MOLECULAR IDENTIFICATION OF STRAINS OF MULTIDRUG RESISTANT BACTERIA (MDRB) ISOLATED FROM WASTE DUMP SOIL AND LIQUID WASTE IN HOSPTIALS WITHIN CALABAR METROPOLIS.

Okpo E. A and Andy I. E.

Department of Microbiology, Faculty of Biological Sciences, University of Calabar, P.M.B. 1115, Calabar, Cross River State, Nigeria.

ABSTRACT

Molecular methods that enable the identification of multidrug resistance determinants are essential surveillance tools that are necessary to aid in curbing the dissemination of multidrug resistance. In this study, multidrug resistant bacteria (MDRB) isolated from waste dump soil and liquid waste in hospitals were subjected to molecular identification. The bacterial isolates were primarily identified using standard biochemical identification methods before subjecting them to molecular characterization. Isolates were subjected to antibiotic susceptibility testing using Kirby Bauer's disc diffusion method. Bacterial isolates capable of resisting at least one member from the three classes of antibiotics examined were selected as MDRB. DNA extraction was done using a nanodrop 1000 spectrophotometer. The PCR reactions of the extracted genomic DNA samples were done using universal primer on ABI 9700 Applied Biosystem Thermal Cycler. Sequencing was done using the BigDye terminator kit on a 3510 ABI sequencer and the obtained sequences were edited using bioinformatics algorithm Trace Edit. The evolutionary distances were computed using the Jukes-Cantor method. Replicate data were analysed using ANOVA. A total of 179 species of bacteria were isolated from samples investigated. The result of the mean count of bacteria obtained showed no significant variation ($p>0.05$) in the sampling points. All the MDRB identified were gram negative. The DNA quantification of the selected MDRB ranged from 98.46ng/µl to 462.85ng/µl. The obtained 16s rRNA sequence from the selected MDRB isolates produced an exact match during the megablast search for highly similar sequence from the NCBI non-redundant nucleotide (nr/nt) database. Molecular identification of the selected MDRB isolates showed percentage similarity of 98% - 100% with gene bank isolates and the isolates were *Pseudomonas aeruginosa* strain AU4850.1, *Providenica vermicola* strain TL1, *Chryseobacterium indologenes* strain Sn6, *Providencia rettgari* strain FDAARGOS-330 and *Escherichia coli* strain E41-1. The high level of antibiotic resistance exhibited by MDRB isolates in this study is a threat to the populace and represents a potential public health risk.

IJSER

KEY WORDS: Molecular, Identification, Strains, Multidrug, Resistant

1. Introduction

Bacterial resistance to antimicrobial agents such as antibiotics is a major threat to public health worldwide. Antibiotics are biologically-active compound produced or secreted by bacteria or fungi which are capable of having a bactericidal or bacteriostatic effect on competing microbial species [6]. It was discovered in the mid nineteenth century and this reduced significantly the threat of infectious diseases which has devastated the human race. The first antibiotic penicillin, produced by a fungus called *Penicillin notatum* was discovered in 1928 by Alexander Flemming [7]. However, soon after the discovery of penicillin, a number of treatment failures and occurance of some bacteria such as *Staphylococi* which were no longer sensitive to penicillin started being noticed. This marked the beginning of the era of antibiotics resistance [8]

IJSER

Bacteria that resist treatment with more than one antibiotic are called Multidrug resistant bacteria [2]. Multidrug resistant bacteria are often found mainly in hospitals and long term care facilities. They often affect people who are older or very ill and can cause severe infection. Multidrug resistant bacteria are often developed when antibiotics are taken longer than necessary, when they are not needed or when complete dose is not taken. At first, only a few bacteria may survive treatment with an antibiotic, but as often as the antibiotics are being used, the more likely it is that resistant bacteria will develop [2].

Multidrug resistant bacteria can spread from patient to patient on the hands of healthcare givers. They can also be spread on objects such as medication cart handles, bed

rails, intravenous poles, catheters, bedside tables etc. They can also be spread from person to person through direct contact. In some cases it is possible for the multidrug resistant bacteria to be present on the body of healthy individual and not cause illness. Such cases are called colonization. For example *Staphylococcus aureus* is commonly found in various parts of the body where the bacteria are not usually found [3].

There are several risk factors that are associated with multidrug resistant bacterial infections, but in general, people who are healthy are at low risk of becoming infected. The risk of infection with multidrug resistant bacteria is increased if an individual has an existing severe illness, an underlying disease or condition such as diabetes, chronic kidney disease, skin lesions, previous prolonged use of antibiotics, invasive procedures such as dialysis and the use of medical devices that enter the body such as tubes used to drain urine (Urinary catheters), repeated contact with the health care system such as frequent/prolonged hospital stay, previous colonization with a multidrug resistant bacteria, and many more cases [4].

IJSER

Multidrug resistant (MDR) bacteria cause infection in any part of the body, including blood stream, lungs, urinary tract, wounds, skin, surgical site etc [5]. Infections caused by MDR bacteria are hard to treat because they do not respond to many commonly used antibiotics, even the most potent ones. But certain antibiotics can still help to control MDR bacteria when diagnosis is made to know the MDR bacteria that are involved in the infection. This study was carried out to identify multidrug resistant bacteria isolated from waste dump site soil and liquid waste generated from major hospitals in Calabar Metropolis.

2. Materials and Methods

2.1. Sample Collection

Hospital waste dump soil was collected from the waste dump site of the hospitals studied. Surface soil were randomly collected using a sterile trowel and was bulked to form a composite sample and then transported to the laboratory for analysis within two hours of collection. The liquid wastes were collected from the outermost chambers of the hospitals before discharging into the drainage system using sterile containers.

2.2. Isolation of Bacteria from samples:

A 10-fold serial dilution of water and soil samples was carried out. 9 milliliters of distilled water was dispensed into test tubes and sterilized by autoclaving at 121^0C for 15 minutes. After sterilization and cooling, 1ml of each of the water samples and 1gram of each of the soil samples were used to carry out serial dilution. For the water samples, 1ml was pipetted aseptically into the first test tube labeled 10^{-1} and mixed homogenously, this was followed by serially diluting the sample to 10^{-10} dilution. For the soil samples, 1gram of the soil sample was weighed into 9ml of sterile water in the test tube labeled 10^{-1} and mixed homogenously, and it was serially diluted using 1ml for each dilution to 10^{-10} .

IJSER

2.3. Enumeration and isolation of pure culture:

After the 24 hours of incubation, the bacterial colonies were counted manually. Plates with 30-300 colonies were counted while plates with more than 300 colonies were regarded as too numerous to count (TNTC). After counting, colonies differing in size, shape, and colour in different plates were selected and further sub-cultured on nutrient agar by streak plate technique and then incubated at 23^0C for 24 hours, after which they were transferred to and maintained on agar slants in the refrigerator till required for characterization and identification

2.4. Antibiotics susceptibility testing:

The resistance of the bacterial isolates from waste water and waste dump soil samples to antibiotics was assayed according to the Kirby-Bauer disc diffusion method [14], on Mueller-Hinton agar plates following the procedures described by clinical and laboratory standard institutes (CLSI).

Briefly, 2-5 colonies of the test organisms were picked using a sterile inoculating loop and suspended in saline after which the inoculum was adjusted to a turbidity equivalent to 0.5 McFarland standard (approximately 1.5×10^8 CFU/ml). The suspension was then vortexed. A fresh sterile cotton-tipped swap was dipped into the suspension; the excess liquid from the swap was removed by pressing it against the side of the tube. The swap was inoculated into a plate containing freshly prepared Mueller-Hinton Agar (MHA), the surface was inoculated with the swap covering the entire plate by spreading back and forth from edge to edge rotating the plate approximately 60^0 and repeating the swabbing procedure thrice to ensure that the entire surface was properly covered. Then the disc containing the antibiotics was applied using a sterile forceps within 15minutes of inoculating the MHA plate and then pressed down firmly to ensure firm contact with the agar. The plate was inverted and incubated at 23^0C for 16-18hours. After the incubation the clear zone around each disc was measured and referred as sensitive, intermediate or resistant. This procedure was carried out on all the bacterial isolates.

The antibiotic discs used were Imipenem (10µg), ceforuxime (30µg), Augmentin (30µg), Levofloxacin (5µg), Gentamicin (10µg), Ramicef (5µg), Cefoxitin (30µg), Grazone (30µg), Vancomycin (30µg) and Ofloxacin (5µg).

IJSER

2.5. Selection of Multidrug resistant Bacteria (MDRB): Bacterial isolates capable of resisting at least one member from the three classes of antibiotics examined were selected as multidrug resistant isolate

2.6. Molecular identification of MDRB:

- **DNA Extraction (boiling method)**

Five millilitres of an overnight broth culture of the bacteria isolate in Luria Bertani (LB) was spun at 14000rpm for 3minutes. The supernatant was decanted and the cells were re-suspended in 500µl of physiological saline (saline) and vortex. It was then heated at 95^0C for 20minutes in a heating block machine. The heated bacterial suspension was cooled on ice and spun for 3minutes at 1400RPM. The supernatant containing the DNA was transferred to a 2ml Eppendorf tube (Micro-centrifuge tube) and stored in the freezer at -2^oC for other downstream reaction.

IJSER

- **DNA quantification**

The extracted genomic DNA was quantified using a Nanodrop 1000 Spectrophotometer with its corresponding software installed in a computer system. The purity and quantity of the DNA extract was read off from the equipment.

- **16S rRNA amplification**

The 16S rRNA region of the rRNA genes of the isolates were amplified using 27F: 5' AGAGTTTGATCMTGGCTCAG-3' (Forward) and 1492R: 5'-CGGTTACCTTGTTACGACTT- 3'(Reversed) primers on AB1 9700 Applied Biosystems thermal cycler at a final volume of 50 microliters for 35cyles. The PCR mix included; the x2 Dream tag master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCL), the primers at a concentration of 0.4M and the extracted DNA as template.

The PCR conditions was set as follows: initial denaturation 95^0C for 5minutes; denaturation 95^0C for 30seconds; annealing 52^0C for 30seconds; extension 72^0C for 30 seconds for 35 cycles and final extension 72^0C for 5minutes. The product was resolved on a 1.5% agarose gel at 120V for 20minutes and visualized on a UV trans-illuminator.

- **Sequencing**

Sequencing was done using the BigDye Terminator Kit on a 3510 ABI sequencer by Inqaba Biotechnology, Pretoria South Africa.

- **Phylogenetic analysis**

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN (Basic Local Alignment Search Tools). The sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbour Joining method in MEGA 6.0 [9]. The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method [11].

IJSER

3. Results and Discussion

Multidrug resistant bacteria (MDRB) are considered to be a major problem due to their implications in nosocomial outbreaks and their tendency to spread rapidly within the environment [1]. MDRB are considered to be a very important clinical markers, therefore, Knowledge of their origin, occurrence and susceptibility will play an important role in the selection of appropriate treatment for infections cause by MDRB.

In this study 179 species of bacteria were isolated from samples investigated. Table 1 shows the coordinates of the sampling sites and sampling points. Each of the sampling stations

(Hospitals) was sampled for 3 days and samples were collected from 3 sampling points in each of the station.

Hospitals	Samples Collected	Sampling Points	Longitude	Latitude

IJSER

TABLE 1

Coordinates of sampling site and sampling points in the hospitals

Hospital 1	Liquid waste (LW)	A	8.3492925	4.9540004
Longitude: 8.3510994		B	8.3493814	4.9534944
Latitude: 4.9553919		C	8.3495023	4.9502109
	Waste Dump Soil (WDS)	A	8.3483941	4.9533241
		B	8.3483311	4.9531817
		C	8.3496395	4.9502978
Hospital 2	Liquid waste (LW)	A	8.3355696	4.9539693
Longitude: 8.3360793		B	8.3360323	4.9531134
Latitude: 4.9536581		C	8.3359156	4.9530387
	Waste Dump Soil (WDS)	A	8.3356783	4.9523700
		B	8.3356047	4.9523585
		C	8.3355185	4.9524448
Hospital 3	Liquid waste (LW)	A	8.3176766	4.9489318
Longitude: 8.3175682		B	8.3172976	4.9487836
Latitude: 4.9491021		C	8.3177150	4.9487762
	Waste Dump Soil (WDS)	A	8.3175858	4.9485456
		B	8.3175891	4.9485059
		C	8.3175323	4.9484964

IJSER

TABLE 2

Mean counts of bacterial isolates in liquid waste and waste dump soil according to sampling points in the hospitals investigated

Hospitals	Samples	Sampling points A	B	C
H1	WDS(CFU/g)	$1.46^{a} \pm 4.00 \times 10^{7}$	$1.46^{b} \pm 7.64 \times 10^{7}$	$1.48^{c} \pm 5.51 \times 10^{7}$
	LW (CFU/ml)	$1.40^{a} \pm 2.00 \times 10^{6}$	$1.39^{b} \pm 3.06 \times 10^{6}$	$1.68^{c} \pm 1.00 \times 10^{6}$
H2	WDS(CFU/g)	$1.89^{a} \pm 4.16 \times 10^{7}$	$1.88^{b} \pm 5.29 \times 10^{7}$	$1.72^{c} \pm 6.93 \times 10^{7}$
	LW (CFU/ml)	$1.67^{a} \pm 1.15 \times 10^{6}$	$1.62^{b} \pm 2.52 \times 10^{6}$	$1.59^{c} \pm 4.16 \times 10^{6}$
H3	WDS(CFU/g)	$1.52^{a} \pm 3.61 \times 10^{7}$	$1.36^{b} \pm 5.29 \times 10^{7}$	$1.33^{c} \pm 2.65 \times 10^{7}$
	LW (CFU/ml)	$8.7^{a} \pm 4.16 \times 10^{6}$	$8.2^{b} \pm 2.00 \times 10^{6}$	$8.2^{c} \pm 2.08 \times 10^{6}$

IJSER

Superscripts a, b and c represents non-significant one way ANOVA p values ($p > 0.05$) across the various rows

Key

H1, H2 & H3: Represents the hospitals

LW: Liquid waste

WDS: Waste dump soil

TABLE 3
Antibiotics susceptibility pattern of bacteria isolated from liquid waste and waste dump soil

Susceptibility pattern	Imipenem	Ceforuxime	Augmentin	Levofloxacin	Gentamicin	Ranicef	Cefoxitin	Graxone	Vancomycin	Ofloxacin
					Pseudomonas aeruginosa (n=23)					
Sensitive (%)	17(73.9)	14(60.9)	20(87.0)	21(91.3)	18(78.3)	3(13.0)	8(34.8)	2(8.7)	1(4.3)	19(82.6)
Resistant (%)	6(26.1)	9(39.1)	3(13.0)	2(8.7)	5(21.7)	20(87.0)	15(65.2)	21(91.3)	22(95.7)	4(17.4)
					Escherichia coli(n=37)					
Sensitive (%)	37(100)	3(8.1)	28(75.7)	31(83.8)	29(78.4)	2(5.4)	7(18.9)	8(21.6)	11(29.7)	35(94.6)
Resistant (%)	0(0.0)	34(91.9)	9(24.3)	6(16.2)	8(21.6)	35(94.6)	30(81.1)	29(78.4)	26(70.3)	2(5.4)
					Chryseobacterium indologenes (n=3)					
Sensitive (%)	0(0.0)	0(0.0)	0(0.0)	1(33.3)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	2(66.7)
Resistant (%)	3(100)	3(100)	3(100)	2(66.7)	3(100)	3(100)	3(100)	3(100)	3(100)	1(33.3)
					Providencia vermicola (n=9)					
Sensitive (%)	6(66.7)	0(0.0)	2(22.2)	8(88.9)	4(44.4)	7(77.8)	2(22.2)	0(0.0)	5(55.6)	8(88.9)
Resistant (%)	3(33.3)	9(100)	7(77.8)	1(11.1)	5(55.6)	2(22.2)	7(77.8)	9(100)	4(44.4)	1(11.1)
					Providencia rettgeri (n=7)					
Sensitive (%)	2(28.6)	0(0.0)	0(0.0)	6(85.7)	4(57.1)	5(71.4)	0(0.0)	0(0.0)	3(42.9)	2(28.6)
Resistant (%)	5(71.4)	7(100)	7(77.8)	1(14.3)	3(42.9)	2(28.6)	7(100)	7(100)	4(57.1)	5(71.4)

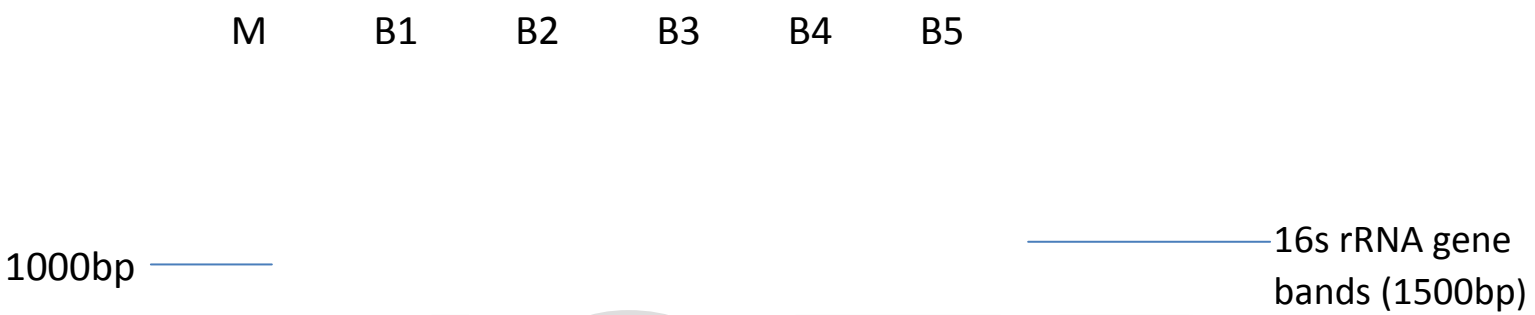
IJSER

TABLE 4

Quantity and purity of genomic DNA extracted from multidrug resistant isolates

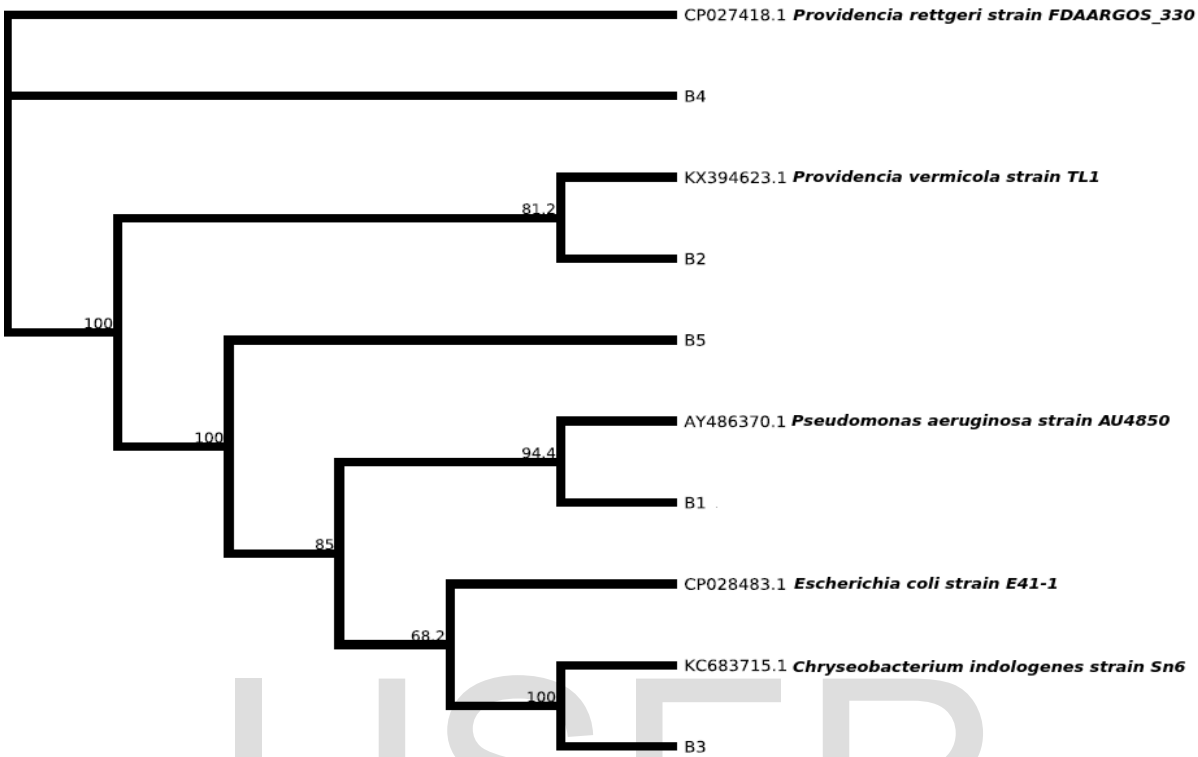
Isolates code	Quantity of bacterial DNA (ng/µl)	Purity(A260/280)
B1	194.24	1.87
B2	98.46	1.61
B3	117.46	1.69
B4	462.85	1.80
B5	128.20	1.62

IJSER



IJSER

PLATE 1: Agarose gel electrophoresis showing the 16S rRNA gene bands of the bacterial isolates. Lane M represents the 100bp DNA ladder



IJSER

FIG. 1: Phylogenetic tree showing the evolutionary distance between the bacterial Isolates

TABLE 5

Identification of the isolates by 16S rRNA genes amplification showing percentage similarities

Isolates code	Gene Bank Isolates	Accession Number	% Similarity
B1	*Pseudomonas aeruginosa* strain AU4850	AY486370.1	99.4
B2	*Providencia vermicola* strain TL1	KX394623.1	100
B3	*Chryseobacterium indologenes* strain Sn6	KC683715.1	99.9
B4	*Providencia rettgeri* strain FDAARGOS_330	CP037418.1	100
B5	*Escherichia coli* strain E41-1	CP028483.1	100

IJSER

Table 2 shows the mean count of bacteria isolated from liquid waste and waste dump soil according to sampling points in the hospitals. The result obtained showed no significant variation ($p>0.05$) in the sampling points.

Table 3 shows the antibiotic susceptibility result of multidrug resistant bacteria that were isolated. Though several other bacterial species were also subjected to susceptibility testing, but those presented in the table showed resistance across the three classes of antibiotics that were used for this study. All the bacterial species identified to be multidrug resistant were all gram negative bacteria. As reported by Andy and Okpo [12], this may be due to their unique outer membrane which excludes certain antibiotics from penetrating the cell. Also the porin channels present on gram negative bacterial outer membrane can also prevent the entry of relatively large hydrophilic antibiotics. Moreso, gram negative bacteria also have a high transformation rate, i.e they have a great facility for exchanging genetic material (DNA) among stains of same species and even among different species. The gram positive bacteria do not have outer membrane, so they are more susceptible to antibiotics.

IJSER

Table 4 shows the quantity and purity of genomic DNA that was extracted from multidrug resistant isolates for molecular identification. Various quantities of genomic DNA ranging from 98.46ng/µl to 462.85ng/µl were extracted for molecular analysis. The PCR reaction of the extracted genomic DNA samples using universal primer on ABI 9700 Applied Biosystem thermal Cycler that amplifies the entire 16S variables region of the DNA at annealing temperature of 52^0c revealed positive amplification of the 16S rRNA gene in all the multidrug resistant isolates on 1.5% agarose gel at 120V for 20minutes as showed in Plate 1.

The obtained 16S rRNA sequence from the multidrug resistant isolates produced an exact match during the megablast search for highly similar sequence from the NCBI

non-redundant nucleotide (nr/nt) database. The 16S rRNA of the isolates showed a percentage similarity to other species ranging from 98.4% - 100% as presented in Table 5. The evolutionary distances computed using the Jukes-cantor method were in agreement with the phylogenetic placement of the 16S rDNA of the isolates as follows: B1 revealed a closely relatedness to *Pseudomonas aeruginosa* strain AU4850 (gb:AY486370.1), B2 to *Providencia vermicola* strain TLI (gb: KX394623.1) B3 to *Chryseobacterium indologenes* strain Sn6 (gb: KC683715.1), B4 to *Provedentia rettgeri* strain FDAARGDS _ 330 (gb :CP037418.1) and B5 to *Escherichia coli* strain E41-1 (gb:CP028483.1) as presented in Figure 1.

The extent of resistance to antibiotics by bacterial isolates in this study may be associated with the extent of antibiotic usage. Bacteria isolated from the hospital waste were highly resistant to some of the antibiotics used, which could be as a result of un-metabolized antibiotics released from the hospital in low concentration and repeated prescription of antibiotics by the medical practitioners can lead to resistant bacteria, which is commonly practiced in Nigeria. Self-medication, counterfeit drugs and inadequate hospital control measures can as well promote the development of resistance in clinical isolates [13]. In developing countries like Nigeria, self-medication is a common practice and could be major causes of antibiotic resistance in clinical isolates since patients only think of going to the hospitals when they are unable to treat themselves.

Multidrug resistance observed in this study may be as a result of r-plasmid transfer within the bacterial population. Some other mechanism of antibacterial resistance include; inability of antibiotics to penetrate cell wall of the bacteria because of alterations in plasma membrane decreased intracellular availability of the drug, production of plasmid or chromosomally encoded enzymes that hydrolyze the drugs. The presence of these multidrug resistant bacteria from liquid waste and waste dump soil in the hospital environments may be

IJSER

transmitted to humans and could result in disease that cannot be treated by conventional antibiotics.

Conclusion

It can be concluded that waste dump soil and liquid waste generated from hospitals are potential source and reservoir of MDRB. This is a threat to the populace in such environment and has a significant effect on the public health, especially due to their high level of resistance to commonly available antibiotics in our locality. It is imperative that waste generated from hospitals be treated properly before discharging into the environment and also science related to environmental dimension should be critically improved in order to efficiently curb further development and spread of multidrug resistant bacteria.

IJSER

References

1. Omoni, V. T., Makinde, O. A. & Abutu, S. O. (2015). Prevalence of multidrug resistant bacteria isolated from biomedical waste generated in Makurdi Metropolis, Benue State, Nigeria. *British Microbiology Research Journal, 10,* 1-10.

2. Moges, F., Mengistu, E., Yeshambel, B. & Walelegn, W. (2014). Isolation and characterization of multiple drug resistance bacterial pathogens from waste water in hospital and non-hospital environments. *BMC Research Note, 7,* 215-221

3. Chee-Sanford, J. C., Mackie, R. I., Koike, S., Krapac, I. G., Lin, Y. F., Yannarell, A. C., Maxwell, S. & Aminov, R. I. (2009). Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. *Journal of Environmental Quality, 38,* 1086-1108.

4. Courvalin, P. (1994). Transfer of Antibiotic Resistance Genes between Gram-Positive and Gram-Negative Bacteria. *Antimicrobial Agents and Chemotherapy, 38,* 1440-1447

5. Zoutman, D. E. & Ford, B. D. (2005). The relationship between hospital infection surveillance and control activities and antibiotic resistant pathogen rates. *American Journal of Infection Control, 33,* 11- 18.

6. Martinez, J. L. (2009). Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environmental Pollution, 157*, 2893-2902.

7. Byarugaba, D. K. (2005). Antimicrobial resistance and its containment in developing countries. In I. Gould & V. Meer (Ed), *Antibiotic policies: Theory and practice* (pp. 617-646). New York: Springer.

8. Dessen, A., Di Guilmi, A. M., Vernet, T. & Dideberg, O. (2011). Molecular mechanisms of antibiotic resistance in gram-positive pathogens. *Current Drug Targets Infectious Disorders, 1*, 63–77.

9. Saitou, N. & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution, 4*,406-425.

10. Felsenstein J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution,* 39, 783-791.

11. Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In H. N, Munro (Ed), *Mammalian Protein Metabolism* (pp. 211-232). New York : Academic Press.

12. Andy, I. E & Okpo, E. A. (2018).Occurrence and Antibiogram of Bacteria Isolated from Effluent and Waste Dump Site Soil of Selected Hospitals in Calabar Metropolis, *Nigeria. Microbiology Research Journal International, 25(5),* 1-9

13. Prescott, L. M., Harley, J. P. & Klein, D. A. (2005). *Microbiology* 6th ed. New York, McGraw- Hill.

14. Bauer, A. W., Kirby, W. M., Sheries, J. C. & Turck, M. (1996). Antibiotic, susceptibility testing by a standardized single disc method. *American Journal of Clinical Pathology, 45*, 49-58

IJSER