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Research Article

Antibiogram and Molecular Characterization of Bacterial from Waste Disposal and Management Sites within Tertiary Hospitals in Port Harcourt, Rivers State, Nigeria

T Sampson*, O Ejims-Enwukwe and LP Peekate

Department of Microbiology, Rivers State University, Nigeria

***Corresponding Author:** T Sampson, Department of Microbiology, Rivers State University, Nigeria.

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Abstract

Over the past few years, waste from hospital environment has been a major concern to the public, as the disposal and management of these wastes portend great danger to the surrounding environment. The study investigated the susceptibility pattern and molecular characterization of bacterial isolates from waste disposal sites within University of Port-Harcourt Teaching Hospital (UPTH) and Rivers State University Teaching Hospital (RSUTH), in Rivers State. About 10g of top soil (0-15 cm) was randomly collected using a sterile trowel from points associated with waste disposal within the hospital premises. The Kirby Bauer disc diffusion method was used to determine the susceptibility pattern of the bacterial isolates to conventional antibiotics. The bacterial organisms were isolated using standard bacteriological media, and further characterized using a Polymerase chain reaction (PCR) based molecular technique. The presence of antibiotics resistance (mecA) gene was probed using specific primers. Data obtained showed that ceftazidime was the least effective antibiotics, as over 80% of both Gram positive and Gram negative isolates were resistant to the antibiotics. The study revealed that most of the isolates from both hospitals had Multiple Antibiotic Resistance (MAR) index greater than 0.2. While UPTH had MAR index that ranged from 0.3 - 0.9, that of RSUTH ranged from 0.3-0.8. Evolutionary relationship between the isolates confirmed that the 16s rRNA sequence were specific to 80% of actual organism; *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus* and *Providencia stuartii*, from the data base. The study detected the presence of mecA gene in 60% of the isolates probed. The research showed that all the isolates (100%) obtained were multi drug resistant (MDR) organism, lurking around waste disposal and management sites as the isolates showed very high resistance pattern between the hospitals studied. It is therefore recommended that since soil acts as a major reservoir to most of these pathogen, the disposal and management sites be properly sited in locations distant from main hospital facility and proper education given to waste handlers on the possible outcome if not adequately taken care of.

Keywords: Anibiogram; Bacteria; Molecular Characterization; Tertiary Hospitals; Waste Disposal

Introduction

Proper management of hospital waste is an important aspect to consider when providing successful healthcare services. Most developing countries like Nigeria are faced with several constraints that may weaken proper management of these waste materials from hospital care.

When waste products are dumped on land, pathogens can proliferate while using up the components in those waste matters as a source of their nutrients and then degrade complex waste materials [1]. These wastes comprises of pathogenic bacterial species that are of high public health importance. One major life threatening public health concern that may result from improper waste disposal and management, is the resistance of bacteria

to normally susceptible antibiotics. The Release of bacteria into the environment aids the exchange of genetic material within microbes that are previously non-resistant thereby increasing the concentration of resistant capacity in the environment [2]. As the abundance and type of antibiotic resistance gene in the environment increases with longer exposure around the environment, the higher the chances of transfer. With that, the natural environment becomes reservoirs of resistant bacteria and resistance genes [3].

Before the use of antibiotics as medicine, the commonness of antibiotic resistance was limited. However, inappropriate use of these antibiotics has resulted in unexpected outcomes necessitating large-scale policy for adjustments and mitigation [4].

Today, multi-drug resistant bacteria, threatens the health system [5]. The development of antibiotic resistant organism in hospitals and health care settings has been a cause of major public health concern [6].

On 30th October 2020, WHO declared antibiotic resistance as a leading worldwide threat facing humanity [7]. Antibiotics and antibiotic-resistant microorganism can have serious repercussions if they accumulate in the environment, humans can get infected with resistant bacteria and become sick. Over the past decade, these resistance of pathogens to more than one antibiotics, have evolved and are referred to as multi-drug resistance organisms known to cause severe infections amongst hospital patients.

The extent of resistance, damage and future outcomes of these bacteria pathogen needs a constant check to avoid more global crises than the world has already witnessed [8]. Hence, the need for proper evaluation on the current status of these isolates to antibiotics and also, the molecular identification of bacteria species from hospital waste disposal and management sites.

The identification of bacteria specie is mainly done through sequencing of their 16S rRNA [9]. In prokaryotic ribosomes, the 16S ribosomal RNA is an important fraction of the 30S Subunit. Interestingly, one of the major aspects to the use of the 16S rRNA gene sequence informatics is that it allows for the identification of genus and specie of an isolate that have not been recognized through an already established biochemical profiles in species that show low identification characteristics when commercial systems are employed or for taxonomy that are not frequently connected with human infectious diseases [10].

Generally, in all the cells, the rRNA gene is the most conserved gene as variability is less likely to occur. Organisms that are distantly related share a variety of similarities in their rRNA sequence. Meaning that sequences of organisms that are less likely related can be accurately matched thereby allowing any difference between their sequences to be easily measured.

As a result, genes encoding the ribosomal RNA (rDNA) have been widely used in the identification of their taxonomy, phylogeny (evolutionary relatedness) and to further estimate the degree of distance in the relationship among bacteria isolates. Hence, when the 16S rRNA sequence are compared, evolutionary relationship of the microbes is revealed [10]. The amplification of some variable region in most isolates has enhanced proper identification of most of these bacteria isolates and allowed for organisms of both known and unknown taxonomy to be effectively identified. As the NCBI (National Center for Biotechnology Information) holds sequences from tens of thousands of environmental and clinical isolates which is readily available on the internet.

Materials and Methods

Study area

The study was carried out at University of Port-Harcourt Teaching Hospital (UPTH), and Rivers State University Teaching Hospital (RSUTH). The hospitals sampled are in Rivers State, Nigeria. Rivers State is one of the 36 states of Nigeria located in the Niger Delta region of the Southern part of the Country. The Nigeria data portal in 2006 ranked Rivers State as one of the most populous (6th) state in Nigeria.

Study period and duration

Soil samples were collected from different points once every month, for each hospital waste disposal and management site for a period of three months (June-August) consecutively.

Sample collection

Soil samples were collected from each hospital disposal site. Top soil (0-15 cm) samples were randomly collected using a sterile trowel. The debris were cleared before digging and collection of soil samples. The trowel was disinfected with a cotton wool and 95% ethanol at intervals before using it to collect sample at the next site. Soil samples collected 30m apart served as controls for each of the study locations.

Sample bottles were labelled with appropriate description of date, time and location. Collected samples were then conveyed to Rivers State University microbiology laboratory, within two hours from when the sample was collected and stored in the refrigerator at 4°C. The samples were collected to cover various points, where different activities were carried out.

Isolation, characterization and identification of bacteria isolates

About four different media; Nutrient agar, Mannitol salt agar, MacConkey agar and Eosin methylene blue agar were used to culture and identify the bacteria species. Isolation of bacterial isolates was carried out following a tenfold serial dilution of samples, where 1g of soil sample was added to 9ml of physiological saline (0.85% w/v sodium chloride (NaCL) solution) to get a 10^{-1} dilution. The suspension was serially diluted up to 10^{-6}. Aliquots (0.1 ml) of selected dilutions were inoculated onto the surface of freshly prepared aforementioned medium using the spread plate method. The plates were incubated aerobically in an inverted position at 37°c for 24hrs; except for EMB plates which were incubated at 44.5°C**.**

Pure culture of bacteria isolates were characterized, based on colonial and cell morphology. Bacteria colonies differing in size, shape and color in different plates on differential selective media were selected and further sub-cultured on nutrient agar by the streak plate technique and then incubated at 37°C for 24hrs. Furthermore, the bacterial colonies were transferred to and maintained on agar slants in a refrigerator till further characterization and identification.

Biochemical tests which included Gram's reaction, indole tests, oxidase test, catalase test, methylred-Voges-Proskauer (MRVP), citrate utilization, motility and carbohydrate fermentation such as glucose, lactose, maltose, mannose, and xylose tests were performed. The bacterial isolates were identified by comparing their characteristics with those of known taxonomy using the schemes of cowan and steel [11].

Antibiotic sensitivity testing by agar disk diffusion (Kirby Bauer) method

The antibiotics resistance pattern of the bacterial isolates to selected antibiotics was assayed according to the Kirby-bauer disc diffusion method [12].

Twenty-four hours culture of the bacterial isolates were inoculated into peptone water and incubated at 37°C for 3-4 hours. The bacteria density was adjusted to 0.5 McFarland standards using additional normal saline. The plates containing Mueller-Hinton Agar were inoculated in a uniformed manner with 0.1 ml of the broth culture of each isolate streaked over the surface of the entire plate in different planes with a swab stick. The plates were left to air dry for a period of 10mins and then with the use of sterile forceps, discs impregnated with antibiotics were placed aseptically on the surface of the Mueller-Hinton Agar medium and then slightly tapped to fit on the surface properly. The discs were placed at equidistance to each other. All inoculated plates were transferred to the incubator at 37°C for 24hrs.

After incubation, the zones of growth inhibition around each disk was measured and used to classify organisms as sensitive or resistant to antibiotic following the interpretive standard of the Clinical and Laboratory Standards Institute [13].

Molecular characterization

The isolates were characterized using 16rRNA gene, and PCR assays were performed. With the use of the agarose gel electrophoresis method, the plasmid profiles of the different isolates were determined.

DNA extraction (Boiling method)

Following the culture of bacteria isolates in Luria Bertani (LB), about 5 ml of the culture was spun for 3 minutes. The cells were further suspended in a 500 µl normal saline and then exposed to high heat of 95°C for 20 minutes. The heated suspension was further spun for 3 minutes at 14000 rpm after cooling on ice. The DNA which contained the supernatant was transferred to a 1.5 ml micro centrifuge tube and kept at -20°C to be used in subsequent experiments.

DNA quantification

The isolated genomic DNA was measured using the Nano drop 1000 spectrophotometer. The equipment's program was launched by double clicking the icon on the Nano drop. Normal saline was used to blank the apparatus after being initiated with 2 µl of sterile distilled water. At the lower pedestal, 2 µl of the extracted DNA was loaded on to it, while the upper pedestal was lowered to establish contact with the DNA that had already been extracted and placed on the lower pedestal. By clicking on the measure button, the DNA concentration was then determined.

16S rRNA amplification

On an ABI 9700 Applied Bio systems thermal cycler, the 16s rRNA section of the isolates' rRNA gene was amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers at a final volume of 40 microliters for 35 cycles. The PCR mix consisted of the inqaba X2 dream taq master mix (taq polymerase, DNT Ps, MgCl), the extracted DNA template and the primers at a concentration of 0.5uM.

The following were the PCR conditions

Initial denaturation took place at around 95°C for 5 minutes; subsequent denaturation at 95°C for 30 seconds; Annealing was at 52°C for 30 seconds; Extension at 72°C for 30 seconds for 35 cycles; and final extension was 72°C for 5 minutes. The product was resolved for 30minutes at 130V on a 1% agarose gel and visualized.

Sequencing

The BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa Sequencing was used to sequence the isolates. At a final volume of 10 ul, the sequencing was carried out. The components used for the process include; 10uM PCR primer, 2-10 ng PCR template per 100 bp, 0.25 ul BigDye® terminator v1.1/v3.1, and 2.25 ul of 5 x BigDye sequencing buffer. The conditions for sequencing were: 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min.

Phylogenetic analysis

With the use of the bioinformatics algorithm Trace edit, the sequences obtained were edited. BLASTN was used to download similar sequences from the National Center for Biotechnology Information (NCBI) data base. While MAFFT was further used to align the sequences. The Neighbor-Joining method in MEGA 6.0 was used in inferring of the evolutionary history [14]. The evolutionary history of the taxa analysed is represented by the bootstrap consensus tree with inference from about 500 replicates [15]. To compute the evolutionary distances, the Jukes-Cantor method was applied [16].

Amplification of MecA and gene

To amplify the methicillin resistant gene (MecA) from the isolates, the mecAF was used. TGGCTATCGTGTCACAATCG mecAR*:* CTGGAACTTGTTGAGCAGAG primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles. The PCR mix included: the X2 Dream Taq Master Mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and 50ng of the DNA that was already extracted and used as template. Initial denaturation took place at around 95°C for 5 minutes; subsequent denaturation at 95°C for 30 seconds; Annealing was at 52°C for 30 seconds; Extension at 72°C for 30 seconds for 35cycles; and final extension was 72°C for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 25 minutes and visualized on a UV trans-illuminator.

Results

Antibiotic susceptibility pattern of the bacterial isolates

Data obtained from the study revealed the isolates were resistant to a number of antibiotics tested in various degree. It was observed that all the isolates (100%) of *Staphylococcus aureus* and *Paenibacillus* spp were resistant to Ceftazidime, while 25% *Bacillus* species isolated were observed to be resistant to the drug Ceftazidime.

Similarly, 60% of the *staphylococcal* isolates were resistant to Ceftriaxone, while 75% of *Bacillus* spp and 100% of *Paenibacillus* spp were resistant to Ceftriaxone. The result of the sensitivity further revealed that for the Gram positive bacterial isolates, *Staphylococcus aureus,* showed the highest resistance and was resistant to almost all the antibiotics tested except for Ofloxacin which it was susceptible to (Figure 1). Also, the Gram negative bacterial isolates showed a high level of resistance to almost all the antibiotics tested (Ceftazidime, Ceftriaxone, Gentamicin, Cloxacillin, Ofloxacin, Augmentin, and Nitrofurantoin) as most of the isolates showed resistance to each antibiotics used (Figure 2), except for *Serratia* spp which was sensitive to about two (2) of the antibiotics (gentamicin and ofloxacin). About 60% of the Gram negative isolates were sensitive to ciprofloxacin. *E. coli* on the other hand was the only gram negative isolate that showed resistance to Nitrofurantoin, as all other gram negative isolates appeared to be susceptible to the drug. Also, from the study, it was observed that *E. coli* showed the highest resistance level with Mar Index of 0.9 (Figure 5).

The data revealed that over 80% and 100% of Gram positive and gram negative isolates, respectively, were resistant to Ceftazidime figure 3 and 4.

While the highest sensitivity to ofloxacin was observed with over 90% of Gram positive isolates and 70% for Gram negative isolates susceptible to the antibiotics (Figure 3 and 4).

Figure 1: Percentage Resistance to Antibiotics for Gram Positive Bacterial isolate.

Figure 2: Percentage Resistance to Antibiotics for Gram Negative Bacterial isolate

Figure 3: Antibiogram of Gram positive bacteria isolated from the Waste sites.

Figure 4: Antibiogram of Gram Negative bacteria isolated from the Waste sites.

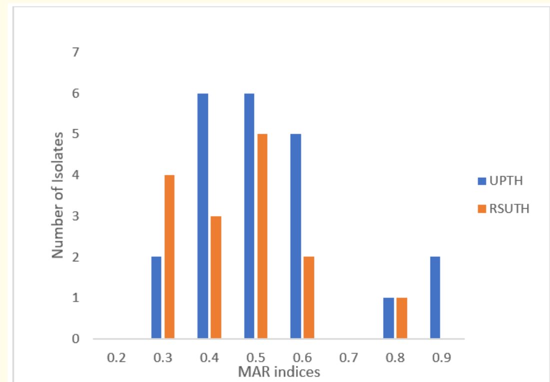

Figure 5: Multiple Antibiotics Resistance Pattern at the Two Hospitals Studied.

Molecular characterization and detection of antibiotics resistance gene of the isolates

The various isolates were sequenced to obtain their 16s rRNA gene, and the megablat search was used to find highly related sequences between the isolates from NCBI non-redundant nucleotide (nr/nt) database. The obtained 16s rRNA sequence from the isolate produced an exact match during the megablast search for highly similar sequences. The 16S rRNA of the isolates showed a percentage similarity to other species at 100% (Plate 1). The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolates within the *Escherichia, Klebsiella, Pseudomonas, Staphylococcus* and *Providencia* genera and revealed a closely relatedness to *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus* and *Providencia stuartii* respectively (Figure 6).

Plate 1: Agarose gel electrophoresis of the 16S rRNA gene of some selected bacterial isolates. Lanes B1-B5 represent the 16SrRNA gene bands (1500 bp), lane L represents the 100 bp molecular ladder.

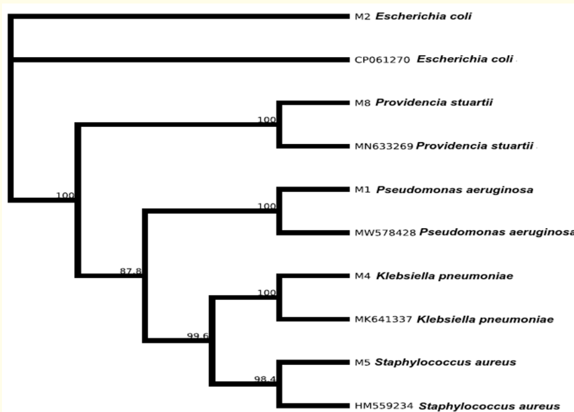

Figure 6: Evolutionary Relationship between the Sampled Bacterial Isolates.

Molecular detection of resistance genes proved that about 60% of the isolates sampled were observed to have the MecA gene present in them (Plate 2).

Plate 2: Agarose gel electrophoresis showing the amplified MecA gene from the Staphylococcal isolates. Lanes 1, 3 and 5 showing the MecA gene bands at 300 bp while lane L represents the 50 bp molecular ladder.

Discussion

Antibiotic susceptibility pattern of the bacterial isolates

From the result of the antibiotics resistance pattern of bacterial isolates, it was observed that the isolates were resistant to most of the antibiotics tested. All the isolates from waste disposal and management site showed multiple drug resistance to Ceftazidime, Cefuroxime, Cefixime, Ceftriaxone and Cloxacillin. However, gram negative organisms showed more resistance to the antibiotics and could be due to the presence of an outer membrane which prevents toxic molecules like antibiotics from getting in [17].

From the study by Sulaimon., *et al*. 2015 [18] there were similarities in the findings observed on the resistance pattern of gram positive isolates to cefuroxime. Hence, these antibiotics cannot be used as alternative options to treat these bacterial infections. A possible cause for the high resistance seen amongst bacterial isolates may be attributed to the indiscriminate use of antibiotics among residents of Rivers State.

However, judging from the study, ofloxacin may be used in treatment of these bacteria species isolated. Mwaikono., *et al*. 2015 [19] reported that ciprofloxacin was effective against enteric bacteria pathogens from a dumpsite in Tanzania, which was in line with the findings from this study except for *E. coli* and *Serratia* spp., which showed 100% and 50% resistance, respectively. The emergence of resistance and spread of these resistance to pathogenic bacteria that may once have been treated with a particular antibiotic but have now grown resistance to that same antibiotics is one great challenge to the use of antibiotics. Furthermore, if these bacterial species continue to develop resistance to antibiotics, it loses its effectiveness and the ability to treat and control these public health threats. In order to successfully treat diseases caused by these microorganisms, it is vital to have a clear knowledge on the antibiotic sensitivity pattern, which continuous to birth more studies on the sensitivity pattern of microbes to antimicrobial agent [20].

Molecular characterization and detection of antibiotics resistance gene of the isolates

The use of traditional phylogenetic characteristics in bacteria identification is a far less accurate method when compared with the genotypic methods which is a preferred method as recent studies have shown. The latter method can identify bacteria with unknown characteristics even up to genus and specie level [21]. For a variety of reasons, the 16S rRNA gene sequences have been the

most widely used, in establishing relatedness between prokaryotic strains [22]. These reasons may include; their presence in relatively all bacteria isolates; the accessibility of various sequences from different organisms and cloned amplicons from a number of varying ecosystems; their high degree of sequence which further aids their detection and the presence of regions that are highly variable making them suitable to differentiate at (sub) specie to higher phylogenetic levels.

In this study, about 80% of the isolates discovered and subjected to megablasting, showed a 100% similarity to the actual organism. The isolates were; *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus* and *Providencia stuartii* respectively. These organisms are of great importance to public health as they are the most leading causes of various illnesses both in hospitals facilities and around the globe. A thorough scrutiny as to the likely behavioral pattern to antimicrobial agents, is needed to circumvent the future outcomes.

Conclusion

The indiscriminate dumping of waste from hospital care poses a serious danger to the health of the public as bacterial species isolated from waste disposal site reveals so. Most of the Pathogens isolated from soil around these study areas are highly infectious organisms and can further leak to surrounding water bodies or food chain, or even contaminate the underground water, which is the main source of water for most of these hospitals and households around. Species of bacteria isolated from this study are responsible for most of the diseases today including the hospital acquired infections (nosocomial infections) by methicillin resistant *Staphylococcus aureus.*

The research showed that all the isolates (100%) obtained were multi drug resistant (MDR) organism, lurking around waste disposal and management sites as the isolates showed MAR index that ranged between and 0.3-0.8% for RSUTH and 0.3 and 0.9% for UPTH.

The use of 16S rRNA to identify and characterize most of these isolates even up to their resistance level is gaining popularity in recent years.

From the *Staphylococcus* isolates screened for the presence of mecA gene which is a major cause of resistance in the bacteria, it can be concluded that not all the species had antibiotics resistance gene, as only 60% of the isolates had the mecA gene present in them.

The high level of resistance is an indication that hospital waste disposed around the environment mainly on soil aids the exchange of these pathogenic bacteria as they serve as reservoirs to these bacteria species and can promote the spread of resistant genes if proper care is not taken. This calls for urgent need to raise awareness, education and management strategies on medical waste issues to ensure health and environmental safety.

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