ABSTRACT

Carbapenem-resistant Enterobacteriaceae (CRE) are a serious new public-health problem. Carbapenem medicines are a class of antibiotics that are often used to treat severe infections caused by bacteria that are antibiotic-resistant. The goal of this study was to use both conventional and molecular methods to isolate, characterize, and identify Carbapenem-resistant Enterobacteriaceae from stool samples of patients at two tertiary hospitals in Port Harcourt, Rivers State, Nigeria. A total of 114 fecal samples were collected and inoculated onto Eosin methylene blue (EMB) agar and MacConkey agar (M.A) plates. The plates were incubated for 24-48 hours. Representative colonies after incubation were isolated and subjected to phenotypic characterisation using Biochemical Test, followed by antibiotic susceptibility testing. Further characterisation was done by subjecting the isolates to molecular analysis. In this study, 52 isolates of 114 stool samples of enteric organisms were identified; 32 were from females while 20 were from males. The gender distribution of these isolates showed that the Enteric organisms were more predominant in females than in males with male subjects having E. coli 7 (35.0%), Klebsiella spp 7 (35.0%), Salmonella spp 2 (10%), Shigella spp 2 (10%) and Pseudomonas spp 2 (10%) while the female subjects had E. coli 12 (37.5%), Klebsiella spp 13 (40.6%), Salmonella spp 3 (9.4%), Shigella spp 3 (9.4%) and Pseudomonas spp 1 (3.1%). Results showed that Klebsiella spp recorded the highest frequency with 20 (38.5%), followed by E. coli with 19 (36.5%), Salmonella spp 5 (9.6%), Shigella spp 5(9.6%) and Pseudomonas spp 3 (5.8%). Results of the antibiotic susceptibility pattern showed that all the
isolates were very susceptible to ciprofloxacin and ofloxacin. Although the meropenem antibiotics showed high sensitivity to the enteric organisms, ciprofloxacin and ofloxacin are recommended for treatment since they have higher antibiotic activities against the enteric bacteria.

Keywords: Carbapenem-resistant; enterobacteriaceae; conventional and molecular methods.

1. INTRODUCTION

Carbapenems are a class of antibiotics that are often used to treat severe infections, especially those caused by bacteria that are resistant to antibiotics [1]. Carbapenem belongs to the β-lactam class of antibiotic compounds [2]. This antibiotic family stops the peptidoglycan layer of bacterial cell walls from being synthesized [3]. They do so by binding to the active sites of penicillin-binding proteins, which are responsible for the last steps in peptidoglycan synthesis. This stops the peptidoglycan layer from crosslinking, preventing normal cell wall production [4].

Carbapenem-resistant Enterobacteriaceae (CRE) or carbapenemase-producing coliforms are Gram-negative bacteria that are resistant to carbapenem antibiotics, which are considered last-line medicines for such infections. Resistance can range from mild to severe [5]. These microbes can be present in the intestines (gut) of healthy people [6]. These bacteria can sometimes move outside the gut, resulting in severe diseases such as urinary tract infections, bloodstream infections, wound infections, and pneumonia. Enterobacteriaceae, which includes Klebsiella sp. and E. coli, is a typical flora of human gut bacteria that can become carbapenem-resistant [7]. Carbapenem resistance can be caused by a number of distinct methods. The development of Klebsiella pneumoniae carbapenemase (KPC) is one of the most prevalent ways that Enterobacteriaceae acquire carbapenem resistant [8]. Certain CRE produce the enzyme Klebsiella pneumoniae carbapenemase (KPC), which was first found in the United States in 2001. The enzyme Klebsiella pneumoniae carbapenemase (KPC) is generated by certain CRE and was initially discovered in the United States in 2001. Carbapenems are broken down by Klebsiella pneumoniae carbapenemase (KPC), rendering them useless. Other enzymes, in addition to KPC, can break down carbapenems and cause CRE to form, although they are uncommon in the US [5].

Carbapenem-resistant Enterobacteriaceae (CRE) are more likely to afflict people with weakened immune systems or who have invasive medical equipment such as tubes inserted into their bodies [9]. The use of certain antibiotics may increase the risk of patients contracting (CRE) Carbapenem-resistant Enterobacteriaceae. Patients who have taken a lot of antibiotics are more likely to get CRE [10]. The reason for this is because the more bacteria are exposed to antibiotics, the more likely they are to develop resistance to the antibiotic, rendering it ineffective.

Carbapenems are a kind of antibiotic that is widely used to treat serious or high-risk bacterial infections. Antibiotics in this class are often used to treat known or suspected multidrug-resistant (MDR) bacterial infections. Carbapenems are antibiotics of the beta lactam class, like penicillin and cephalosporins, that kill bacteria by attaching to penicillin-binding proteins and blocking bacterial cell wall formation. However, as compared to most cephalosporins and penicillins, these medicines have a larger spectrum of action. Furthermore, carbapenems, unlike other beta-lactam antibiotics, are usually unaffected by growing antibiotic resistance. Merck & Co. produced carbapenem antibiotics from the carbapenem thienamycin, a naturally occurring product of Streptomyces cattleya [11]. CRE has been dubbed “nightmare bacterium” by Tom Frieden, the former director of the Centers for Disease Control and Prevention. KPC (Klebsiella pneumoniae carbapenemase) and NDM (Klebsiella pneumoniae carbapenemase) are two types of CRE (New Delhi Metallo-beta-lactamase). KPC and NDM are enzymes that break down carbapenems and render them ineffective in Klebsiella pneumoniae [12].

The rising resistance rate of Carbapenem-resistant Enterobacteriaceae (CRE) patients in Nigeria is a major concern, as patients with these infections die on a daily basis due to a lack of effective drugs or treatment to combat the deadly infections [13], potentially leading to higher morbidity and mortality rates.

Carbapenem-resistant Enterobacteriaceae (CRE), particularly Klebsiella pneumoniae carbapenemases (KPC) Enterobacteriaceae, has spread globally in the last decade, posing a
serious public health threat because it causes varying levels of carbapenem resistance and leaves few therapeutic options for treating such infections [14].

This study used both conventional and molecular methods to characterize Carbapenem-resistant Enterobacteriaceae from stool samples of patients at two tertiary hospitals in Port Harcourt, Rivers State, Nigeria.

2. METHODOLOGY

2.1 Study Area

This study took place in the University of Port Harcourt Teaching Hospital and the Save a Life Mission Hospital, both of which are tertiary hospitals. The hospitals are in Nigeria’s Rivers State. The research lasted three months, from January to March of this year. Using sterile feces sample vials, faeces samples were collected. The sterile containers were carefully numbered and labeled, and they were promptly delivered to Rivers State University’s microbiology laboratory in refrigerators with ice packs for analysis. Over the course of three months, one hundred and fourteen (114) faeces samples were obtained from 45 male and 69 female patients. The samples were tested as soon as they were collected.

2.2 Enteric Organism Isolation

Enteric organisms were isolated using the streak plate technique using Eosin methylene blue (EMB) and MacConkey (MA) agar and cultured for 18 to 24 hours at 37°C before being tested for growth. Colonies were characterized and subcultured onto nutrient agar plates, incubated for additional 24 hours for pure cultures (for subsequent testing), and then stored at 4°C in sterile 10% glycerol broth. According to Cheesbrough, Gram stain response and biochemical tests such as indole, catalase, methyl red, sugar fermentation, and citrate assays were performed.

2.3 Antimicrobial Susceptibility Testing

Susceptibility testing was done using the Kirby-Bauer disc technique for all clinical isolates identified according to the Clinical and Laboratory Standards Institute [15] guideline. The test organisms’ colonies were emulsified in test tubes containing 0.5 McFarland standard of normal saline using sterile wire loop [16]. The suspension’s turbidity was matched to the turbidity of 0.5 McFarland generated in good light right before use. A sterile swab was used to infect a plate of Mueller Hinton agar (Oxoid, UK) with the test organism, which was then prepared according to the manufacturer’s instructions. Excess fluid was removed by pressing and rotating the swab against the edge of the tube above the level of the suspension. To achieve uniform dispersion, the medium’s surface was streaked in three directions while rotating the plate around 360°C. The surface of the agar was allowed to dry for 3-5 minutes with the petri-dish cover in place. The antibiotic discs were put onto the agar using sterile forceps. The plates were inverted 30 minutes after applying the discs and incubated at 37°C for 18 to 24 hours. The test plates were evaluated after an overnight incubation period. The diameter of each zone of inhibition, as well as the end point of inhibition, was measured in mm using a ruler on the underside of the plate. [17] showed how to construct the MAR index, which measures the number of drugs the isolates were resistant to.

2.4 Molecular Identification

DNA Extraction (Boiling technique), DNA Quantification, 16S rRNA Amplification, Sequencing, and Genotypic Characterization were utilized to further examine all of the clinical isolates shown to be resistant to the antibiotics administered, particularly Carbapenem.

2.4.1 DNA Extraction (Boiling method)

Five milliliters of the bacterial isolate’s overnight broth culture in Luria Bertani (LB) were spun at 14000rpm for three minutes. The cells were resuspended in 500ul normal saline and heated for 20 minutes at 95°C. The hot bacterial solution was spun for 3 minutes at 14000rpm after cooling on ice. The DNA-containing supernatant was transferred to a 1.5ml microcentrifuge tube and kept at -20°C for use in subsequent procedures [18].

2.4.2 DNA Quantification

The Nanodrop 1000 spectrophotometer was used to measure the isolated genomic DNA. By double-clicking the Nanodrop icon, the equipment’s program was launched. The apparatus was blanked with normal saline after being initiated with 2ul of sterile distilled water. The lower pedestal was filled with two microlitres of extracted DNA, and the higher pedestal was
lowered to make contact with the extracted DNA on the lower pedestal. The concentration of DNA was determined by pressing the "measure" button [19].

2.4.3 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 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 0.5uM 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 extension, 72ºC for 5 minutes.

2.4.4 Sequencing 16S

Inqaba Biotechnological, Pretoria, South Africa, used the BigDye Terminator kit on a 3510 ABI sequencer for sequencing. 0.25 l BigDye® terminator v1.1/v3.1, 2.25 l 5 x BigDye sequencing buffer, 10 l Primer PCR primer, and 2-10ng PCR template per 100bp were used in the sequencing at a final volume of 10 l. The following were the conditions for sequencing: 32 cycles of 96ºC for 10 seconds, 55ºC for 5 seconds, and 60ºC for 4 minutes [20].

2.4.5 Genotypic Characterization

According to the manufacturer's instructions, deoxyribo nucleic acid (DNA) was extracted using the spin column technique. The purified DNA was subjected to multiplex PCR in a 20-liter total volume, with 5 liters being the primer mix (HELINI ready to use Gene Primer Mix), 10 liters being the master mix (which contains 2U of Taq DNA polymerase, 10X Taq reaction buffer, 2 mM MgCl2, 1 liter of 10 mM dNTPs mix, and Red Dye PCR additives), and 5 liters being purified. The following was programmed into the PCR machine with the total volume: Denaturation: 95ºC for 5 minutes, Denaturation: 94ºC for 30 seconds, Annealing: 58ºC for 30 seconds 35 cycles, Extension: 72ºC for 30 seconds, Final extension: 72ºC for 5 minutes. PCR products were loaded after combining with gel loading dye and 10 L HELINI 100 bp DNA Ladder as a size marker on an agarose gel electrophoresis. During electrophoresis at 50V, we waited until the dye reached three-fourths of the gel's distance before viewing the gel with a UV Transilluminator and observing the bands pattern [18].

3. RESULTS AND DISCUSSION

The study looked at 114 feces samples from individuals, including both male and female patients. Table 1 shows the number of enteric isolates and their proportion of incidence. E. coli was found in 19 isolates (36.5%), Klebsiella spp. in 20 isolates (38.5%), Salmonella spp. in 5 isolates (9.6%), Shigella spp. in 5 isolates (9.6%), and Pseudomonas spp. in 3 isolates (5.8%).

During a megablast search for extremely similar sequences from the NCBI non-redundant nucleotide (nr/nt) database, the isolate's 16S rRNA sequence returned a perfect match. The isolate K2's 16S rRNA has a percentage similarity to other species of 100 percent. The evolutionary distances calculated using the Jukes-Cantor method agreed with the phylogenetic placement of the 16S rRNA of the isolate K2 within the Klebsiella spp. and revealed a close relationship to Klebsiella pneumoniae, while K1, K4, K3, and K5 were closely related to Escherichia coli, Shigella flexneri, Pseudomonas aeruginosa, and Salmonella ty (Fig. 1). Salmonella spp. were 100 percent resistant to Ceftazidime, Cefuroxime, and Augmentin, followed by Cefixime (80 percent), Nitrofurantoin (60 percent), and Meropenem (20 percent), with Gentamicin, Ofloxacine, and Ciprofloxacin having the least resistance (0 percent). Also, Salmonella spp. also showed (100%) susceptibility to Gentamicin and Ofloxacine, followed by Ciprofloxacin and meropenem (80%), then Cefixime and Nitrofurantoin (20%), while Ceftazidime, Cefuroxime and Augmentin with (0%) susceptibility had the least (Table 4). The antibiotic susceptibility pattern of Shigella spp showed (100%) resistant to Cefuroxime, Augmentin and Cefixime, followed by Ceftazidime (80%), Nitrofurantoin and meropenem (20%), while Gentamicin, Ofloxacine and Ciprofloxacin had the least with (0%) resistance each. On the contrary, Gentamicin, Ofloxacine and Ciprofloxacin proved active (i.e. 100%) against Shigella spp followed by Nitrofurantoin and meropenem (80%), while
Ceftazidime, Cefuroxime, Augmentin and Cefixime recorded the least with (0%) susceptibility each (Table 3).

Susceptibility pattern of *Pseudomonas spp* revealed (100%) resistant to Cefuroxime, Augmentin and Cefixime than other antibiotics used followed by Ceftazidime (66.7%), Nitrofurantoin and meropenem (33.3%) each while Gentamicin, Ofloxacin and Ciprofloxacin had the least with (0%) resistant each but on the contrary, Gentamicin, Ofloxacin and Ciprofloxacin proved active (i.e. 100%) against *Pseudomonas spp* followed by Nitrofurantoin and meropenem (66.7%), then Ceftazidime (33.6%). Whereas Cefuroxime, Augmentin and Cefixime recorded the least with (0%) susceptibility each (Table 4).

The antibiotic susceptibility results showed that most of the Enterobacteriaceae (*Salmonella spp* Shigella spp and *Pseudomonas spp*) isolated in this study were susceptible to meropenem while very few were resistant to meropenem. The presence of CRE in human stool is a probable factor for the development of infections caused by them. Faecal carriage of CRE have been observed in the studies carried out in different parts of the world [21]. The stockpiling of CRE in faeces opportunistically behaves as a source for its transmission and spread [22]. Overuse and misuse of various class of antibiotics are reasons for man-made antibiotics pressure which also led to the emergence and spread of (CRE) Carbapenem-resistant *Enterobacteriaceae* [23]. In addition, global spread of Carbapenem resistance *Enterobacteriaceae* (CRE) is facilitated by poor sanitation, bad hygiene in community and in hospital [24]. The faecal carriage of CRE acts as a reservoir for dissemination of these multidrug-resistant pathogens through cross-transmission and studies have been observed in different parts of the world [8].

Table 1. Number of Enteric Isolates and their Percentage (%) Occurrence

| Enterobacteriaceae isolated | No. of organism isolates | Organisms (%) |
|-----------------------------|-------------------------|---------------|
| *E. coli*                   | 19                      | 36.5          |
| *Klebsiella spp.*           | 20                      | 38.5          |
| *Salmonella spp.*           | 5                       | 9.6           |
| *Shigella spp.*             | 5                       | 9.6           |
| *Pseudomonas spp.*          | 3                       | 5.8           |
| **Total**                   | **52**                  | **100.0**     |

Table 2. Susceptibility pattern of *Salmonella spp* to various antibiotics

| Antibiotics (Conc.)        | Resistant n (%) | Intermediate n (%) | Susceptible n (%) |
|----------------------------|-----------------|--------------------|-------------------|
| Ceftazidime (30μg)         | 5 (100.0)       | 0 (0.00)           | 0 (0.00)          |
| Cefuroxime (30μg)          | 5 (100.0)       | 0 (0.00)           | 0 (0.00)          |
| Gentamicin (10μg)          | 0 (0.00)        | 0 (0.00)           | 5 (100.0)         |
| Ofloxacin (5μg)            | 0 (0.00)        | 0 (0.00)           | 5 (100.0)         |
| Augmentin (30μg)           | 5 (100.0)       | 0 (0.00)           | 0 (0.00)          |
| Cefixime (5μg)             | 4 (80.0)        | 0 (0.00)           | 1 (20.0)          |
| Nitrofurantoin (300μg)     | 30 (60.0)       | 1 (20.0)           | 1 (20.0)          |
| Ciprofloxacin (5μg)        | 0 (0.00)        | 1 (20.0)           | 4 (80.0)          |
| Meropenem (10μg)           | 1 (20.0)        | 0 (0.00)           | 4 (80.0)          |

Table 3. Susceptibility pattern of *Shigella spp* to various antibiotics

| Antibiotics (Conc.)        | Resistant n (%) | Intermediate n (%) | Susceptible n (%) |
|----------------------------|-----------------|--------------------|-------------------|
| Ceftazidime (30μg)         | 4 (80.0)        | 1 (20.0)           | 0 (0.00)          |
| Cefuroxime (30μg)          | 5 (100.0)       | 0 (0.00)           | 0 (0.00)          |
| Gentamicin (10μg)          | 0 (0.00)        | 0 (0.00)           | 5 (100.0)         |
| Ofloxacin (5μg)            | 0 (0.00)        | 0 (0.00)           | 5 (100.0)         |
Table 4. Susceptibility pattern of *Pseudomonas spp* to various antibiotics

| Antibiotics (Conc.)       | Resistant n (%) | Intermediate n (%) | Susceptible n (%) |
|---------------------------|-----------------|--------------------|-------------------|
| Augmentin (30μg)          | 5 (100.0)       | 0 (0.00)           | 0 (0.00)          |
| Cefixime (5μg)            | 5 (100.0)       | 0 (0.00)           | 0 (0.00)          |
| Nitrofurantoin (300μg)    | 1 (20.0)        | 0 (0.00)           | 4 (80.0)          |
| Ciprofloxacin (5μg)       | 0 (0.00)        | 0 (0.00)           | 5 (100.0)         |
| Meropenem (10μg)          | 1 (20.0)        | 0 (0.00)           | 4 (80.0)          |

Table 5. Mar index of enteric organisms identified

| MAR INDEX | *Salmonella spp.* (%) | *Shigella spp.* (%) | *Pseudomonas spp.* (%) |
|-----------|-----------------------|---------------------|------------------------|
| 0.0       | 0.00 (0.00)           | 1 (20.0)            | 1 (33.3)               |
| 0.1       | 2 (40.0)              | 4 (80.0)            | 2 (66.7)               |
| 0.2       | 3 (60.0)              | 0.00 (0.00)         | 0.00 (0.00)            |
| 0.3       | 0.00 (0.00)           | 0.00 (0.00)         | 0.00 (0.00)            |
| 0.4       | 0.00 (0.00)           | 0.00 (0.00)         | 0.00 (0.00)            |
| 0.5       | 0.00 (0.00)           | 0.00 (0.00)         | 0.00 (0.00)            |
| 0.6       | 0.00 (0.00)           | 0.00 (0.00)         | 0.00 (0.00)            |
| 0.7       | 0.00 (0.00)           | 0.00 (0.00)         | 0.00 (0.00)            |
| 0.8       | 0.00 (0.00)           | 0.00 (0.00)         | 0.00 (0.00)            |
| 0.9       | 0.00 (0.00)           | 0.00 (0.00)         | 0.00 (0.00)            |
| 1.0       | 0.00 (0.00)           | 0.00 (0.00)         | 0.00 (0.00)            |

N = 5  N = 5  N = 3
When the MAR index is >0.2 is suggestive of an area where drugs are taken indiscriminately. The Multiple Antibiotic Resistance (MAR) indicates areas where drugs were strongly abused with Salmonella spp having 60%. (Table 5).

4. CONCLUSION

In conclusion, the present study isolated E. coli, Klebsiella spp., Salmonella spp., Shigella spp. 5 and Pseudomonas spp from stool samples and these isolates exhibited multidrug resistance. This study also showed that antimicrobial resistance is still very much a paramount problem globally and the multidrug resistance exhibited in this study is a clear indication of overuse of antibiotics.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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