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Detection of bacterial load in drinking water samples by 16s rRNA ribotyping and RAPD analysis

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Key words:
Drinking water, Bacteriological contamination, RAPD analysis, 16S rRNA gene amplification

Abstract:

Background: Safe and healthy drinking water is inaccessible to more than 20% of the world population. Among some major risks to safety of potable water, contamination with pathogenic microorganisms is the most alarming and harmful Therefore, it is needed to develop and implement fast and accurate methods for the detection of bacterial contamination in water.

Methods: Biological analysis of drinking water samples obtained from nine different collection points of Lahore city was carried out and total of six different bacterial strains were isolated. Biochemical characterization was done under standard laboratory conditions. Molecular identification of these isolates was done by using random amplified polymorphic DNA (RAPD) analysis.

Results: The drinking water sample collected from Punjab University showed highest bacterial count 1066/0.5 ml of drinking water while residential area of University of the Punjab contained least number of bacterial counts i.e., 38/0.5 ml of drinking water. Amplification patterns of isolates SZ1, SZ3, SZ4 and SZ6 obtained by RAPD were found similar to genus Bacillus. While, SZ2 and SZ5 had unique amplification patterns identical to Bacillus megaterium. All the six bacterial strains were tested for the presence of protease, lipase, cellulase, and amylase. Strain SZ2 gave positive result for all of them except amylase.

Conclusion: Tube well water of Punjab University area of Lahore is safe for drinking purpose except admin block tube. It is recommended to monitor the bacteriological load of drinking water at regular intervals in order to control water borne bacterial diseases.

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Introduction

Access to safe drinking water is one of the major challenges of the 21st century [1]. About 20% of the world population does not have access to pure drinking water and the death rate associated with inadequate sanitation and impure drinking water consumption is more than 1.7 million/year [2]. In Asian and African countries, children under five years of age are mostly affected by water borne microbial diseases. In Pakistan, bacteriological contamination of drinking water is also a serious problem. Every year some major water borne gastro intestinal diseases break out due to consumption of this water [3]. The microbiological contamination of drinking water in Punjab is highest as compared to chemical and physical pollution [4]. Ground water is one of the important natural sources of drinking water and is usually considered pure for drinking purpose but several sources could be the cause of its contamination [5]. The major sources of ground water microbial contamination are seepage from the sewage system pipelines into boreholes due to cracked casings, poor water distribution supply networks and poor construction of water supply systems [6]. Excessive growth of bacteria in drinking water distribution systems can lead to bio-fouling of distribution pipes, bio-corrosion and clogging of filters [7].

Verification of microbial quality is necessary to manage the health impacts associated with the potential microbial hazards which include analysis of source water, water present in the distribution system, household water or water received after treatment [2]. In order to minimize the potential re-growth of bacteria the best approach is the addition of disinfectants in the distribution systems such as chlorine, chlorine dioxide or monochloramine [8]. Regular monitoring of microbial quantity in drinking water is also very important to control disease outbreaks [9]. Besides all these measures testing of drinking water microbiological quality plays an essential role in the monitoring of drinking water quality and assessing protection of public health [1].

In the current research, we have performed biological analysis of drinking water samples from Punjab University (Quaid-e-Azam Campus) to assess the bacterial load as the source of contamination by molecular and microbiological techniques.

Methods

Drinking water samples

Simple random sampling (SRS) technique was followed for collecting drinking water samples by using sterilized plastic sampling bottles. Randomly nine drinking water samples were collected from two locations viz. staff colonies and tube wells. Five water samples were collected from different blocks of staff colony viz. B-Block, C-Block, D-Block, E-Block and GH-Block while four tube well water samples were collected from the areas including Botanical Garden, E-Type block, Community Centre and Admin Block. The samples were stored at 4°C.

Microbiological analysis of drinking water samples

Ten sterile autoclaved LB agar solid media plates were prepared to allow growth of bacterial colonies for 24 hours at 37°C while one plate was used as negative control. LB agar medium contained 10 g tryptone, 5 g yeast extract and 5 g NaCl and 15 g agar per 1000 ml of the medium. Under aseptic conditions, 0.5 ml of each drinking water sample was dropped on the agar plates and spreaded with subsequent incubation at 37°C for 24 hours. The overnight incubated LB agar plates were observed visually under colony counter to determine following morphological parameters of bacterial colonies viz. shape, color, size and total number of colonies per sample. Average colony size was measured by setting a suitable scale.

Six morphologically distinct bacterial colonies (Table 2) viz. SZ1, SZ2, SZ3, SZ4, SZ5 and SZ6 were selected from the solid media plate and inoculated into the 10 ml LB broth and incubated for 24-72 hours in 37°C shaker for determination of the growth rate and subsequent biochemical as well as molecular analysis. Bacterial growth rate was determined by taking the optical density (OD) at 600 nm of the each bacterial strain culture after 12, 24, 48 and 72 hours by using spectrophotometer.

Biochemical analysis and Gram staining of bacterial strains

All the six bacterial strains (SZ1-SZ6) were screened in plate assays for the production of protease [10], lipase [11], cellulase [12] and amylase [13]. The level of comparative enzyme production was determined in terms of zone formation around each spot for bacterial strains (SZ1-SZ6). The gram staining of all six bacterial strains was carried out by standard procedure [14].

Bacterial genomic DNA isolation

Bacterial genomic DNA was isolated from all six bacterial strains by using GF-1 bacterial genomic DNA extraction kit, Vivantis, USA. 1.5 ml of liquid culture of each bacterial strain was used for DNA extraction. The DNA pellet of each bacterial strain was dissolved in 50 µl of deionized autoclaved water and stored at -20°C for further molecular analysis. DNA quality was determined by agarose gel electrophoresis.

Random Amplified Polymorphic DNA (RAPD) analysis

RAPD analysis of all six bacterial strains was carried out by using two different primers, P1 5'GGGTAACGCC3' and P2 5'CCCGTCAGCA3' in separate reaction mixtures. Each RAPD reaction mixture contained 1X PCR buffer 1.5 mM $MgCl_2$, 2 mM dNTPs, 100 picomoles P1 and P2 primers in separate reactions each, Taq polymerase 2.5 units, genomic DNA 3 µl and total volume of the reaction mixture was adjusted to 50 µl for each bacterial strain. The cyclic conditions for RAPD were: initial denaturation: at 95°C for 2 min, denaturation: at 95°C for 30 sec, annealing at: (25°C, 30°C and 35°C) for 2 min, elongation at 72°C for 2 min, and total cycles were 40 coupled with final extension at 72°C for 7 min and final termination at 4°C for 30 min. The amplified RAPD products were resolved on 1% agarose gel.

Ribotyping

The 16S rRNA gene of SZ2 was amplified by using the primers and conditions as described earlier [15]. The amplified 1400 bp fragment of rRNA gene was retrieved by gene clean by gene clean kit, Fermentas, USA and subjected to sequencing by using primers as described earlier [15]. The sequencing results were analyzed through Basic Local Alignment Search Tool or BLAST analysis for bacterial strain characterization at species level.

Statistical analysis

Analysis of variance for microbial load for quantitative data was carried out using the method of Steel *et al.* [16].

Results

The evaluation of drinking water for presence of bacteria was carried out from nine different localities of University of the Punjab. The samples were first spreaded on bacterial growth media and separated into six different strains viz., SZ1-SZ6 based on morphological features. These strains were further characterized by determining their growth rates, and subjected subsequently to molecular and biochemical analysis. Significant results were found for all studied features (Table 1a, 3a, 3b, 3c, 3d).

The water sample obtained from Admin block tube well showed maximum number of bacterial colonies (1243/0.5 ml of sample) while water sample collected from tube well from E-type houses showed least number of bacterial colonies (38/0.5 ml of sample). The water samples from B-Block, D-Block and community centre tube wells had not good water quality having 301, 163 and 160 bacterial colonies respectively (Table 1).

Locality	Bacterial Colonies*
Admin Block Tube well	1243
B-Block	300
D-Block	163
Community Centre Tube well	160
Botanical	92
GH-Block	49
C-Block	42
E-Block	40
E-Block	38

***Total no of bacterial colonies/0.5 ml sample**

Table 1: Bacterial load determination in drinking water samples

Source	DF	SS	MS	F	P
Replication	1	0.02	0.02		
Locality	8	2400273	300034	3636777*	0
Error	8	0.66	0.0825		
Total	17	2400274			

* = Significant at 5% probability level

Table 1a: ANOVA for total bacterial colonies

The bacterial strain SZ1, SZ2 and SZ5 were found to be gram positive while all remaining strains were gram negative (Table 2).

All six purified bacterial strains viz., SZ1-SZ6 were subjected to genomic DNA isolation by kit method. The results indicated that in case of all bacterial strains intact DNA band of ~20 kb was observed (Fig. 1).

In RAPD analysis, primer 1 amplification patterns of SZ1, SZ3, SZ4 and SZ6 were found to be similar, while SZ2 and SZ5 had unique amplification patterns (Fig. 2).

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Strain	Colony Size	Shape	Color	Gram Staining	Locality
SZ1	Small	Round	Yellow	Positive	E-Type Tube well
SZ2	Large	Irregular	White	Positive	E-Type Tube well
SZ3	Large	Round	White	Negative	E-Type Tube well
SZ4	Medium	Round	White	Negative	Admin Block Tube well
SZ5	Small	Round	White	Positive	Community Centre Tube well
SZ6	Medium	Round	Yellow	Negative	C-Block

Key: Small: Less than 0.3 cm diameter; Medium: 0.3-0.5 cm diameter; Large: 0.6-1.5 cm diameter.

Table 2: Morphological features and gram staining of bacterial strains

Strains	OD at 600 nm after 12 hours	Strains	OD at 600 nm after 24 hours	Strains	OD at 600 nm after 48 hours	Strains	OD at 600 nm after 72 hours	Strains	General Growth Features
SZ6	1.395 A	**SZ6**	2.795 A	**SZ6**	3.05 A	**SZ3**	2.895 A	**SZ1**	Fast growing
SZ1	1.15 B	**SZ1**	2.605 B	**SZ1**	2.995 B	**SZ1**	2.79 B	**SZ2**	Slow growing
SZ5	0.895 C	**SZ3**	1.705 C	**SZ3**	2.415 C	**SZ5**	2.705 C	**SZ3**	Medium growth
SZ3	0.81 D	**SZ5**	1.49 D	**SZ5**	2.05 D	**SZ6**	2.61 D	**SZ4**	Slow growth
SZ4	0.495 E	**SZ2**	0.805 E	**SZ2**	1.215 E	**SZ2**	1.895 E	**SZ5**	Medium growth
SZ2	0.405 F	**SZ4**	0.695 F	**SZ4**	0.895 F	**SZ4**	1.41 F	**SZ6**	Fast growth

Key: Fast growth: 1-1.5 OD in first 12 hours; Medium growth: 0.6-0.9 OD in first 12 hours; Slow growth: Up to 0.5 OD in first 12 hours

Table 3: Determination of bacterial strains growth rates and OD at 600 nm

Source	DF	SS	MS	F	P
Replication	1	0.00083	0.00083		
Strains	5	1.42857	0.28571	312.82*	0
Error	5	0.00457	0.00091		
Total	11	1.43397			

* = Significant at 5% probability level

Table 3a: ANOVA for determination of bacterial strains OD at 600nm after 12 hours

Source	DF	SS	MS	F	P
Replication	1	0.00001	0.00001		
Strains	5	7.74278	1.54856	17530.8*	0
Error	5	0.00044	0.00009		
Total	11	7.74323			

* = Significant at 5% probability level

Table 3b: ANOVA for determination of bacterial strains OD at 600 nm after 24 hours

Source	DF	SS	MS	F	P
Replication	1	0.00013	0.00013		
Strains	5	8.08087	1.61617	743.64*	0
Error	5	0.01087	0.00217		
Total	11	8.09187			

* = Significant at 5% probability level

Table 3c: ANOVA for determination of bacterial strains OD at 600 nm after 48 hours

Source	DF	SS	MS	F	P
Replication	1	0.00001	0.00001		
Strains	5	3.53574	0.70715	4767.29*	0
Error	5	0.00074	0.00015		
Total	11	3.53649			

* = Significant at 5% probability level

Table 3d: ANOVA for determination of bacterial strains OD at 600nm after 72 hours

Bacterial Strain	Protease	Lipase	Cellulase	Amylase
SZ1	+	-	+	+
SZ2	++	++	++	+
SZ3	+	-	+	+
SZ4	+	-	+	-
SZ5	+	-	-	-
SZ6	+	-	+	+

Key: ++: High level of enzyme production (zone diameter equal or above 2 cm); +: Low level of enzyme production (zone diameter below 1cm); -: Negative regarding enzyme production (no zone observed).

Table 4: Enzymatic profile of different bacterial strains

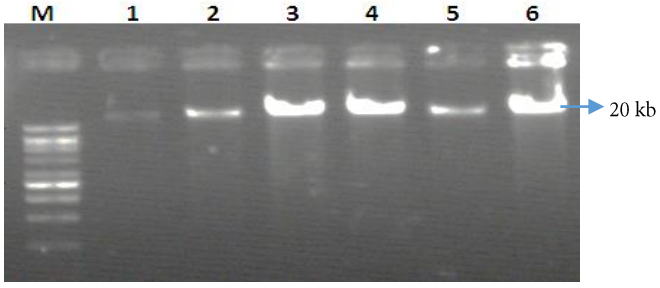

Figure 1: Genomic DNA isolation from six bacterial strains. Key: 1-6: Bacterial strains SZ1-SZ6, M: Marker DNA (Fermentas 1 Kb ladder mix).

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Bacterial contamination/ Locations	Admin Block Tube well	B-Block	Botanical Garden Tube well	C-Block	Community	D-Block	E-Block Tube well	E-Block
B-Block	0							
P<0.05	1							
Botanical Garden Tube well	0.6707*	-0.6286*						
P<0.05	0.4022	0.5672						
C-Block	0.9912*	0	0.6074*					
P<0.05	0	1	0.4328					
Community Centre Tube well	0.9321*	0	0.7577*	0.2902*				
P<0.05	0	1	0.5323	0				
D-Block	0.866*	0.5	0.3592	-0.0812	-0.0043			
P<0.05	0.3333	0.6667	0.7661	0.3333	0.3333			
E-Block Tube well	0.9078*	0.4193	-0.0996	0.0907	00978	-0.5766		
P<0.05	0.2755	0.7245	0.1573	0.2755	0.2755	0.6088		
E-Block	0.6412*	0	-0.0777	0.0022	0.0011	-0.0216	0.0918	
P<0.05	0	1	0.4328	0	0	0.3333	0.2755	
GH-Block	0	0.7231*	-0.06286	0	0	0.015	0.4193	0
P<0.05	1	0	0.5672	1	1	0.0667	0.7245	1

Table 5: Correlation among the locations for bacterial contamination

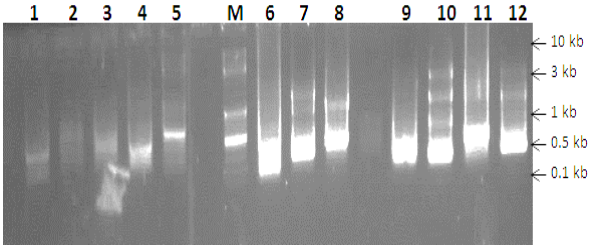

Figure 2: RAPD analysis of six purified bacterial strains (SZ1-SZ6). Key: 1-6: Bacterial strains SZ1-SZ6 samples amplified with primer P1; 7-12: Bacterial strains SZ1-SZ6 samples amplified with primer P2; M: Marker DNA (Fermentas 1 Kb ladder mix).

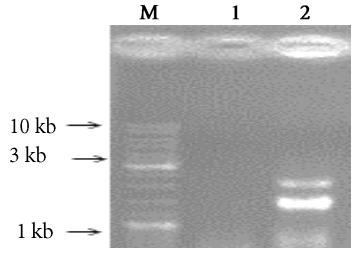

Figure 3: rRNA gene amplification of bacterial strain SZ2. 1: Negative control; 2: Bacterial strain SZ2; M: Marker DNA (Fermentas 1 Kb ladder mix).

In order to further validate the results, the genomic DNA of all six bacterial strains was further subjected to RAPD analysis by primer 2 and same results were observed as earlier mentioned by primer 1. Bacterial strains SZ1, SZ3, SZ4 and SZ6 amplified a prominent band of 0.5 kb while strains SZ2 and SZ5 showed a prominent amplification of 0.6 kb (Fig. 2). Therefore, it is concluded that bacterial strains SZ1, SZ3, SZ4 and SZ6 genomes have not much significant genetic variation from each other and placed in the same group similarly genomes of strains SZ2 and SZ5 have genetic similarity therefore placed in second group based on RAPD analysis (Fig. 2).

All the six bacterial strains were evaluated for the production of four enzymes viz., protease, lipase, cellulase and amylase. The strain SZ2 showed overall most efficient in enzymes production except for amylase while strain SZ5 was found to be least efficient regarding all enzymes production (Table 4). Amplification of 16S rRNA gene and its subsequent sequencing was performed to characterize the bacterial strain SZ2 at specie level which was most efficient regarding enzymes production. The band of rRNA gene of 1.4 kb was amplified and purified for further sequencing reactions (Fig. 3). The BLAST analysis of sequence indicated that the sequence has absolute homology with *Bacillus megaterium* strain SZ2 (GenBank: JQ864207.1). It was persuasive form table 5 that the correlation among the bacterial strains of different locations was significant for admin block location with all locations except B-block and GH-block.

Discussion

The current study was carried out to determine the quality of drinking water collected from nine different sources inside University of the Punjab (Quaid-e-Azam campus, Lahore). The bacterial cultures were initially

characterized based on their morphological features and subsequently subjected to biochemical and molecular analysis. In the current study, the water sample obtained from Admin block tube well showed maximum number of bacterial colonies (2128) while water sample collected from tube well from E-type houses showed least number of bacterial colonies viz. 38 only which showed that this drinking water has comparatively good quality for drinking purpose. The variability of bacterial load in drinking water as observed in current study was also described by other workers as well in their researches on drinking waters obtained from other geographic regions [17]. The variability in the bacterial load in drinking water is attributed to the improper cleaning of drinking water supply pipes and in sufficient chlorination of water and this finding is also in agreement an earlier study, which proposed the same fact while working on determining the quality analysis of drinking water [5].

The growth rates of six purified bacterial strains were determined for differentiation from each other regarding their generation time. The bacterial strains were divided into three categories based on growth results and these were fast growth bacteria (1-1.5 OD in first 12 hours), medium growth bacteria (0.6-0.9 OD in first 12 hours) and slow growth bacteria (0.5 OD in first 12 hours). These patterns of bacterial growth rates were observed by different workers while characterizing the novel bacterial strains isolated from different sources [18]. All six bacterial strains were screened for four enzymes viz., protease, lipase, cellulase and amylase. The strain SZ2 showed overall most efficient in enzymes production except for amylase while strain SZ5 was found to be least efficient regarding all enzymes production while remaining strains (SZ1, SZ3, SZ4 and SZ6) showed almost same efficiency regarding different enzymes production and these strains genomes also showed similarity as observed in RAPD analysis.

RAPD analysis, by using primer P1 and P2, amplified similar patterns of bacterial strains SZ1, SZ3, SZ4 and SZ6 while, SZ2 and SZ5 had unique amplification pattern. So, in RAPD analysis bacterial strains were differentiated in two separate groups as also witnessed in another study [11]. The bacterial strain SZ2 found most efficient regarding all enzymes except amylase was subjected to ribotyping by amplifying rRNA gene of 1.4 kb with subsequent direct sequencing coupled with BLAST. Sequence alignment analysis it was found to be *Bacillus megaterium*. As this bacterial strain is efficient in protease, lipase and cellulase production so, it can be exploited further for these enzymes production. It is suggested to screen the drinking water samples for bacteriological load at regular intervals in order to control water borne bacterial diseases.

In conclusion from this study, this can be prescribed that tube well water of Punjab University area of Lahore is safe for drinking purpose except admin block tube. It is suggested to monitor the bacteriological load of drinking water at regular intervals in order to control water borne bacterial diseases.

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