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Random Amplified Polymorphic DNA (RAPD) Markers Protocol of Bacterial Isolates from Two selected General Hospitals Wastewater (HWW)

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Abstract

In this research work, Random amplified polymorphic DNA (RAPD) markers Protocol was used to characterize bacterial isolates from two selected General Hospitals Wastewater (HWW). The hospital environment and its wastes accumulate diseases from both inward and outward patients. It is pertinent to investigate the wastewater and study its microbial community. Wastewater samples were collected from two major General Hospitals in Akoko area, namely, Ikare and Akungba-Akoko General Hospitals. Samples were microbiologically examined for the presence of bacterial colonies. Isolated bacterial species were preliminarily identified through conventional biochemical tests and were further characterized using 16s rRNA sequencing protocol. The bacterial gene was amplified using selected primers. DNA was isolated, purified, and amplified using 16s rRNA gene sequence protocol, and the results were analyzed and assessed with the standard bacteria sequence in the NCBI database. Molecular evolutionary analyses and Phylogenetic trees were mapped out from the results obtained. it was observed that the Bacterial counts in Cfu/ml range between 10.0 x 10^3 Cfu/ml to 24.0 x 10^3 Cfu/ml. The highest bacterial count was observed from the test sample from Ikare followed by Akungba Akoko General Hospitals with the Cfu/ml value of 24.0 x 10^3 and 18.0 x10^3 Cfu/ml respectively. Molecular data sequence, when compared with NCBI database using BLAST showed 99.60 –99.87% bactaria phylogenic identity and E-value equal to 0 for all closely related taxa. The following bacteria were characterized using Random amplified polymorphic DNA (RAPD) markers protocol namely: Streptococcus pneumoniae, Staphylococcus aureus, Bacillus cereus, and Bacillus subtilis. this study has revealed the presence of different bacterial species in hospital wastewater which pose threat to public health. Hence, proper monitoring and sewage treatment should be encouraged in different hospital settings before the disposal of these wastewaters to the public water runways.

Keywords: *Hospital Wastewater (HWW), 16SrRNA Molecular sequencing*

Introduction

Hospital wastewater (HWW) is the liquid waste generated by hospitals and other healthcare facilities. It differs significantly from domestic wastewater due to the presence of a wider range of contaminants, including: (1) Pathogenic microorganisms: Bacteria, viruses, and parasites that can cause disease. (2) Pharmaceutical residues: Unused or expired medications, including antibiotics, hormones, and chemotherapeutic agents. (3)Chemical disinfectants: Used to sterilize medical equipment and surfaces. (4) Radionuclides: Isotopes used in medical imaging and therapy. (5) Heavy metals: Trace elements such as lead, mercury, and copper (1).

Improper treatment of hospital wastewater may pose a major threat to public health and the environment as a whole. Pathogens can spread to waterways and contaminate drinking water supplies. Pharmaceutical residues maybe harmful to aquatic life and contribute to the development of multiple resistant bacteria. Hospitals have a responsibility to ensure their wastewater is treated effectively before it is released into the environment. There are a number of treatment methods available in the hospital settings can maybe practiced to improve hospital waste disposal, this including: (1) Primary treatment, which involves the Removes solids and organic matter through physical processes such as screening and sedimentation.(2) Secondary treatment, which involves the using biological processes to break down organic matter using bacteria.(3)Tertiary treatment, which involves providing additional removal of pollutants, such as nutrients and pathogens.(4) Advanced treatment processes,May be used to remove specific contaminants, such as pharmaceutical residues and heavy metals. The specific treatment methods used by a hospital will depend on the nature and volume of its wastewater, as well as local regulations (2,3).

It should be mentioned that, the hospital waste water constitute a major public heath hazard, if it is not properly treated. It may become major health inpediment. Most of the hospital may be polluted and pendemic health challenges may emanate from the environmental pollution caused by waste water. For the third world country like Nigeria, we should establish a structure to treate our hospital waste(4,5).

Random amplified polymorphic DNA (RAPD) Markers protocol is rapid method of identifying and characterizing of bacteria isolate.

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It makes use of ther abundant informations in their genetic pool to classify the bacterial isolate, by harvesting the molecular information in the 16s rRNA , it give us a leverage to rapidly identify a bacterial isolate especially where we have multiple number of isolates . this rapid techniques for identification is a robust analytical method to identify microorganism to the sub species level by generation the phylogenetic tree and compare the result obtained with the data bank(6).. In addition, 16s rRNA molecular sequencing methods have revolutionized bacterial identification and taxonomy studies, allowing microbiologist and bacteriologists alike, to classify prokaryotes based on their phylogenetic similarities(7).

Material and methods

Study area

Study area include two different General Hospitals, Ondo State. namely Ikare and Akungba Akoko Ondo State, Nigeria. Ondo state shares its border with other cities such as Benin, Ekiti etc. The geographic location Greenwich of Meridian Latitude and 8'15 North of the equator of Ondo State , Nigeria.

Collection of two General hospitals waste water sample

Sample bottles were rinsed and sterilized at 121°C for 15 minutes using an autoclave before sampling. The hospitals wastewater sample were collected from the effluent waste water channels, at the tip of drainage tube and transport to the laboratory for further microbiological assessment. After the wastewater sample collected from each sampling point, samples were labeled, transported to Adekunle Ajasin University Akungba Akoko, Microbiology Laboratory for bacterial analysis.

Isolation of Bacterial isolates from two different General Hospitals waste water samples

9ml of distilled water was serially diluted and dispensed into 7 test tubes and the mouth of the test tubes were corked with cotton wool, wrapped with aluminum foil and then sterilized at 121^0C for 15 minutes using an autoclave. After sterilization, the hospital waste water samples were allowed to cool at ambient temperature, for few minutes. Each test tube was then labeled as 10^2- 10^6 respectively. 1 ml of the hospital waste water samples was dispensed into 9 ml of sterile test tube. 1 ml of the stock culture was then transfer to 9ml sterile distilled water in a test tube and serially diluted in an aliquot manner up to the sixth diluents (8).

Identification of Bacterial Isolates from two selected General Hospitals waste water samples using Gram staining of bacterial isolates.

A flamed wire loop was used to pick a colony from a plate and a thin film smear was made on a clean grease free slide. The film was allowed to dry and was heat fixed by waving over flame of a Bunsen burner. It was then covered with crystal violet reagent for one (1) minute. The slide was placed on a rack over a sink and rinse in a slowly running tap for 5seconds. The film was flooded with iodine solution for 1 minute rinsed slowly running water for 5 seconds. It was decolorized with alcohol reagent slowly until no 43 more dye runs out. The smear was covered with Saffranin reagent for 30 seconds and rinsed in slowly running water. It was then air dried before viewing under the microscope. The stained slide was viewed with oil immersion lens x100 of the microscope. Gram negative cells appeared pink or red (9).

Biochemical tests for identification of bacterial isolates from two selected General Hospitals waste water sample

The bacteria isolates from the hospital waste water sample were identified by conventional methods. Briefly, a sterile wire loop a drop of normal saline were added to the center of grease-free slide and a portion of the colony were emulsified into the center of a glass slide and allowed to air dry before air fixing. Crystal violet was then applied after 3min. It was then replaced with a Gram's iodine for one minute, prior to rinsing with water and application of 95% alcohol until no color appeared. The Slides were then rinsed with water and Safranin for 1-2min. this was followed by rinsing and air-drying before being observed microscopically under ×100 oil immersion lens. Where interpreted that purple and blue color indicated the presence of Gram-positive bacteria and pink or red color identify the presence of Gram negative bacteria. All slants of test organisms were kept at -4^0 C prior to the bioassay of the extracts. Extensive series of biochemical tests were carried out to further confirm all the test bacterial strains. Biochemical tests done includes; Indole test, Catalase, Citrate, Methyl Red-Voges Proskaeur (MR-VP), Triple Sugar Iron (TSI), Urease, Motility Test, Oxidase Test.

Molecular identification using Random Amplified Polymorphic DNA (RAPD) Markers of bacterial isolates from two selected General Hospitals waste water sample

DNA extraction and PCR of bacterial isolates from two selected General Hospitals waste water sample

DNA was extracted from the cultured bacteria in broth using the Quick-DNA™, Bacteria Mini-prep Kit (Zymo Research) using the manufacturers procedures. Extracted DNA was then stored in -20°C till PCR. PCR sequencing preparation cocktail was prepared using (per reaction) 25 µlTaq 2X Master Mix | NEB, 4 µl of-AGA10GTT TGApmolTCMTGGeachCTC AG27F-3' 5'-and 1525R,-AAGGAGGTGATCCAGCC5′-3′ primers and made up to 4 water to which 8µl DNA template was then add. PCR System Thermalcycler (Applied Biosystem Inc., USA) with a Pcr profile consisting of an initial denaturation at 94°C for 5 min; followed by a 30 cycles consisting of 94°C for 30 s, 50°C for 60s and 72°C for 1 minute 30 seconds ; and a final termination at 72°C for 10 mins and chilled at 4°C GEL.

Purification of Amplified Product of bacterial isolates from two selected General Hospitals waste water sample

After gel integrity, the amplified fragments were ethanol purified in order to remove the PCR reagents. Briefly, 7.6 µl of Na acetate 3M and 240 µl of 95% ethanol were added to each about 40µl PCR amplified product in a new sterile 1.5 µl tube eppendorf, mixed thoroughly by vortexing and kept at -20°C for at least 30 min. Centrifugation for 10 min at 13000 g and 4°C followed by removal of supernatant (invert tube on trash once) after which the pellet were washed by adding 150 µl of 70% ethanol and mixed, then centrifuged for 15 min at 7500 g and 4°C. Again all supernatant (invert tube on trash) were removedand tube inverted on paper tissue and allowed to dry in the fume hood at room temperature for 10-15 min. Then suspended with 20 µl of sterile distilled water and kept in -20°C prior to sequencing. The purified fragment was checked on a 1.5% Agarose gel ran on a voltage of 110V for about 1hr as previous, to confirm the presence of the purified productand quantified using ananodrop of model 2000 from thermo scientific (10). The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems

using manufacturers' man Big Dye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA 6 were used for all genetic analysis

Results

Table 1 shows the sampling location, codes, number of sample and types of samples collected from Ikare and Owo general hospitals. The total number of nine (9) waste water samples were collected.

Table 2 showed the total bacterial count from the two sampling locations. Bacterial counts in cfu/ml ranges from 10.0 x 10^{-3}cfu/ml to 24.0 x 10^{-3}cfu/ml. Highest bacterial count was observed in waste water samples from Ikare general hospital with the cfu/ml value of 24.0 x 10^{-3}cfu/ml.

Table 3 shows the plate characteristics of bacterial isolates. In this table, isolates were observed for their color, appearance, elevation and opacity. Isolate O1, O2 and O3 showed white-grey, large yellow and large yellow color on agar, with smooth, raised and convex opacity respectively. Isolate I 1, I2 and I3 showed large yellow, slight yellow respectively with smooth surface, raised and flat elevation and convex and slightly convex opacity respectively.

Table 4 shows the Gram staining and biochemical characteristics of the isolated bacteria species. All isolates as observed in this table were +ve Rod for Gram staining with singly arrangements. Isolate O1, O2, O3 and I 1 were negative while I2 and I 3 were positive for oxidase. Isolate O1, O2, O3, I1, I2 and I3 were positive for catalase, citrate, indole motility, Triple sugar iron respectively.

Table 2: Total bacterial count from two selected General Hospitals waste water sample

Isolates codes	Total bacterial counts cfu/ml
O 1	10.0 x 10^3
O 2	20.0 x 10^3
O 3	12.0 x 10^3
I 1	16.0 x 10^3
I 2	24.0 x 10^3
I 3	22.0 x 10^3

Table 1: Sampling code, location, number of sample collected from two selected General Hospitals waste water sample

Sample code	Number of samples collected	Sample type	Location
Ik 1	3	Waste-water	Ikare, Ondo State
Ow	3	Waste-water	Owo, Ondo State
Ik 2	3	Waste-water	Ikare, Ondo State
Ow 2	3	Waste-water	Owo, Ondo State
Ik 1	3	Waste-water	Ikare, Ondo State
Ow	3	Waste-water	Owo, Ondo State

Table 3: Cultural characteristics of Bacterial isolated from two selected General Hospitals waste water sample

Isolate codes	Color	Appearance	Elevation	Opacity
O 1	White-grey	Smooth	Raised	Convex
O 2	Large yellow	Smooth	Raised	Convex
O 3	Large yellow	Smooth	Raised	Convex
I 1	Large yellow	Smooth	Raised	Convex
I 2	Slight yellow	Smooth	Flat	Slightly convex
I 3	Slight yellow	Smooth	Flat	Slightly convex

Table 4: Biochemical characteristics of bacterial isolates from two selected General Hospitals waste water sample

Isolate codes	*Gram staining*	*Arrangements*	*Oxidase*	*Catalase*	*Citrate*	*Indole*	*Motility*	*TIS*					
								Gas	*Glucose*	*Lactose*	*Maltose*	*Sucrose*	*H_2S*
O 1	+ve Rod	Singly	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve
O 2	+ve Rod	Singly	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
O 3	+ve Rod	Singly	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
I 1	+ve Rod	Singly	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
I 2	+ve Rod	Singly	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve
I 3	+ve Rod	Singly	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve

Molecular identification of bacterial isolates from two selected General Hospitals waste water sample

Table 6 shows the sample ID, scientific name, max score, Total score, query cover, E value and Percentage identity of bacterial isolates, characterized. Four (4) bacterial isolated were identified manely; (1) *Streptococcus pneumoniae,* (2) *Staphylococcus aureus,* (3) *Bacillus cereus* and (4)*Bacillus subtilis.*

Figure 1 to 3 presented the Agarose gel electrophoresis photograph of bacterial isolates against 1500 bp marker. In figure 1 and 2 agarose gel electrophoresis revealed the presence of different strains of *Staphylococcus* species against 1500bp ladder marker. Figure 1 Lane 1, depicted the band sequence of *Streptococcus pneumoniae* while lane showed the band sequence of *Staphylococcus aureus.* Figure 2 lane 1 and 2 shows the band sequences of *Staphylococcus aureu* and *Staphylococcus aureus.* Figure 3 shows the electrophoresis analysis of selected bacterial isolates. In this figure, band sequence of selected bacterial isolates were compared against the gene marker of 1500bp. Agarose gel electrophoresis showed the presence of *Bacillus cereus* and *Bacillus subtilis* (Lane 1 and two) respectively.

Figure 4 shows the phylogenic tree of bacterial isolates from two selected General Hospitals waste water sample

Molecular identification of bacterial isolates from two selected General Hospitals waste water sample

Table 6 shows the sample ID, scientific name, max score, Total score, query cover, E value, Percentage identity of bacterial isolates characterized using 16S rRNA sequencing protocol. Six (6) bacterial isolates were characterized and were identified as; *Streptococcus pneumoniae, Staphylococcus aureus, Bacillus cereus* and *Bacillus subtilis* Isolates were observed to have at least 99.60 percentage identities.

Figure 1 to 3 presented the Agarose gel electrophoresis photograph of bacterial isolate against 1500 bp marker. In figure 1 and 2 agarose gel electrophoresis revealed the presence of different strains of *Staphylococcus* species against 1500bp ladder marker. Figure 1 Lane 1, depicted the band sequence of *Streptococcus pneumoniae* while lane showed the band sequence of *Staphylococcus aureus.* Figure 2 lane 1 and 2 shows the band sequences of *Staphylococcus aureu* and *Staphylococcus aureus.* Figure 3 shows the electrophoresis analysis of selected bacterial isolates. In this figure, band sequence of selected bacterial isolates were compared against the gene marker of 1500bp. Agarose gel electrophoresis showed the presence of *Bacillus cereus* and *Bacillus subtilis* (Lane 1 and two) respectively.

Figure 4 shows the phylogenic tree of bacterial isolates **from two selected General Hospitals waste water sample**

Table 5: Sample ID Scientific Name, Max Score, Total Score, Query Cover, E value and Percentage identity of Isolates

Sample ID	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident
1	*Streptococcus pneumoniae*	2724	2724	100%	0	99.66%
15	*Staphylococcus aureus*	2719	2719	99%	0	99.60%
5EMKISS	*Staphylococcus aureus*	2756	2756	100%	0	99.87%
2 COF	*Staphylococcus aureus*	2724	2724	100%	0	99.87%
MSA7	*Bacillus cereus*	2754	2754	100%	0	99.87%
MSA3	*Bacillus subtilis*	2691	2691	100%	0	99.93%

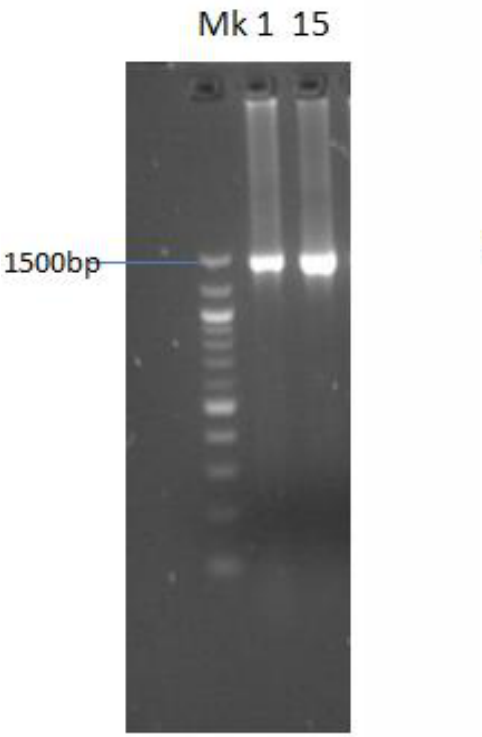

Fig 1: Photograph of based Random Amplified Polymorphic DNA (RAPD) for two different strains: Streptococcus pneumoniae and Staphylococcus aureus (Lanes 2 and 3) against 1500bp ladder DNA marker (Lane 1).

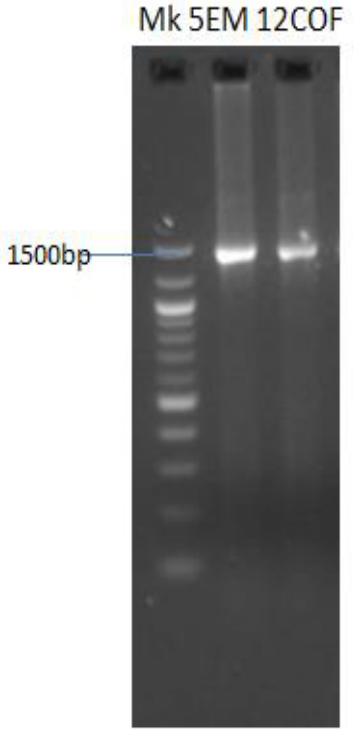

Fig 2: Photograph of Random Amplified Polymorphic DNA (RAPD) for two strains: Staphylococcus aureus and Staphylococcus aureus (Lanes 2 and 3) against 1500bp ladder DNA marker (Lane 1).

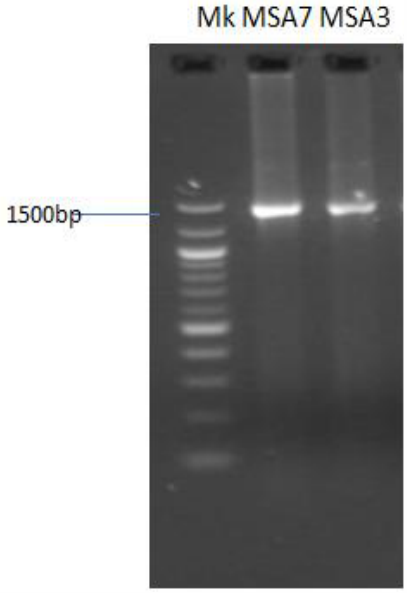

Fig 3: Photograph of Random Amplified Polymorphic DNA (RAPD) for two strains: Bacillus cereus and Bacillus subtilis. (Lanes 2 and 3) against 1500 bp ladder DNA marker (Lane 1).

Phylogenic tree of bacterial isolates recovered from two selected General Hospitals waste water sample

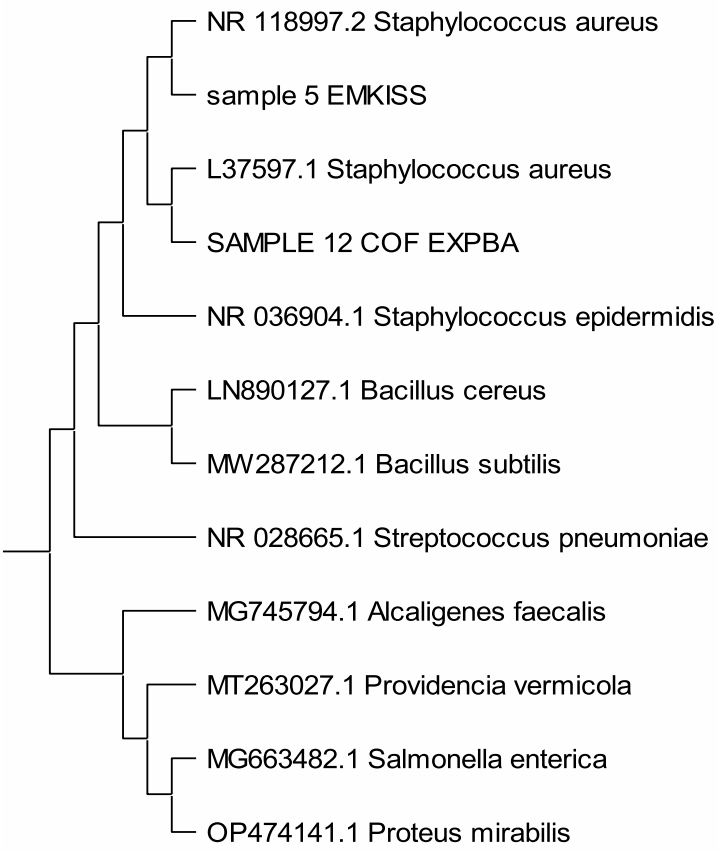

Fig 4 Phylogenic tree for isolates 16S rRNA gene sequencing from two selected General Hospitals waste water sample

Discussion

This research work may be an eye-opener to the various sharp practices in the pretreatment of hospital waste and the probable organisms found in the waste. Large numbers of water-related microbes can cause diseases in humans taking cognizant to home and hospital environments. The transmission of these diseases through waste disposal and hospital waste water for example is a subject to discuss and a constant threat to our public life, this has been a big problem in the third world and developing countries like Nigeria (2). A drastic measure must be taken but we need to understand the threat before robust countermeasures. This research is an eyes opener to the constant menace of Hospital wastewater and its management and other measures of waste disposal practice. To avoid major health catastrophes in our health facilities and environment alike. Bacteria are the most versatile group of microorganisms implicated in wastewater challenges since they are responsible for the structural and functional activity of the waste found in the hospital waste canal (7).

Hospital wastewater contains numerous quantities of pathogenic microorganisms. It is rightly said that Hospital wastewater (HWW) contains many microbes and emerging infectious particles, which are derivatives of pharmaceutical waste used in the hospital, that are hazardous to the environment, and ultimately human health. In many countries, Hospital wastewater (HWW) is directly discharged into sewage water without pretreatment, this will eventually cause a major health crisis. In places like Nigeria, Hospital wastewater (HWW) is a health hazard because of poor treatment facilities, poor education on waste management especially in the hospital, and poor government policies in our various hospitals and health facilities (12).

It should be noted that if there is the presence of pathogenic organisms in the hospital wastewater. such as our finding, it denotes that in a place like Nigeria and Ondo State as a case study, we have hospitals but the waste management system needs urgent revamping, it was observed that samples examined from two main General hospitals in Akoko communities of Ondo State, a total bacterial count ranging from 10.0 x 10^3cfu/ml to 24.0 x 10^3cfu/ml were found in the wastewater. This result is a little bit high from a local hospital, and drastic measures must be put in place to avoid major health problems. if major pharmaceutical and radioactive ingredients which include are not properly disposed practices, the number of organism in the wastewater may also increase drastically and the number of microbe will continue to soar astronomically.

The highest bacterial count was observed in wastewater samples from Ikare General Hospital with the Cfu/ml value of 24.0 x 10^{-3}cfu/ml. The high bacterial load observed in this study is similar to a study conducted by Dagnew *et al*. (2019). It should be noted that due to the large number of populace visiting the Ikare General Hospital daily, there is a large accumulation of microbes because residual quantities of disinfectants used for the treatment of skin microbial infection and to disinfect instruments and surfaces of hospitals, also end up in the HWW, increasing pathogenic microbes. Also, pathogenic microflora present in HWWs come from medical devices, the atmosphere, and water used in the hospital practice, and the pathogens are released mainly in the form of excreta of patients (13). It was reported by Abranches *et al*. (2018), a high bacterial load of 15.0 x 10^{-2} in hospital wastewater samples, corroborates the result of this research work. Urgent steps need to be taken to avert major health crises in our local hospitals., especially in third-world countries, where governmental policies are inadequate and pollution is at a high increase.

Rapid and accurate identification of bacteria is important to understand a precise method of identifying the bacteria to the species level, not only in the hospital wastewater but in all samples in the hospital setting. The conventional method may is necessary but may be slow and less accurate, to identify the natural organism in the waste water. Random Amplified Polymorphic DNA (RAPD) Markers Protocol is another accurate method of identifying bacteria, it is accurate precise, and fast, once all primers are available, bacteria are identified within hours. It was observed that in the case of Hospital Waste Water consists of a mixture of pathogenic microbes including bacteria, fungi, yeasts, algae, viruses, protozoa, parasites, and bacteriophages. More rapid identification methods are necessary.

The finding of this research revealed the amplified sequences of 1500 base pairs in the length of 16s rRNA of the isolated bacteria from the hospital wastewater sample. Subsequent 16s rRNA analysis used suggested the universal primers may then be applied to select more specific bacterial DNA sequences to the subspecies level, which may be more appropriate for molecular analysis of bacterial compositions of clinical wastewater isolates(14).

Bacterial isolates in this study by the findings of Kim *et al*. (2021), where the species of *Streptococcus pneumoniae* and *Escherichia coli* were the most frequently occurring isolates in wastewater of hospital environments. Similarly, Ragan *et al*. (2014) reported the occurrence of *Bacillus* spp. *Staphylococcus* spp. and *Pseudomonas* sp. in effluents of different hospitals. The sources of these isolates including *Bacillus subtilis,*

Streptococcus pneumoniae include leaking septic systems, stormwater run-off, sewage discharged or dumping from the hospital environments. It is perhaps not surprising that they were recovered from hospital effluents. *Staphylococcus aureus, Bacillus* spp in soil and water. These bacteria produce enzymes including DNase, hyluronidase, staphylokinase, staphylolysin, streptokinsase, etc. that help degrade wastewater material (16)

Conclusion and Recommendation

In conclusion, the bacterial load suggests that the activities of hospital wastes in the environment is a major health and environmental threat, which therefore call for a proper regulatory system on disposal of hospital waste water, especially in the developing countries like Nigeria. Adequate governmental policies on waste disposal and management of water pollution should be encouraged, to avoid major health crisis.

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Competing Interests

Authors have declared that no competing interests exist.

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References

1. Wong, C., Jelacic, S. and Habeeb, R. (2000). Risk of the hemolytic-uremic syndrome after antibiotic treatment of *Escherichia coli* O157: H7 infections. *N Engl J Med*.;342(26):1930– 1936.

2. Zhou, Y., Zhu, X., Hou, H., Lu, Y., Yu, J., Mao, L. (2018), Characteristics of diarrheagenic Escherichia coli among children under 5 years of age with acute diarrhea: A hospital-based study, *BMC Infect. Dis.* 18:63.

3. Roy, S., Shamsuzzaman, S.M. and Mamun K.Z., Molecular detection of diarrheagenic Escherichia coli in children with Acute Diarrhea in Tertiary Care Hospitals of Dhaka, Bangladesh, *Asian J Med Sci.* 5 (2017) 59–66.

4. Spano, L., Cunha, K. and Monfardini, M. (2017). High prevalence of diarrheagenic*Escherichia coli* carrying toxin-encoding genes isolated from children and adults in southeastern Brazil. *BMC Infect Dis*.;17:773.

5. Abranches, J., Zeng, L., Kajfasz, J.K., Palmer, S.R., Chakraborty, B., Wen, Z.T., Richards, V.P., Brady, L.J. and Lemos, J.A. (2018). Biology of oral streptococci. *MicrobiolSpectr* 6: 6.5.11.

6. Tassew, A. (2018). *Isolation, Identification, Antimicrobial Profile and MolecularCharacterization of E. coli O157: H7 at DebrezeitElfora Export Abattoir and Addis Ababa Abattoirs Enterprise, Ethiopia* (MSc dissertation); 2015.22. BDAO.*Bedele District Agricultural Office Report*. Bedele, Ethiopia: BDAO.

7. Abdissa, R., Haile, W. and Feyisa, A. (2017). Prevalence of E. coli O157: H7 in beef cattle at slaughter and beef carcass at retailer shop in Ethiopia. *BMC Infect Dis.*;17:277.

8. Jiang, S., Gao, X., Jin, L. and Lo, E.C. (2016). Salivary microbiome diversity in cariesfree and caries-affected children. *Int J MolSci* 17:1978.

9. Fuller, C., Pellino, C. and Flagler, J. (2011). Shiga toxin subtypes display dramatic differences in potency. *Infect Immun*;79:1329–1337.

10. Hamid, M., Tefera, T. and Eguale, T. (2018). E. coli O157: H7: prevalence, Identification and antimicrobial resistance at Addis Ababa Municipal abattoir, Ethiopia. *Int J Adv Res BiolSci.*;10:136–146.

11. Dagnew, A.B., Tewabe, T., Miskir, Y., Eshetu, T., Kefelegn, W. and Zerihun, K. (2019), Prevalence of diarrhea and associated factors among under-five children in Bahir Dar city, Northwest Ethiopia, 2016: A crosssectional study, *BMC Infect. Dis.* 19: 417.

12. Akin B.S. (2016)Contaminant properties of hospital clinical laboratory wastewater: a physiochemical and microbiological assessment. *J. Environ. Protect.* 2016;7(05):635.

13. Nunez L., Moretton J.(2007) Disinfectant-resistant bacteria in Buenos Aires city hospital wastewater. *Braz. J. Microbiol.* 2007;38(4):644–648.

14. Chonova T., Keck F., Labanowski J., Montuelle B., Rimet F., Bouchez A. Separate treatment of hospital and urban wastewaters: a real scale comparison of effluents and their effect on microbial communities. *Sci. Total Environ.* 2016;542:965–975.

15. Chonova T., Keck F., Labanowski J., Montuelle B., Rimet F., Bouchez A(2016). Separate treatment of hospital and urban wastewaters: a real scale comparison of effluents and their effect on microbial communities. *Sci. Total Environ.* 2016;542:965–975

16. Quan P.-L., Sauzade M., BrouzesE(2018). dPCR: a technology review. *Sensors.* 2018;18(4):1271