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REGULAR ARTICLE

ASSESSMENT OF 16S rRNA SEQUENCE AND HETEROTROPHIC PLATE COUNT (HPC) METHODS OF IDENTIFYING BACTERIA FROM DRINKING WATER SYSTEMS IN BENIN CITY METROPOLIS

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ABSTRACT

A variety of simple culture-based tests which are proposed to recover a wide range of microorganisms from water are collectively known as heterotrophic plate count (HPC) and used as an indirect indicator to give information about water quality. The aim of this study was to assess the heterotrophic plate count (HPC) culture-based dependent and 16S rRNA independent techniques of identifying bacteria. The HPC was conducted by incubating a filtered sample of water on R2A agar plates and enumerating the number of resultant bacterial colonies that grow on each plate. The molecular analysis was performed by extracting the deoxyribonucleic acid (DNA) from bacterial isolate and polymerase chain reaction (PCR) was done to obtain the amplicons (PCR products). Purified PCR products were sequenced by ABI V3.1 Big dye kit and the analysis of sequence was conducted by the basic local alignment search tool (BLAST) to identify their closest relatives. A total number of 17 isolates of *Pseudomonas*, *Bacillus* and *Proteus* were phenotypically identified, while the nucleotide sequence revealed the presence of 59 diverse strains with distribution (percentage) of *Pseudomonas* (25) 42.2 %, *Bacillus* 17 (28.8 %) and *Proteus* (17) 28.8 %. The investigated isolates when compared from gene data-base recorded genetic relatedness with similarity index of 61 to 100 %. The 16S rRNA gene sequences from DNA extractions showed microbial consortia in drinking water to comprise of a broad array of bacterial diversity, including those of critical concern to human health such as *Pseudomonas* and *Bacillus.* Finally, there was no significant correlation between the HPC test and 16S rRNA sequence analysis.

Keywords: HPC, DNA extraction, 16S rRNA gene, PCR, Sequencing and Blast

INTRODUCTION

The basic duty of the Water Authority is to evaluate the challenge of microbial contamination for drinking water in relation to Total Coliform Rule (TCR). This requires the assessment of coliform group of bacteria in drinking water distribution system (**WHO, 2002**). The heterotrophic plate count (HPC) test is applied as indirect indicator of water safety and consists of a variety of simple culture-based approaches that are intended to obtain a wide range of bacteria from water consortia and pollutant sources.

The HPC is also employed as a marker for investigating aesthetic quality causes and challenges and provide data concerning water quality by estimating the levels of heterotrophic bacteria in water samples (**Bartram, Cotruvo, Exner, Fricker & Glasmacher, 2003).** However, the HPC is limited in spectrum and accuracy of information it reveals because, the test does not virtually detect all virulent bacteria. More so, the use of bacterial inactivation (chlorination and chemotherapeutic agents) seldom permits the growth of some potentially pathogenic species not observed by total coliform and HPC tests, such as *Pseudomonas*, *Bacillus* and *Mycobacterium***.**

Consequently, improvements in culture-based independent sequencing techniques now allows the evaluation of microbial consortia in much greater extent than was earlier performed from culture-based dependent approach, such as practiced in HPC (**Farcas, 2012**). The previous understanding of bacterial identification relied on growth in enrichment culture should be accepted with caution. Nevertheless, there is currently a paradigm shift whereby, the identity of a microbe in an environmental sample can be inferred by the sequences of its 16S ribosomal ribonucleic acid (rRNA) genes (**Medlar *et al*., 2014**).

The ribosome is found in all living organisms and the genetic sequences that encode the rRNA are relatively preserved in the domains of life. The multiple nucleotide sequences of gene can exist in a single bacterium, from which specific section can be targeted for replication. The 16S rRNA gene plays structural role in protein synthesis and annealing capacity of universal primers. Applying the molecular approach, it is now possible to investigate the microbial load and conduct detailed evaluation of broad network of environments, determine greater spectra of bacterial phylogeny, genetic similarity and identify uncommon bacterial in samples (**Stamatakis, 2014**).

MATERIALS AND METHODS

Sample Collection

Drinking water samples were collected thrice from different storage tanks from four locations in Benin City metropolis (Sapele, Auchi, Oko Central and Ugbowo Roads axis) in the months of March to May 2018. Samples were also obtained from the end-user point (taps) by allowing the taps to run for I minute before capturing 1Litre of the water sample in sterile containers. Forty milliliters of water were transferred into 50 ml conical tube prior to performing the heterotrophic plate count (HPC) test and deoxyribonucleic acid (DNA) extraction.

Heterotrophic plate counts (HPC)

The HPC tests were conducted according to protocols described in standard methods for the examination of water and waste water (**APHA, 2005**). Water samples were diluted in sterile peptone water (Zymo Research, Johannesburg, S/A) and filtered through 0.45 µm diameter Millipore membrane filters. The filters were then transferred onto R2A agar plates and incubated at 28 ºC for 48 hr. The number of colonies on each plate was then counted prior to the colour and morphology of each colony.

Identification and characterization of bacteria

Three 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**).

16S rRNA gene sequencing from HPC colonies

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 (**Ghatak, Muthukumara & Nachimuthu, 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 ºC 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, 5.0 µL. The process was performed in Gene PCR Thermo Cycler with the recommended guidelines: Initial denaturation at 94 ºC for 30 min; denaturation at 94 ºC for 1 min; annealing at 50 ºC for 1 min, extension at 72 ºC for 1 min; final extension at 72 ºC for 7 min and hold at 4 ºC.

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 ºC for 30 min and the reaction was stopped by heating the mixture at 95 ºC for 5 min. The purified PCR products were eluted in 5 µL nuclease free water for 30 min and stored at -20 ºC 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 likelihood and Neighbour-joining analyses. The bootstrap analysis was performed to estimate the confidence of the 16S rRNA gene-tree topology.

RESULTS

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

Table Phenotypic characterization of isolates obtained from biofilm water samples

Morphological Examination			**Biochemical Examination**							**Isolates**
Colonial Characteristics on nutrient agar (NA)	**Colonial Characteristics on MacConkey agar (MA)**	**Gram Staining**	**Ca**	**Ox**	**In**	**Mr**	**Ci**	**Vp**	**La**	
Greenish pigmented colony with an entire margin	Colourless colony with an entire margin	negative rods	+	+	-	-	+	-	-	Pseudomonas 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.

Legend: Ca: Catalase test; Ox: Oxidase test; In: Indole test; Mr: Methyl red test; Vp: Voges Proskauer test; Ci: Citrate test; La: Lactase test; -: negative reaction; +: positive reaction; sp. represents species.

Table 2 Distribution Percentage of bacterial isolates by the corresponding methods of identification

Isolates	**Phenotypic: Frequency (%)**	**Sequencing: Frequency (%)**
Pseudomonas	7 (41.2)	25 (42.2)
Bacillus	6 (35.3)	17 (28.8)
Proteus	4 (23.5)	17 (28.8)
Total	17	59

The phenotypic distribution of bacterial isolates recorded the highest number (7) for *Pseudomonas* and the least (4) was reported for *Proteus*

Table 3 Identification of bacterial isolates using 16S rRNA gene sequencing

Samples	**Isolates**	**Closest relative in gene data base**	**Similarity (%)**	**Accession number**
SA-R-1	*Pseudomonas aeruginosa* strain 907-R-AO8-02	*Pseudomonas aeruginosa*	80	KX860116
OK-T-4	*Proteus penneri* strain 907-R – B08-05	*Proteus penneri* strain FFL8	100	JN092595
AC-R-8	*Pseudomonas aeruginosa* strain 907-R-F07-16	*Pseudomonas aeruginosa* strain KCTC 23843	61	MG009256
OK-R-9	*Proteus* sp. strain 907-R-GO7-19	*Proteus* sp. strain KL11	62	KP313867
UB-R-12	*Bacillus* sp. strain 907-H07-22	*Bacillus* sp. strain B-3-35	100	KT583528
UB-T-13	*Bacillus cereus* strain 907-R-G07-19	*Bacillus cereus* strain D12-2	100	CP016315
AC-T-15	*Pseudomonas hibiscicola* strain 907-R- F06-24	*Pseudomonas hibiscicola strain IHBB 6867*	61	KF668478

Legend: OK (Oko Central road), UB (Ugbowo Road), SA (Sapele Road), AC (Auchi Road), R (Reservoir), ne), T (Tap)

The results of the nucleotide sequence of part of 16S rRNA gene analysis of bacterial isolates revealed other strains with closest identity with their accession numbers when compared from gene data-base by Neighbour joining method proframme to include: *Pseudomonas aeruginosa* (KX860116), *Proteus penneri* strain FFL8 (JN092595), *Pseudomonas aeruginosa* strain KCTC 23843 (MG009256), Proteus sp. strain KL11 (KP313867), *Bacillus* sp. strain 3-35 (KT583528) *Bacillus cereus* strain D12-2 (CP016315), *Pseudomonas hibiscicola* strain IHBB 6867 (KF668478),

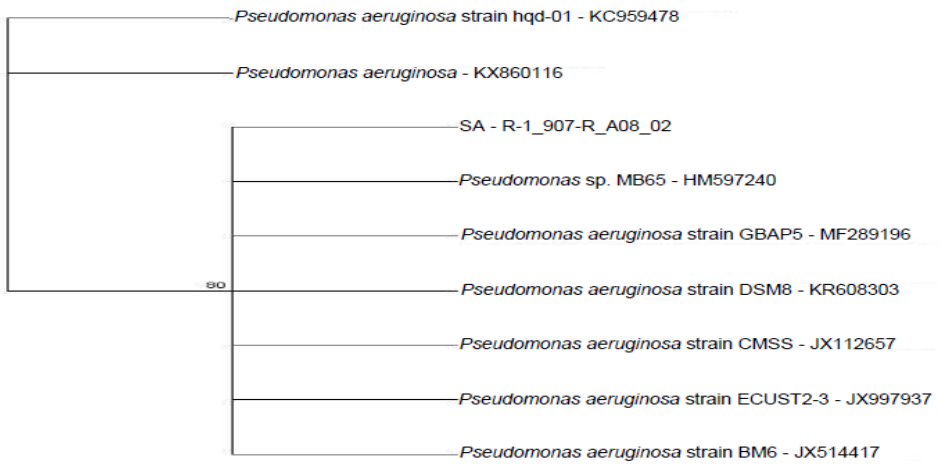

Figure 1.1 Phylogenetic analysis of clinical sample based on the nucleotide sequence of part of the 16S rRNA nucleotide sequence of Bacteria. The phylogenetic tree was constructed by the Neighbor-Joining method program in the Geneious package (version 9.0.5). The numbers at the forks show the numbers 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

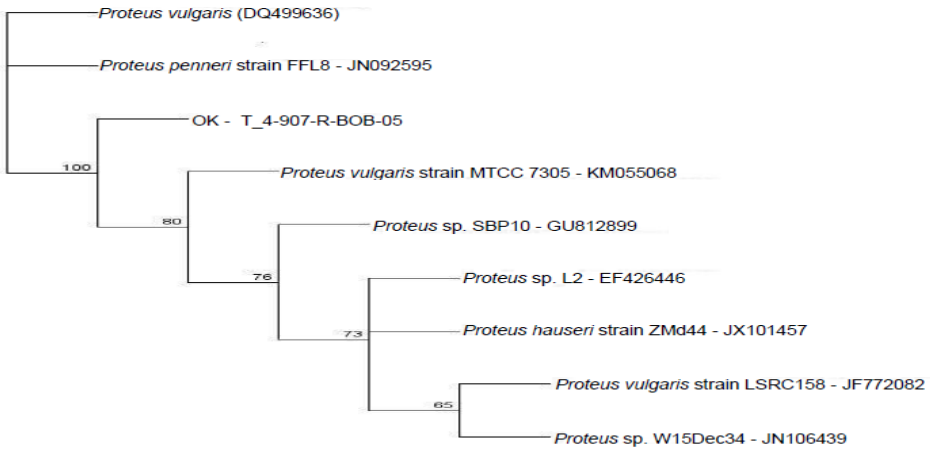

Figure 1.2 Phylogenetic analysis of clinical sample based on the nucleotide sequence of part of the 16S rRNA nucleotide sequence of Bacteria. The phylogenetic tree was constructed by the Neighbor-Joining method program in the Geneious package (version 9.0.5). The numbers at the forks show the numbers 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

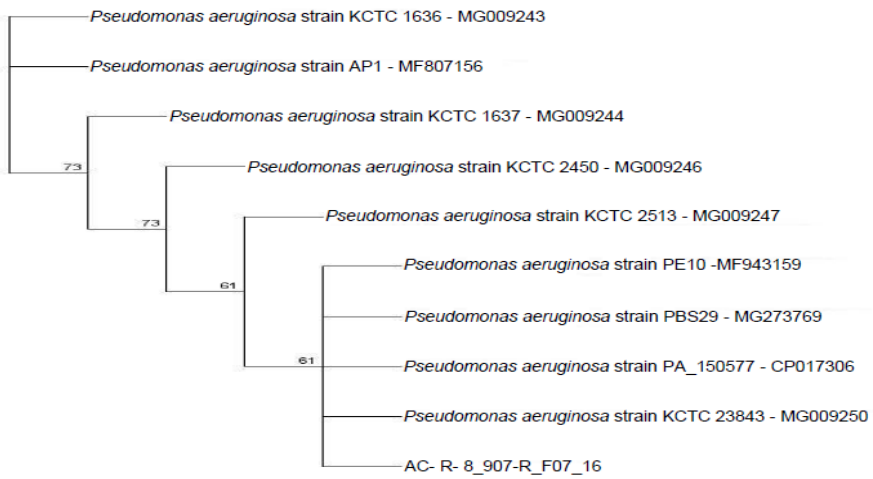

Figure 1.3 Phylogenetic analysis of clinical sample based on the nucleotide sequence of part of the 16S rRNA nucleotide sequence of Bacteria. The phylogenetic tree was constructed by the Neighbor-Joining method program in the Geneious package (version 9.0.5). The numbers at the forks show the numbers 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

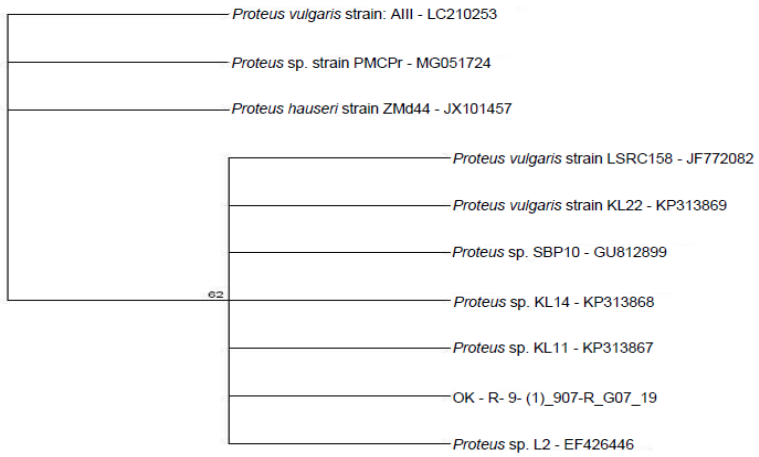

Figure 1.4 Phylogenetic analysis of clinical sample based on the nucleotide sequence of part of the 16S rRNA nucleotide sequence of Bacteria. The phylogenetic tree was constructed by the Neighbor-Joining method program in the Geneious package (version 9.0.5). The numbers at the forks show the numbers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

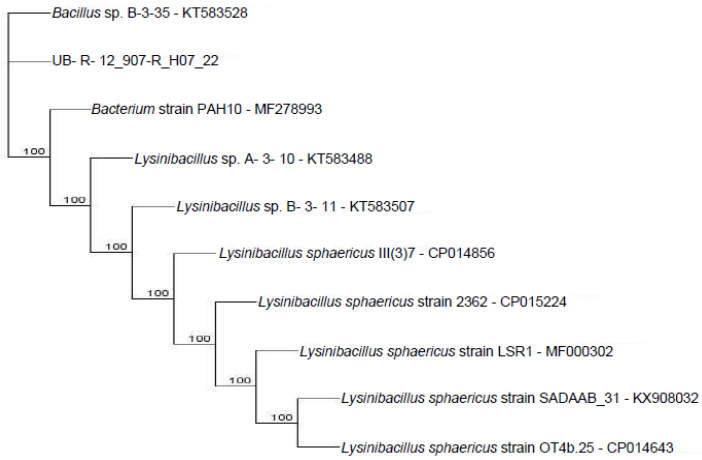

Figure 1.5 Phylogenetic analysis of clinical sample based on the nucleotide sequence of part of the 16S rRNA nucleotide sequence of Bacteria. The phylogenetic tree was constructed by the Neighbor-Joining method program in the Geneious package (version 9.0.5). The numbers at the forks show the numbers of occurrences of the repetitive groups to the right out of 100 bootstrap samples. The isolate **UB- R- 12** has similar sequence with *Bacillus sp.* B-3-35 with accession number KT583528

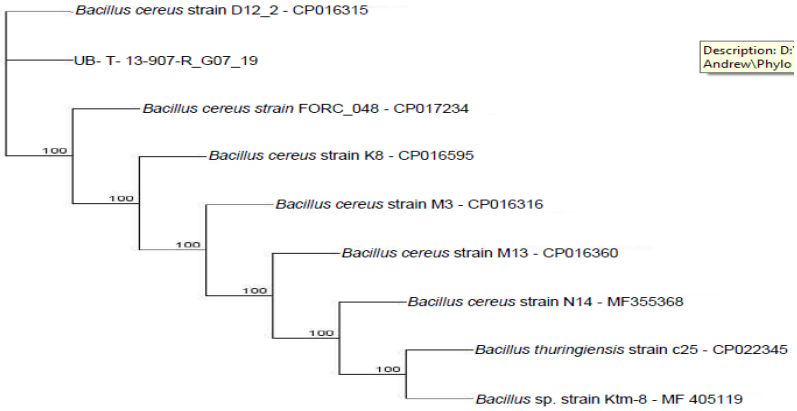

Figure 1.6 Phylogenetic analysis of clinical sample based on the nucleotide sequence of part of the 16S rRNA nucleotide sequence of Bacteria. The phylogenetic tree was constructed by the Neighbor-Joining method program in the Geneious package (version 9.0.5). The numbers at the forks show the numbers of occurrences of the repetitive groups to the right out of 100 bootstrap samples. The isolate **UB- T- 13** has similar sequence with *Bacillus cereus* strain D12_2 with accession number CP016315

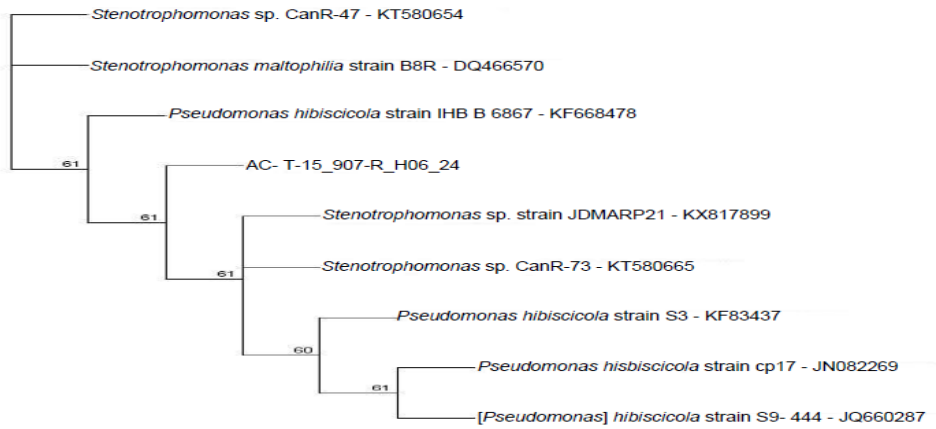

Figure 1.7 Phylogenetic analysis of clinical sample based on the nucleotide sequence of part of the 16S rRNA nucleotide sequence of Bacteria. The phylogenetic tree was constructed by the Neighbor-Joining method program in the Geneious package (version 9.0.5). The numbers at the forks show the numbers 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

DISCUSSION

The data collected in this study indicated no significant relationship between the heterotrophic plate counts and sequences, with the bacterial isolates phenotypically identified were *Bacillus, Pseudomonas and Proteus.* The highest frequency (percentage) was recorded for *Pseudomonas* (41.2%) and *Bacillus* 35.3% and *Proteus* 23.5% (Table 2). The genus *Pseudomonas* is of particular concern because of the diversity of obligate and opportunistic pathogens contained within the genus that caused nosocomial infections in susceptible patients and their high intrinsic resistance to a variety of antibiotics, including β - lactams, aminoglycosides and fluroquinolones that made them very difficult to eliminate. The B*acillus* produced putative virulent factors capable for triggering infections, responsible for endocarditis and neurological cases (**Farcas, 2012**).

In the phylogenetic analyses of biofilm water samples, all the isolates had similar sequences when compared with those from gene data-base which include: *Pseudomonas aeruginosa* (KX860116), *Proteus penneri* strain FFL8 (JN092595), *Pseudomonas aeruginosa* strain KCTC 23843 (MG009256), Proteus sp. strain KL11 (KP313867), *Bacillus* sp. strain 3-35 (KT583528) *Bacillus cereus* strain D12-2 (CP016315), *Pseudomonas hibiscicola* strain IHBB 6867 (KF668478).

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 (**Ghatak *et al*., 2013**). 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.* 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. Lavender and kinzelman compared agar-based count of *Escherichia coli* in water samples to detection by quantitative polymerase chain reaction (qPCR), wherein an *E. coli*-specific gene was amplified to quantify the *E. coli* in the sample and discovered qPCR to be more sensitive than culture-based methods (**Lavender & Kinzelman, 2009**). Approximately, nearly a third of sequences analyzed in this study showed 100 % homologous similarity with sequences in the currently available data bases (Table 3). 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. 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. More so, 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 due to the presence of the proteinaceous surface (**USEPA, 2012**).

The alignment with sequences available within gene data-base 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.* (25) 42.2 %, B*acillus* (17) 28.8 % and *Proteus (*17*)* 28.8 % (Table 2). It should be noted that the percentage (frequency) of *Pseudomonas, Bacillus* and *Proteus* obtained in sequencing were relatively higher than bacterial distribution from HPC. Comparatively, the sequencing revealed total number of 59 homologous isolates, while 17 was recorded from the HPC (thrice the value of HPC). This further confirmed that molecular approach gives more detail and broad spectrum of bacterial investigation than standard methods.

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 is 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 (HPC) monitoring technique, but which might be of potential human concern (**APHA, 2005**).

CONCLUSION

The obtained results in this study reveal a high possibility of cross contamination of food from food handlers and contact surfaces sampled. Organisms like *Enterobacter* sp., *Bacillus* sp, *Streptococcus* sp., *Micrococcus* sp., *Staphylococcus aureus*, *Streptococcus* sp. were isolated from the food and contact surfaces. It is recommended that some preventive measures be adopted to avoid contamination of cooked food like the routine examination of cooked food in restaurants by the relevant authorities and consequent sanctions if minimum standard are violated, hygiene awareness for personnel who prepare and handle ready to eat cooked food, training on the adoption on the integration of Hazard Analysis Critical Control Point (HACCP) procedures into the food preparation and production process.

REFERENCES

APHA (2005). *Standard Methods for Examination of Water and Wastewater*, 21st ed., American Public Health Association WWA, Washington, D.C., USA. p. 311. https://ajph.aphapublications.org/doi/pdf/10.2105/AJPH.85.8_

Barrow, G. I. & Feltham, R. K. A. (2003). *Cowan and Steel's Manual of Medical Bacteria.* 3rd ed. p. 352. Cambridge University Press. Doi: https://doi.org/10.1017/CBO9780511527104

Bartram, J., Cotruvo, J., Exner, M., Fricker, C. & Glasmacher, A. (Ed) (2003). *Heterotrophic Plate Counts and Drinking-water Safety*. IWA Publishing, Alliance House, 12 Caxton Street, London SW1H 0QS, UK. https://www.who.int/water_sanitation_health/dwq/HPCFull.pdf

Ghatak, S., Muthukumara, R.B. & Nachimuthu, S.K. (2013). A simple method of genomic DNA extraction from human samples for PCR-RFLP analysis. *J Biomol Tech.*, **24**(4), 224-31. Doi: https://doi.org/10.7171/jbt.13-2404-001.

Farcas, A. (2012). *Biofilms Impact on Drinking Water Quality Assessmen*t. (3rd edition). Rijeka Press, Croatia. pp. 246. Doi: https://doi.org/10.5772/32808

Lavender, J. S. & Kinzelman, J. L. (2009). A cross comparison of QPCR to agar-based or defined substrate test methods for the determination of Escherichia coli and enterococci in municipal water quality monitoring programs. *Water Res.*, **43**, 4967–4979. Doi: https://doi.org/10.1016/j.watres.2009.08.010

Letunic, I. Bork, P. (2007). Interactive Tree of Life: an online tool for phylogenetic tree display and annotation. *Bioinformatics*, **23**(1), 127-128. DOI: https//doi.org/10.1093/bioinformatics/btl529

Medlar, A., Tuomas, A. & Ari, L. (2014). Science: Reference-based phylogenetic analysis for 18S rRNA studies. *Evolutionary Biology*, **14**, 45 – 50. Doi: https://doi.org/10.1186/s12862-014-0235-7

Stamataklis, A. (2014). A tool for phylogenetic analysis and post analysis of large phylogenes. *Bioinformatics*, **033**, 100 – 109. Do: https://doi.org/10.1093/bioinformatics/btu033

United States Environmental Protection Agency (USEPA) 2012. Guidelines for Drinking-water Reuse. Office of Research and Development, Washington, D.C. pp. 294. http://nepis.epa.gov/Adobe/PDF/P100FS7K.pdf

World Health Organization (2002). Heterotrophic plate count measurement in drinking water system management. *Water, Sanitation and Health Bulletin*, **2**, 10 - 15. https://www.who.int/water_sanitation_health/dwq/WSH02.10.pdf