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Identification and Characterization of Bacteria Isolated from Pipe-borne Water Samples in Selected Areas of Lagos Using Api kits and RAPD-PCR

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ABSTRACT

Pipe-borne water is a major source of drinking water and contamination of such water source could lead to an epidemic. The aim of this present study is to isolate, identify and characterize microorganisms in pipe-borne water in different locations of Lagos metropolis. Six microorganisms were isolated from water samples collected at five locations. The isolates were subjected to morphological as well as biochemical tests using gram staining and Analytical Profile Index (API) tests respectively. Three primers (S gene, RAPD 1 and RAPD 2) were used for the PCR amplification of DNA samples extracted from the isolates and were identified as bacterial species: *Bacillus polymyxa, Bacillus alvei, Bacillus mycoides, Pasteurella aerogenes, Pasteurella pneumotropica* and *Staphylococcus aureus*. Gel electrophoresis of the amplified DNA samples generated a total of 128 bands which were subjected to statistical analysis using the NTSYS software (version 2.02j). The results as shown in the UPGMA dendrograms revealed a similarity coefficient of 0.66 among *B. polymyxa, B. alvei* and *B. mycoides* as well as a high similarity coefficient of 0.72 between *P. pneumotropica* and *S. aureus.* The presence of spore-forming bacteria, *B. mycoides B. polymyxa* and *B. alvei* is considered a biohazard as they can survive for long periods while the presence of *P. aerogenes, P. pneumotropica* and *S. aureus*, known as opportunistic pathogens is equally hazardous and can be a potential cause of water borne disease. This present study, using RAPD-PCR technique has improved the identification and characterization of microorganisms isolated from pipe-borne water.

INTRODUCTION

Water is an essential natural resource that supports all forms of life. It covers over 70% of the earth surface, 97.5% of which is salt water and 2.5 % is fresh water, which is renewable. Clean drinking water is essential to humans and other life forms as it can be used for agricultural, industrial, household and recreational activities. Olorunfemi and Gbadegesin (2007) reported that an average man is two thirds water and can survive for a longer period without food if water is present. Most diseases that occur in developing countries are as a result of inadequate safe and wholesome water supply. Water should be easily accessible, adequate and free from contamination and readily available throughout the year. Aderibigbe *et al*. (2008) estimated that each individual requires at least two liters of water per day. Plants need water for photosynthesis and can only absorb nutrients in soluble forms.

As stated by Olorunfemi and Gbadegesin (2007) many Nigerian households purchase water from private vendors because public water supplies are unreliable, erratic and of low quality. The major source of public water supply in Nigeria is known as pipe-borne water however most are located in urban areas. According to Egwari and Aboaba (2002), safe potable water is considered a luxury to over 80% of the low socio-economic class of Lagos residents. Different organisms have been associated with contamination of water supply. Fecal contamination is a major problem in the maintenance of good water supply as this is caused by both humans and animals (El-Jakee *et al*. (2009). The use of rapid techniques such as the API kits and a molecular technique known as Random Amplified Polymorphic DNA (RAPD) helped to provide a faster and more efficient identification of the isolated organisms. Also the research did not focus identification of only enteric pathogens but an all round identification and analysis was used.

Drinking water is exposed to contamination by microorganisms at every stage of its movement through the distribution systems. Some of the pipes pass through gutters, there is corrosion of pipes and leakages; therefore, the 'clean water' might not be as clean when it reaches its consumers. Also, there is more attention given to organisms that cause major water-borne disease outbreaks whereas there are other organisms that tend to accumulate for a long time in water and are not easily noticeable.

MATERIALS AND METHODS

Cultural Identification and Gram Staining

Isolation and sub-culturing was performed on nutrient agar (Wade Road, Basingstoke Hants, England). The isolates were sub-cultured using the streaking method. The colonies were observed with respect to size, margin, elevation, pigmentation, shape, cell size and colour. Gram staining was done to further classify the isolates to Gram positive and Gram negative.

Biochemical Identification Using Analytical Profile Index (API) Kits-

API KITS were produced by bioMerieux Inc. 100, Roldophe Street Durham NC27712. All the API kits were inoculated, 2-3 drops of the bacterial suspension were introduced into each micro ampoule and was incubated for 24 hours at 37°C. API 20E was carried out on five of the six isolates and API 50 CHB on three isolates. API Staph System was carried out on one isolate; a saline suspension of the isolate was used. All reactions were read according to the Reading Table and the identification was obtained by referring to the Analytical Profile Index.

Chromosomal DNA Extraction

DNA extraction was carried out directly from the samples by boiling using the procedure outlined by Chachaty and Saulnier, 2000. The concentration and purity of the extracted DNA was estimated using a Nanodrop spectrophotometer.

Identification Using RAPD-PCR

PCR amplification was carried out using the primer S (5^1 TCACGATGCA 3^1), RAPD 1(5^1 TGAGCATAGACCTCA 3^1) and RAPD 2 (5^1 CCCGTCAGCA 3^1). The 25µl reaction mixture consisted of x1 PCR buffer, 1.5mM Magnesium Chloride, 200µM of each dNTP, 20pmol of each primer and 1U Taq DNA polymerase (Fermentas).

Amplification was carried out in an Eppendorf Mastercycler model (850. Lincoln Centre Drive, Foster City, California, USA) using the following cycling parameters: An initial denaturation at 95ºC for 5 minutes and 40 cycles of 95ºC for 30 seconds, 36ºC for 1 minute and 72ºC for 1 minute 30 seconds. This was followed by a final extension of 72ºC for 10 minutes. The PCR products were separated on a 1.5 % agarose gel and 100bp DNA ladder was used as DNA molecular weight standard.

Statistical Analysis

The fragment sizes of PCR amplified products were estimated from the gel by

comparison with standard molecular weight markers (100 b DNA ladder, Fermentas. Pair wise distance matrices using sequential, hierarchical and nested (SAHN) clustering option of the Numerical Taxonomy System of Multivariate Program (NTSYS) software package, Version 2.02j (Rohlf, 1993). The program generated the dendrogram which grouped the test lines on the basis of Nei genetic distance (Nei, 1972) using un-weighted pair group method with arithmetic average (UPMGA) cluster analysis.

RESULTS

The samples were collected from five pipe-borne water sources and were subjected to microbiological, biochemical and molecular analysis. Six different bacteria species (AII, B, CI, CII, CIV and DI) were isolated from five sources as seen in Table 1. Fig. 1 shows the viable count at each location in colony forming unit per ml (cfu/ml).

Table 1: Isolates obtained from the different water samples.

Water Sample	CODE	Isolates
University of Lagos, Akoka, Faculty of Science	UL	AII, CIV
Yaba College of Technology, Yaba, Lagos, Akata Hall	YT	B, CII, CIV, CI
Federal College of Education, Akoka, Lagos School, Business Education	FC	CI, CII, CIV
Johnson Bus stop, Ladilak, Lagos	LK	DI, B
11, Teslim Nosiru Street, Ilaje, Bariga, Lagos	TN	CI

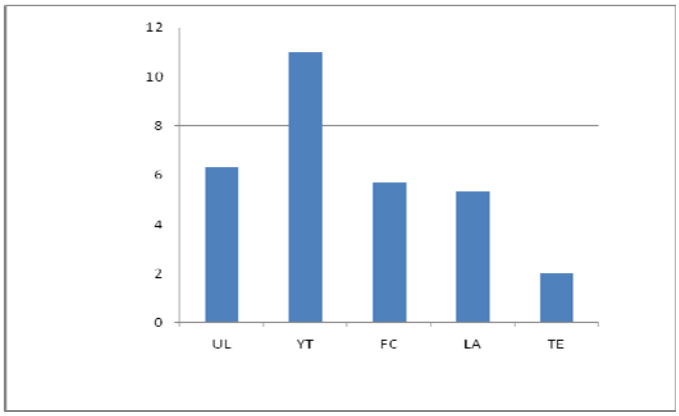


Fig. 1: Bar chart showing the viable count of the isolates in cfu/ml

Cultural Characteristics and Gram Staining Results:

Table 2 shows the cultural characteristics of the isolates, their reaction to Gram staining and the presence or absence of spores. The Gram reaction determined the API kits to be used on each isolates.

Table 2: Cultural Characteristics of Isolates

Isolates	Colour	Size	Elevation	Description	Gram stain	Spore
A II	Cream	Large	Flat	Rhizoid	+ve rods	Present
B	Grey	Small	Convex	Irregular	-ve rods	Absent
C I	Cream	Large	Flat	Undulate margin	+ve rods	Present
C II	Grayish-yellow	Small	Slightly raised	Smooth	-ve coccobacillus	Absent
C IV	Cream	Large	Flat	Swarming growth	+ve rods	Present
D I	Golden yellow)	Tiny	Convex	Smooth	+ve cocci	Absent

API Results

API 50CH and API 20E were used for isolates AII, CI and CIV while API 20E was used for isolates B and CII and API Staph for isolate DI. Table 3 shows the result of API 50CH and API 20E carried out on isolates AII, CI and CIV.

Table 3: API results for Isolates AII, CI and CIV

		ISOLATE	AII		ISOLATE CI				ISOLATE CIV			
	50CH		**20E**		**50CH**		**20E**		50CH		20E	
0	0	–	O	–	0	–	O	–	0	–	O	–
1	GLY	–	ONPG	+	GLY	+	ONPG	+	GLY	+	ONPG	+
2	ERY	–	ADH	+	ERY	–	ADH	–	ERY	–	ADH	–
3	DARA	–	LDC	–	DARA	–	LDC	–	DARA	–	LDC	–
4	LARA	–	ODC	–	LARA	+	ODC	–	LARA	–	ODC	–
5	RIB	–	CIT	+	RIB	+	CIT	+	RIB	+	CIT	–
6	DXYL	+	H_2S	–	DXYL	+	H_2S	–	DXYL	–	H_2S	–
7	LXYL	–	URE	–	LXYL	–	URE	–	LXYL	–	URE	–
8	ADO	–	TDA	–	ADO	–	TDA	–	ADO	+	TDA	–
9	MDX	–	IND	–	MDX	+	IND	–	MDX	–	IND	+
10	GAL	–	VP	+	GAL	+	VP	+	GAL	+	VP	+
11	GLU	+	GEL	+	GLU	+	GEL	+	GLU	+	GEL	–
12	FRU	+	GLU	+	FRU	+	GLU	+	FRU	–	GLU	+
13	MNE	–	MAN	–	MNE	+	MAN	–	MNE	–	MAN	–
14	SBE	–	INO	–	SBE	–	INO	–	SBE	–	INO	–
15	RHA	–	SOR	–	RHA	–	SOR	–	RHA	–	SOR	–
16	DUL	–	RHA	–	DUL	–	RHA	–	DUL	–	RHA	–
17	INO	–	SAC	+	INO	–	SAC	+	INO	–	SAC	–
18	MAN	–	MEL	–	MAN	+	MEL	+	MAN	–	MEL	–
19	SOR	–	AMY	–	SOR	–	AMY	+	SOR	–	AMY	–
20	MDM	–	ARA	–	MDM	–	ARA	+	MDM	–	ARA	–
21	MDG	–	NO_2	+	MDG	+	NO_2	+	MDG	–	NO_2	+
22	NAG	+			NAG	–			NAG	+		
23	AMY	+			AMY	+			AMY	–		
24	ARB	+			ARB	+			ARB	+		
25	ESC	+			ESC	+			ESC	+		
26	SAL	+			SAL	+			SAL	+		
27	CEL	–			CEL	+			CEL	–		
28	MAL	+			MAL	+			MAL	+		
29	LAC	–			LAC	+			LAC	–		
30	MEL	–			MEL	+			MEL	–		
31	SAC	+			SAC	+			SAC	–		
32	TRE	+			TRE	+			TRE	–		
33	INU	–			INU	–			INU	–		
34	MLZ	–			MLZ	–			MLZ	–		
35	RAF	–			RAF	+			RAF	–		
36	AMD	+			AMD	+			AMD	–		
37	GLYG	+			GLYG	+			GLYG	–		
38	XLT	–			XLT	–			XLT	–		
39	GEN	–			GEN	+			GEN	–		
40	TUR	–			TUR	+			TUR	–		
41	LYX	–			LYX	–			LYX	–		
42	TAG	–			TAG	–			TAG	–		
43	DFUC	–			DFUC	–			DFUC	–		
44	LFUC	–			LFUC	–			LFUC	–		
45	DARL	–			DARL	–			DARL	–		
46	LARL	–			LARL	–			LARL	–		
47	GNT	–			GNT	+			GNT	–		
48	2KG	–			2KG	–			2KG	–		
49	5KG	–			5KG	–			5KG	–		
		Bacillus mycoides				*Bacillus polymyxa*				*Bacillus alvei*		

Table 4 shows the result of API 20E carried out on isolates CII and B while API Staph was carried out on isolate DI. The resulting data were used in identifying the organisms to the species level. Three Bacillus strains were identified; *Bacillus mycoides* (AII), *Bacillus polymyxa* (CI) and *Bacillus alvei* (CIV). Two Pasteurella strains were also identified; *Pasteurella aerogenes* (CII) and *Pasteurella pneumotropica* (B). *Staphylococcus aureus* (DI) was also identified.

Table 4: API results for Isolates CII, B and DI

ISOLATE CII			ISOLATE B		ISOLATE DI	
		20E		API 20E	API Staph	
0	O	–	O	–	O	–
1	ONPG	+	ONPG	–	GLU	+
2	ADH	–	ADH	–	FRU	+
3	LDC	–	LDC	–	MNE	+
4	ODC	+	ODC	–	MAL	+
5	CIT	–	CIT	–	LAC	+
6	H_2S	–	H_2S	–	TRE	+
7	URE	+	URE	–	MAN	+
8	TDA	–	TDA	–	XLT	–
9	IND	–	IND	–	MEL	–
10	VP	–	VP	+	NIT	+
11	GEL	–	GEL	–	PAL	+
12	GLU	+	GLU	+	VP	+
13	MAN	–	MAN	–	RAF	–
14	INO	+	INO	–	XYL	–
15	SOR	–	SOR	–	SAC	+
16	RHA	–	RHA	–	MDG	–
17	SAC	+	SAC	+	NAG	+
18	MEL	–	MEL	–	ADH	+
19	AMY	–	AMY	–	URE	+
20	ARA	+	ARA	–	LSTR	–
21	OX	+	OX	–	***Staphylococcus aureus***	
22	NO_2	+	NO_2	–		
23	N_2	–	N_2	–		
24	MOB	–	MOB	–		
25	McC	+	McC	–		
26	OF-O	+	OF-O	–		
27	OF-F	+	OF-F	–		
28	***Pasteurella aerogenes***		***Pasteurella pneumotropica***			

DNA Quantification

The extracted DNA retrieved from the isolates using a Nanodrop spectro-photometer. The absorbance ratio (260/280) value ranged from 1.45-2.14 as seen Fig. 2 which shows the absorbance ratio (260/280) of each isolate. Isolates AII, B and CI showed an absorbance ratio (260/280) value of 2.04, 2.04 and 2.14 respectively while CII has the lowest value of 1.45.

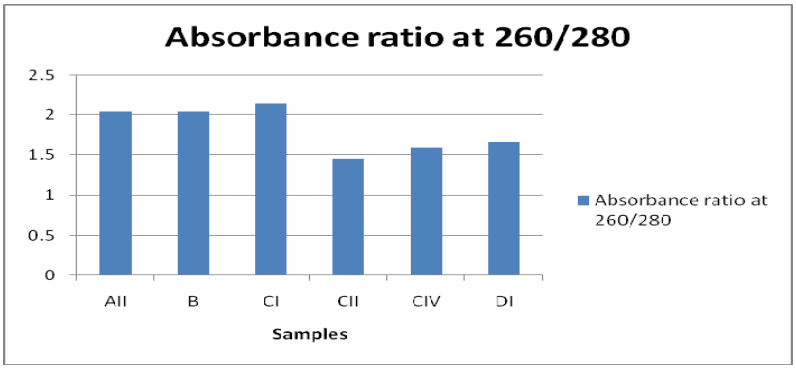
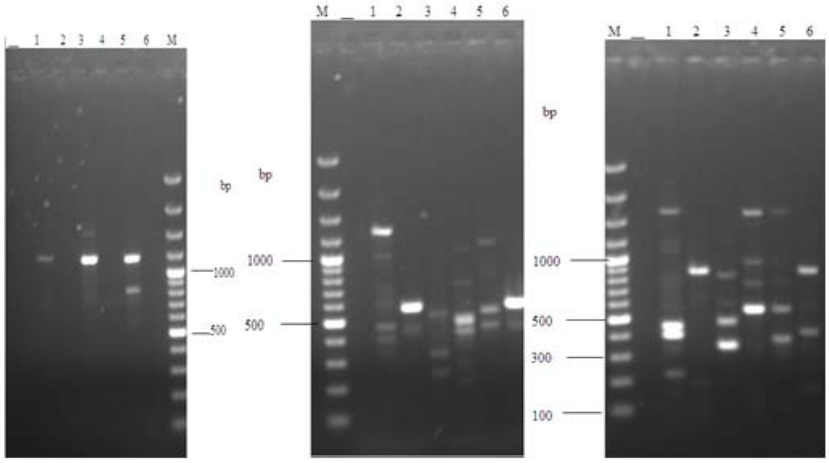


Fig. 2: Bar chart showing the absorbance ratio at 260/280

RAPD-PCR

Figs. 3-5 are the agarose gel results from the use of three RAPD primers. The primers were used for all six isolates. Three decamer primers were used for the RAPD-PCR analysis. The S primer showed banding patterns in isolates AII, B, CI, CII and CIV, 15 bands were present with G+C content of 50%. RAPD 1 primer showed banding patterns in all isolates with 52 bands present with G+C content of 70%. RAPD 2 primer showed banding patterns in all isolates with

61 bands present with G+C content of 70%. The primers that generated higher number of bands had a higher G+C content compared to the primer that had a lower G+C content. A total of 128 bands were generated within the range of 200-4500bp.



Figs. 3, 4 and 5: RAPD profile using S gene, RAPD 1 and RAPD 2 primers. The gel lane marked M is the primer marker, 100bp DNA ladder while the lane labelled – is the negative control. Lanes 1-6 represent isolates AII, B, CI, CII, CIV and D.

Data Analysis for the three RAPD primers

To provide a more accurate and reliable result, an UPMGA dendrogram which combines the results of the three primers- S gene, RAPD 1 and RAPD 2- was generated (Figure 6). The dendrogram formed two clusters (1 and 2) at a similarity coefficient of 0.58. Cluster 1 is made up of isolates *B. mycoides, B. polymyxa, B. alvei* and *P. aerogenes* while cluster 2 is made up of *S.* aureus and P. *pneumotropica*. A sub-cluster was formed in cluster 1 made up of *B. mycoides* and *B. polymyxa* with a similarity coefficient of 0.70. In cluster two B and DI have the highest similarity coefficient of 0.72.

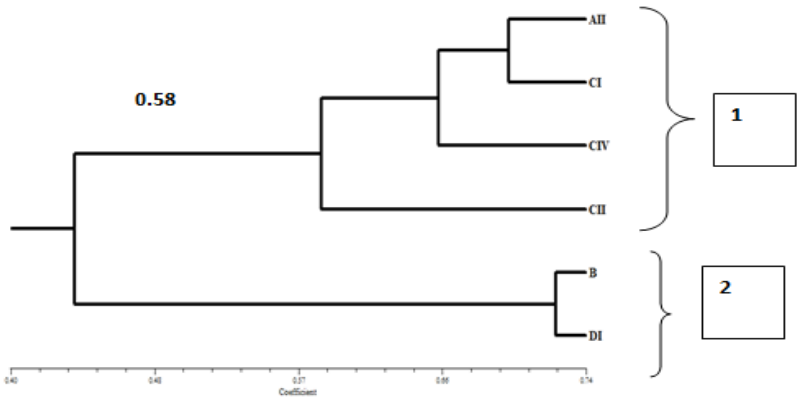


Fig. 6: UPMGA dendrogram showing the relationship among the isolates using the RAPD profile produced by the S gene primer, RAPD 1 primer and RAPD 2 primer. AII represents *B. mycoides*, B represents *P. pneumotropica*, CI represents *B. polymyxa*, CII represents *P. aerogenes*, CIV represents *B. alvei,* and DI represents *S. aureus.*

DISCUSSION

Viable Count

Sample YT has the highest viable count in cfu/ml as shown in Figure 1 while sample TE had the lowest. The level of contamination in the YT sample could be as a result of leakage of pipes which could lead to contamination (Egwari and Aboaba, 2002)

and because it is located within a female hostel, the soil around the environment might have been exposed to fecal contamination. The low level of contamination in the sample collected in TE could be as a result of the proper maintenance and handling of the water pipes as was observed during sample collection. Also, the water source was the major supplier of water to most households within the environment, hence the microorganisms might not have been able to adhere to pipes and form biofilms.

Representative Isolates in Each Sample

Isolate CIV (*Bacillus alvei*) occurred in 3 of the 5 samples collected- UL, YT and FC. Based on the locations of samples collection, the isolate seems to be present in the same area which could mean that the three locations have the same water source. All the locations had at least one *Bacillus* strain present except the sample from LA. *Bacillus* species are spore-forming bacteria and can therefore survive in unfavourable conditions.

Cultural Characteristics and Gram Staining Results

The cultural characteristics of the isolates, their reaction to Gram staining and the presence or absence of spores helped to select the API kits used to identify the isolates. The *Bacillus* strains were first identified by their reaction to Gram stain (Gram positive rods with spores). Two of the isolates were Gram negative. Isolate DI was a Gram positive cocci the golden colour of its colonies was also used to identify the organism as a *Staphylococcus* strains.

API Tests

Based on the results provided by the cultural and morphological observations of the different colonies and also the Gram staining results, API 20E was used for the gram negative organisms and the isolates B and CII were identified as *Pasteurella pneumotropica* and *Pasteurella aerogenes* respectively. API Staph was used to identify Isolate DI and with was found to be *Staphylococcus aureus*. API 50 CHB and API 20E were used to identify isolates A, CI and CIV as *Bacillus mycoides, B. polymyxa* and *Bacillus alvei* respectively. This combination was used because some sugars required for *Bacillus* identification are present in API 20 E and not in API 50CHB.

Staphylococcus aureus has been found to be present in biofilms which could explain its presence in the LA location (Olson *et al.*, 2002). *Pasteurella* species have been associated with the saliva and waste product of pet animals. *Pasteurella* species are opportunistic pathogens and are found in the upper respiratory tracts of both wild and domestic animals. Pasteurellosis is a common disease in cattle but can occur in pets. It can lead to high mortality in animals. It could also affect humans. The presence of these organisms in pipe-borne water is hazardous.

Data analysis for the RAPD primers

When the three primers were combined they gave a more concise result for the characterization of the organisms. The highest similarity coefficient of 0.72 occurred between *S. aureus* and *P. pneumotropica* showing a high similarity coefficient of 72%. The two organisms do not belong to the same genus and as such a high level of similarity could be as a result of genetic recombination especially if they existed in the same biofilm. There could also be the presence of similar nucleotides in the organisms. *B. mycoides and B. polymyxa* also showed a high similarity coefficient of 0.70.

CONCLUSION AND RECOMMENDATION

The presence of organisms like *Bacillus spp.* which can survive for long periods because of their ability to produce spores is a biohazard. Also the presence of *Pasteurella spp.* and *S. aureus* which are known opportunistic pathogens is also hazardous and can cause outbreaks within the environment. The presence of these organisms might have gone unnoticed because most tests are based on the detection of coliforms. The use of RAPD-PCR molecular technique has enhanced the identification and characterization of the microorganisms isolated from pipe-borne

water. To prevent disease outbreaks, routine tests should be done, the pipes in which these water flows into different houses should be thoroughly flushed and damaged pipes should be replaced.

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