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INVESTIGATION OF SOME CARBAPENEM RESISTANCE GENES IN *ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE* FROM HOSPITAL WASTE

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ABSTRACT

The hospital waste is a reservoir for multidrug resistant *Escherichia coli* and *Klebsiella pneumoniae* and it leads to an increase in the frequency of these resistance genes creating a critical predicament for the society. The aim of this study was to identify some resistance genes found in *Escherichia coli* and *Klebsiella pneumoniae* obtained from hospital waste. A total of 100 samples of wastes which were hazardous and non-hazardous were collected from identified waste dumps sites in the Rivers State University hospital into sterile bottles. These were transported to the microbiology laboratory of the Rivers State University using cold chain. The conventional method of culturing, isolating and identifying was used. The samples were cultured on prepared agar plates Cysteine Lactose Electrolyte Deficient (CLED), MacConkey (MAC), Eosin Methylene Blue (EMB), Mueller-Hinton Agar (MHA), Blood Agar (BA) and nutrient agar (NA) and incubated at 37°c for 24hours. The culture plates were examined, the isolates subcultured and used for identification. Gram staining and other biochemical tests were employed for the identification of the isolates. Results showed isolates of *Klebsiella pneumoniae* constituting a majority of the isolated bacteria forming (27.5%). *Escherichia coli*, *Staphylococcus aureus* and *Bacillus spp*. constituted 25%, 22.5% and 15% respectively while *Pseudomonas* spp. was 10%. The isolated and identified organisms (*E.coli* and *K. pneumoniae*) were subjected to sensitivity tests and the resistant forms were now taken for molecular identification of resistant genes to carbapenem. Molecularly the DNA of the organisms were extracted and quantified using nanodrop 1000 spectrophotometer after which they were amplified using the polymerase chain reaction (PCR) and they were checked using agarose Gel electrophoretic system to be sure they possessed enough DNA and the 16srRNA against the standard known as the ladder. It was then stored frozen in vial form and sent to South Africa where 3510 ABI sequencer was used. The sequences were lodged into the gene bank National Center for Biotechnology Information (NCBI) data base using BLASTN where the organisms were identified molecularly and the genes associated with carbapenems were also identified. The results of the molecular techniques showed the identification of *Escherichia coli* and *Klebsiella pneumoniae* and the resistant genes to carbapenems. The New Delhi Metallo-Beta-Lactamase (NDM) resistant gene was detected in three of the isolates of *Escherichia coli* and *Klebsiella pneumoniae* as against the other three *Klebsiella pneumoniae* carbapenemases (KPC), Oxacillinase (OXA) and Verona Integron-encoded Metallo-Beta-Lactamase (VIM) carbapenem genes. This study therefore has identified a gene that is resistant to carbapenem.

KEYWORDS: Carbapenem, Resistance Genes, Hospital waste, organisms, 16srRNA.

1.0 INTRODUCTION

Hospital waste is any waste that is generated in the diagnosis, treatment, or immunization of human beings and animals and it is gotten from research or testing of biological materials including but not limited to soil or blood soaked bandages, culture dishes and other glassware.[1] In the hospital, diverse types of therapeutic operations such as chemotherapy, dialysis, surgery, deliveries, autopsy, biopsy paraclinical exams, injections and laboratory works are accomplished with the outcome being the production of infectious wastes, sharp objects contaminated with patient's blood and secretions, radioactive wastes and chemical materials which are considered to be hazardous waste.[2] Inadequate waste management will cause environment pollution, unpleasant smell, growth and multiplication of insects, rodents and worms and may lead to the transmission of diseases such as hepatitis, typhoid, cholera and AIDS through injuries from syringes and needles infected with human blood.[3] This places certain groups of people at risks which include healthcare workers (nurses, doctors, laboratorians, administrators etc), patients, visitors, waste disposal crew and scavengers at waste dumpsites.[4]

Unfortunately the adverse effects of healthcare hazardous wastes are not usually attributed to them

unless a careful and thorough investigation is carried out. Usually the effects of these exposures are not felt immediately until many years later. Therefore, it is very important that hazardous hospital waste be thoroughly managed to protect lives.

Escherichia coli and *Klebsiella pneumoniae* are gram negative bacteria seen mostly in the guts of humans and animals, even if some species can also be identified in water, soil or sewage. Some species are harmless while some others are familiar pathogenic microorganisms.

Antibiotics are antimicrobial agents used to annihilate or suppress the growth of bacteria. Recently, antibiotics resistance has spread to a very alarming rate as there has been continuous deployment of antimicrobial drugs in treating infections.[5] Multidrug resistance (MDR) is defined as antimicrobial resistance shown by a species of microorganism to at least one antimicrobial drug in three or more antimicrobial categories.[6]

Carbapenem-producing Enterobacteriacea of which *Escherichia coli* and *Klebsiella pneumonia* are the most significant are the most pervasive antibiotic resistant threats to health services worldwide. In this study, *Escherichia coli* and *Klebsiella pneumoniae* in hospital wastes were been characterized using molecular techniques and multidrug resistance of these organisms was examined. The aim of this study is to detect carbapenem resistant genes in *Escherichia coli* and *Klebsiella pneumoniae* from isolates in wastes in Rivers State teaching hospital, Port Harcourt.

2.0 MATERIALS AND METHODS

2.1 Study area /design

The study area where waste samples were collected was the Rivers State University Teaching Hospital, a tertiary Hospital in Port Harcourt which is the largest city in Rivers State according to census done in 2006 with its attendant massive waste generation. The design of this study is a descriptive design. Ten (10) randomly selected sites were chosen for the collection of samples from the Rivers State University Teaching Hospital Port Harcourt. This Hospital was chosen because it is one of the busiest facilities in the State and it is accessible to people of different ethnic groups that live in and around the State.

2.2 Sample collection

Waste samples were collected from the Rivers State University Teaching Hospital from different unit. Family medicine (unit 1); Office waste (unit 2); Open dump (unit 3); Surgical Clinic (unit 4); Theatre (unit 5); Children Emergency (unit 6); Male Medical Ward (unit 7); Gynecological Ward (unit 8); Laboratory (unit 9) and General Waste (unit 10).

Three sets of waste samples were collected over a period of six months ranging from July to December. The waste samples were both hazardous and non-hazardous.

Samples were collected into sterile dry screw top specimen bottles labeled with sample number, date and time of collection and then immediately transported under standard conditions (cold chain) to the laboratory for analysis. Samples were processed for the isolation and identification of pathogenic bacteria. Microbiological processing was done by standard laboratory methods.[7]

2.4.1 CULTIVATION OF MICROORGANISMS

2.4.1.1 Serial Dilution and Total Heterotrophic Count

Serial dilution was made with 1ml of the leachate from the waste in each unit. Five test tubes and media plates were labeled from 10^1 to $10^{5.}$ Nine (9mls) millilitre of sterile normal saline was pipette into each of the test tubes. One (I) ml of the waste sample is pipette into nine (9) mls of normal saline into the test tube labeled 10^1. 1 ml from the test tube labeled 10^1 is pipette out and into the test tube labeled 10^2 and this is continued until the test tube labeled 10^5 is used. 1ml is taken from the test tube labeled 10^5 and placed on the agar plate and this spread uniformly using a sterile spreader. After incubation at 37^OC for 24 hours the individual and total bacterial count for each bacterium from the different wastes from the different units in the hospital were counted.

2.4.1.2 Isolation of Bacteria

The manufacturer's instructions were followed in the preparation of the media. The spread plate technique was used as an aliquot (0.1ml) of the diluted sample was inoculated on the following agar plates; MacConkey, Salmonella Shigella agar, Deoxycholate Citrate agar, Chocolate agar, Nutrient agar, Eosin Methylene Blue agar, Cysteine-Lactose-Electrolyte-Deficient Agar and Thioglycholate Citrate Bile Salt agar. A sterile spreader was used to spread the 0.1 ml evenly on the surface of the plates and then incubated at 37^OC for 24-48 hours in aerobic and anaerobic conditions for the growth of pure single colonies according to the methods of Cheesbrough.[7] Presumptive identification of target colonies was done. All colonies were sub cultured unto freshly prepared nutrient agar medium to obtain pure colonies of the organisms for identification using standard microbiological methods from Cowan and Steel.[8]

2.5 Antimicrobial Susceptibility Testing

Using the Kirby-Bauer disk diffusion method, antimicrobial susceptibility testing was done using oxoid single discs. Three colonies of the test organisms were added into 3mls of tryptose phosphate and compared visually to an equivalent 0.5 MacFarland standard (0.5mls of $Bacl_2$ to 9.5 mls of 1 % H_2SO_4). It was incubated for 2 hours. Cotton tipped swab stick was used to spread the suspension unto the MHA plate. Using sterilized forceps, the single discs were placed on the inoculated plates and incubated overnight at 37°C. The following antimicrobials were used for the susceptibility

testing; Azithromycin 15µg, Gentamicin 30 µg, Norfloxacin 10 µg, Ceftazidine 30 µg, Ciprofloxacin 5 µg, Imipenem 10 µg, Ertapenem 10 µg, Cefpodoxime 10 µg, Colistin 10 µg and Cefotaxime 30 µg. Results of resistance (R) and sensitive (S) were determined for the various antimicrobials and interpreted according to Clinical and Laboratory Standard Institute guidelines.

2.6 Molecular Methods

The microorganism of interest which was *E. coli* and *K. pneumoniae* was stored in 10% glycerol at -20^{O}C until ready for molecular analysis which was carried out in the Department of Medical Laboratory Science, Niger Delta University Bayelsa.

Ten isolates were analyzed in the molecular laboratory and consisted of seven (7) *E. coli* and three (3) *K. pneumonia*e.

2.6.1 DNA Extraction and quantification

Five milliliters of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) in a sterile eppendorf microcentrifuge tube was spun at 14000rpm for 3 min. The cells were re-suspended in 500ul of normal saline and mixed using the voltex machine. This was repeated three times. Elution buffer was added to the cells and heated at 95^{O}C for 20 min. The heated bacterial suspension was cooled on ice at -3^{O}C for 10minutes and spun for 3 min at 14000rpm. The supernatant containing the DNA was transferred to a 1.5ml eppendorf microcentrifuge tube and stored at -20^{O}C for other downstream reactions.

The extracted genomic DNA was quantified using the Nano-drop 1000 spectrophotometer. The software of the equipment was launched by double clicking on the Nano-drop icon. The equipment was initialized with 2µl of sterile distilled water and blanked using normal saline. 2 µl of the extracted DNA was loaded onto the lower pedestal; the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the "measure" button.

The result was in the range for standard DNA quantification and purity. The standard DNA concentration and purity required for every PCR is 5-100 µg/ml and 1.0 – 2.5 nano respectively.

2.6.2 16S rRNA Amplification

The 16s rRNA region of the rRNA gene of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 µl 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 0.5uM and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation; 95ºC for 5 minutes, denaturation; 95ºC for 30 seconds, annealing; 52ºC for 30 seconds, extension; 72ºC for 30 seconds for 35 cycles and final extention; 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 30 minutes and visualized on a blue light transilluminator.

2.6.3 Detection of Resistance Genes

Plasmid mediated Carbapenem Resistance gene amplification was done with specific forward and reverse primers for KPC, NDM, OXA and VIM to detect the presence of resistance genes.

2.6.3a KPC Gene Amplification

Amplification of KPC genes from the isolates were carried out using the Uni-KPCF: 5'-ATGTCACTGTATCGCCGTCT-3'; Uni-KPCR: 5'-TTACTGCCCGTTGACGCCC-3′ 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, $MgCl_2$), the primers at a concentration of 0.4M and 50ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 55ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel tinted with E-Z vision dye at 120V for 25 minutes and visualized on a UV transilluminator.

2.6.3b NDM Gene Amplification

Amplification of NDM genes from the isolates were amplified using the NDM F: 5'-ATGGAATTGCCCAATATTATGCAC-3'; NDMR: 5'-TCAGCGCAGCTTGTCGGC-3′ 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 extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 56ºC for 30 seconds; extension, 72ºC for 40 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel tinted with E-Z vision dye at 120V for 25 minutes and visualized on a UV transilluminator.

2.6.3c OXA gene amplification

Amplification of OXA genes from the isolates were amplified using the OXA F:5'-TCTTTCGAGTACGGCATTAGC-3' and OXA R:5'-AATGATGCCCTCACTTTCC-3'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 extracted DNA as template. The PCR conditions were as

follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 58ºC for 30 seconds; extension, 72ºC for 40 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel tinted with E-Z vision dye at 120V for 25 minutes and visualized on a UV transilluminator for a 700bp product size.

2.6.3d VIM gene amplification

VIM genes from the isolates were amplified using the VIMF: 5'-ATGCAGCCAACGACAAAGG -3'andVIMR: 5'-CGCCCTCGCGAGCGCGCTTC-3'primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microliters 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 0.4uM and 50ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 58ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 25 minutes and visualized on a blue trans-illuminator for a 1243bp product size.

2.6.4 Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10µl, the components included 0.25µ l BigDye® terminator v1.1/v3.1, 2.25ul of 5 x BigDye sequencing buffer, 10µM Primer PCR primer and 2-10ng PCR template per 100bp. The sequencing conditions were as follows: 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min.

2.6.5 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. These sequences were aligned using MAFFT. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0. The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method.

3.0 RESULTS

3.1 Isolation of bacteria using conventional methods

Bacteria isolated from the one hundred (100) waste samples were eighty (80) in numbers. Cellular morphology revealed they were *Klebsiella pneumoniae* 22 (27.5%), *Pseudomonas* spp. 8(10%), *E. coli* 20 (25%), *Bacillus spp*. 12 (15%) and *Staphylococcus aureus 18 (22.5%) (Table1). Klebsiella pneumoniae was found to be* highest in prevalence followed by *E. coli*, then *Staphylococcus aureus* and *Bacillus spp*. As seen in Figure 1. Table 2 and Table 3 represents Individual Bacteria Count in Serial Dilution of 10^5 and Total Heterotrophic Bacteria Count.

3.2 Antimicrobial Susceptibility Pattern of *E. coli* and *K. pneumoniae* Isolated

Antibiotics sensitivity testing was done for the forty-two (42) isolates of interest which are *E. coli* (20) and *K. pneumoniae* (22). This is seen in Table 4.

3.3 Bacteria Isolated Using Molecular Methods

The extraction of 16S genomic DNA is represented in plate 4.1. Lanes 1, 3-10 represent the 16SrRNA gene bands (1500bp), Lane 2, failed amplification, Lane N represents the negative control, lane L represents the 100bp molecular ladder. This is seen in plate 1.

3.4 Resistant Genes from the Waste

The detection of antimicrobial resistance genes of NDM was carried out using specific forward and reverse primers.

3.4.1 KPC Gene Detection

In all ten (10) isolates tested for the presence of KPC Gene resistance, of which two were *E. coli* isolates from the children emergency ward and Men's Medical ward and one was *K. pneumoniae* isolates from the surgical clinic, none had the presence of the KPC gene as shown in plate 2. Lanes 1 -10 represents the negative bands. Lane M is the 50bp Molecular DNA ladder. On the molecular ladder, 600bp represents the supposed KPC gene if it were to be positive.

3.4.2 NDM Gene Detection

NDM resistance gene was detected in three (3) of the ten isolates as shown in plate 3. Lanes 4, 7 & 9 represents the positive bands at 500bp while lane M represents the 1000bp Quick-Load DNA molecular ladder. All the three (3) (30%) multidrug resistant strains harboured NDM resistance gene with size 500bp. They are represented in plates 3. These three resistant genes were detected in two *E. coli* isolates from the children emergency ward and Men's Medical ward and one *K. pneumoniae* isolates from the surgical clinic.

3.4.3 OXA Gene Detection

In all ten (10) isolates tested for the presence of OXA Gene resistance of which two were *E. coli* isolates from the children emergency ward and Men's Medical ward and one was *K. pneumoniae* isolates from the surgical clinic, none had the OXA gene as shown in plate 4. Lanes 1 -10 represents the negative bands. Lane M is the 500bp Molecular DNA ladder. On the molecular ladder, 700bp represents the supposed KPC gene if it were to be positive. This is seen in plate 4.

3.4.4 VIM Gene Detection

In all ten (10) isolates tested for the presence of VIM Gene resistance, of which two were *E. coli* isolates from the children emergency ward and Men's Medical ward

and one was *K. pneumoniae* isolates from the surgical clinic, none had the presence of the VIM gene as shown in plate 5.Lanes 1 -10 represents the negative bands. Lane M is the 50bp Molecular DNA ladder. On the molecular ladder, 391bp represents the supposed KPC gene if it were to be positive.

3.5 Sequencing /Phylogenetic analysis

The obtained 16s rRNA sequence from the isolate produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The 16S rRNA of the isolate W1 showed a percentage similarity to other species at 100.

The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolate B1-B10 within the *Escherichia, Proteus, Pseudomonas, Klebsiella, Enterobacter, Escherichia, Providencia, Klebsiella, Alcaligenes and Enterobacter* sp and revealed a closely relatedness to *Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Klebsiella pneomoniae, Enterobacter sp, Escherichia coli, Providencia rettgeri, Klebsiella pneumoniae, Alcaligenes feacalis and Enterobacter sp.RD DAROS_04* than other sp respectively. This is seen in figure 2

Table 1: Bacteria Isolated using the Conventional Method.

Bacteria	Number of isolates (%)
Escherichia coli	20 (25%)
Staphylococcus aureus	18 (22.5%)
Pseudomonas spp	8 (10%)
Klebsiella pneumoniae	22 (27.5%)
Bacillus spp	12(15%)

Table 2: Individual Bacteria Count in Serial Dilution of 10^5.

Month	*E. coli*	*Staphylococcus sp*	*Pseudomonas sp*	*Klebsiella pneumoniae*	*Bacillus sp*	Total Cfu/ml
July/August	7	5	2	8	5	27
September/October	5	6	3	10	3	27
November/ December	8	7	3	4	4	26
Total	20	18	8	22	12	80

Table 3: Total Heterotrophic Bacteria Count.

Bacteria	colony count		Cfu/ml
E. coli	20	20×10^5	2.0×10^6
Staphylococcus sp	18	18×10^5	1.8×10^6
Pseudomonas sp	8	8×10^5	0.8×10^6
Klebsiella pneumonia	22	22×10^5	2.2×10^6
Bacillus sp	12	12×10^5	1.2×10^6

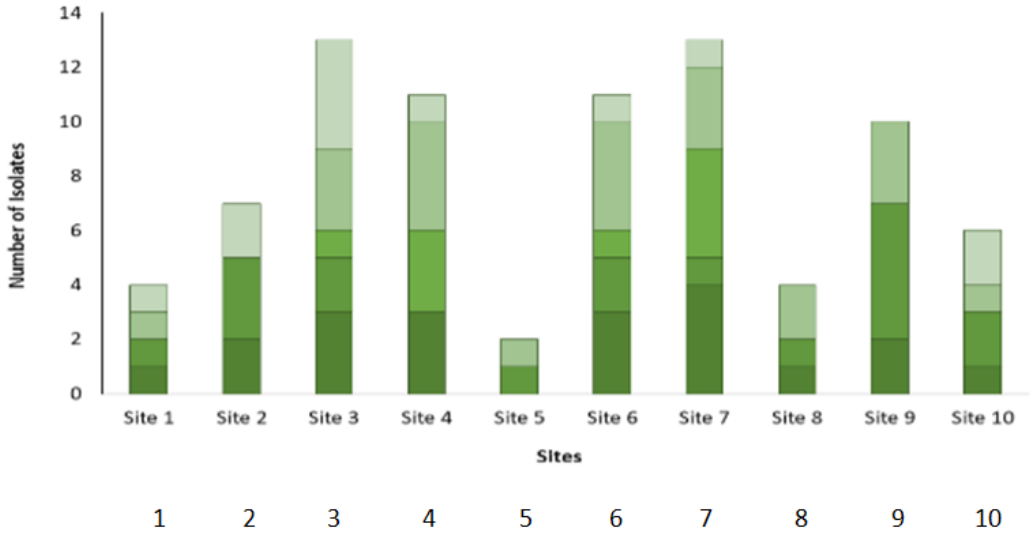

Figure 1: **Number of isolates seen on each site.**

Table 4: Antimicrobial Susceptibility Testing of *E. coli* and *K. pneumoniae* Isolates.

Antimicrobial agent	*E. coli* (n=20)		*k. pneumoniae* (n=22)	
	S	R	S	R
Imipenem(10µg)	18	0	22	0
Ertapenem (10µg)	19	1	22	0
Cefpodoxime (10µg)	3	17	2	20
Colistin (10µg)	5	15	3	19
Ceftazidine(30µg)	3	17	11	11
Cefotaxime(30µg)	2	18	6	16
Azithromycin(15µg)	1	19	3	19
Ciprofloxacin(5µg)	7	13	17	5
Norflaxacin (10µg)	8	12	9	13
Gentamicin (10µg)	0	20	0	22

Key
S - Sensitive
R - Resistant

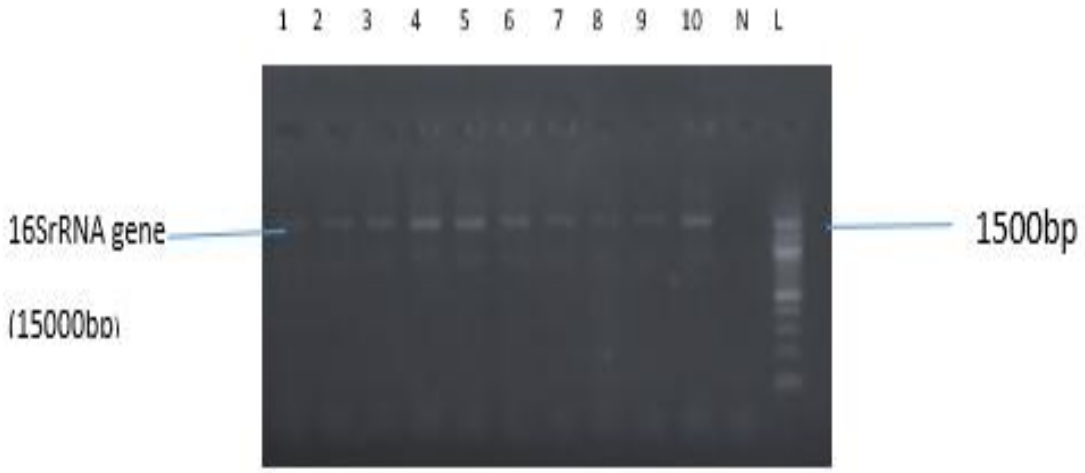

Plate 1: Agarose Gel Electrophoresis of 16S rRNA Genes of ten Bacteria Isolates.

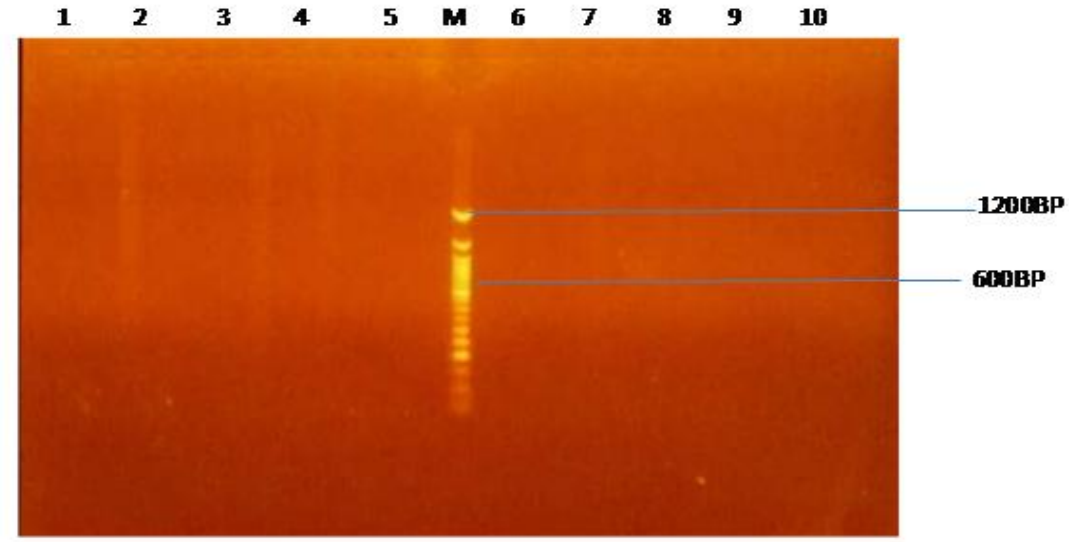

Plate 2: Agarose Gel Electrophoresis of Isolates of Negative KPC Genes.

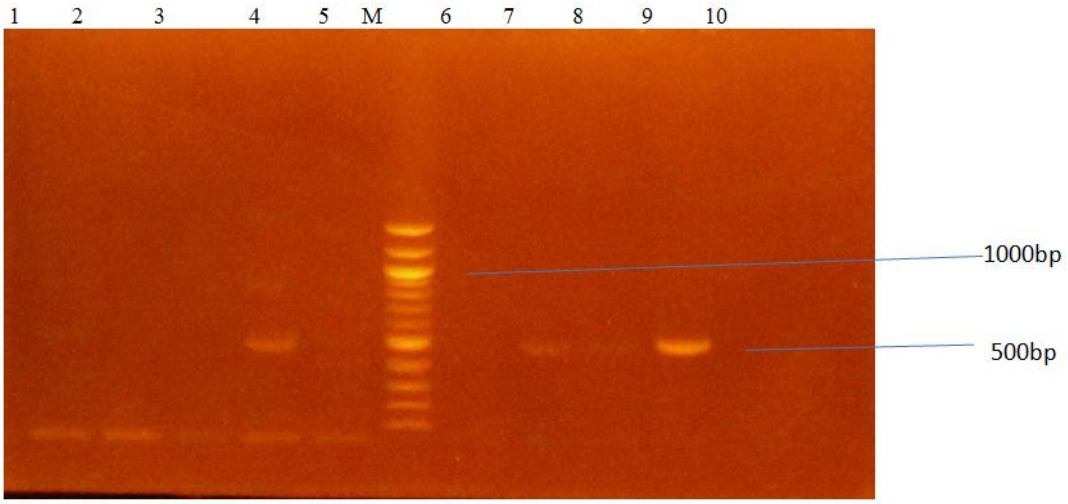

Plate 3: Agarose Gel Electrophoresis of Isolates of Positive and Negative NDM Genes.

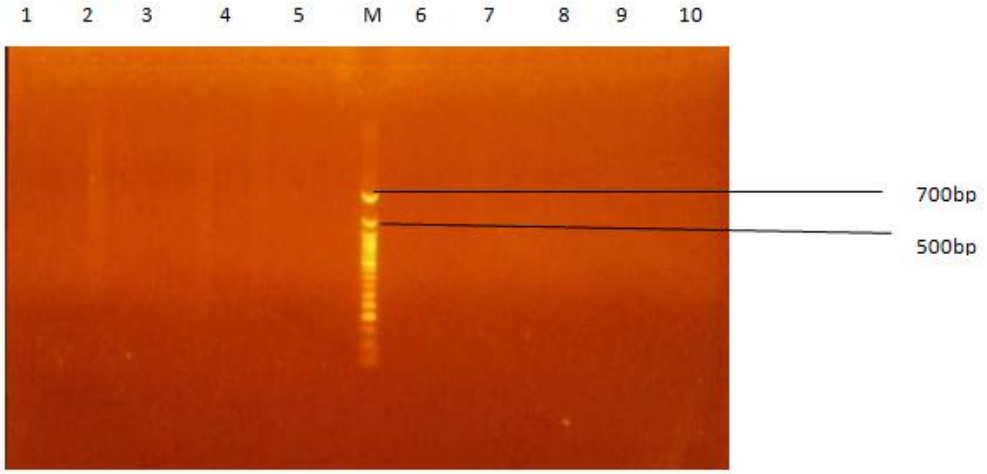

Plate4: Agarose Gel Electrophoresis of Isolates of Negative OXA Genes

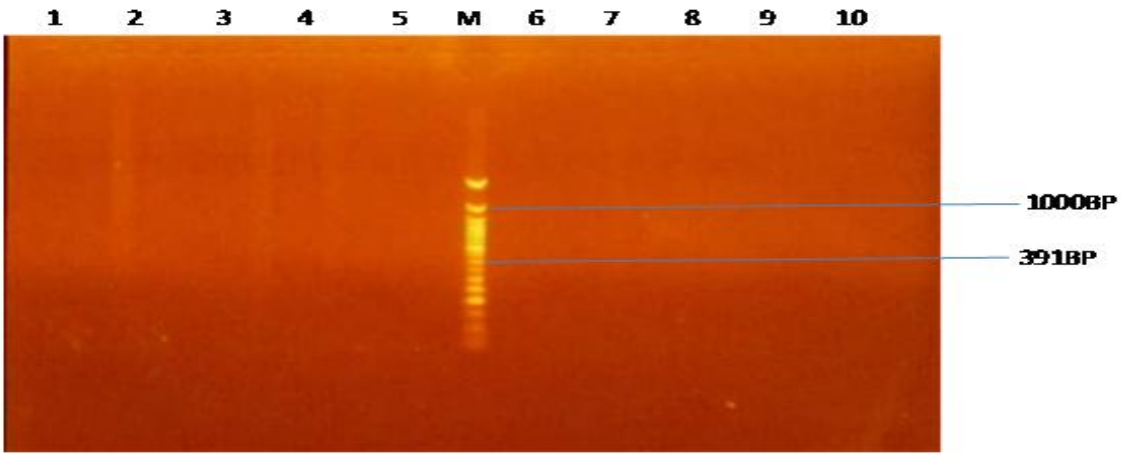

Plate 5: Agarose gel Electrophoresis of Isolates of Negative VIM Genes.

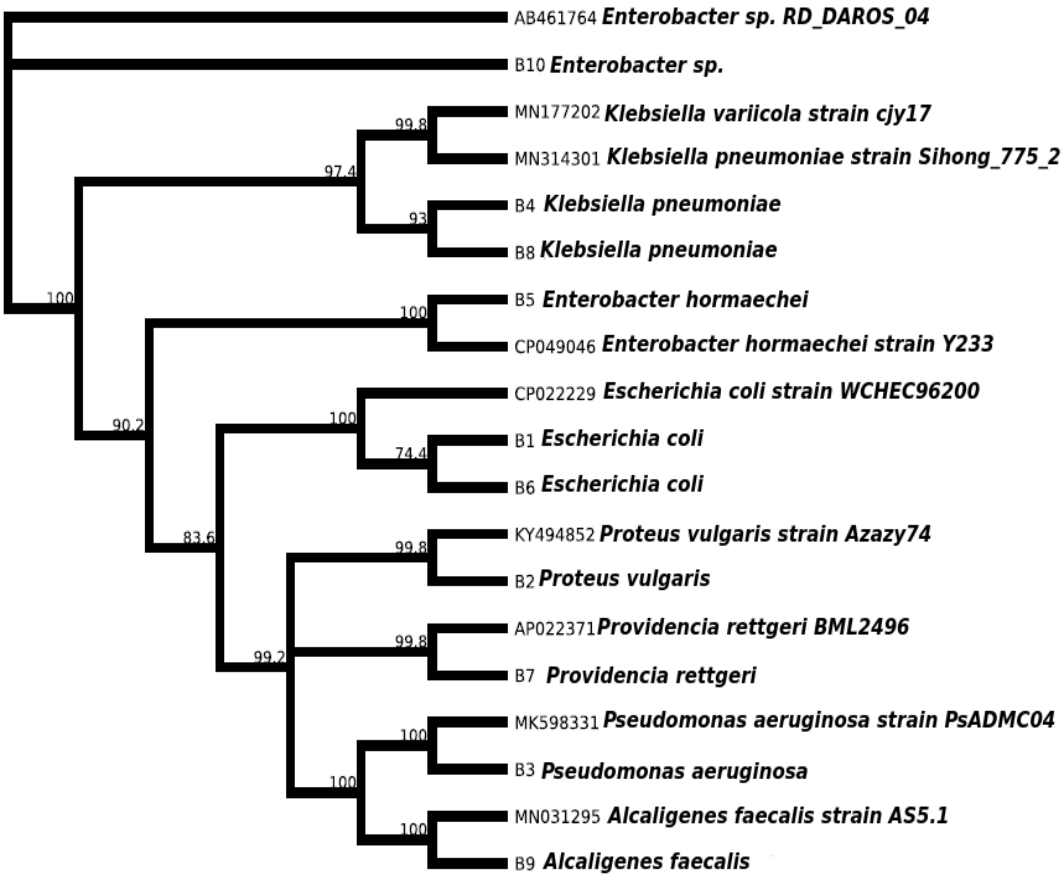

Fig 2: Phylogenetic Analysis showing the Evolutionary Relationship among the Bacterial Isolate.

4.0 DISCUSSION

4.1. Isolation of bacteria using conventional methods

Klebsiella pneumoniae was found to be highest (27.5%) in prevalence followed by *E. coli (25%)*, then *Staphylococcus aureus* (22.5%) and *Bacillus spp* (15%) with *Pseudomonas* spp. (10%) being the least in this study. This is in agreement with studies in the past that revealed the presence of high levels of *E. coli* and *K. pneumoniae* in hospital waste.[9,10]

The overall prevalence of bacteria was seen in Sites 3 and 7 with 13 isolates (16%) of the total. The high incidence of *E. coli* in males in contrast to females might be as a result of benign prostatic enlargement in men which is the most predisposing factor. This is so because of the frequency and methods in which catheters are introduced to ease urination. *E.coli* being a normal flora in the gastrointestinal tract may mistakenly be introduced into the patient when established protocol designed for sterility is not followed and this result agrees with study carried out in Edinburgh.[11] The least prevalence was in site 5 which had just the *S. aureus* and *K. pneumoniae* isolates present. *E. coli*, *S. aureus* and *K. pneumoniae* were seen on nine (9) different sites while *Bacillus spp* was found in 7 sites and *Pseudomonas spp* was present in only four (4) sites. This is seen in figure 1. Similar studies have revealed the presence of *K. pneumoniae, E. coli, Staphyloccus aureus, Bacillus species* and *Pseudomonas spp* in hospital wastes from different units of the hospital.[12,13]

4.2 Antimicrobial susceptibility pattern of *E. coli* and *K. pneumoniae* isolated

Antibiotics sensitivity testing was done for all isolates from the organisms of interest which are *E. coli* (20) and *Klebsiella pneumoniae* (22). Isolates showed different levels of sensitivity and resistance with Carbapenem antimicrobials showing very high levels of sensitivity as compared to the other classes of antimicrobials used. The highest level of resistance in all isolates was seen in gentamicin. However, ertapenem revealed a better performance index to all isolates followed by imipenem. Other researchers have reported carbapenem antibiotics to have a broad antimicrobial spectrum with activities against almost all pathogens.[14] A significant level of resistance was detected in most isolates to at least three of the antimicrobials used (Table 4).Studies have shown high level of multi drug resistance to some commonly used antibiotics in Rivers State.[15] Studies have also revealed that environments where antibiotics are high in concentration, indiscriminate use of antibiotics and plasmids are major causes in the development of resistant genes.[16,17]

Most of the isolates from the theatre were sensitive to most of the antibiotics which could be as a result of the highly aseptic conditions in which their procedures are

performed such as hand disinfection of the operating team prior to surgery, disinfection of surgical sites and wearing of sterile gloves by scrubbed personnel [18].Right from the entrance of the theatre, there is zero tolerance to sepsis.

4.3 Detection of resistant genes

From this study, it was observed that three (3) out of the ten (10) isolates tested positive for the NDM resistant genes while the other seven (7) isolates did not harbor the other carbapenem genes which are VIM, OXA and KPC. That is to say, only NDM Carbapenem resistance genes was detected out of the four carbapenem genes KPC, NDM, OXA and VIM

This present study revealed that the three isolates were *E. coli* and *Klebsiella pneumoniae* isolates, each possessing the NDM carbapenem resistance genes which enable them to be resistant to carbapenems like Imipenem and Ertapenem. The two (2) *E. coli* isolates were from the children's emergency ward and male medical ward while the *K. pneumoniae* was isolated from the surgical clinic, therefore, present in the wastes from the Rivers State University Teaching Hospital. This is worthy to note because carpenem antimicrobials have become drugs of last resort therefore its detection from these hospital wastes will be a source of life threatening nosocomial infections. Although, Studies have revealed that Carbapenem resistant enterobacteriaceae remain uncommon in our health care system their genes were detected in both *E. coli* and *K pneumonia*.[19]

Studies have also shown that NDM positive strains have been found worldwide, representing a noteworthy challenge for clinical management and public health.[20,21] In this study, the three NDM positive isolates were phenotypically sensitive to imipenem and ertapenem antimicrobials. This could be as result of gene not fully expressed at the antimicrobial sensitivity level but overtime could be expressed because of the resistance genes detected in them at the molecular level. Also, mutations in gene which play a significant role in contributing to resistance in these isolates may have occured. However, this needs to be studied further. The three NDM positive isolates being phenotypically sensitive to imipenem and ertapenem antimicrobials could also be as a result of the lack of a consensus among existing recommendations put forth by expert advisory groups like CLSI and EUCAST bringing about differences in the breakpoint cut off recommended by them.[22,23] Resistance genes from isolates in the children emergency ward could be because most of the care givers believe that the waste of children are not infectious thereby keeping wastes from children longer than they should before discarding. Secondly, most of the wastes could be mixed wastes as patient's caregivers do not adhere strictly to proper waste disposal methods. Most cases also present in the children emergency unit are cases of acute infections which are highly infectious.

4.4 Phylogenetic analysis

The obtained 16s rRNA sequence from the isolate produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The 16S rRNA of the isolate W1 showed a percentage similarity to other species at 100%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolate B1-B10 within the *Escherichia, Proteus, Pseudomonas, Klebsiella, Enterobacter, Escherichia, Providencia, Klebsiella, Alcaligenes and Enterobacter* sp and revealed a closely relatedness to *Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Klebsiella pneomoniae, Enterobacter sp, Escherichia coli, Providencia rettgeri, Klebsiella pneumoniae, Alcaligenes feacalis and Enterobacter sp.RD DAROS_04.* This is relatable as *Escherichia coli* and *Klebsiella pneumoniae* was isolated using the conventional method of bacteria identification.

5.0 CONCLUSION

The hospital environment is a potential reservoir of bacteria with plasmids conferring carbapenem resistance, more bothersome is hospital wastes in our climes.

From this study, it can be concluded that *Escherichia coli,* and *Klebsiella pneumoniae,* are found in wastes in Rivers State University Teaching Hospital. Poor hospital waste management practices are critical and very important for reducing if not totally eliminating nosocomial infections. This study showed high level of susceptibility to imipenem and ertapenem against all isolates of *E.coli* and *K. Pneumoniae*. A significant observation in this study was that there was NDM carbapenem resistant gene detected in three (3) of the isolates of which one (1) was *E. coli a*nd two (2), *K pneumoniae*. The present study suggests that Rivers State University Teaching Hospital waste acted as an important source of NDM producing bacteria, and untreated hospital waste is a significant source of these bacteria. The proposition of this finding is likely to be important from a public health perspective. A comparable situation may exist in other hospital settings where there is a high incidence of multidrug resistant organisms among patients as a result of lack of adequate sanitation and hygiene, unregulated use of antibiotics and most importantly pitiable hospital waste management guidelines although this study was focused primarily on waste samples collected from Rivers State University Teaching Hospital.

In conclusion there was a low relative abundance of *E. coli* and *K. pneumoniae* that harbored carbapenem gene in wastes in the Rivers State University Teaching Hospital. NDM resistant genes were also present in some isolates from wastes obtained from the Male Medical Ward, Surgical Clinic and Children's Emergency Ward in the Rivers State University Teaching Hospital.

Although there was a low relative abundance of carbapenem resistant *E. coli* and *K. pneumoniae* in this study, there is need to improve the quality of waste disposal treatments in order to reduce the emergence of carbapenem resistant infections from these organisms and the incidence of infections from other enterobacteriaceae. It is recommended that hospitals should treat and decontaminate their wastes and effluents properly to avoid transmission of carbapenem resistant infections through wastes. Hospital personnels, Patient contacts and patients should sanitize their hands on contact with waste bins, incidence of nosocomial infection with carbapenem resistant organisms should be prevented by continuous supervision of hospital waste disposal and treatment. The Authorities concerned should promote health education campaigns to raise awareness about transmission of carbapenem infections, danger of poor hygienic practices, the hazard in improper waste treatment and the risks associated with the abuse of antibiotics.

REFERENCES

1. Environmental Protection Agency (2005). Medical waste, Available at www.epa.gov.epaoswer/other/medical/sharps. Retrieved on the 19th February, 2019.
2. Pruss, A., Cirouit, E., Rushbrook, P. Definition and characterization of health-care waste. *Safe Management of Wastes from Health-Care Activities*, 1999; 2: 46.
3. Mathur, P., Patan, S. & Shobhawat, A. S. Need of biomedical waste management system in hospitals-An emerging issue-a review. *Current World Environment*, 2012; *7*(1): 117.
4. Ngwuluka, N., Ochekpe, N., Odumosu, P., & John, S. A. Waste management in healthcare establishments within Jos Metropolis, Nigeria. *African Journal of Environmental Science and Technology*, 2009; *3*(12): 459-465.
5. Levy, J. I. Impact of residential nitrogen dioxide exposure on personal exposure: an international study. *Journal of the Air & Waste Management Association*, 1998; *48*(6): 553-560.
6. Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G. & Paterson, D. L. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection*, 2012; *18*(3): 268-281.
7. Cheesebrough, M. District laboratory practice in tropical countries Part 2. Cambridge: Cambridge University Press, 2000; 63-70.
8. Cowan, S. T. & Steel, K. J. *Cowan and Steel's Manual for the Identification of Medical Bacteria*. England: Cambridge University Press, 2003.
9. Omoni, V. T., Makinde, O. A. & Abutu, S. O. Prevalence of multidrug resistant bacteria isolated from biomedical waste generated in Makurdi Metropolis, Benue State, Nigeria. *Microbiology Research Journal International*, 2015; 10: 1-10.
10. Andy, I. E. & Okpo, E. A. Occurrence and Antibiogram of Bacteria Isolated from Effluent and Waste Dump Site Soil of Selected Hospitals in Calabar Metropolis, Nigeria. *Microbiology Research Journal International*, 2018; 25(5): 1-9.
11. Karabey, S., Ay, P., Derbently, S., Nagipoglu, Y. & Esen, F. Hand washing frequencies in an intensive care unit. *Journal of Hospital Infection*, 2012; 50(1): 36-41.
12. Ekhaise, F. O., & Omavwoya, B. P. Influence of hospital wastewater discharged from University of Benin Teaching Hospital (UBTH), Benin City on its receiving environment. *American-Eurasian Journal of Agriculture and Environmental Science*, 2008; 4(4): 484-488.
13. Hossain, M. D., Rahman, N. N. N. A., Balakrishnan, V., Puvanesuaran, V. R., Sarker, M., Islam, Z., & Kadir, M. O. A. Infectious risk assessment of unsafe handling practices and management of clinical solid waste. *International Journal of Environmental Research and Public Health*, 2013; *10*(2): 556-567.
14. Vardakas, K. Z., Matthaiou, D. K., Falagas, M. E., Antypa, E., Koteli, A., & Antoniadou, E. Characteristics, risk factors and outcomes of carbapenem-resistant Klebsiella pneumoniae infections in the intensive care unit. *Journal of Infection*, 2015; *70*(6): 592-599.
15. Nkemkanma, A. V., Abbey, S. D., Wachukwu, C. K., Nwokah, E. G., & Amba, O. O. Enteric Bacteria associated with Diarrhoea and their susceptibility pattern in Rivers State. *World Journal of Pharmaceutical and Life Sciences*, 2017; 3(8): 28-33.
16. Greenwood, D., Slack, R. C., Barer, M. R. & Irving, W. L. (2012). *Medical Microbiology E-Book: A Guide to Microbial Infections: Pathogenesis, Immunity, Laboratory Diagnosis and Control*. Churchill Livingston, England.
17. Hudson, C. M., Bent, Z. W., Meagher, R. J., & Williams, K. P. Resistance determinants and mobile genetic elements of an NDM-1-encoding Klebsiella pneumoniae strain. *Public Library of Science One*, 2014; *9*(6): 1-14.
18. Krediet, A. C., Kalkman, C. J., Bonten, M. J., Gigengack, A. C. M. & Barach, P. Hand-hygiene practices in the operating theatre: an observational study. *British Journal of Anaesthesia*, 2011; *107*(4): 553-558.
19. Jacob, J. T., Klein, E., Laxminarayan, R., Beldavs, Z., Lynfield, R., Kallen, A. J. & Rasheed, J. K. Vital signs: carbapenem-resistant Enterobacteriaceae. *Morbidity and Mortality Weekly Report*, 2013; 62(9): 165.
20. Moellering Jr, R. C. NDM-1—a cause for worldwide concern. *New England Journal of Medicine*, 2010; *363*(25): 2377-2379.
21. Dortet, L., Poirel, L. & Nordmann, P. (2014).Worldwide dissemination of the NDM-type

carbapenemases in Gram-negative bacteria. *BioMed Research International*, *2014*.

22. Giske, C. G., Monnet, D. L., Cars, O. & Carmeli, Y. Clinical and economic impact of common multidrug-resistant gram-negative bacilli. *Antimicrob*ial *Agents of Chemotherapy*, 2008; 52(3): 813–821.
23. Wayne PA. Performance standards for antimicrobial susceptibility testing. Clinical and Laboratory Standards Institute. 2014. Twenty third Informational Supplement. CLSI document M100-24. Available at file:///C:/Downloads/molecules-23-00795.pdf. Retrieved on 22nd august, 2019.