Molecular Characterization of Bacteria Associated with a Dumpsite Soil in University of Port Harcourt, Rivers State, Nigeria

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Abstract: This study identified the bacterial species associated with dumpsite soil using both cultural and molecular techniques. Soil samples were obtained from the University of Port Harcourt, Rivers State. Bacteria associated with dumpsite soil were isolated using both Nutrient agar and serial dilution method. Deoxyribonucleic acid (DNA) was extracted from pure cultures of bacterial isolates using Quick-DNA Fungal/Bacterial MiniPrep™ Kit and the concentration determined using Nanadrop 2000c Spectrophotometer. Polymerase chain reaction (PCR) amplification of I6S rRNA gene of the extracted DNA was carried out using bacteria universal primer pair; 16SF and 16SR. Sequencing was performed in an ABI3500 Genetic analyzer. The sequences were aligned and compared with some sequences on the National Centre for Biotechnology Information (NCBI) database for species identification and evolutionary trend. Four bacterial species were obtained. The result of the nucleotide sequence analysis revealed that isolate 1 with 1129bp had a 100% similarity to *Enterobacter ludwigii*; isolate 2 with 1235bp had a 91% similarity to *Acinetobacter baumannii*; isolate 3 having 1033bp had a 98% similarity to *Lysinibacillus fusiformis*; while isolate 4 with 1129bp had a 95% similarity to *Burkholderia latens*. The accession numbers MN310508, MK719842, MN396184 and MN207011 were assigned to isolates 1 to 4 respectively. This study provided more information on the bacteria species associated with dumpsite soils.
Key words: Dumpsite, sequencing, phylogeny, bacteria

INTRODUCTION

Soil is a mixture of organic matter, minerals, gases, liquids, and organisms that together support life (Pong, 2015). Soil is a product of several factors: the influence of climate, relief (elevation, orientation, and slope of terrain), organisms, and the soil's parent materials (original minerals) interacting over time. It continually undergoes development by way of numerous physical, chemical and biological processes, which include weathering with associated erosion (Yu *et al*., 2015).

A land used for dumping wastes is called a dumpsite. Dumpsite is an old traditional method of waste disposal similar to landfill method of waste management. The problems of the Municipal Solid Wastes (MSW) are particularly acute in developing countries where the socio-economic changes have not been met with improvements in waste management technologies (Zhou *et al*., 2014). In developing countries, waste is deposited on land or discharged into water bodies. When waste is dumped on land, microorganisms such as bacteria and fungi proliferate using the components of the waste materials as source of nutrient for growth as well as degrading the organic materials in the waste (Kumar *et al*., 2016). The sheer volumes of wastes produced in the fast-growing urban agglomerations, their hazards to the environment and human health, as well as the costs associated with urban waste systems have increasingly necessitated new and more cost-effective strategies in waste management (Afon, 2012).

In the University, the common types of waste generated are paper, plastics cans and food remnants. The microbial ecology of dumpsite soil can help us predict the consequences of such waste dump on soil living organisms and activities. This could also be of public concern as reports on the spread of diseases through dumpsites have been well documented (Alexander, 2016). According to Karen *et al*. (2015), health is at risk for those who live within five kilometers of a landfill site (dumpsite) as they are being exposed to air pollutants emitted by the waste causing lung cancer, respiratory diseases as well as deaths and these are prominent especially in children.

Microorganisms are important to humans as they are unavoidably associated with food, medicine, genetic engineering, industrial processes and many other fields of life. Molecular methods have been found to provide more significant and acceptable data than the traditional techniques in the identification of microorganisms. Over the last few decades, a variety of detection technologies have been developed in order to provide more knowledge and yield more understanding of the ecology of microorganisms (Roh *et al*., 2010; Zhou *et al*., 2015). To detect and characterize broader groups of organisms, highly conserved degenerate primers are designed, such as those used for amplifying 16S rRNA genes for bacteria and archaea (Fischer *et al*., 2016). Extensive information has been obtained from several ecosystems important to human health (Alivisatos *et al*., 2015) using molecular methods. This study was designed to isolate and characterize the bacteria associated with dumpsite soil using molecular tool. PCR amplicon sequencing of the 16S ribosomal RNA (rRNA) gene, which is the most conserved region in bacteria, was analyzed and was the basis of characterization of the isolates.

MATERIALS AND METHODS

Study Area and Collection of Samples

The study was conducted at the Regional Center for Biotechnology and Bioresources Research Laboratory, University of Port Harcourt Rivers State. The dumpsite is an open dumpsite located in University of Port Harcourt and consisted of food remnants, poyethene bags, plastics, leathers, disposed papers and grasses. Soil samples were collected from five different points at a distancee of 6 feet apart. Samples were collected under the refuse pile at a depth of 10cm and transferred into a sterile container. 10 grammes of soil was collected. The soil was immediately moved to the laboratory for analysis.

Isolation and Enumeration of Bacteria

Isolation of bacteria was done using serial dilution and spread plate method (Hartman, 2011). Five-fold serial dilution was prepared and 0.1ml was collected from each of the 10^{-2}, $10^{-3}$and 10^{-4} dilutions and inoculated aseptically into sterile freshly prepared nutrient agar plates in duplicates, using the spread plate method. The inoculated plates were incubated at 37^{o}C for 48hrs and observed for growth. Discreet colonies were counted and the morphological characteristics of the different bacteria isolates were identified. The bacteria isolates were further sub-cultured and purified with freshly prepared sterile nutrient agar. Pure cultures of the bacteria were preserved in agar slants for further use.

Bacterial DNA Extraction, Amplification and Sequencing

DNA was extracted using Zymo Quick DNA Fungal/Bacterial Mini prep kit protocol with slight modification as described by Iyanyi *et al*. (2019) and obtainable at the Regional Centre for Biotechnology and Bioresources Research (RCBBR) Laboratory, University of Port Harcourt, Rivers State, Nigeria. DNA quantity and purity was measured using Nano Drop 2000c spectrophotometer. (Thermo Fisher Scientific Inc. Wilmington Delaware, USA). Purity was measured as a ratio of absorbance at 260nm to that of 280nm. Agarose gel electrophoresis was used to ascertain the quality of the DNA.

Bacterial universal primers for the amplification of the 16S rRNA gene; 16SF forward: (5'GTGCCAGCAGCCGCGCTAA 3') and 16SR reverse: (5' AGACCCGGGAACGTATTCAC 3') were used to amplify fragments of the nuclear ribosomal DNA (rDNA) of the bacterial isolates through Polymerase Chain Reaction (PCR). The PCR cocktail mix consisted of 2.5µl of 10x PCR buffer,1µl of 25mM $MgCl_2$, 1ul each of forward primer and reverse primer, 1µl of DMSO, 2µl of 2.5mMDNTPs, 0.1µl of 5µ/µl Taq DNA polymerase, and 3µl of 10ng/µl DNA. The total reaction volume was made up to 25µl using 13.4µl Nuclease free water.

The PCR cycling parameters were initial denaturation at 94˚C for 5mins, followed by 36 cycles of denaturation at 94˚C for 30sec, annealing at 56˚C for 30secs and elongation at 72˚C for 45sec. Followed by a final elongation step at 72˚C for 7 minutes and hold temperature at 10˚C. The PCR thermal cycler used was GeneAmp PCR system 9700 (Applied Biosystems, France). PCR products were visualized on safe view-stained 1.5% agarose gel. The PCR products were loaded on gel prepared with agarose powder and 1X TrisBoris EDTA (Ethylene Diamine Tetra-acetic Acid). Electrophoresis was carried out at 100 volts for 40 mins. After the run time, the gel was transferred to a gel documentation system (Gel Documentation microDOCTM, Cleaver Scientific Ltd, UK). The gel documentation system consists of a gel holing area, a digital camera and a UV transiluminator. Amplification was captured by the digital camera in form of bands as illuminated by the UV transiluminator. Amplified samples were sequenced using ABI 3500 Genetic analyzer (Thermo Fisher Scientific, Massachusetts, U.S.A.).

Phylogenetic Analysis of the 16S rRNA Genes

Sequence were edited and trimmed on MEGA X. Sequences were blasted on National Centre for Biotechnology Information (NCBI) database for identification of the organisms. The sequences obtained were compared with sequences in GenBank. Sequences were aligned using Clustal W. Best BLAST hits were used for the construction of neighbor-joining phylogenetic tree using the Maximum composite likelihood method; and evolutionary analysis was conducted on MEGA X software (Kumar *et al*., 2018).

RESULTS

Bacteria Isolated from Dumpsite Soil

Four bacteria were isolated from the dumpsite soil. The pure cultures of the isolates are presented in Figure 1. The frequency of occurrence of each bacterium colony on nutrient agar was determined. Isolate No. 4 had the highest frequency of occurrence (2.5) followed by isolate No. 2 (2.25) as shown in Table 1.

Table 1: Frequency of occurrence of bacteria isolated from dumpsite soil

Isolate ID	Frequency of occurrence
1	2±0.3
2	2.25±0.46
3	1.75±0.46
4	2.5±0.15

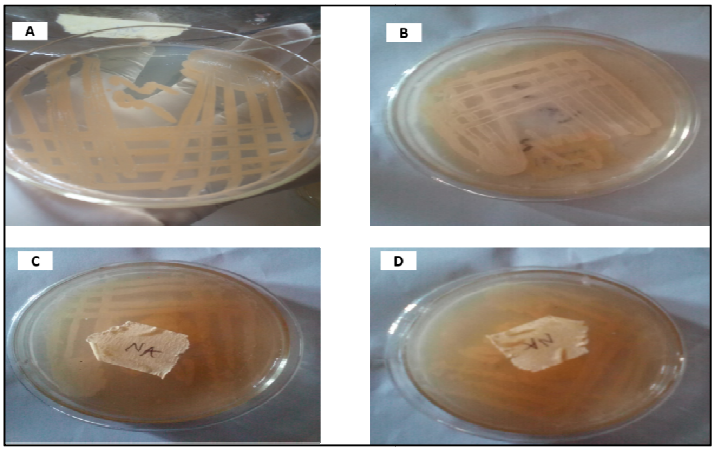

Figure 1: Pure cultures of bacterial isolates obtained from dumpsite soil Alphabets A to D represent isolates 1 to 4.

Concentration, Purity and Quality of Bacterial DNA

The Concentrations of DNA from the various isolates is presented in Table 2. The result of the gel electrophoresis showed that the extracted DNA were of good quality and suitable for polymerase chain reaction as presented in Figure 2.

Table 2: Concentration of DNA from bacteria isolates obtained from dumpsite soil

Isolate ID	Nucleic acid conc. (ng/µl)	Absorbance at 260nm/280nm (Purity)
1	153.6	1.87
2	66.1	1.89
3	189.3	1.83
4	57.3	1.92

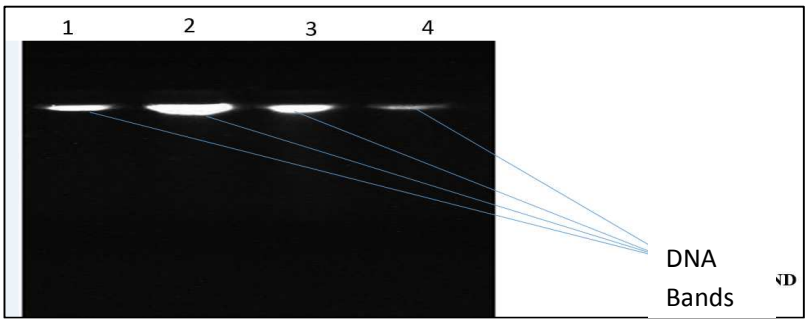

Figure 2: Gel electrophoresis of genomic DNA obtained from the bacterial isolates

The numbers 1 to 4 represent DNA from the respective isolates.

Polymerase Chain Reaction (PCR)

Amplified DNA showed bands on gel when viewed under UV light. The size of the PCR product obtained from each isolate can be seen in Figure 3.

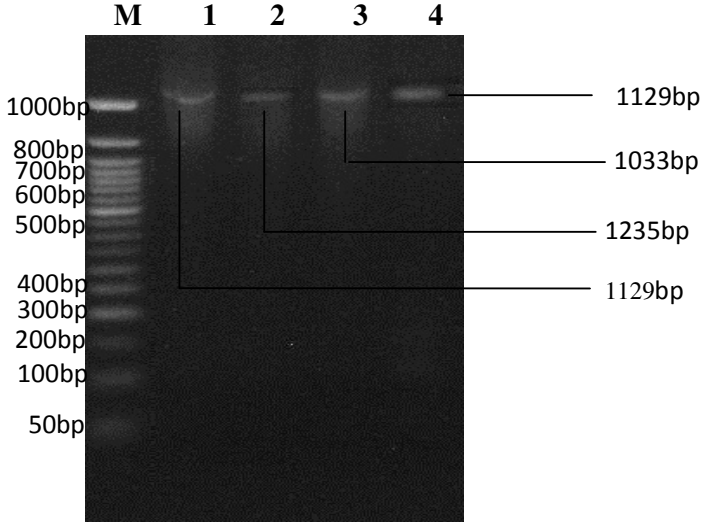

Figure 3: Gel electrophoresis of PCR amplification of 16S gene sequences
M- 50bp DNA ladder; the numbers 1 to 4 represent the bacterial isolates.

DNA Sequences Obtained from Isolates

After sequencing, 1129, 1235, 1033 and 1129 base pairs were obtained from isolates 1 to 4 respectively. The alignment of the isolates (Figures 4a to 4d) as represented by the red and pink lines, exceeded 200 base pairs which is ideal for the identification of microorganisms.

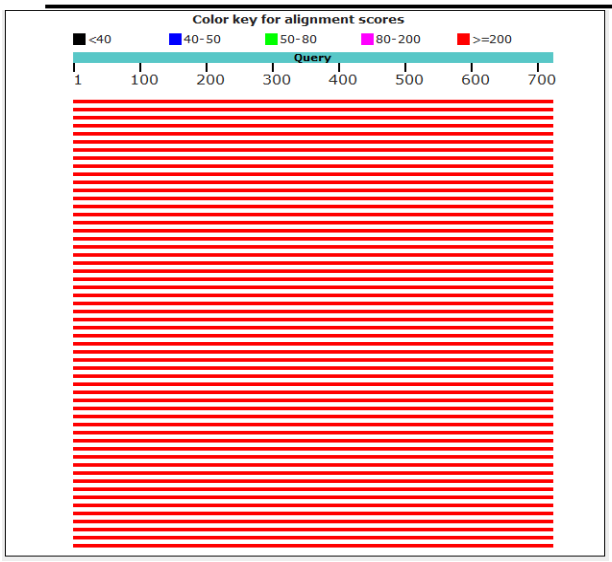

Figure 4a: Sequence alignment of isolate 1

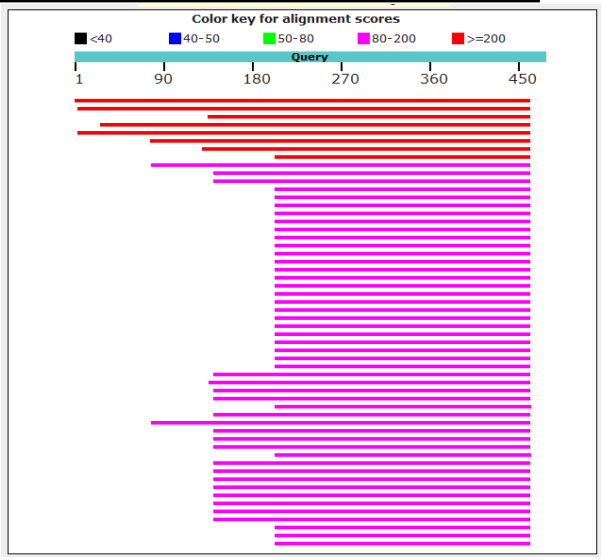

Figure 4b: Sequence alignment of isolate 2

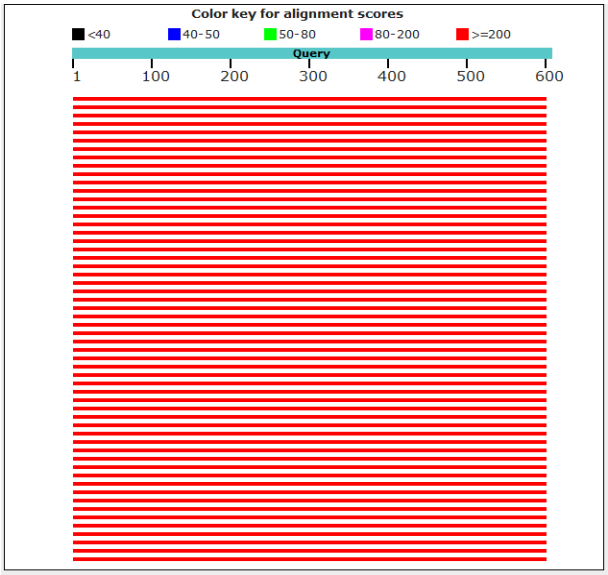

Figure 4c: Sequence alignment of isolate 3

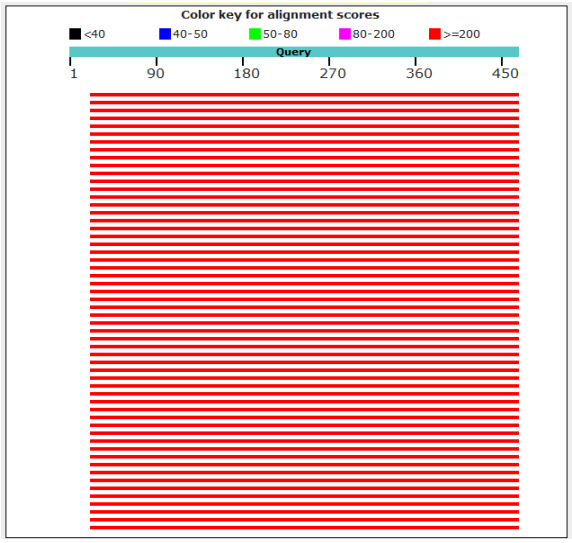

Figure 4d: Sequence alignment of isolate 4

BLAST results revealed the species identity of the bacteria isolates as *Enterobacter ludwigii, Acinetobacter baumanni, Lysinbaccillus fusiformis* and *Burkholderia latens* respectively. Table 3 shows the Taxonomic affinities of the isolates from BLAST Searches.

Table 3: Taxonomic affinities of 16S sequences inferred from BLAST search

Isolate ID	Taxonomic affinity (GenBank no)	Similarity (%)
1	*Enterobacter ludwigii* (MN310508)	100
2	*Acinetobacter baumanni* (MK719842)	91
3	*Lysinibacillus fusiformis* (MN396184)	98
4	*Burkholderia latens* (MN207011)	95

The sequences generated have been submitted to GenBank under the accession numbers MN310508, MK719842, MN396184 and MN207011 corresponding to the strain numbers RCBBR_AEAMI1, RCBBR_AEAMI2, RCBBR_AEAMI3 andRCBBR_AEAMI4 for samples 1 to 4 respectively.

Phylogenetic Analysis

The phylogenetic trees of the sequences obtained from the various isolates are presented in Figure 5. Isolates 1, 2, 3 and 4 were identified as *Enterobacter ludwigii*, *Acinetobacter baumannii*, *Lysinibacillus fusiformis* and *Burkholderia lanterns* respectively. These isolates were found to be closely related to: *Enterobacter cloacae, Acinetobacter pitii*, *Lysinibacillus rhizophila* and *Burkholderia territorii* respectively.

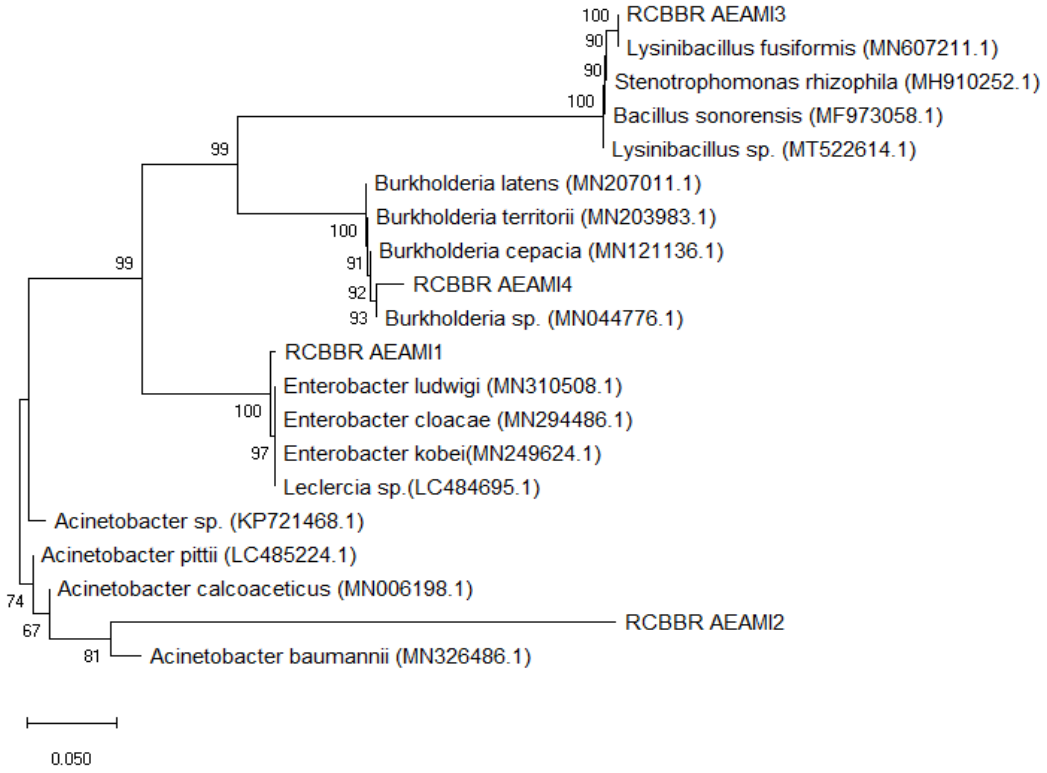

Figure 4: Neighbor-joining phylogenetic tree showing the isolates obtained from dumpsite soil

DISCUSSION

Dumpsite soils are associated with diverse bacterial genera. The mircroorganisms present in dumpsites aid in the detoxification of complex organic molecules (Osazee *et al*., 2013). Open dumpsites are breeding spaces for a diversity of microorganisms which can cause different health implications to man. This study was aimed at isolating bacterial isolates from dumpsite soil using cultural techniques and characterizing the isolates using molecular techniques. PCR and sequencing of the 16S gene led to the identity of four (4) bacterial isolates from the dumpsite soil to the species level. This study revealed the identity of the bacteria isolates as: *Enterobacter ludwigii*, *Acinetobacter baumannii*, *Lysinibacillus fusiformis* and *Burkholderia latens*. Osazee *et al*., (2013) isolated and characterized 10 microbial isolates consisting of 6 bacteria and 4 fungi from waste dumpsites in Benin City, Nigeria. The bacteria were *Klebsiella* sp., *Pseudomonas* sp., *Bacillus* sp., *Staphylococcus* sp., *Aeromonas* sp. and *Enterobacter* sp. Eleanya *et al*., (2017) isolated *Staphylococcus* sp., *Klebsiella* sp., *Bacillus* sp., *Pseudomonas* sp., *Salmonella* sp. *Escherichia coli* and *Citrobacter* sp. from dumpsite soils in Bwari, Federal Capital Territory, Nigeria.

Enterobacter ludwigii is a gram negative, facultatively anaerobic, rod-shaped, non-sporing bacteria. It survives in the skin and dry surfaces replicating in contaminated environment such as water (mostly waste water) and inhabit soil ubiquitous in nature(Yousaf *et al*., 2011). *E. ludwigii* has been reportedly responsible for infections in urinary tract, abdominal, and surgical site infections, respiratory tract infection, endocarditis, intra-abdominal infection, ophthalmic infection and prolonged hospitalization (Hooper *et al*., 2012). It has also been reported to effect the development and causes age-dependent neurodegeneration, non-occlusive thrombosis of the superior Vena cava and atrium and nosocomial infection (Erny and Prinz, 2017).

Acinetobacter baumannii is a gram negative bacillus that is aerobic, pleomorphic and non-motile; an opportunistic pathogen. It can survive over long periods and the disease can spread through contact with contaminated surfaces; and is able to survive in adverse environmental conditions (Fishbian and Peleg, 2010). *A. baumannii* causes infections in blood, meningitis, urinary tract and lungs causing pneumonia; in wounds and other parts of the body. It can colonize or live in a patient without causing infections or symptoms especially in respiratory secretion (sputum) (Cerqueira and Peleg, 2011). It normally leads to prolonged hospitalization of patients and breaches in skin and airway protection. It also has multi-drug resistance pattern. It's an emerging pathogen and when contacted, it can lead to serious nosocomial infection because of the ability to attach to any substrate (Vimercah *et al*., 2016).

Lysinibacillus fusiformis is a gram positive, rod-shaped bacteria. It possess the ability to form biofilms which are "microbial-population-formed-cells" with high density communities. Biofilms are beneficial in crop protection (Flemming *et al*., 2016). *L. fusiformis* causes tropical ulcer formations, severe sepsis, dermal or respiratory infections. It has tetradoxin-producing ability. Tetradoxin are highly fatal neurotoxin that destroys the central nervous systems of humans causing paralysis (Wang *et al*., 2010). *L. fusiformis* can utilize oxygen to metabolize various sugar and other simple carbohydrates. It can hydrolyze casein and gelatin, urea to produce ammonia and CO_2. The bacterium has the ability to reduce chromate to chromium and is resistant to multiple metals such as copper, nickel, cobalt, mercury, silver and cadmium (He *et al*., 2010).

Burkholderia latens is a gram negative, aerobic, non-spores forming bacterium with colonies that are moist and are ubiquitous (Oren and Garrity, 2015). *B. latens* are plant growth promoters, bioremediators and bio control strain. It also causes diseases in plant, horses and immunocompromised individuals such as cystics fibroses (Gupta, 2016).

The molecular methods (PCR and Sanger sequencing) employed in this study were only able to identify four bacterial organisms. This method of identification of microorganisms is better than cultural method which only deals with the morphological and microscopic examination of the isolates. Though identification using PCR amplification and Sanger sequencing has limitations as it is laborious and can only identify limited number of organisms because these organisms must be purely cultured on media before DNA extraction can be done. But using other molecular methods such as shotgun metagenomics, several bacteria and other microbial communities of organisms can be identified; though these methods are expensive and also require complex data analysis.

CONCLUSION

Molecular techniques have proven to be more reliable than traditional methods. Molecular characterization through polymerase chain reaction amplification and sequencing of the 16S rRNA (ribosomonal RNA) gene lead to the identification of the bacterial isolates. Four bacterial isolates namely *Enterobacter ludwigii*, *Acinetobacter baumannii*, *Lysinibacillus fusiformis* and *Burkholderia lateens* were

characterized in this study. These bacterial isolates have been routinely isolated from the soil. This study has highlighted the economic importance associated with some of the bacterial species present in dumpsite soils which will help enlighten the public on the need for effective management of dumpsites.

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