metalloprotease gene (*Pr596*) and AHL synthase gene (*SpnI*) were also amplified and sequenced (Fig. 2). The BLAST results of 16S rRNA gene, *Pr596* and *SpnI* showed that the isolate shared 99.5, 98 and 99% similarity respectively with *S. marcescens*. This is the first description of *S. marcescens* strains as pathogens of guppy *Poecilia reticulata*. To date, only limited scientific reports on *Serratia* sp. associated fish diseases are documented. Nieto et al. (1990) isolated *S. plymuthica* from moribund rainbow trout in north-western Spain, McIntosh and Austin (1990) isolated bacteria resembling *S. liquefaciens* from salmonids in Australia and Scotland and Baya et al. (1992) isolated *S. marcescens* from white perch, *Morone americanus* in USA. Although the origin of the *S. marcescens* in the diseased guppy is not known, it is possible that the bacteria may have originated from one of the farm personnel in close contact with the fish. *S. marcescens* is a well known human pathogen causing respiratory tract and urinary tract infection as well as endocarditis, osteomyelitis, pneumonia and meningitis (Hejazi and Falkiner, 1997).

### Table 1. Biochemical characteristics of *Serratia marcescens* (NPSM-1) bacterial strains isolated from guppy mass mortality in this study

| Biochemical tests | *S. marcescens* (NPSM-1) isolated in this study | *S. marcescens* ATCC 8100 isolated from experimentally challenged fish | *S. marcescens* isolated from white perch, *Morone americanus* in USA. |
|-------------------|-----------------------------------------------|--------------------------------------------------------------------|---------------------------------------------------------------------|
| Gram stain        | -                                             | -                                                                  | -                                                                   |
| Motility          | +                                             | +                                                                  | +                                                                   |
| Oxidase           | -                                             | -                                                                  | -                                                                   |
| Catalase          | +                                             | +                                                                  | +                                                                   |
| Oxidative/        | F                                             | F                                                                  | F                                                                   |
| Fermentative glucose |                                             |                                                                    |                                                                     |
| Methyl red        | -                                             | -                                                                  | -                                                                   |
| Voges-Proskauer   | +                                             | +                                                                  | +                                                                   |
| Indole production | -                                             | -                                                                  | -                                                                   |
| Nitrate reduction | +                                             | +                                                                  | +                                                                   |
| Citrate utilisation |                                             | -                                                                  | +                                                                   |
| Growth at 15°C    | +                                             | +                                                                  | +                                                                   |
| Growth at 25°C    | +                                             | +                                                                  | +                                                                   |
| Growth at 37°C    | +                                             | +                                                                  | +                                                                   |
| Growth at 0% NaCl | +                                             | +                                                                  | +                                                                   |
| Growth at 3% NaCl | +                                             | +                                                                  | +                                                                   |
| Growth at 6% NaCl | +                                             | +                                                                  | +                                                                   |
| Arginine decarboxylase - | -                               | -                                                                  | -                                                                   |
| Lysine decarboxylase |                                             | +                                                                  | +                                                                   |
| Ornithine decarboxylase+ |                                             | -                                                                  | +                                                                   |
| Haemolysis on sheep blood agar | β | β | β |
| Sugar utilisation |                                               |                                                                    |                                                                     |
| Mannose           | +                                             | +                                                                  | +                                                                   |
| Galactose         | +                                             | +                                                                  | +                                                                   |
| Fructose          | +                                             | +                                                                  | +                                                                   |
| Maltose           | +                                             | +                                                                  | +                                                                   |
| Sucrose           | +                                             | +                                                                  | +                                                                   |
| Rahmnnose         | -                                             | -                                                                  | -                                                                   |
| Arabinose         | -                                             | -                                                                  | -                                                                   |
| Salicin           | +                                             | +                                                                  | +                                                                   |
| Trehalose         | +                                             | +                                                                  | +                                                                   |
| Lactose           | -                                             | -                                                                  | -                                                                   |
| Xylose            | -                                             | -                                                                  | -                                                                   |
| Cellobiose        | -                                             | -                                                                  | -                                                                   |
| Raffinose         | -                                             | -                                                                  | -                                                                   |
| Mannitol          | +                                             | +                                                                  | +                                                                   |
| Sorbitol          | +                                             | +                                                                  | +                                                                   |
Virulent *Serratia marcescens* from guppy fish

Extracellular metalloproteases, *Pr596* are mostly associated with pathogenic bacteria or bacteria that have industrial significance (Hase and Finkelstein, 1993). Many Gram negative pathogens control the expression of virulence factors, secretion of extracellular protease, pectinase and rhamnolipid and biofilm formation via the quorum-sensing system (de Kievit and Iglewski, 2000). The regulation of flagellum independent populational surface migration, the synthesis of biosurfactant and production of prodigiosin and nuclease in *S. marcescens* SS-1 are co-ordinately negatively monitored by *spnIR* (Horng et al., 2002). It has been shown that *Serratia* strains employ quorum sensing for the regulation of genes encoding extracellular virulence factors. In *Serratia*, at least four different LuxR/AHL quorum sensing systems viz., *SprIR* from *Serratia proteamaculans*, *SwrIR* from *Serratia liquefaciens* MGI (now renamed as *S. marcescens* MG-1) *SpnIR* from *S. marcescens* SS-1 and *SmaIR* from *Serratia* sp. ATCC39006 have been described (Wei and Lai, 2006).

**Phylogenetic tree**

The phylogenetic tree was constructed based on 1450 bp of 16S rRNA gene sequences (Fig. 3). The 16S rRNA sequence of NPSM-1 was compared with published sequences of *S. marcescens* and sequences of other species viz., *S. liquefaciens*, *S. plymuthica*, *S. odorifera*, *S. glossinae* and *S. ficania*. Distantly related bacteria *V. cholerae* was taken as outgroup. It generated clusters, which were supported by bootstrap values of 93 and 100.

**Haemolytic assay**

*S. marcescens* produces a range of secreted products, including lipases, proteases, chitinases, nucleases, biosurfactants and haemolysin (Hejazi and Falkiner, 1997). In our study, when *S. marcescens* colonies were spotted on 10% sheep blood agar, they did not form haemolysis zones at 24 h of incubation at 28°C, but after incubation for an additional 12 h at 4°C the haemolytic zones were observed (Fig. 4). Haemolysin production is a common attribute of *S. marcescens* strains and has been shown to be involved in the virulence of this pathogen.

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**Fig. 2.** Agarose gel image showing PCR-amplified 1408 bp and 1486 bp bands of metalloprotease gene (*Pr596*) from *Serratia marcescens* (NPSM-1). Lanes 1 and 2 - metalloprotease gene (*Pr596*); Lanes 3 and 4 - AHL synthase gene (*Spn*); Lane 5 - DNA template from *Aeromonas hydrophila* as negative control. Lane L: Low range DNA ruler, 100 bp to 3 kbp (GeNei)

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**Fig. 3.** Phylogenetic tree based on the concatenated sequences of 16S rRNA of *Serratia marcescens* (NPSM-1) isolated from guppy and related *Serratia* species. The neighbour-joining algorithm was used to construct the tree with genetic distance computed by Kimura’s 2-parameter method. Bootstrap values of 1000 simulations are indicated at the branches. The bar indicates percentage difference
(Hilger and Braun, 1995). *S. marcescens* secretes a haemolysin that also acts as a cytotoxin and the haemolytic activity is determined by the *shlA* and *shlB* genes (Poole et al., 1988). In the absence of *ShlB*, inactive *ShlA* of *S. marcescens* remains in the periplasm and displays less haemolytic activity with small haemolysis zone (Schiebel et al., 1989).

**Cytotoxicity**

The ECP from the NPSM-1 displayed a positive cytotoxic response on the CCKF cell line tested within 24 h. The cytotoxic changes started appearing in the CCKF cells within 6 h post-inoculation (hpi) and followed by lysis of cells after 24 hpi. No morphological changes could be detected in the control cells inoculated with PBS. Cytotoxic changes viz., granulation, vacuolation, rounding and dislodgement of cells, were recorded on microscopic examination of the infected CCKF cell line (Fig. 5a, b, c and d). Vacuoles in different epithelial cell lines viz., adherent HEp-2, RT112, HeLa, Chang and HeC1B cells were observed when treated with supernatant culture of *S. marcescens* within 15 min, followed by lysis after 40 min (Hertle et al., 1999). A similar cytotoxic change to that of *ShlA*- induced vacuolation has been observed with aerolysin from *Aeromonas hydrophila* (Abrami et al., 1998). *S. marcescens* exerts haemolytic and vacuolating cytotoxic activities mainly in direct contact with the different target cells (Braun et al., 1985).

**Antibiotic sensitivity**

The susceptibility of *S. marcescens* to antibacterial agents varied. The bacteria was resistant to ampicillin, amoxicillin, cefalaxein, furazolidone, kanamycin, nitrofuratoin and oxetacycline while susceptible to cefixime, chloramphenicol, ciprofloxacin, erythromycin and gentamycin. Similar observations concerning *S. marcescens* isolated from clinical cases of marine and freshwater fish were also previously described (Baya et al., 1992). Resistance of *S. marcescens* to 11 out of 16 tested antimicrobial agents was observed. This suggests development of multidrug resistance by this bacteria and need for identifying appropriate antibiotic treatment against this bacterium, supported by antibiotic susceptibility testing. The fish farmer was advised to administer ciprofloxacin in the feed to affected fish at the dose rate of 5 mg g⁻¹ feed twice daily for five days. After administering the antibiotic, fish recovered from the disease and no further mortality of guppy was noticed in the farm.

**Experimental challenge tests**

Fish infected with NPSM-1 showed the following external signs viz., dark skin, necrotic skin lesions and fin rot. *S. marcescens* was reisolated in pure cultures from infected fish. No mortality or any disease signs were observed in fish injected with sterile PBS. The mortality...
rate of the experimentally challenged group was 55% as reported in earlier studies (Baya et al., 1992). In this study, the reisolation of S. marcescens from freshly dead experimental fish and its confirmation by sequencing fulfilled Koch’s postulates. The results suggest that the present S. marcescens strain could be considered a potential bacterial pathogen for fish.

It has been demonstrated (Buras et al., 1985) that a high bacterial load in water, stresses the fish immune system and result in invasion and proliferation of environmental bacteria in the fish tissues. There is a possibility for dissemination of S. marcescens to other geographic areas through the water and fish trade. Chan et al. (2013) isolated S. marcescens W2.3, a suspected causal agent of an endemic disease outbreak along with other bacteria from the tilapia fish farms of Malaysia during 2009. The emergence of multidrug resistant Serratia sp., has been distressing in the human medical field and also in aquaculture and agriculture sectors (Kurz et al., 2003). In addition, the potential pathogenic capability and multidrug resistance of this isolate may be of public health concern since S. marcescens is a well recognised opportunistic pathogen causing important human infections.

The isolation of the highly virulent and multidrug resistant zoonotically important bacterium S. marcescens, from freshwater ornamental fish in India poses a threat that this pathogen could cause infections to the farm personnel. This may be given due attention as they might cause zoonotic diseases. Further epidemiological investigations together with studies on S. marcescens pathogenicity are necessary to elucidate the public health significance of S. marcescens.

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