International Journal of Innovative Research and Advanced Studies (IJIRAS)
Volume 6 Issue 4, April 2019

ISSN: 2394-4404

Molecular Characterization Of Bacterial Isolates From Drinking Water Distribution Systems Of Some Higher Institutions In Edo State, Nigeria

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Abstract: ***In drinking water systems, a large number of microorganisms live in complex communities in a self-produced extracellular polymeric matrix associated with inner surfaces of pipes, walls and floors of storage tanks, joints and faucets. This study was aimed at characterizing the bacterial isolates of drinking water distribution systems using the molecular approaches. The enumeration and isolation of bacteria from the water samples were carried out by the standard heterotrophic bacterial count methods. The molecular identification was performed by the extraction of the bacterial DNA from isolates, using the polymerase chain reaction (PCR) to obtain the PCR product, which were purified and sequenced. The sequences were aligned by importing closely related sequences from Gene-bank data base and compared by using the basic local alignment search tool to identify their closest relative. The phenotypically identified bacteria were Citrobacter, Klebsiella, Bacillus, Pseudomonas, Proteus and Providencia. Nucleotide sequence revealed the presence of Proteus penneri R-B08-05, Pseudomonas hibiscicola R-H06-24, Pseudomonas aeruginosa R- F07-16, Bacillus cereus R-G07-19, Bacillus sp. R-H07-22, Pseudomonas aeruginosa R- A08-02, Bacillus cereus R-G07-19, Pseudomonas aeruginosa R-F07-16 and Proteus penneri R-G07-19 with a similarity index of 61% to 100 %. The phylum Proteobacteria constituted 85.7 % of the bacterial isolates identified, while phylum Firmicutes represented 14.3 % of isolates from all the University communities surveyed. However, the Pseudomonas strains recorded the highest (frequency) percentage (43.0 %), Bacillus (35.0 %) and Proteus (23.0 %). The study revealed that drinking water biofilm is a reservoir for microorganisms that could cause public health concerns and molecular approach gives more indebt analysis of bacterial consortia that would not have been realized by other conventional methods.***

Keywords: Biofilms, isolation, polymerase chain reaction, sequencing and blast.

I. INTRODUCTION

Periodic monitoring conducted to ascertain for water quality involves only the bulk water that undermine and not proffer the actual representation of the preponderance of species in the distribution system. Biofilm, therefore, represents the appropriate source for the examination of microbiota consortium because high concentration and varied types of microorganisms are enmeshed in the biofilm (Farcas, 2012).

In characterizing drinking water bacterial biofilms, culture-based methods and biochemical techniques are commonly used. The phenotypic methods though acceptable, are severely limited in scope and specificity of the result and tends to grossly underestimate the microbial population within water samples In order to avoid such challenges, the introduction of advanced molecular techniques have enhanced the characterization of natural microbial consortia without the use of culture-based technique and has revealed new

perceptions into the microbial ecology of different ecosystems (Gillings *et al*., 2008).

Distinctive assessment of established techniques involving bacterial growth on specific media and molecular approach based on 16S rRNA sequence identity reveals a high inconsistency between what was expected to grow and the species isolated from growth media. Therefore, bacterial analysis based on selective isolation and culturing is proposed to be reported with caution (Gestaland, 1999).

Recently, studies have depended on the application of 16S rRNA sequence analysis to examine microbial communities and ascertain phylogenetic connection of bacterial populations (Letunic and Bork, 2007). Using this approach, it is now possible to determine the microbial network and perform indebt analysis of a wide array of environment, determine broad-scale microbial phylogeny, genetic relatedness, detect rare and unknown microorganisms in environmental samples (Gillings *et al*., 2008; Liarrull *et al.*, 2009).

II. MATERIALS AND METHODS

SAMPLES COLLECTIONS

The biofilm water samples were collected from the reservoir stations (storage tanks), distribution pipelines and collection points (taps) from the corresponding location sites by scrapping the surfaces or walls in contact with water with sterile steel blades.

MICROBIOLOGICAL ANALYSES

ISOLATION AND ENUMERATION OF BACTERIA

Isolation of bacteria from biofilm water samples was performed by standard methods of pour plating using nutrient agar and MacConkey agar (Barrow and Feltham, 2003). The plates were incubated at 28 ± 2 ^{o}C for 24 hr and distinct bacterial colonies in the nutrient agar and MacConkey agar plates were used to respectively deduce the heterotrophic bacteria counts (HBC) and total coliform counts (TCC).

IDENTIFICATION AND CHARACTERIZATION OF BACTERIA

Six bacterial colonies were picked based on their different colonial morphologies and each of them was phenotypically characterized with prescribed standard methods (Barrow and Feltham, 2003).

MOLECULAR CHARACTERIZATION

Further identification of the pure culture of the bacterial isolates from the biofilm water samples was achieved by polymerase chain reaction (PCR), amplification, purification and sequencing of 16S rRNA gene (Chen, 2013).

The following outlines the key steps in the methods taken.

DNA EXTRACTION

The chromosomal DNA was extracted using Zymo Pure Miniprep Kit, as prescribed by the manufacturer (Zymo Research Centre, Johannesburg, South Africa). The DNA extract was purified by adding 5.0 µL nuclease-free water and incubated for 30 min. The extracted DNA product was eluted in 25 µL DNA elution buffer and stored at -20 ^{o}C as DNA template, ready for use in PCR process.

AMPLIFICATION OF 16S RRNA GENE

The protocol for DNA amplification using the genomic guilded sequence method was employed

The 16S rRNA gene from the chromosomal DNA was PCR amplified using universal primer sets (27F (5 AGA GTT TGA TCC TGG CTC AG-3) and 1492R 5 TAC GGT CTA CTT GTT ACG TA-3). The PCR master mix contained the following components of up to 25 µL: One taq master mix, 12.5 µL; Forward and Reverse primers, 1.25 µL; Nuclease free water, 5.0 µL and DNA template, 5.0µL. The process was performed in Gene PCR Thermo Cycler with the recommended guidelines: Initial denaturation at 94 ^{o}C for 30 min; denaturation at 94 ^{o}C for 1 min; annealing at 50 ^{o}C for 1 min, extension at 72 ^{o}C for 1 min; final extension at 72 ^{o}C for 7 min and hold at 4 ^{o}C.

PURIFICATION OF THE PCR PRODUCTS

The PCR products were cleaned using Exo SAP PCR master mix (South Africa) as per manufacturer protocol: PCR Mixture 10.0 µL; Exo SAP Mix (Exonuclease 50.0 µl and Shrimp Alkaline Phosphate 200 µl). The mixture was incubated at 37 ^{o}C for 30 min and the reaction was stopped by heating the mixture at 95 ^{o}C for 5 min. The purified PCR products were eluted in 5 µL nuclease free water for 30 min and stored at -20 ^{o}C until used for sequencing.

SEQUENCING OF THE 16SRRNA GENE

The purified PCR products were sequenced by using universal primers 27F and 1492R. To obtain the full length sequence of the 16S rRNA gene, the sequencing was done by the ABI V3.1 Big dye kit according to manufacturer's instructions.

ANALYSIS OF SEQUENCES BY BASIC LOCAL ALIGNMENT SEARCH TOOL (BLAST) AND PHYLOGENETIC ANALYSIS

The sequences were compared using the BLAST to identify their closest relatives. A detailed phylogenetic analysis was conducted using the Geneious package (version 9.0.5) program (South Africa). The sequences were aligned by importing closely related sequences from GeneBank (htpp;/ncbi.nlm.nih.gov/genebank) and the aligned sequences were subjected to maximum likelihold and Neighbour-joining analyses. The bootstrap analysis was performed to estimate the confidence of the 16S rRNA gene-tree topology.

III. RESULTS

The bacterial isolates phenotypically identified were *Citrobacter*, *Klebsiella*, *Bacillus*, *Pseudomonas*, *Proteus* and *Providencia* species.

Morphological Examination			Biochemical Examination							
Colonial characteristics on nutrient agar (NA)	Colonial characteristics on MacConkey agar (MA)	Gram Staining	Catalase	Oxidase	Indole	Methyl Red	Citrate	Voges proskauer	Lactose	Isolates
Large gray colony with serrated margin	Colourless colony with serrated margin	negative rods	+	-	+	+	+	+	-	*Providencia* sp.
Opaque colony with serrated margin	Pinkish colony with serrated margin	negative rods	+	-	-	+	+	-	+	*Citrobacter* sp.
Dry colony with serrated margin	Pinkish colony with serrated margin	positive rods	+	+	-	-	+	+	+	*Bacillus* sp.
Greenish pigmented colony with an entire margin	Colourless colony with an entire margin	negative rods	+	+	-	-	+	-	-	*Pseudomonas* sp.
Mucoid swarming colony with an entire margin	Colourless colony with an entire margin	negative rods.	+	-	-	+	+	-	-	*Proteus* sp
Mucoid colony with an entire margin	Pinkish colony with an entire margin	negative rods	+	-	-	-	-	+	+	*Klebsiella* sp.
Mucoid colony with entire margin	Colourless colony with entire margin	positive rods	+	+	-	-	+	+	+	*Bacillus* sp.
Mucoid swarming colony with an entire margin	Colourless colony with an entire margin	negative rods	+	-	-	+	+	-	-	*Proteus* sp.

Key: R (Reservoir), D (Distribution pipeline), T (Tap).

Table1: Phenotypic characterization of isolates obtained from biofilm water samples

Organisms	Frequency	Percentage (%)
Klebsiella sp.	4	19.0
Citrobacter sp.	3	14.3
Pseudomonas sp.	5	23.8
Proteus sp.	3	14.3
Providencia sp.	1	4.78
Bacillus sp.	5	23.8
Total	21	100

Table 2: Percentage (distribution) of the bacterial isolates

The results of the phylogenetic analysis based on nucleotide sequence as showed in figures 1.1 to 1.9 revealed a total number of 84 homologous isolates with percentage (distribution) of *Pseudomonas* (43.0 %), *Bacillus* (35.0 %) and *Proteus* (23.0 %).

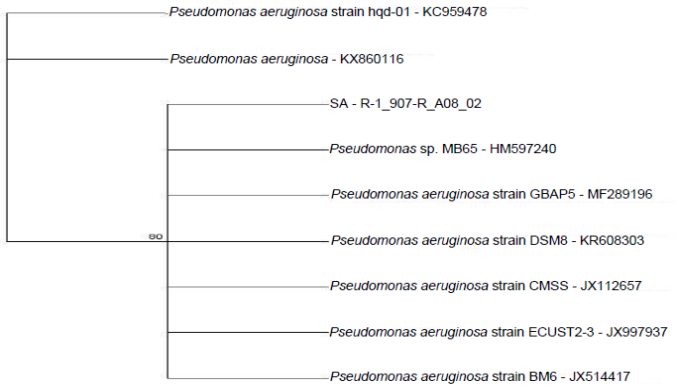


Figure 1.1: Phylogenetic analysis of clinical sample based on the nucleotide sequence of part of the 16S rRNA nucleotide sequence of Bacteria. The phylogenetic tree was constructed by the Neighbor-Joining method program in the Geneious package (version 9.0.5). The numbers at the forks show the numbers of occurrences of the repetitive groups to the right out of 100 bootstrap samples. The isolate SA- R- 1 has similar sequence with Pseudomonas aeruginosa with accession number KX860116

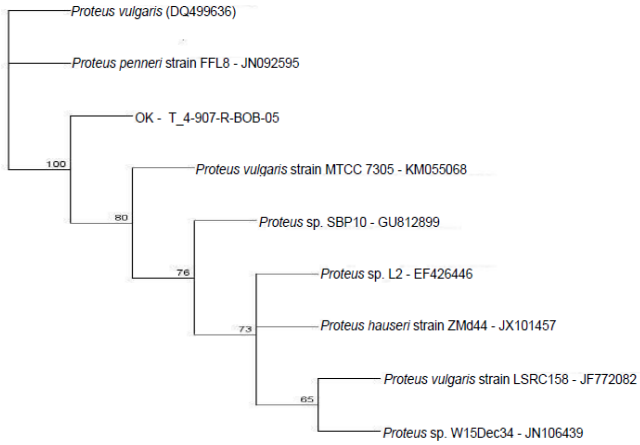


Figure 1.2: Phylogenetic analysis of clinical sample based on the nucleotide sequence of part of the 16S rRNA nucleotide sequence of Bacteria. The phylogenetic tree was constructed by the Neighbor-Joining method program in the Geneious package (version 9.0.5). The numbers at the forks show the numbers of occurrences of the repetitive groups to the right out of 100 bootstrap samples. The isolate OK - T-4 has similar sequence with Proteus penneri strain FFL8 with accession number JN092595

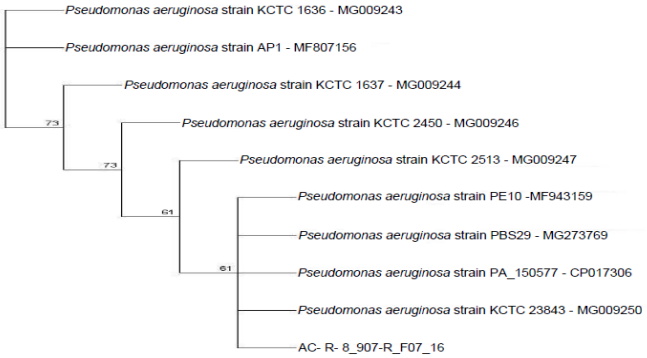


Figure 1.3: Phylogenetic analysis of clinical sample based on the nucleotide sequence of part of the 16S rRNA nucleotide

sequence of Bacteria. The phylogenetic tree was constructed by the Neighbor-Joining method program in the Geneious package (version 9.0.5). The numbers at the forks show the numbers of occurrences of the repetitive groups to the right out of 100 bootstrap samples. The isolate AC- R-8 has similar sequence with Pseudomonas aeruginosa strain KCTC 23843\ with accession number MG009250

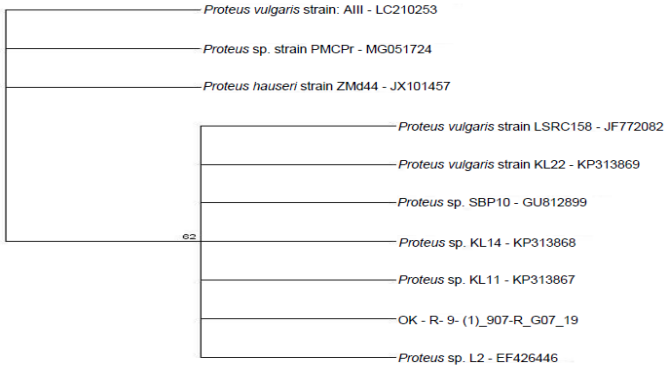


Figure 1.4: Phylogenetic analysis of clinical sample based on the nucleotide sequence of part of the 16S rRNA nucleotide sequence of Bacteria. The phylogenetic tree was constructed by the Neighbor-Joining method program in the Geneious package (version 9.0.5). The numbers at the forks show the numbers of occurrences of the repetitive groups to the right out of 100 bootstrap samples. The isolate OK- R- 9 (1) has similar sequence with Proteus sp. KL11 with accession number KP313867

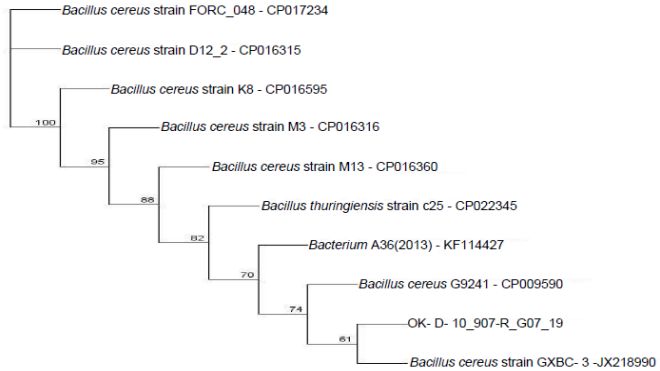


Figure 1.5: Phylogenetic analysis of clinical sample based on the nucleotide sequence of part of the 16S rRNA nucleotide sequence of Bacteria. The phylogenetic tree was constructed by the Neighbor-Joining method program in the Geneious package (version 9.0.5). The numbers at the forks show the numbers of occurrences of the repetitive groups to the right out of 100 bootstrap samples. The isolate OK- D-10 has similar sequence with Bacillus cereus G9241 with accession number CP009590

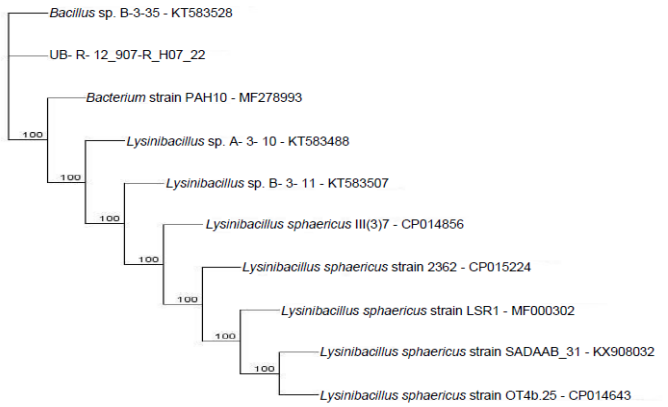


Figure 1.6: Phylogenetic analysis of clinical sample based on the nucleotide sequence of part of the 16S rRNA nucleotide sequence of Bacteria. The phylogenetic tree was constructed by the Neighbor-Joining method program in the Geneious package (version 9.0.5). The numbers at the forks show the numbers of occurrences of the repetitive groups to the right out of 100 bootstrap samples. The isolate UB- R- 12 has similar sequence with Bacillus sp. B-3-35 with accession number KT583528

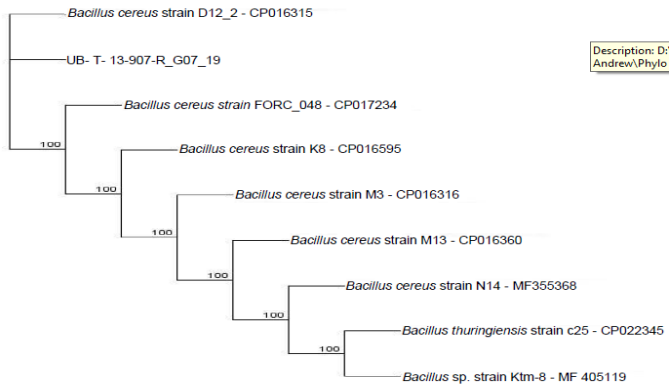


Figure 1.8: Phylogenetic analysis of clinical sample based on the nucleotide sequence of part of the 16S rRNA nucleotide sequence of Bacteria. The phylogenetic tree was constructed by the Neighbor-Joining method program in the Geneious package (version 9.0.5). The numbers at the forks show the numbers of occurrences of the repetitive groups to the right out of 100 bootstrap samples. The isolate UB- T- 13 has similar sequence with Bacillus cereus strain D12_2 with accession number CP016315

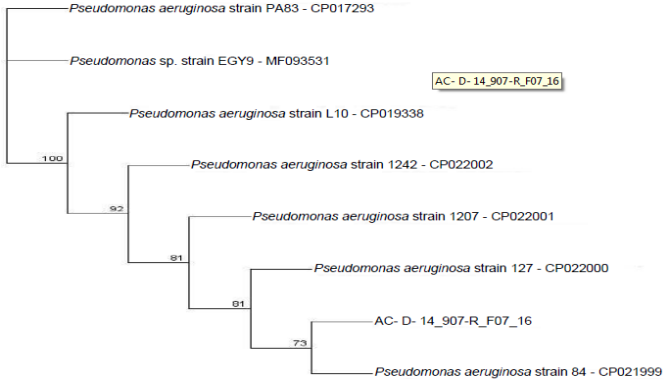


Figure 1.8: Phylogenetic analysis of clinical sample based on the nucleotide sequence of part of the 16S rRNA nucleotide sequence of Bacteria. The phylogenetic tree was constructed

by the Neighbor-Joining method program in the Geneious package (version 9.0.5). The numbers at the forks show the numbers of occurrences of the repetitive groups to the right out of 100 bootstrap samples. The isolate AC- D-14 has similar sequence with Pseudomonas aeruginosa strain 127 with accession number CP0220000

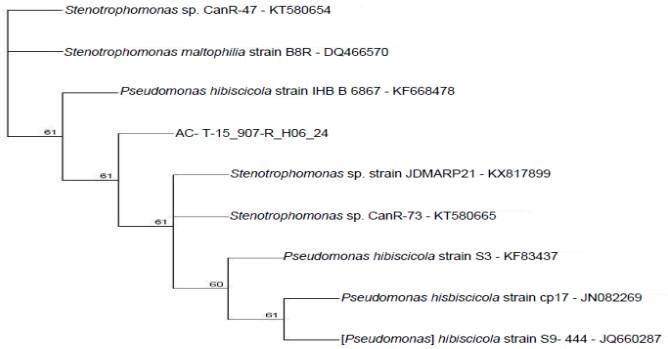
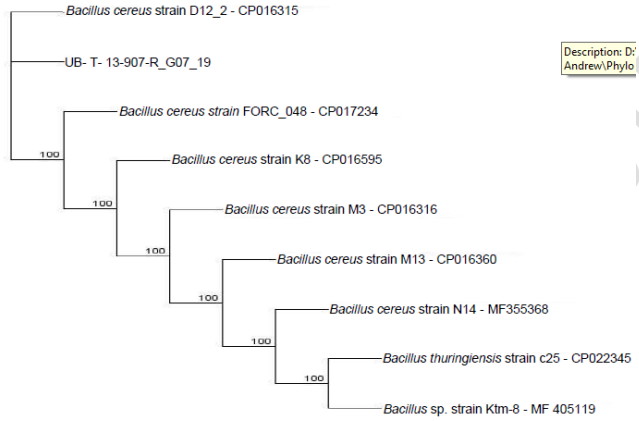


Figure 1.9: Phylogenetic analysis of clinical sample based on the nucleotide sequence of part of the 16S rRNA nucleotide sequence of Bacteria. The phylogenetic tree was constructed by the Neighbor-Joining method program in the Geneious package (version 9.0.5). The numbers at the forks show the numbers of occurrences of the repetitive groups to the right out of 100 bootstrap samples. The isolate AC- T-15 has similar sequence with Pseudomonas hibiscicola strain IHB B 6867 with accession number KF668478



The nucleotide sequence revealed identity of isolates with similarity index of 61 % to 100 %, relatively, to the closest in gene data base.

These include, *Proteus penneri* R-B08-05, *Pseudomonas hibiscicola* R-H06-24, *Pseudomonas aeruginosa* R- F07-16, *Bacillus cereus* R-G07-19, *Bacillus* sp. R-H07-22, *Pseudomonas aeruginosa* R-A08-02, *Bacillus cereus* R-G07-19, *Pseudomonas aeruginosa R*-F07-16 and *Proteus penneri R*-G07-19 (Table4).

Samples	Isolates	Closest relative in gene data base	Similarity (%)	Accession number	Samples
OK-T-4	*Proteus penneri* strain 907-R – B08-05	*Proteus penneri* strain FFL8	100%	JN092595	OK-T-4
AC-T-15	*Pseudomonas hibiscicola* strain 907-R-F06-24	*Pseudomonas hibiscicola* Strain IHBB 6867	61%	KF668478	AC-T-15
AC-D-14	*Pseudomonas aeruginosa* strain 907-R-F07-16	*Pseudomonas aeruginosa* strain *127*	81	CP021999	AC-D-14
UB-T-13	*Bacillus cereus* strain 907-R-G07-19	*Bacillus cereus* strain B 12-2	100	CP016315	UB-T-13
UB-R-12	Bacillus sp. strain 907-R-H07-22	*Bacillus* sp .strain B-3-35	100	KT583528	UB-R-12
SA-R-1	*Pseudomonas aeruginosa* strain 907-R-AO8-02	*Pseudomonas aeruginosa*	80	KX860116	SA-R-1
OK-D-10(1)	*Bacillus cereus* strain 907- R-GO7-19	*Bacillus cereus* strain – G9241	81%	CP009590	OK-D-10(1)

Key: SA (OK (Igbinedion University, Okada), UB (University of Benin, Benin City), AA (Ambrose Alli University, Ekpoma), SA (Samuel Adeboyega University, Ogwa), AC (Auchi Polytechnic, Auchi), R (Reservoir), D (Distribution pipeline), T (Tap)

Table 4: Identification of bacterial isolates using 16S rRNA gene sequencing

The gamma Proteobacter was reported to be the numerically dominant group in the phylum with percentage (occurrence) (85.7 %) and the Firmicutes (14.3 %).

Phylum	Class	Order	Family	Genus	Species
Proteobacter	Gamma proteobacter	Pseudomonadales	Pseudomonaceae	Pseudomonas	*P. aeruginosa, P.maltophila P.hibiscicola*
Firmicutes	Bacilli	Bacilales	Bacillaceae	*Bacillus*	*B. thuringiensis, B. cereus, L. sphaericus*
Proteobacter	Gamma proteobacter	Enterobacteriales	Enterobacteriaceae	*Proteus*	*P. penneri, P. hauseri, P. vulgaris*
Proteobacter	Gamma proteobacter	Enterobacteriales	Enterobacteriaceae	*Klebsiella*	-
Proteobacter	Gamma proteobacter	Enterobacteriales	Enterobacteriaceae	*Citrobacter*	-
Proteobacter	Gamma proteobacter	Enterobacteriales	Enterobacteriaceae	*Providencia*	-

Table 5: The HBC colonies classified to genus /species level (Bacterial lineage)

IV. DISCUSSION

The bacterial isolates phenotypically identified were *Citrobacter, Klebsiella, Bacillus, Pseudomonas, Proteus* and *Providencia.* The highest frequency (percentage) was recorded for *Bacillus* and *Pseudomonas* (23.8%) and *Providencia*, the least (4.80%) (Table2). The investigated biofilms proved to be extremely active bacteria consortia with high concentrations of cultivable bacteria: heterotrophs and opportunistic bacteria.

The *Pseudomonas* species are opportunistic pathogens that caused nosocomial infections in susceptible patients and their high intrinsic resistance to a variety of antibiotics,

including β - lactams, aminoglycosides and fluroquinolones made them very hard to eliminate. The B*acillus* produced putative virulent factors capable for triggering infections, responsible for endocarditis and neurological cases (Gillings *et al*., 2008).

In the phylogenetic analyses of biofilm water samples, all the isolates had similar sequences when compared with those from gene data-base which include: *Proteus penneri* strain FFL8 (JN092595), *Pseudomonas hibiscicola* strain IHBB 6867 (KF668478), *Pseudomonas aeruginosa* strain 127 (CP0220000), *Bacillus cereus* strain D12-2 (CP016315), B*acillus* sp. strain 3-35 (KT583528), *Pseudomonas aeruginosa* (KX860116), *Bacillus cereus* strain G9241 (CP009590), *Pseudomonas aeruginosa* strain KCTC 23843 (MG009250) and *Proteus* sp. strain KL11 (KP313867).

The phylogenetic analysis of strains examined in this study, illustrated many similarities to previous studies pertaining to drinking water biofilms. The 16s rRNA sequences affiliated to *Pseudomonas, Bacillus* and *Proteus*-like organisms have been previously shown to be present in drinking water biofilms (Gestaland, 1999).

In general, the results from sequences, found significant levels of *Stenotrophomonas maltophila, Pseudomonas hibiscicola, Pseudomonas aerugniosa, Bacillus cereus, Bacillus thuringienis and Lysinbacillus sphaericus.* Others *were proteus penneri, Proteus vulgaris and Proteus hauseri.*

The sequence comparisons with existing data-bases revealed that gamma Proteobacter and Firmicutes represented nearly 85.7 % and 14.3% of total strains examined, respectively. In addition, all sequences were closely related and numerically more than the cultured bacteria thereby, supported the notion that culture-based methods can under-estimate the bacteria diversity of drinking water systems. Approximately, nearly a third of sequences analyzed in this study showed 100 % homologous similarity with sequences in the currently available data bases (Table 6). This suggested that some drinking water bacteria represent novel bacterial species.

The phylogenetic analysis further revealed other sequences which were closely related to *Sternotrophomonas maltophila* such as, *Pseudomonas hibiscicola*, which has been implicated in production of Laccase, an oxidase enzyme useful in Environmental pollution Control Programmes, was earlier reported in plants, has now been revealed in this sequencing. Importantly, bacterial Laccase has become a new biocatalyst, increasing in use because they are highly active, much more stable at high temperature and pH values (USEPA, 2012). The *Lysinbacillus sphaericus* identified, also produces α and β proteins (Binary toxins) that act following ingestions. These toxins can be used in insect control programs to reduce the population of disease vector species (anophelex and culex mosquitoes) that transmit diseases such as malaria, yellow fever and West Nile Virus. In addition, the bioremediation potential of the identified strain A-3-10 (Fig. 1.6) is able to reversibly bind heavy metals such as lead, cadmium, uranium and due to the presence of the proteinaceous surface (USEPA, 2012).

The alignment with sequences available within National Centre for Biotechnological Information (NCBI) database (Gene Bank) showed that, there was no significant correlation between the sequences and heterotrophic bacterial counts (HBC) and revealed little similarity in over-all community diversity, as well as significant distortion in relative abundance particularly for *Pseudomonas spp.* 43.0 %, B*acillus* 35.0 % and *Proteus* 23.0 %. It should be noted that the percentage (frequency) of *Pseudomonas* and *Bacillus* obtained in this study were relatively high. The data suggested that some conditions in the water distribution system (WDS) might selectively contribute for their growth, which included the amount of dissolved oxygen, total and assimilable organic carbon and growth elements.

These results implied that, heterotrophic bacterial count (HBC) has little relevance for determining parameters of drinking water quality regarding microbial communities. This is particularly striking given that HBC may not detect the potential presence of pathogenic microbes of concern to human health. Furthermore, that drinking water ecosystem are much more microbiologically complex than culture-based surveys would suggest. Therefore, such a determination would infer that low HBC imply the presence of microbes that are not detected by conventional monitoring technique, but which might be of potential human concern (APHA, 2005).

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