Original Research Article

Characterization of Carbapenem-resistant *Enterobacteriaceae* from Patients Stool in Tertiary Hospitals, Port Harcourt, Rivers State, Nigeria

ABSTRACT

Carbapenem-resistant Enterobacteriacaeae (CRE) are an important emerging threat to public health. Carbapenem are group of antibiotics that are usually reserved to treat serious infections particularly when these infections are caused by microorganisms that are highly resistant to antibiotics. The aim of this study was to Isolate, Characterize and Identify Carbapenem-resistant *Enterobacteriaceae* from Stool Samples of Patients in Two Tertiary Hospitals in Port Harcourt, Rivers State, Nigeria, Using Both Conventional and Molecular Methods. A Total of 114 feacal samples were collected and inoculated onto Eosine methylene blue (EMB) agar and MacConkey agar (M.A). Representative colonies were subjected to Biochemical Test for phenotypic characterization and identification, and subsequently antimicrobial susceptibility tests. These Enteric isolates were also subjected to molecular analysis for further characterization. In this study, 52 isolates of 114 stool samples of enteric organisms was identified; 32 were females while 20 were 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%).

Key words: Carbapenem-resistant, *Enterobacteriaceae*, Conventional and Molecular Methods

1. INTRODUCTION

Carbapenems are a group of antibiotics that are usually reserved to treat serious infections, particularly when these infections are caused by germs that are highly resistant to antibiotics [1]. Carbapenem is a type of ß-lactam, a family of diverse antibiotic molecules [2]. This family of antibiotics prevents the synthesis of the peptidoglycan layer of bacterial cell walls [3] they do this by binding to the active sites of penicillin-binding proteins, which are responsible for the final steps in peptidoglycan synthesis. This prevents the cross-linking of the peptidoglycan layer and thus prevents proper cell wall synthesis [4].

Carbapenem-resistant Enterobacteriaceae (CRE) or carbapenemase-producing coliforms are Gram-negative bacteria that are resistant to the carbapenem class of antibiotics, which are considered the drugs of last resort for such infections. They are resistant because they produce an enzyme called carbapenemase that disables the drug molecule. The resistance can vary from moderate to severe [5]. These microorganisms are found in normal human intestines (gut) [6].

Sometimes these bacteria can spread outside the gut and cause serious infections, such as urinary tract infections, bloodstream infections, wound infections, and pneumonia. *Klebsiella* sp and *E. coli* are common examples of Enterobacteriaceae, a normal flora of the human gut bacteria that can become carbapenem-resistant [7]. Resistance to carbapenems can be due to a few different mechanisms. One of the more common ways that *Enterobacteriaceae* become resistant to carbapenems is due to production of *Klebsiella pneumoniae* carbapenemase (KPC) [8]. *Klebsiella pneumoniae* carbapenemase (KPC) is an enzyme that is produced by some CRE that was first identified in the United States around 2001. *Klebsiella pneumoniae* carbapenemase (KPC) breaks down carbapenems making them ineffective. Other enzymes, in addition to KPC, can breakdown carbapenems and lead to the development of CRE, but they are uncommon in the United States [5].

Carbapenem-resistant Enterobacteriaceae (CRE) are more likely to affect those patients who have compromised immune systems or have invasive devices like tubes going into their body [9]. Use of certain types of antibiotics might also make it more likely for patients to get (CRE) Carbapenem-resistant Enterobacteriaceae. Patients who have already taken lots of antibiotics are more at risk of picking up CRE [10]. The reason for this is that the more bugs are exposed to antibiotics, the more likely they will develop 'resistance' to that antibiotic so that the antibiotic won't be effective any longer.

Carbapenems are a class of highly effective antibiotic agents commonly used for the treatment of severe or high-risk bacterial infections. This class of antibiotics is usually reserved for known or suspected multidrug-resistant (MDR) bacterial infections. Similar to penicillins and cephalosporins, carbapenems are members of the beta lactam class of antibiotics, which kill bacteria by binding to penicillin-binding proteins, thus inhibiting bacterial cell wall synthesis. However, these agents individually exhibit a broader spectrum of activity compared to most cephalosporins and penicillins. Furthermore, carbapenems are typically unaffected by emerging antibiotic resistance, even to other beta-lactams. Carbapenem antibiotics were originally developed at Merck & Co. from the carbapenem thienamycin, a naturally derived product of Streptomyces cattleya [11]. Tom Frieden, former head of the Centers for Disease Control and Prevention has referred to CRE as "nightmare bacteria". Types of CRE are sometimes known as KPC *(Klebsiella pneumoniae carbapenemase)* and NDM (New Delhi Metallo-beta-lactamase). *Klebsiella pneumoniae carbapenemase* (KPC) and NDM are enzymes that break down carbapenems and make them ineffective [12].

The increasingly resistant rate of patients with Carbapenem-resistance Enterobacteriaceae (CRE) in Nigeria is a major concern as patients with these infections die on a daily basis as a result of effective drugs or treatment to combat the deadly ailments [13], this could lead to higher morbidity or mortality rate.

The global spread of Carbapenem-resistance Enterobacteriaceae (CRE) especially *Klebsiella pneumoniae* carbapenemases (KPC) *Enterobacteriaceae* in the last decade represents a serious public health threat, since it leads to variable levels of carbapenem resistance and few therapeutic options remain available for treating such infections [14].

This study was carried to Characterize Carbapenem-resistant *Enterobacteriaceae* from Stool Samples of Patients in two Tertiary Hospitals in Port Harcourt, Rivers State, Nigeria, Using Both Conventional and Molecular Methods.

2. METHODOLOGY

2.1 Study Area

This research study was carried out in two tertiary hospitals-the University of Port Harcourt Teaching Hospital and Save a Life Mission Hospital. The hospitals are located in Rivers State, Nigeria. The study was for a period of three months between January, 2019 to March 2019. Faecal samples were collected using sterile faecal sample bottles. The sterile containers were properly numbered and labelled, and also transported in coolers containing ice pack to the microbiology laboratory in Rivers State University immediately after collection for analysis. A total of one hundred and fourteen (114) faecal samples were collected from (45) male and (69) female patients for a period of three months. Samples were analyzed immediately after collection.

2.2 Isolation of Enteric Organisms

Enteric organisms were Isolated with Eosin methylene blue (EMB) and MacConkey (MA) agar using streak plate method and incubated for 18 to 24 hrs at 37°C before they were examined for growth. Characteristics colonies were described and subcultured onto nutrient agar plates and incubated for another 24hours for pure cultures (for further tests) were then preserved in sterile 10% glycerol broth at 4°C. Gram stain reaction and biochemical tests Such as indole, catalase, methyl red, sugar fermentation and citrate tests were carried out according to Cheesbrough.

2.3 Antimicrobial Susceptibility Testing

Susceptibility testing was performed for all the identified clinical isolates according to the Clinical and Laboratory Standards Institute [15] guideline using the Kirby-Bauer disc method. Using sterile wire loop, colonies of the test organisms were emulsified into test-tubes containing 0.5 McFarland standard of normal saline [16]. In a good light, the turbidity of suspension was matched with the turbidity of 0.5McFarland prepared immediately before use. Using sterile swab, a plate of Mueller Hinton agar (Oxoid, UK) prepared to manufacturer's instructions, was inoculated with the test organism. Excess fluid was removed by pressing and rotating the swab against the side of the tube above the level of the suspension. The surface of the medium was streaked in three directions, rotating the plate approximately 360°C to ensure even distribution. With the petri-dish lid in place, 3-5 minutes was given to allow the surface of the agar to dry. Using sterile forceps, the respective antibiotic discs were placed onto the agar. Each disc was slightly pressed down to ensure its contact with the agar. Within 30 minutes of applying the discs, the plates were inverted and incubated at 37^{o}C for 18 to 24hrs. After overnight incubation, the test plates were examined. Using a ruler on the underside of the plate, the diameter of each zone of inhibition was measured in mm, the end point of inhibition. The MAR index which represents the number of antibiotics the isolates were resistant to were calculated as described by [17].

2.4 Molecular Identification

Molecular testing was further performed for all the identified clinical isolates resistant to the antibiotics used especially Carbapenem using DNA Extraction (Boiling method), DNA Quantification, 16S rRNA Amplification, Sequencing and Genotypic Characterization.

2.4.1 DNA Extraction (Boiling method)

Five milliliters of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) was spun at 14000rpm for 3 min. The cells were re-suspended in 500ul of normal saline and heated at 95^0C for 20 min. The heated bacterial suspension was cooled on ice and spun for 3 min at 14000rpm. The supernatant containing the DNA was transferred to a 1.5ml microcentrifuge tube and stored at -20^{o}C for other downstream reactions [18].

2.4.2 DNA Quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was lunched by double clicking on the Nanodrop icon. The equipment was initialized with 2ul of sterile distilled water and blanked using normal saline. Two microlitre 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 [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 a 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. The product was resolved on a 1% agarose gel at 130V for 30 minutes and visualized on a blue light transilluminator [18]

2.4.4 Sequencing 16S

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 10 µl, the components included 0.25 µl BigDye® terminator v1.1/v3.1, 2.25 µl of 5 x BigDye sequencing buffer, 10 µl 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 4mins [20]

2.4.5 Genotypic Characterization

Deoxyribonucleic Acid (DNA) was extracted using spin column method as per manufacturer's instructions. The purified DNA was put through multiplex PCR in a 20 µL total volume, in which, 5 µL was the primer mix (HELINI ready to use Gene Primer Mix), 10 µL was master mix (It contains 2U of Taq DNA polymerase, 10X Taq reaction buffer, 2 mM MgCl2, 1 µL of 10 mM dNTPs mix and Red Dye PCR additives), and 5 µL was purified bacterial DNA. Total volume was placed into PCR machine and was programmed as follows, Initial 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. Agarose gel electrophoresis was carried out where, PCR products were loaded after mixing with gel loading dye along with 10 µL HELINI 100 bp DNA Ladder as a size marker. We waited till the dye reaches three fourth distances of the gel during Electrophoresis at 50V and Gel was viewed in UV Transilluminator and the bands pattern was observed [18].

3. RESULTS AND DISCUSSION

The study examined 114 stool samples collected from patients which is composed of both male and female. Number of enteric isolates and their percentage occurrence is illustrated in Table 1. 52 isolates were identified which includes, *E. coli* 19 (36.5%), *Klebsiella spp.* 20 (38.5%), *Salmonella spp.* 5 (9.6%), *Shigella spp.* 5 (9.6%) and 3 (5.8%) *Pseudomonas spp*.

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 K2 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 K2 within the *Klebsiella spp.* and revealed a closely relatedness to *Klebsiella pneumoniae*, K1, K4, K3 and K5 were closely related to *Escherichia coli*, *Shigella flexeneri*, *Pseudomonas aeruginosa* and *Salmonella typhi* respectively (Figure1). From the antibiotic susceptibility results obtained in Table 2, *Salmonella spp* were (100%) resistant to Ceftazidime, Cefuroxime and Augmentin, followed by Cefixime (80%), Nitrofurantoin (60%) and meropenem (20%), while Gentamicin, Ofloxacin and Ciprofloxacin had the least with (0%) resistant each. Also, *Salmonella spp.* also showed (100%) susceptibility to Gentamicin and Ofloxacin, 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, Ofloxacin and Ciprofloxacin had the least with (0%) resistance each. On the contrary, Gentamicin, Ofloxacin 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].

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).

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

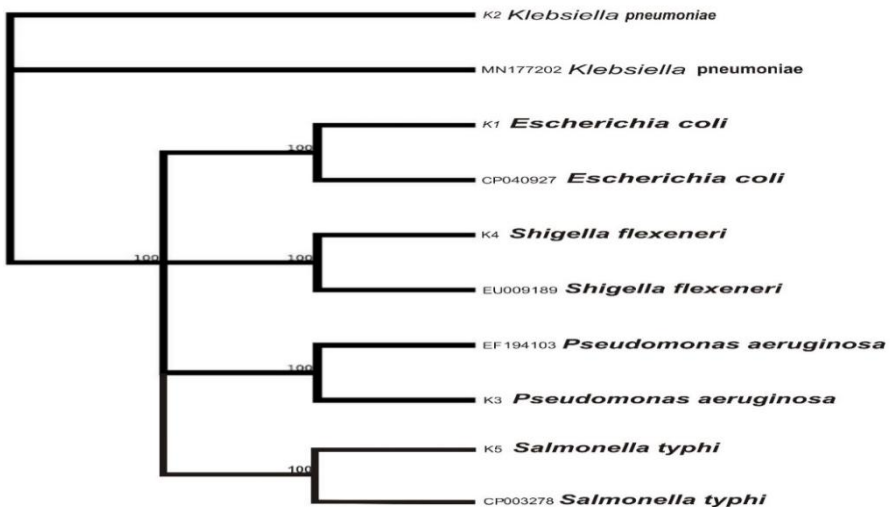

Figure 1: Phylogenic Tree/Evolutionary Relationship Between Bacterial Isolates

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)
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 4: Susceptibility Pattern of *Pseudomonas spp* to Various Antibiotics

Antibiotics (Conc.)	Resistant n (%)	Intermediate n (%)	Susceptible n (%)
Ceftazidime (30µg)	2 (66.7)	0 (0.00)	1 (33.3)
Cefuroxime (30µg)	3 (100.0)	0 (0.00)	0 (0.00)
Gentamicin (10µg)	0 (0.00)	0 (0.00)	3 (100.0)
Ofloxacin (5µg)	0 (0.00)	0 (0.00)	3(100.0)
Augmentin (30µg)	3 (100.0)	0 (0.00)	0 (0.00)
Cefixime (5µg)	3 (100.0)	0 (0.00)	0 (0.00)
Nitrofurantoin (300µg)	1 (33.3)	0 (0.00)	2 (66.7)
Ciprofloxacin (5µg)	0 (0.00)	0 (0.00)	3 (100.0)
Meropenem (10µg)	1 (33.3)	0 (0.00)	2 (66.7)

Table 5: Mar Index of Enteric Organisms Identified

MAR INDEX	*Salmonella spp.* (%)	*Shigella spp.* (%)	*Pseudomonas spp.* (%)
0.0			
0.1	0.00 (0.00)	1 (20.0)	1 (33.3)
0.2	2 (40.0)	4 (80.0)	2 (66.7)
0.3	3 (60.0)	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

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.

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