Environmental Research & Technology, Vol. 1(2), pp. 69-74, 2018

Environmental Research & Technology

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RESEARCH ARTICLE

Isolation and characterization of fluoride resistant bacteria from groundwaters in Dindigul, Tamilnadu, India

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ABSTRACT

The use of microbes to remove fluoride from water and soil is an extent of applied research and development. This is the first attempt have been made to examine physiochemical characteristics and also isolate fluoride resistant bacteria from ground waters in selected villages at Dindigul district, Tamil nadu, India. Based on high fluoride resistance (200 mM), three bacterial isolates were selected for further studies. The isolates authentically identified as genus *Pseudomonas*. Biochemical and 16S rRNA sequencing analysis of the isolates revealed that they are closely related (97%) to *Pseudomonas* sp. (98%) to *Pseudomonas* aeruginosa and (97%) to *Pseudomonas* sp. The 16S rRNA sequences were submitted in the NCBI under accession numbers MF481852, MF481853, MG751413. The fluoride resistant bacterial strains were resistant to antibiotics such as amoxicillin, ampicillin, chloramphenicol, kanamycin and streptomycin.

Keywords: Ground water, Fluoride resistant bacteria, Antibiotic resistance, *Pseudomonas*

1. INTRODUCTION

Groundwater is a most valuable natural source that is essential for human health, socio-economic development and functioning of ecosystems [1]. The rapid urbanization, agricultural activities and other environmental fluctuations are continuously deteriorating the quality of various water resources [2, 3]. Fluoride pollution occurs in the environment through natural and anthropogenic sources [4]. The natural source of fluoride in waters are fluoride bearing minerals and soil consisting clays [5]. The wastewater released from semiconductor, aluminium and glass manufacturing industries, also contributes fluoride water pollution especially in groundwater [4]. The permissible limit of fluoride is 1.5 mg L^{-1} in water [6]. It is harmful when it exceeds the acceptable limit and it can lead to various diseases for example osteoporosis, arthritis, and brittle bones cancer, infertility, brain damage, and thyroid disorder [7]. The fluoride problem has reached alarming proportion affecting at least 19 states of India [8, 9, 10]. Most of the countries are affected by fluorosis including China, India, Srilanka, Mexico, Argentina, U.S.A, New Zealand, Japan, Egypt, Jordan, Turkey, Iran, Iraq, Kenya, Tanzania, South Africa, Australia, Thailand, Canada, Saudi Arabia, Persian Gulf, and Syria [11, 12, 13, 14]. In this study to assess the physicochemical parameters of some open wells, hand pumps and bore well samples were collected from three different villages of Dindigul district. In addition, to screen and isolate fluoride resistant bacteria from the groundwater samples.

2. MATERIALS AND METHOD

2.1. Sample Collection and analysis

Groundwater samples were collected from 22 different sites including bore wells, hand pumps, and wells covering three villages of Dindigul district (Fig 1). Dindigul is one of the district in the state of Tamil Nadu. The geological longitude of the sampling site of Tamilnadu is 11.1271° N, 78.6569° E. For

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Received 7 March 2018; Received in revised form 10 April 2018; Accepted 10 April 2018
Available Online 20 April 2018
Doi: ISSN:

physicochemical characteristics, water samples were collected in sterile plastic bottles and then carefully sealed, labelled and transferred to laboratory for the analysis. Portable device (PCSTestr 35, Eutech), was employed to record pH, electrical conductivity(EC) and total dissolved solid (TDS). The other physiochemical parameters such as total hardness (TH), residual (free) chlorine, chloride, iron and nitrate were determined by titration method as recommended by manufacturer instructions (Himedia, Mumbai, India). The fluoride concentration was estimated by LABMAN ion meter (lumion-40) with fluoride electrode combination.

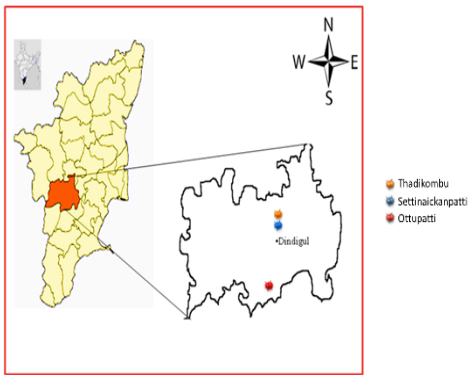

Fig 1. Groundwater sampling sites of three different villages in Dindigul district

2.2. Isolation of fluoride resistant bacteria

For isolation and enumeration of fluoride resistant bacteria, 0.1 ml of undiluted water samples were separately plated on Luria-Bertani agar (LB) (Himedia, Mumbai, India) supplemented with Sodium fluoride (NaF) (SRL, Mumbai, India). The initial concentration of 10 and 70 mM NaF was used to screen the fluoride resistant bacteria from Thadikombu, Settinaickanpatti and Ottupatti water samples. Plates were incubated at 37°C for 72 h. Fluoride resistant colonies differing in morphological, physiological and biochemical characteristics were isolated and used for further studies.

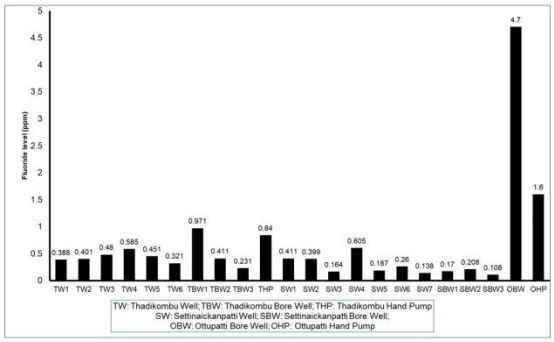

Fig 2. Fluoride values in groundwater analysed by ion selective electrode method. The concentration of fluoride is mentioned in parts per million (ppm) units

2.3. Determination of fluoride resistance

Fluoride resistance was determined on LB agar plates supplemented with different concentration of NaF starting from 10 to 250 mM. The working concentrations of NaF were prepared from 1 M stock solution of Sodium fluoride. The stock solution of fluoride was prepared in sterilized double distilled water. Minimum inhibitory concentration (MIC) was evaluated until the selected isolates were unable to grow on fluoride containing LB agar plates. Based on this analysis, MIC was determined in five days at 37 °C.

2.4. Biochemical Characterization of fluoride resistant bacteria

Selected fluoride resistant isolates were checked their growth on MacConkey agar, Eosin Methylene Blue agar (EMB), and *Pseudomonas* isolation agar media (Himedia, Mumbai, India). The shape and colour of the colonies were examined under the microscope after Gram staining. The isolates were identified according to Bergey's Manual of Systematic Bacteriology [15]. The optimal growth conditions with reference to pH and temperature were determined. The selected isolates were grown in LB medium, in the presence and absence of fluoride with varying pH values, i.e., 4, 5, 6, 7, 8, 9 and incubated at 25, 30, 37 and 44 oC. The optical density of the growing cultures in all the above-mentioned conditions was observed at 570 nm using a photo colorimeter (Deep vision, model 312) to determine the optimum growth.

2.5. Polymerase chain reaction (PCR) amplification

One colony or toothpick of every bacterial culture was suspended in 10 µl sterile double distilled water containing 1.5 ml Eppendorf tube. The tubes were kept for 15 min at 95 °C in boiling water bath and short spin at 10,000 rpm for 2 min. From the supernatant, 1µl was used as a template for PCR reaction. Amplification of 16S rRNA was carried out by using the universal bacterial 16S rRNA primers, 27 F 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1429 R 5'-GGT TACC TTG TTA CGA CTT-3' [16] in thermal cycler under the following cyclic conditions as follows: 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s and final extension at 72°C for 5 min. Polymerase chain reaction was performed in Agilent Technologies, SureCycler 8800. PCR product was analysed in 1.0% agarose gel electrophoresis. The amplified PCR products were eluted by GeneJET gel extraction kit (ThermoScientific, USA) and then carry out for sequencing.

2.6. 16S rRNA sequencing and blast analysis

The 16S rRNA gene sequencing of fluoride resistant isolates was carried out in 48-capillary ABI 3730 DNA analyser by direct sequencing of the PCR-amplified 16S rRNA gene. The sequences obtained were

compiled and compared to the sequences in the Genbank databases using BLAST analysis [17].

2.7. Determination of antibiotic resistance

Antibiotic sensitivity of the fluoride resistant isolates was determined by disc diffusion method. Antibiotic-impregnated discs (Himedia, Mumbai, India) were placed on Mueller Hinton (MH) agar plates swabbed with individual isolates and incubated at 37^{o}C for 24-48 h. The diameter of the inhibition zones around the discs was measured. The antibiotic concentrations of the disc used were Amikacin (AK, 30mcg), Amoxyclav (AMC, 30mcg), ampicillin (AMP, 10mcg), chloramphenicol (C, 30mcg), ciprofloxacin (CIP, 5mcg) gentamycin (GEN, 10mcg) kanamycin (K, 30mcg) streptomycin (S, 10mcg) and tetracycline (TE, 30mcg) respectively.

2.8. Salt tolerance

The salt tolerance was determined in LB agar plates supplemented with different concentration of Sodium chloride (1-10%). The growth was monitored after 48 h incubation at 37°C.

3. RESULTS & DISCUSSION

3.1. Physiochemical characteristics

The physicochemical parameters and statistical measures of groundwater samples are mentioned in Table 1. In this study, pH values of all groundwater samples were ranging from 6.78 to 8.28. According to the WHO, safe limit of pH in water is 6.5-8.5 [18]. So that collected water samples pH values were within the permissible limit. The most desirable limit of EC in drinking water is prescribed as 300 µS cm^{-1} [19]. The average value of EC is 1863 µS cm^{-1} and maximum number of samples showed above the desirable limit EC values. The WHO most desirable and maximum allowable limit of TDS is 500 and 1500 mg L^{-1} respectively. The TDS values of all samples ranges from 1.09 to 935 mg L^{-1} and also within the acceptable limit. The highest and average value of salinity was found to be at 942 and 491.5 mg L^{-1} respectively. There were iron and residual free chlorine values not detected in all type of water samples.

In regards to total hardness, the highest values were obtained at 800 and 750 mg L^{-1} for Thadikombu (SW3) and Settinaickanpatti (SW7) well waters. The permissible limit of chloride (Cl-) in water is 1000 mg L^{-1} as recommended by the BIS. The Cl- content of all samples values were (100-750 mg L^{-1}) within the permissible limit. In general nitrate level in drinking water can also be an indicator of overall water quality. But in this study, tested nitrate values (0-25 mg L^{-1}) were below the desirable limit, that is 45 mg L^{-1} [20]. Fluoride concertation was estimated by LABMAN ion meter and their values are shown in Fig. 2. The range (0.108-0.971 ppm) of fluoride concentrations were perceived from Thadikombu and Settinaickanpatti villages. In this analysis, maximum number of samples showed below fluoride level <0.5 ppm and it causes dental caries [21, 22]. In contrast, high fluoride values (1.6 and 4.7 ppm) were tested in Ottupatti village. Likewise, high fluoride contamination (4.34 ppm) in drinking water was reported in Ottapidaram block, Thoothukudi District [23]. Fluoride is harmful, when it exceeds the permissible limit of 1.5 ppm [24]. The concentration of fluoride above 1.5 ppm may cause dental fluorosis, intake of fluoride concentration above 3.0 ppm may cause skeletal fluorosis respectively [19].

Table 1. Statistical measures like, maximum, minimum, average and standard deviation of water samples in the study area

Water quality parameters	Units	Maximum concentration	Minimum concentration	Average	Standard deviation (SD)	WHO/ISI permissible limit
pH	-	8.28	6.78	7.49	0.39	6.5-8.5
EC	µS cm^{-1}	1863	2.24	983.61	723.56	300
TDS	mg L^{-1}	935	1.09	215.36	335.20	500
Salinity	mg L^{-1}	942	1.14	491.50	364.01	-
Nitrate	mg L^{-1}	25	0	23.86	5.33	45(ISI)
TH	mg L^{-1}	800	50	393.18	318.64	500
Chloride	mg L^{-1}	750	40	318.64	172.91	200

3.2. Screening and isolation of fluoride resistant bacteria

Five hundred ninety-four colonies were screened from initial level of NaF containing LB agar plates. After secondary screening, the colonies were transferred consecutively from 100-200 mM NaF supplemented LB agar plates. Finally, one colony was selected based on high growth in 200 mM NaF supplemented LB broth and agar medium. In addition, an attempt was made to screen fluoride resistant bacteria from low desirable limit fluoride (1.0 ppm) in Thadikombu and Settinaickanpatti water samples. After screening, two high fluoride resistance (200 mM) bacterial isolates were identified only from Thadikombu water samples. The fluoride resistance (200 mM) is equivalent to the bacterium, that was isolated from high fluoride contaminated (1.6 and 4.7 ppm) Ottupatti water samples. Although, no high NaF resistance colonies were identified from Settinaickanpatti water samples. Three bacterial isolates were selected (THP6, THP41 and OHP5) and

used for further studies. The fluoride resistant isolates were made growth on MacConkey, EMB, and *Pseudomonas* isolation agar. They were gram negative, rod shaped bacteria (Fig 3). The optimum growth was observed at 37 oC and pH 7.

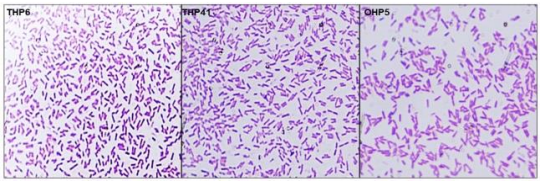

Fig 3. Microscopic view of fluoride resistant bacteria

Bacterial 16S rRNA gene (1.5 kb) was amplified successfully using 16S rRNA universal primers and then eluted by gel extraction kit (Fig 4). The eluted products were sequenced. Based on the morphological, biochemical and 16S rRNA sequencing analysis showed that the strains were close to the members of genus *Pseudomonas*. The highest sequences similarities were observed for THP6 (97%), THP41(97%) and OHP5 (98%) and matched highly homology to *Pseudomonas* sp. MB65 (HM597240), *Pseudomonas* sp. (GU966668) and *Pseudomonas aeruginosa* DN1 (KP119458) respectively. The 16S rRNA sequences were submitted in the NCBI database under accession numbers (MF481852, MF481853, MG751413). The evolutionary history was inferred using the UPGMA method [25]. The evolutionary distances were computed using the Maximum Composite Likelihood method [26] and phylogenetic tree was created in MEGA7 [27]. Phylogenetic analysis of fluoride resistant bacteria is shown in Fig 5. In this study, three *Pseudomonas* strains exhibited 200 mM NaF resistance was determined on LB agar plates. Previously testified fluoride resistant bacteria including *P. aeruginosa* and *Acinetobacter* sp. RH5 showed only 100 mM and 100 mg L^{-1} fluoride resistance was isolated from soil and groundwater [28, 9]. Heterotrophic bacteria (Particularly *Pseudomonas* species) are ubiquitous and common bacterial species in ground water mainly because of their phenotypic plasticity [29, 30]. Bruins et al. [31] also proved that, *Pseudomonas* species exhibit resistance to a variety of heavy metals, antibiotics, toxic substances, and can use various compounds as carbon sources. Therefore, they have generated a high degree of interest in the area of environmental bioremediation.

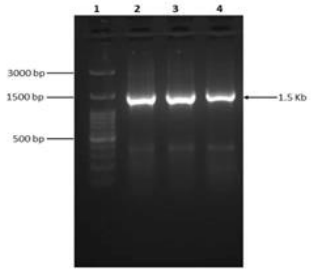

Fig 4. Agarose gel electrophoresis of 16r RNA gene amplification. Lane: 100bp DNA ladder H3 RTU; Lanes 2, 3 & 4: PCR products (1.5 kb) amplified from fluoride resistant bacterial strains HP6, HP41 and OHP5 isolated from groundwaters

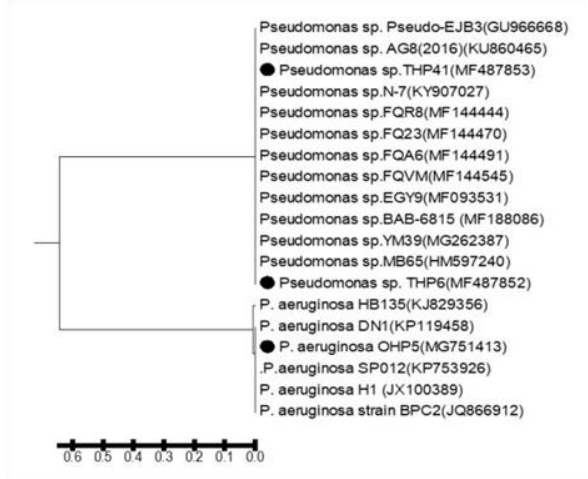

Fig 5. Phylogenetic analysis of fluoride resistant *Pseudomonas* and related species obtained from Genbank based on 16S rRNA sequences. The optimal tree with the sum of branch length = 1.29429242 is shown in the figure. The scale bar represents the units of the number of base substitutions per site. A total of 19 sequences involved in the analysis

3.3. Resistance to Salt and Antibiotics

Fluoride resistant isolates (THP6, THP41 and OHP5) exhibited 5% (w/v) salt resistance in LB agar plates. They were resistant to antibiotics such as amoxicillin, ampicillin, chloramphenicol, kanamycin and streptomycin. But they sensitive to amikacin and gentamycin antibiotics (Table 2). Isolates THP6, OHP5 was sensitive to ciproflaxin but THP41 is resistant, in other way, THP6 and OHP5 was resistant to Tetracycline in contrast THP41 is sensitive to it. Similarly, heavy metal resistant *Pseudomonas aeruginosa* BC15 showed resistance to many antibiotics such as ampicillin, tetracycline, chloramphenicol, erythromycin, kanamycin and streptomycin [32]. In contrast fluoride tolerant *Bacillus flexus* NM25 was sensitive to recommended doses of ofloxacin, kanamycin, rifampicin, levofloxacin, vancomycin, gatifloxacin, gentamicin, doxycycline, streptomycin, and nalidixic acid but only resistant to ampicillin respectively [33].

Table 2. Antibiotic sensitivity of Fluoride resistant isolates

Antibiotics	Disc content (mcg)	THP6	THP41	OHP5
Amikacin (Ak)	30	21(S)	18(S)	18(S)
Amoxicillin (AMC)	30	NZ(R)	8(R)	NZ(R)
Ampicillin (AMP)	10	NZ(R)	NZ(R)	NZ(R)
Chloramphenicol (C)	30	12(R)	8(R)	12(R)
Ciprofloxacin (CIP)	5	32(S)	9(R)	28(S)
Gentamycin (GEN)	10	24(S)	21(S)	20(S)
Kanamycin (K)	30	9(R)	9(R)	8(R)
Streptomycin (S)	10	19(R)	19(R)	18(R)
Tetracycline (TE)	30	9(R)	8(S)	NZ(R)

Note: S-sensitive, I-Intermediate, R- Resistance, NZ-No Zone, Zone of inhibition noted in (mm)

4. CONCLUSIONS

The study proves a variation in the Physico-chemical characteristics of groundwater. In concerning with fluoride contamination, less than desirable and higher than permissible values were detected in Thadikombu, Settinaickanpatti and Ottupatti villages. To our knowledge, this is the first report for identifying high fluoride resistant bacteria especially from Dindigul district, Tamilnadu. Fluoride resistant bacterial isolates exhibited 5% salt tolerance and also associated with resistant to multiple antibiotics. Future work will explore in terms of bacterial fluoride bioremoval, characterize fluoride resistant gene in *Pseudomonas* species and to develop biosensor for fluoride detection.

ACKNOWLEDGEMENT

This work was supported by grant from SERB, Department of Science & Technology, New Delhi, DST Ramanujan Fellowship to C. Edward Raja.

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