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Research Article

Characterization of high fluoride resistant *Pseudomonas aeruginosa* species isolated from water samples

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ABSTRACT

Groundwater fluoride contamination is one of the most serious toxicological environmental issues in India and around the world. Water samples were taken from Natham taluk and screened for fluoride resistant bacteria. Initially, twenty-four fluoride resistant colonies were selected from 50 mM NaF supplemented LB agar plates. On blood agar plates, all isolates showed β-haemolysis, confirming their status as pathogens. Virulence factors (*algD*, *plcH*, *toxA*, *gyrB*, *rhlC*, *lasB*) and biofilm-forming genes (*ppyR*, *pelA*, *pslA*) were identified in these isolates by PCR analysis. The fluoride ion transporter '*crcB*' was successfully amplified from these isolates by gene-specific PCR. Genus *Pseudomonas* and *P. aeruginosa* species-specific PCR analysis identified that all strains as belonging to the *P. aeruginosa* species. Besides, three high fluoride resistance strains were selected based on high fluoride resistance and confirmed as *P. aeruginosa* species by 16S rRNA sequencing and NCBI blast analysis.

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INTRODUCTION

Fluorine is the first element in the halogen group with the symbol 'F' [1]. Fluoride (F^-) exists in the groundwater due to natural or anthropogenic sources [2]. Fluorine occurs in nature as fluoride anion (F^-) in water. The natural sources of F^- are clay minerals, fluoride-bearing minerals and F^- replacing OH^- in the ferromagnesium silicates [3]. The industrial causes of F^- arise from phosphate-based fertilizer plants, mining operations and iron, glass, ceramics and oil refineries [4]. In drinking water, the allowable limit of fluoride is 1.5 mg/L fixed by the World Health Organization [5] and the Bureau of Indian Standards [6]. The F^- concentration in drinking water exceeds the permissible limit, it can lead to various health issues [7]. Pseudomonads are highly versatile and adapt to survive various environments [8, 9]. *P. aeruginosa* is ubiquitous, isolated from various water sources for example treated effluent wastewater [10], healthcare facilities [11, 12], municipal drinking water systems [13], and accommodation facilities [14], swimming pools [15] recreational water [16] and groundwater [17]. Several authors have investigated the F^- concentration in the Dindigul district's groundwater [18–22].

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Table 1. The various oligonucleotide sequences used in the present study

Genes	Primer sequences (5'-3')	Anneal at °C	Size (bp)	References
PA-GS-F	GACGGGTGAGTAATGCCTA			[25]
PA-GS-R	CACTGGTGTTCCTTCCTATA	54	618	"
PA-SS-F	GGGGGATCTTCGGACCTCA			"
PA-SS-R	TCCTTAGAGTGCCCACCCG	58	956	"
GyrPA-398	CCTGACCATCCGTCGCCACAAC			[26]
GyrPA-620	CGCAGCAGGATGCCGACGCC	66	222	"
ETA1	GACAACGCCCTCAGCATCACCAGC			"
ETA2	CGCTGGCCCATTCGCTCCAGCGCT	66	397	"
algDF	ATGCGAATCAGCATCTTTGGT			[27]
algDR	CTACCAGCAGATGCCCTCGGC	55	1311	"
lasBF	GGAATGAACGAAGCGTTCTC			"
lasBR	GGTCCAGTAGTAGCGGTTGG	55	300	"
plcHF	GAAGCCATGGGCTACTTCAA			"
plcHR	AGAGTGACGAGGAGCGGTAG	55	307	"
rhlCF	ACCGGATAGACATGGGCGT			[28]
rhlCR	GCAGGCTGTATTCGGTGTC	61	570	"
pslA	TCCCTACCTCAGCAGCAAGC			[24]
pslAR	TGTTGTAGCCGTAGCGTTTCTG	65	656	"
pelAF	CATACCTTCAGCCATCCGTTCTTC			"
pelAR	CGCATTCGCCGCACTCAG	65	786	"
ppyF	CGTGATCGCCGCCTATTTCC			"
ppyR	ACAGCAGACCTCCCAACCG	65	160	"
FlucPF	AAGCTTATGTGGAAATCCATTCTCG			Present study
FlucPR	CTGCAGCTATTTGCCCAGCATCCA	66	384	"

In this work, water samples were taken from Natham taluk and isolate the F^- resistant bacteria, determine their F^- resistance, and assess the antibiotic susceptibility, investigate the presence of virulence genes profile and identify the F^- resistant gene '*crcB*' by gene specific PCR analysis.

MATERIALS AND METHODS

Isolation and Characterization of F^- Resistant Bacteria

Groundwater samples from borehole, handpump were taken from Natham Taluk, Tamilnadu, India. All the water samples were serially diluted and plated on LB agar (g/L): peptone 10, yeast extract 5, NaCl 10, agar 15 (HiMedia, Mumbai, India) plates added with 50 mM Sodium Fluoride (NaF). All the plates were incubated at 37 °C for 48 h incubation. After the duration, the selected resistant colonies transferred to selective chromogenic media like EMB (Eosin Methylene Blue) agar, MacConkey agar, and *Pseudomonas* isolation agar (HiMedia, Mumbai, India). All the plates were incubated at 37 °C for 24 h to check their growth. For this purpose, *P. aeruginosa* MTCC 2453 was used as the reference strain. Besides, F^- resistant isolates were identified with different characteristics.

Haemolysis

To determine the haemolytic activity, the selected F^- resistant isolates were streaked on blood agar plates (HiMedia, Mumbai, India) and kept at 37 °C for 24 h. After incubation, results were observed based on their haemolytic property.

Casein and Starch Hydrolysis

The selected F^- resistant isolates were streaked on Skim milk agar plates (HiMedia, Mumbai, India) and incubated at 37 °C for 24 h. The presence of a clear zone surrounding the colony indicated the proteolysis of casein. In this test *P. aeruginosa* MTCC 2453 was used as the reference strain. Starch hydrolysis was carried out with LB agar plates containing 1% starch and incubated at 37 °C for 24 h. Iodine crystals was used to confirm the starch hydrolysis test.

Determination of F– Resistance
The minimum inhibitory concentration (MIC) was determined on LB agar plates containing different concentrations of NaF. The concentration of NaF was used starting from 50 to 300 mM. MIC was evaluated until the selected isolates were unable to grow on F^- containing media. Based on this analysis, MIC was found at 37 °C in five days.

Antibiotic Resistance
Antibiotic resistance of F^- tolerant isolates was determined by the disc diffusion method. A sterile swab used for culture swabbing and 24 h broth culture was prepared as inoculum. The sterile antibiotic discs (HiMedia, Mumbai, India) were placed on the surface of MH (Muller Hinton) agar plates and incubated at 37 °C for 24 h. Twenty-Six antibiotics were used in this study is mentioned in Table 4 and 5. Antibiotic sensitivity was classified as resistant, intermediate or sensitive. The zone of inhibition (mm) was measured from each disc as suggested by Clinical & Laboratory Standards Institute (CLSI) and The European Union Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines.

Salt Tolerance
The salt tolerance of F^- resistant isolates was determined on LB agar plates supplemented with different concentrations of NaCl (Sodium Chloride). Based on an earlier report [23], the working concentration of NaCl selected from 0 to 18% (V/V) was prepared from 20% NaCl stock solution (W/V). The growth was monitored at 37 °C for 120 h.

Biofilm Production and Screening of Biofilm Forming Genes
Microtiter plate assay was used to check the biofilm production. The 1% inoculum was transferred to 96-well plate and incubated for 24 h at 37 °C. The biofilm formation is assessed into four ways 1. Non-adherent (0), 2. Weak (+), 3. Moderate (++) and 4. Strong (+++) production [24]. Tests were performed in 3 times. Besides, biofilm forming genes are identified by PCR using specific primers [24]. The primers detail is shown in Table 1. PCR was performed in Veriti 96 well thermal cycler and amplified products were separated on 2% agarose gel.

Virulence Genes Profile
The identification of virulence genes in fluoride resistant *Pseudomonas* was evaluated by PCR using gene-specific primers. The virulent factors 1) alginate (*algD*), 2) elastase (*lasB*), 3) haemolytic phospholipase (*plcH*), 4) rhamnolipid C (*rhlC*), 5) DNA gyrase (*gyrB*), and 6) exotoxin A (*toxA*) were used in this study. For this purpose, *P. aeruginosa* MTCC 2453 acted as the positive control. The priming details and annealing conditions are shown in Table 1.

Fluoride Resistant Gene Amplification
F^- resistant gene '*crcB*' was amplified using gene-specific primers, and genomic DNA was used as a template. The primers were designed to contain *Bam*HI and *Nco*I restriction sites used for the amplification of *crcB* gene. PCR was performed on a Veriti 96 well thermal cycler (Applied Biosystems, Foster City, CA, USA). The amplified PCR products were separated on 1.5% agarose gel electrophoresis.

Molecular Identification of *Pseudomonas* by PCR
In this study, PCR based assay was used for the identification and differentiation of *P. aeruginosa* from other *Pseudomonas* species [25]. The primer details are mentioned in Table 1. PCR was carried out in Veriti 96 well thermal cycler. The amplified PCR products were separated on 1.5% agarose gel electrophoresis.

PCR Amplification and 16S rRNA Sequencing
The 16S rRNA gene amplification was carried out Using the universal bacterial 16S rRNA primers, 27 F 5'-AGA GTT TGA TCM TGG CTC AG-3' and 1429 R 5'-CGGT TACC TTG TTA CGA CTT-3' in thermal cycler under the following cyclic conditions as follows: 95 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec, extension at 72 °C for 1.5 min and final extension at 72 °C for 5 min. The polymerase chain reaction was performed in Veriti 96 well thermal cycler Applied Biosystems. Genomic DNA was used as template for PCR and products were separated on 1% agarose gel electrophoresis. The amplified PCR products were eluted by GeneJET gel extraction kit (ThermoScientific, USA) and then carried out for sequencing. The sequences obtained were compiled and compared to the sequences in the GenBank databases using BLAST analysis [29].

RESULTS AND DISCUSSION

Isolation and Characterization of F– Resistant Bacteria
Twenty-four colonies were selected from 50 mM NaF containing LB agar plates used for further studies. The selected isolates were grown on EMB and MacConkey agar plates, which may belong to Gram-negative bacteria. The biochemical characteristics and carbon utilization are listed in Tables 2 and 3. F– resistant isolates tested positive for the phenylalanine deamination test. All isolates utilized citrate and malonate and were involved in casein hydrolysis and production of glucose, xylose, and oxidase. Based on haemolysis, all strains exhibited a clear colourless zone surrounding the growing colonies and were found to have beta (β)- haemolytic activity. Based on morphological characteristics, the selected isolates were grown on specific selective media for *Pseudomonas*.

Table 2. Biochemical characteristics of F^- resistant isolates

Tests/ isolates	Blood agar	ONPG	Lysine utilization	Ornithine utilization	Urease	Phenylalanine deamination	Nitrate reduction	H_2S production	Citrate	VP test	Methyl red	Indole	Malonate utilization	Esculin hydrolysis	Oxidase	Starch hydrolysis	Casein hydrolysis
S6-2	β	N^-	N^-	N^-	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	P^+	N^-	P^+	N^-	P^+
S6-4	β	N^-	N^-	N^-	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	P^+	N^-	P^+	N^-	P^+
S15-6	β	N^-	N^-	N^-	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	P^+	N^-	P^+	N^-	P^+
S18	β	N^-	N^-	N^-	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	P^+	N^-	P^+	N^-	P^+
S6-6	β	N^-	N^-	N^-	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	P^+	N^-	P^+	N^-	P^+
S6-7	β	N^-	N^-	N^-	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	P^+	N^-	P^+	N^-	P^+
S29	β	N^-	N^-	N^-	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	P^+	N^-	P^+	N^-	P^+
S28-25	β	N^-	N^-	N^-	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	P^+	N^-	P^+	N^-	P^+
S6-1	β	N^-	N^-	N^-	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	P^+	N^-	P^+	N^-	P^+
S6-3	β	N^-	N^-	N^-	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	P^+	N^-	P^+
S6-5	β	N^-	N^-	N^-	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	P^+	N^-	P^+	N^-	P^+
S9-1	β	N^-	N^-	N^-	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	P^+	N^-	P^+	N^-	P^+
S9-2	β	N^-	N^-	N^-	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	P^+	N^-	P^+	N^-	P^+
S15	β	N^-	N^-	N^-	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	P^+	N^-	P^+	N^-	P^+
S15-1	β	N^-	N^-	N^-	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	P^+	N^-	P^+	N^-	P^+
S15-2	β	N^-	N^-	N^-	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	P^+	N^-	P^+	N^-	P^+
S15-3	β	N^-	N^-	N^-	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	P^+	N^-	P^+	N^-	P^+
S15-4	β	N^-	N^-	N^-	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	P^+	N^-	P^+
S15-5	β	N^-	N^-	N^-	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	P^+	N^-	P^+
S15'	β	N^-	N^-	N^-	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	P^+	N^-	P^+	N^-	P^+
S18-18	β	N^-	N^-	N^-	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	P^+	N^-	P^+	N^-	P^+
S19	β	N^-	N^-	N^-	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	P^+	N^-	P^+	N^-	P^+
S28-12	β	N^-	N^-	N^-	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	P^+	N^-	P^+	N^-	P^+
S28-27	β	N^-	N^-	N^-	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	P^+	N^-	P^+	N^-	P^+
MTCC	β	N^-	N^-	P^+	N^-	P^+	N^-	N^-	P^+	N^-	N^-	N^-	P^+	P^+	P^+	N^-	P^+

Table 3. Utilization of carbon sources by F^- resistant isolates

Tests/ isolates	Xyl.	Ado.	Rha.	Cell.	Mel.	Sacc.	Raff.	Tre.	Glu.	Lac.	Inu.	Sodium gluconate	Gly.	Sal.	Dul.	Ino.	Sor.	Mann.	Ado.	Ara.	Ery.	α Methyl D- Glucoside
S6-2	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-
S6-4	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-
S15-6	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-
S18	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-
S6-6	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-
S6-7	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-
S29	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-
S28-25	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-
S6-1	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-
S6-3	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-
S6-5	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-
S9-1	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-
S9-2	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-
S15	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-
S15-1	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-
S15-2	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-
S15-3	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-
S15-4	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-
S15-5	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-
S15'	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-
S18-18	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-
S19	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-
S28-12	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-
S28-27	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-
MTCC	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	P^+	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-	N^-

Xyl: Xylose; Ado: Adonitol; Rha: Rhamnose; Cell: Cellobiose; Mel: Melibiose; Sacc: Saccharose; Raff: Raffinose; Tre: Trehalose; Glu: Glucose; Lac: Lactose; Inu: Inulin; Gly: Glycerol; Sal: Salicin; Dul: Dulcitol; Ino: Inositol; Sor: Sorbitol; Mann: Mannitol; Ara: Arabitol; Ery: Erythritol.

Table 4. Antibiotic sensitivity of F^- resistant isolates

DISC	mcg	S15-1	S15-2	S15-3	S15-4	S15-5	S15'	S9-1	S9-2	S18-18	S19	S28-27	S28-12	MTCC
Amikacin (AK)	30	28(S)	27(S)	23(S)	23(S)	29(S)	22(S)	23(S)	23(S)	22(S)	29(S)	23(S)	23(S)	18(S)
Amoxyclav (AMC)	30	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Ampicillin (AMP)	10	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Chloramphenicol (C)	30	13(I)	10(S)	10(S)	12(S)	14(I)	10(S)	10(S)	10(S)	13(I)	12(R)	9(R)	15(I)	13(I)
Ciprofloxacin (CIP)	5	35(S)	32(S)	31(S)	36(S)	37(S)	35(S)	36(S)	33(S)	33(S)	37(S)	36(S)	35(S)	32(S)
Cefoperazone (CPZ)	75	19(I)	22(S)	18(I)	20(I)	22(S)	20(I)	19(I)	18(I)	17(I)	15(R)	22(S)	22(S)	19(I)
Cefotaxime (CTX)	30	20(I)	20(I)	18(R)	18(R)	18(R)	18(R)	17(R)	19(R)	18(R)	19(R)	20(I)	21(I)	20(I)
Doxycycline-Hcl(DO)	30	NZ	NZ	NZ	NZ	16(S)	NZ	NZ	NZ	NZ	15(S)	NZ	NZ	NZ
Fosfomycin (FO)	200	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Gentamycin (G)	10	33(S)	31(S)	24(S)	23(S)	31(S)	24(S)	25(S)	25(S)	27(S)	32(S)	26(S)	28(S)	19(S)
Imipenem (IPM)	10	32(S)	31(S)	29(S)	30(S)	30(S)	29(S)	30(S)	29(S)	29(S)	30(S)	31(S)	30(S)	25(S)
Kanamycin (K)	30	15(I)	17(I)	9(R)	8(R)	17(I)	9(R)	11(R)	12(R)	10(R)	18(R)	10(R)	12(R)	15(I)
Minocycline (MI)	30	16(S)	13(I)	11(R)	14(I)	16(S)	10(R)	11(R)	12(R)	11(R)	17(S)	12(R)	11(R)	NZ
Meropenem (MRP)	10	33(S)	29(S)	35(S)	35(S)	30(S)	35(S)	33(S)	34(S)	34(S)	33(S)	33(S)	34(S)	30(S)
Nalidixic Acid (NA)	30	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Netillin (NET)	30	34(S)	31(S)	26(S)	26(S)	33(S)	26(S)	24(S)	25(S)	25(S)	34(S)	26(S)	24(S)	19(S)
Nitrofurantoin (NIT)	300	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Norfloxacin (NX)	10	30	30(S)	30(S)	30(S)	32(S)	30(S)	31(S)	31(S)	29(S)	33(S)	31(S)	31(S)	27(S)
Piperacillin (PI)	100	25(S)	25(S)	22(S)	25(S)	25(S)	25(S)	24(S)	22(S)	21(S)	23(S)	22(S)	25(S)	23(S)
Streptomycin (S)	10	24(S)	25(S)	22(S)	13(S)	26(S)	14(S)	17(S)	15(S)	15(S)	28(S)	14(S)	18(S)	17(S)
Tetracycline (TE)	30	15(S)	15(S)	12(I)	16(S)	18(S)	15(S)	20(S)	18(S)	15(S)	19(S)	14(I)	15(S)	9(R)
Trimethoprim (TR)	10	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Methicillin (MET)	5	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Penicillin (P)	2	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Rifampicin (RIF)	2	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Vancomycin (VA)	10	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ

NZ: No zone; R: Resistance; I: Intermediate; S: Sensitive.

Table 5. Antibiotic sensitivity of F^- resistant isolates

DISC	mcg	S6-2	S6-4	S6-6	S6-7	S15-6	S18	S29	S28-25	S6-1	S6-3	S6-5	S15	MTCC
Amikacin (AK)	30	17 (S)	16(I)	17(S)	17(S)	17(S)	24(S)	21(S)	18(S)	16(S)	18(S)	18(S)	22(S)	18(S)
Amoxyclav (AMC)	30	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Ampicillin (AMP)	10	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Chloramphenicol (C)	30	11(R)	14(I)	15(I)	12(I)	15(I)	12(I)	13(I)	13(I)	12(I)	14(I)	13(I)	14(I)	13(I)
Ciprofloxacin (CIP)	5	30(S)	30(S)	30(S)	29(S)	34(S)	35(S)	30(S)	30(S)	28(S)	31(S)	31(S)	34(S)	32(S)
Cefoperazone (CPZ)	75	20(I)	18(I)	19(I)	20(I)	20(I)	23(S)	21(S)	21(S)	18(I)	18(I)	18(I)	19(I)	19(I)
Cefotaxime (CTX)	30	19(R)	18(R)	19(R)	18(R)	18(R)	20(R)	14(R)	18(R)	19(R)	18(R)	19(R)	17(R)	20(R)
Doxycycline-Hcl (DO)	30	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Fosfomycin (FO)	200	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Gentamycin (G)	10	18(S)	18(S)	18(S)	18(S)	20(S)	27(S)	21(S)	18(S)	18(S)	18(S)	18(S)	23(S)	19(S)
Imipenem (IPM)	10	27(S)	26(S)	26(S)	28(S)	25(S)	30(S)	27(S)	26(S)	27(S)	26(S))	26(S)	28(S)	25(S)
Kanamycin (K)	30	9(R)	9(R)	9(R)	9(R)	8(R)	15(I)	13(S)	8(R)	9(R)	9(R)	9(R)	13(R)	15(I)
Minocycline (MI)	30	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Meropenem (MRP)	10	34(S)	33(S)	33(S)	33(S)	33(S)	33(S)	33(S)	31(S)	35(S)	33(S)	34(S)	34(S)	30(S)
Nalidixic Acid (NA)	30	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Netillin (NET)	30	18(S)	18(S)	18(S)	19(S)	19(S)	26(S)	23(S)	19(S)	19(S)	18(S)	18(S)	24(S)	19(S)
Nitrofurantoin (NIT)	300	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Norfloxacin (NX)	10	27(S)	27(S)	27(S)	26(S)	27(S)	30(S)	26(S)	25(S)	27(S)	30(S)	27(S)	28(S)	27(S)
Piperacillin (PI)	100	23(S)	22(S)	21(S)	23(S)	22(S)	26(S)	22(S)	22(S)	23(S)	22(S)	23(S)	22(S)	23(S)
Streptomycin (S)	10	18(S)	17(S)	16(S)	16(S)	19(S)	22(S)	19(S)	14(S)	19(S)	19(S)	17(S)	22(S)	17(S)
Tetracycline (TE)	30	9(R)	NZ	9(R)	11(R)	10(R)	12(R)	10(R)	8(R)	8(R)	10(R)	10(R)	9(R)	9(R)
Trimethoprim (TR)	10	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Methicillin (MET)	5	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Penicillin (P)	2	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Rifampicin (RIF)	2	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
Vancomycin (VA)	10	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ

NZ: No zone; R: Resistance; I: Intermediate; S: Sensitive.

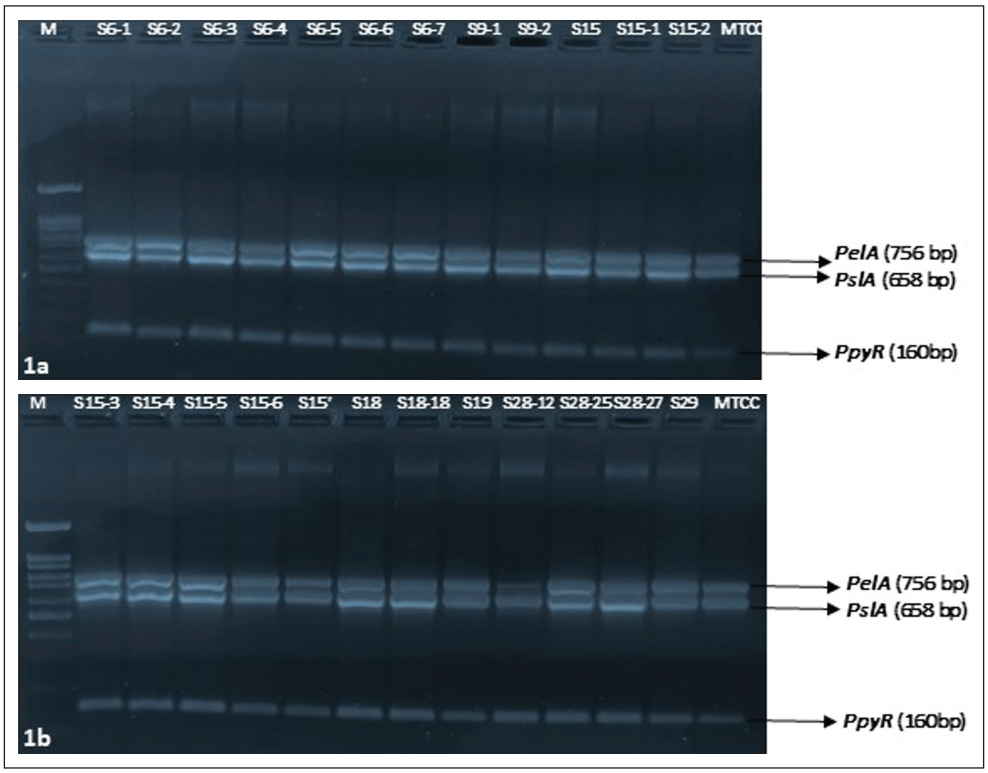


Figure 1. Detection of biofilm forming genes (*pslA*, *pelA*, *ppyR*) by PCR analysis. Lanes M: 100 bp DNA ladder RTU (Gene DireX); **(a)** Lanes (S6-1, S6-2, S6-3, S6-4, S6-5, S6-6, S6-7, S9-1, S9-2, S15, S15-1, S15-2) and **(b)** Lanes (S15-3, S15-4, S15-5, S15-6, S15, S18, S18-18, S28-12, S28-25, S28-2, S29) biofilm genes amplified by F– resistant isolates; Lanes MTCC: positive (+) control gene amplification by *P. aeruginosa* MTCC 2453.

Antibiotic Resistance

Fluoride tolerant isolates showed resistance to chloramphenicol, cefotaxime, doxycycline hydrochloride, fosfomycin, minocycline, nalidixic acid, nitrofurantoin, and trimethoprim (Tables 4, 5). In particularly, isolates S19, S15-5 was sensitive only to doxycycline hydrochloride. In other side, all isolates including positive controls were showed sensitive to gentamycin, imipenem, meropenem, netillin, and norfloxacin (Tables 4, 5). Similarly, many *Pseudomonas* species retrieved from water were resistant to various antimicrobial agents [30]. Moreover, *P. aeruginosa* showed resistance to amoxillin when isolated from sewage and sputum samples [31]. Recently, the WHO announced 12 species of waterborne antibiotic-resistant bacteria, including *Pseudomonas*, *Acinetobacter*, and various *Enterobacteriaceae*, which may cause a threat to human health [32]. According to the WHO report, we identified antibiotic-resistant *Pseudomonas* with fluoride tolerance that was isolated from water sources.

Salt Tolerance

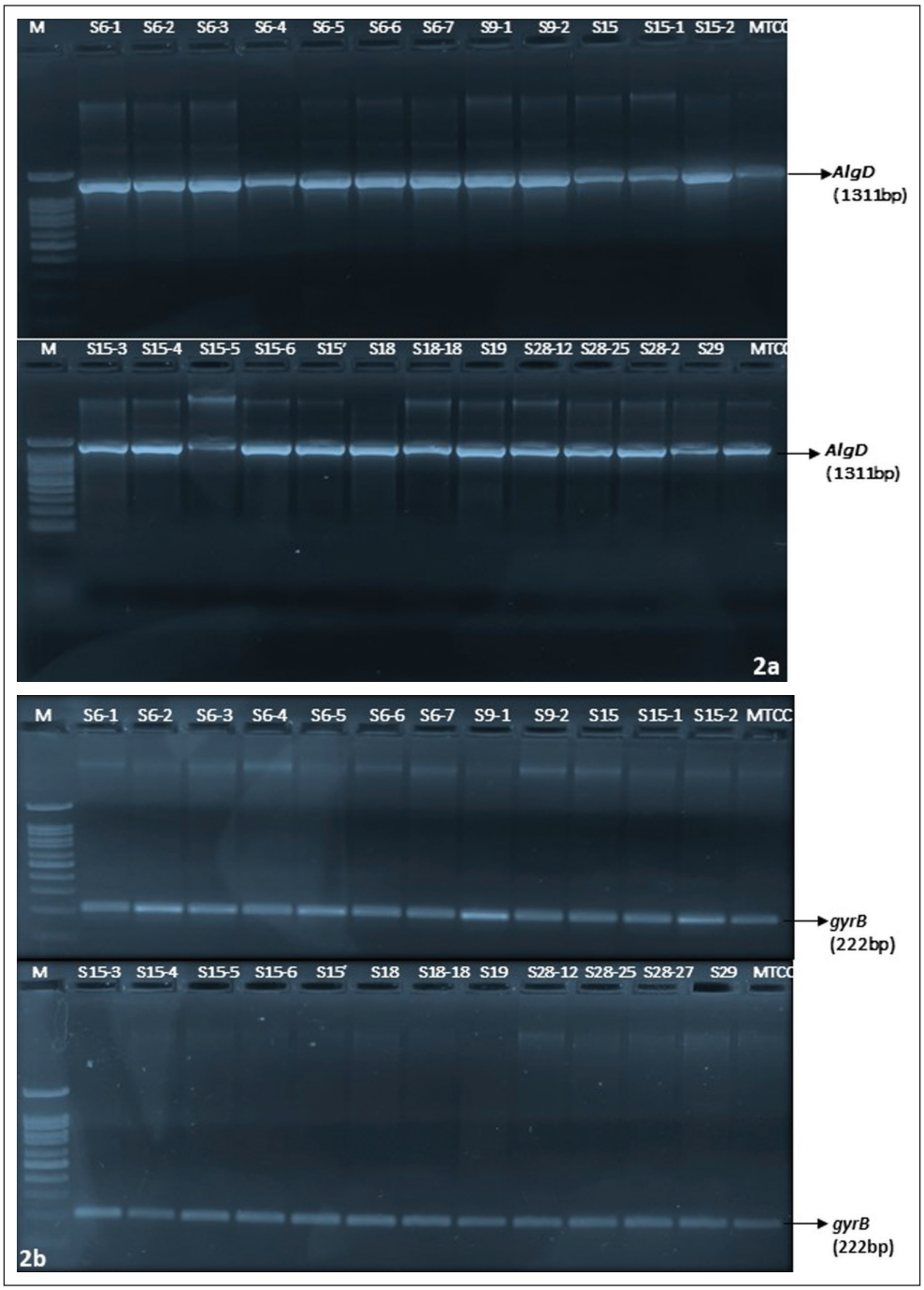
The F^- resistant isolates were able to survive upto 5% NaCl (V/V) augmented LB agar plates. Similarly, Kirupa Sree et al. [33] reported that F^- resistant *Pseudomonas* strains THP6, THP41 and OPH5 grow upto 5% NaCl. In another study, F^- resistant isolates DWC1, DWC2 and DWC5 showed their tolerance only 2.5% and 4% NaCl [34]. But, F^- resistant *Bacillus flexus* NM25 showed upto 20% NaCl high salt tolerance was isolated from soil [23].

Biofilm Forming Genes

Tissue culture method is one of a standard method for biofilm detection [24]. Fluoride resistant isolates exhibited non-adherent, weak and moderate biofilm producer. The biofilm producing genes *ppyR*, *pslA* and *pelA* was present in these *Pseudomonas* isolates amplified by PCR analysis (Fig. 1a, b). Earlier these kinds of biofilm genes have been identified in the clinical isolates of *P. aeruginosa* [35]. Hou et al. [36] stated that no *P. aeruginosa* isolates were phenotypically positive for biofilm formation.

Virulence Profile of *Pseudomonas*

P. aeruginosa has secreted multiple virulence factors including EPS, lipopolysaccharides, elastases and proteases, lipases, exotoxin A, exoS, and type IV pili, rhamnolipids and pyocyanin production [37]. In this work, six different (*algD*, *gyrB*, *lasB*, *plcH*, *toxA*, *rhlC*) virulence factors were amplified by PCR (Fig. 2a–f). The gene "*gyrB*" gene PCR is one of the important target for the detection and identification of *P. aeruginosa* was isolated from water [38]. Under environmental stress conditions, *P. aeruginosa* able to secrete '*algD*' and '*lasB*' genes can influence

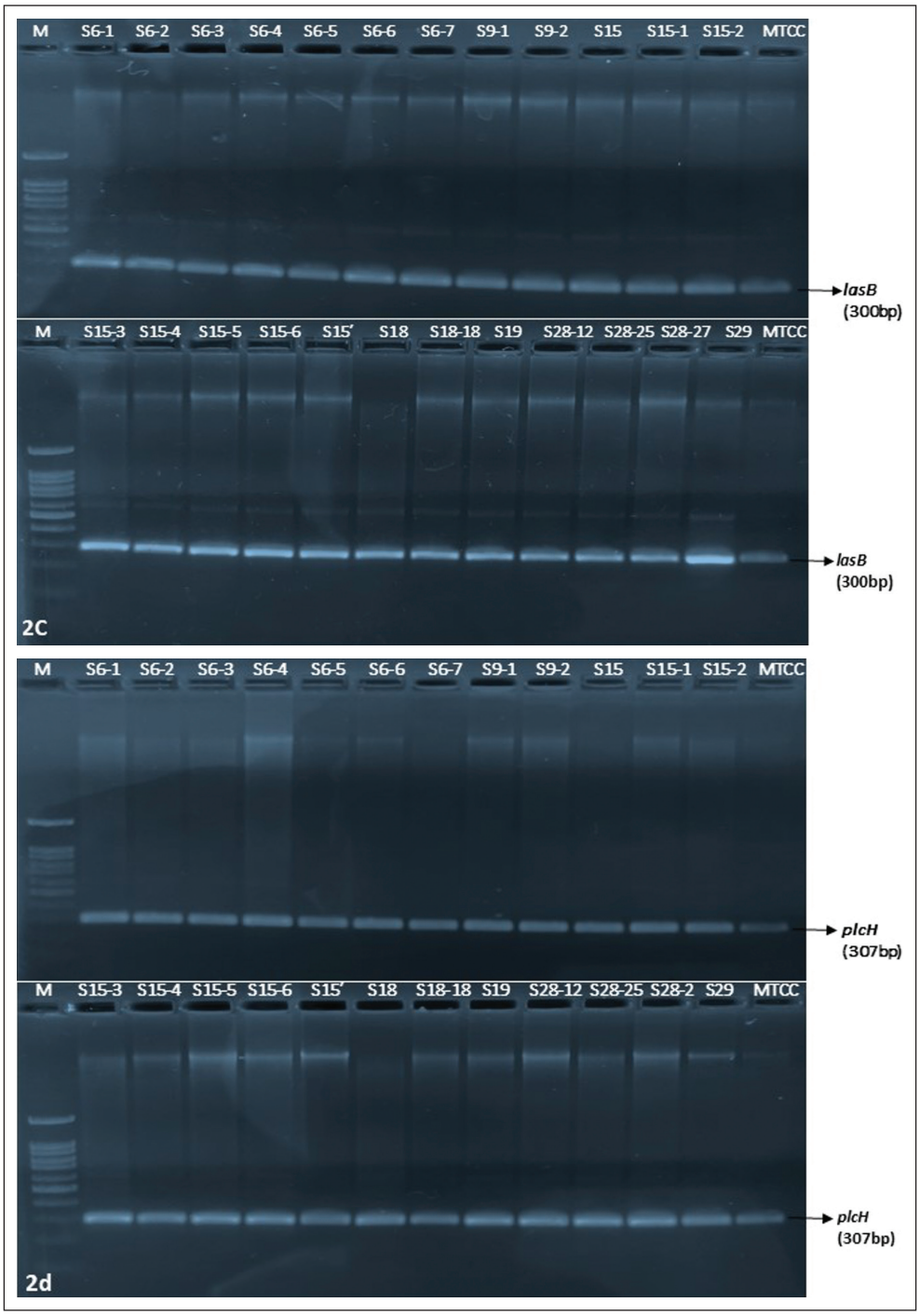


pathogenesis by enhancing adhesion, colonization, and invasion of tissues causing chronic pulmonary inflammation [27]. Likewise, Martins et al. [39] reported that the most common virulent genes (*algD*, *lasB*, and *plcH*) existing in *P. aeruginosa* were isolated from clinical and environmental origins.

Exotoxin A (*toxA*) was identified in this study. The '*toxA*' gene inhibits protein biosynthesis, and contributes to the colonization process [40]. In general, *rhlA*, *rhlB* and *rhlC* are key enzymes found in *Burkholderia* and *Pseudomonas* species responsible for rhamnolipid biosynthesis [41, 42]. In this study, we found only '*rhlC*' was amplified in these isolates by PCR (Fig. 2f).

Fluoride Resistant Gene Amplification

In *Pseudomonas* species, fluoride resistant gene was amplified using gene specific PCR. For this purpose, we designed the primers from the target sequence of *P. aeruginosa* AR_0111 was obtained from NCBI database. The 384 bp product was successfully amplified from *Pseudomonas* species (Fig. 3a, b).



Confirmation of *Pseudomonas* by PCR

Two primers were used for the specific identification of genus *Pseudomonas* and *P. aeruginosa* species [25]. In this work, we amplified 618 bp fragment from fluoride resistant isolates and identified as *Pseudomonas* species (Fig. 4a). Besides, 956 bp fragment amplified from all isolates were further confirmed as *P. aeruginosa* (Fig. 4b). The 16S rDNA variable region and '*toxA*' coding gene PCR are simple, and rapid identification of environmental *P. aeruginosa* strains [43].

Selection of Potent Strains

In plate assay studies, all the *Pseudomonas* isolates were efficiently growing in 200 mM NaF containing LB agar plates. Further, three strains (S6-3, S15', and S28-25) were selected based on their growth performances on NaF-supplemented liquid media. The selected strains are able to tolerate 150 mM NaF in LB broth. Based on their growth performances in fluoride-containing liquid media, potent fluoride-resistant strains were selected for further identification.

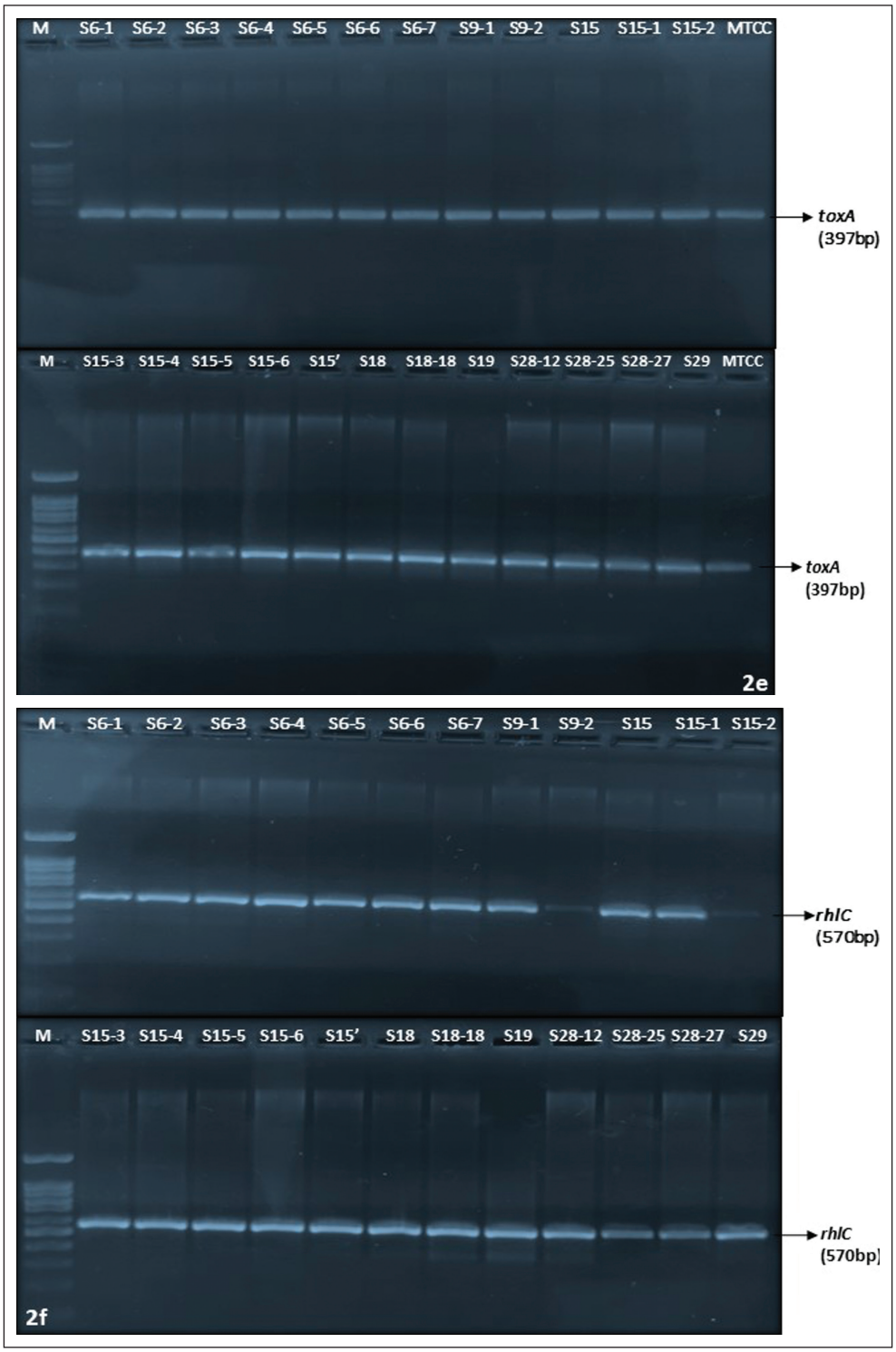


Figure 2. Virulence profile of F^- resistant *Pseudomonas* isolates. Lanes M: 100bp DNA ladder; lanes MTCC: (+) control *P. aeruginosa* 2453; The selected virulence genes **(a)** *algD* **(b)** *gyrB* **(c)** *lasB* **(d)** *plcH* **(e)** *toxA* **(f)** *rhlC* amplifications confirmed by gene specific primers.

16SrRNA Sequencing Analysis

Genomic DNA was extracted from these strains (S6-3, S15", S28-25) used as templates for PCR reactions. The 1.5 kb PCR products were successfully amplified from each isolate using 16S rRNA universal primers. The amplified products were gel excised and eluted by gel extraction kit (Thermofisher Scientific, USA). The purified PCR products was used as template for Sequencing process. The sequencing analysis was performed

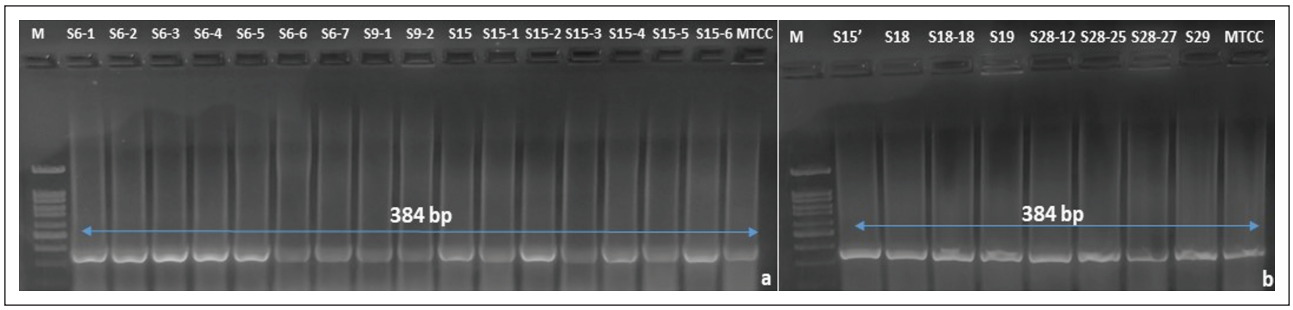


Figure 3. **(a, b)** The '*crcB*' gene amplification by F^- resistant *Pseudomonas* isolates. Lanes M: 100 bp DNA ladder; Lanes MTCC: (+) control *P. aeruginosa* 2453.

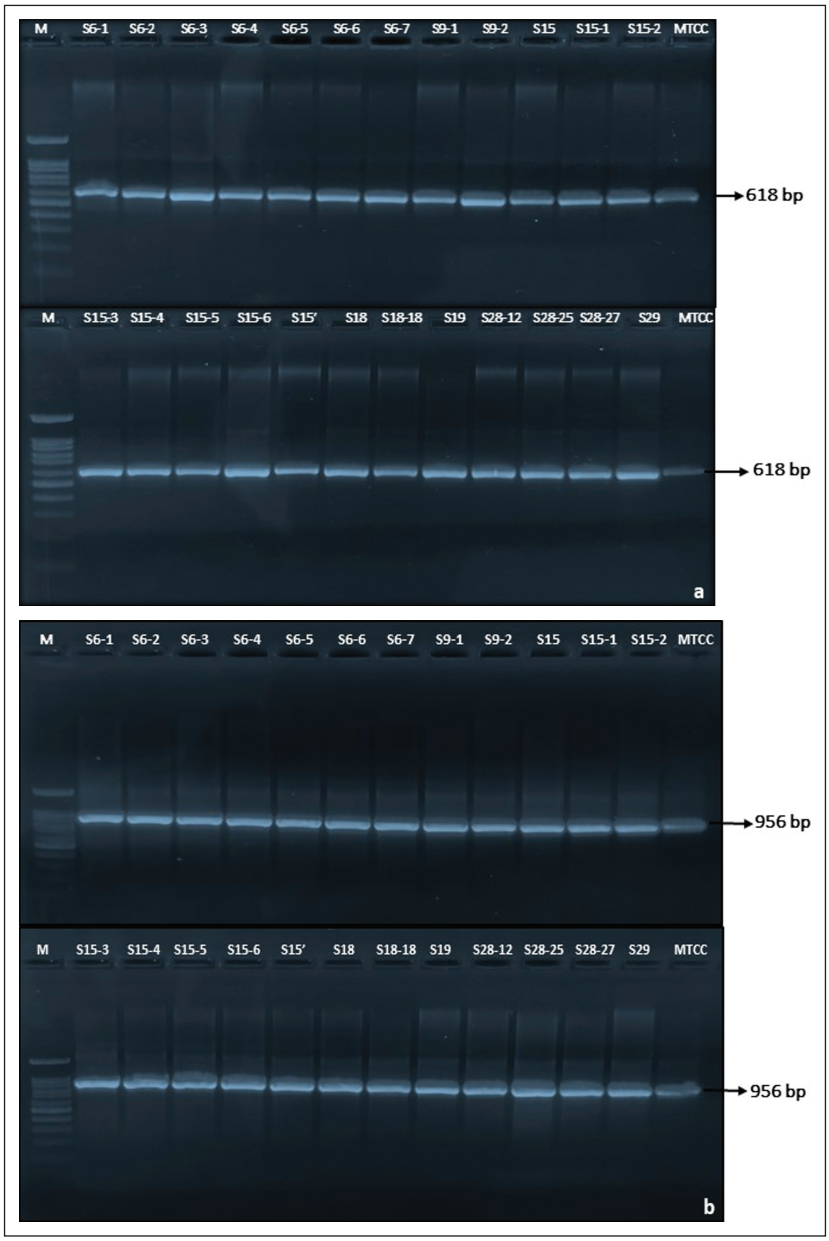


Figure 4. Genus and species-specific PCR analysis. **(a)** F^- resistant isolates amplified (618 bp) PCR products and identifying as genus *Pseudomonas*. **(b)** The PCR products (956 bp) shown by *Pseudomonas* isolates confirmed as *Pseudomonas aeruginosa* species. Lanes M: 100 bp DNA ladder; Lanes MTCC: (+) control *P. aeruginosa* 2453.

in AgriGenome Pvt Ltd, Cochin, Kerala. Comparative analysis of the 16S rRNA sequences with already available database showed that the selected isolates were closed to the family of Pseudomonadaceae. The present result also coincides with previous studies have shown that *Pseudomonas* was one of the bacterial species commonly found in groundwater [44].

Earlier studies shown that various F– resistant bacterial species isolated from various environment regimens [33, 34, 45–48]. In this study, *Pseudomonas* was recognized as F– resistant bacteria confirmed by genus, species-specific PCR and 16S rRNA sequencing analysis.

CONCLUSIONS

Genus, species-specific PCR, 16S rRNA sequencing and blast analysis concluded that twenty-two F– resistant strains were identified as *Pseudomonas* species. All the strains showed 200 mM NaF resistance determined in LB agar plates. They exhibited β-haemolytic activity on blood agar plates. Virulence genes and biofilm-forming genes were present in these isolates confirmed by Gene-specific PCR analysis. In addition, β-haemolytic *Pseudomonas* showed resistance to multiple antibiotics evidenced by a disc diffusion method. We identified the *crcB* domain in *Pseudomonas* using gene-specific primers. In near future, genetically modified fluoride resistant bacteria will be used in fluoride bioremediation studies.

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DATA AVAILABILITY STATEMENT

The authors confirm that the data that supports the findings of this study are available within the article. Raw data that support the finding of this study are available from the corresponding author, upon reasonable request.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICS

There are no ethical issues with the publication of this manuscript.

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