Carbapenemase Producers among Gram Negative Bacteria from Environmental and Clinical Samples in Makurdi, Nigeria

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Authors’ contributions

This work was carried out in collaboration between both authors. Author FBO designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author OEF Supervised the study. Both authors read and approved the final manuscript.

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ABSTRACT

Carbapenem-resistant Gram negative bacteria in healthcare setting is an important medical problem and a major threat. These Gram negative bacteria are rapidly spreading worldwide. Clinical bacteria isolates were obtained from clinical samples Urine, High Vaginal Swab (HVS), Wound Swab (WS), Stools, Ear Swab (ES), Endocervical Swab (ECS), Sputum and Blood, from two tertiary hospitals. Environmental samples were also collected from both hospitals using standard sampling techniques. Multiple drug resistant (MDR) patterns were determined using disc diffusion technique. Biochemical tests were used in the identification. Antibiotics sensitivity of clinical and environmental isolates was verified using disc diffusion method. Detection of carbapenemase producing bacteria was done using double disc synergy test and modified Hodge test. Data obtained were analysed using descriptive statistics. Clinical bacteria (403) obtained were distributed 271 Federal Medical Centre(FMC) and 132 Benue State University Teaching Hospital (BSUTH) out of which 299 were confirmed Gram negative, 218 (FMC) and 81 (BSUTH), respectively. Thirty-nine Gram negative bacteria were also isolated from the environmental samples. A higher percentage of carbapenemase producers (12.8%) were found in environmental samples.
isolates compared to 2.7% in clinical isolates. Any levels of resistance to carbapenems calls for the need to reduce the indiscriminate use of this group of antibiotics and monitoring both in the hospitals and environment.

Keywords: Gram negative bacteria; antibiotic resistance; carbapenemase.

1. INTRODUCTION

The emergence of resistant bacteria decades after the first patients were treated with antibiotics led to concerns on the efficacy of antibiotics even though its discovery transformed medicine and saved millions of lives [1]. The challenge has been traced to overuse and misuse of medications and slow development of new drugs by the pharmaceutical industries [2,3]. The Centre for Disease Control and Prevention (CDC) classified a number of bacteria based on urgent, serious, and concern they pose to human health leading to coordinated efforts in research to finding solutions to the menace [4,5].

In hospitals, antibiotics are widely used both to treat pre-existing bacterial infections and prevent infection of surgical incisions. Antibiotics can affect the normal, friendly bacterial populations in patients. The population dynamics of the flora changes by chance mutations of drug-resistant bacteria cells when they first entered the hospital. Antibiotic treatment eliminates most or all of the sensitive bacterial cells from these patients. Freed from competition with these sensitive strains, drug-resistant cells can rise to high frequency. For an individual patient, emergence of antibiotic resistance is bad news [6].

A patient with a resistant strain is also bad news for the other patients. Through no fault of her own, but as a result of cross infection which could occur through the hands of care givers and other sources, a hospitalized patient may not keep resistant strains to him/herself. Medical staffs often visit multiple patients without washing their hands, clothing and equipment [7]. As a result, healthcare workers often serve as vectors, carrying resistant strains from infected patients to patients who’s normal, drug sensitive flora have been killed by antibiotic treatment. The resistant strains do not encounter competitors in these flora-free patients, and easily establish new, resistant infections. Transmission of such resistant strains among hospitalized patients accounts for a large fraction of new resistant infections. Patients who would otherwise have recovered from surgery with very few complications sometimes acquire resistant infections that significantly prolong their stay in the hospital. The hospital patients carrying resistant bacteria sometimes transmit those resistant strains to family members which can lead to community infection.

The global spread of carbapenem-resistant Enterobacteriaceae (CRE) is a major challenge in clinical and public health settings. Infections with CRE organisms that are multidrug-resistant (that is, non-susceptible to at least one antimicrobial in at least three antimicrobial classes), extensively drug-resistant, or pan-drug-resistant are difficult to treat resulting in severe infections [8,9]. Examples of the carbapenems are, ertapenem, imipenem, meropenem, and doripenem used often as antimicrobials of last resort to treat infections due to extended-spectrum beta-lactamase (ESBL) or plasmid-mediated Amp C (pAmpC) - producing organisms of the Enterobacteriaceae family. Such pathogens are frequently also resistant to other antibiotic classes including quinolones, aminoglycosides, trimethoprim sulfamethoxazole, and other classes [10]. To ensure their containment, wide dissemination of information and robust multifaceted strategies involving microbiologists, clinicians, and decision makers are essential. Carbapenem-resistant Enterobacteriaceae (CRE) are a group of bacteria that have become resistant to nearly all available antibiotics, including carbapenems.

Phenotypic detection of carbapenemase production in bacteria is achieved by performing either; i) the modified Hodge test (MHT) [11,12,13,14]. Or ii) the modified carbapenem inactivation method (mCIM), a modification of the carbapenem inactivation method (CIM) [15,16,17]. These methods have not only been documented to be simple and cost effective but have also been documented to have high sensitivity [11,12,13,14]. Despite the high sensitivity of MHT, it has been linked to a high frequency of false-positive results especially in carbapenem resistant Enterobacteriaceae that are producers of ESBLs and AmpC β-lactamases and low sensitivity in regards the detection of NDM-1 producing bacteria [18,19]. In
addition to this, the use of E.coli ATCC 25922 has also been implicated with low sensitivity, specificity and repeatability of MHT [18]. However, replacing E. coli ATCC 25922 with Klebsiella pneumoniae ATCC 700603 has been shown to provide high sensitivity, specificity and repeatability of the test [20]. MHT has also been associated with inability to discriminate between the different classes of carbapenemases (i.e. Klebsiella pneumoniae carbapenemase, Metallo β-lactamase and Oxacillinases) and also difficulty in interpretation of results has been reported [21]. Despite these, MHT remains a phenotypic reference method for confirmation of carbapenemase production [21]. The aim of this study was to isolate and characterized β-lactam resistant Gram-negative bacteria from clinical, and environmental sources and to phenotypically determined carbapenemase- producers among them in two tertiary healthcare facilities.

2. MATERIALS AND METHODS

2.1 Sample Site/Collection

This study was conducted to study carbapenemases in Gram negative bacteria from wastewater, sediments and clinical bacterial isolates from two hospitals in Makurdi, Benue State, Nigeria. The two hospitals involved were Federal Medical Centre (FMC) and Benue State Teaching Hospital (BSUTH). Approval was obtained from the two hospitals before the commencement of the study. Ethical approval was obtained from the government of Benue State of Nigeria. Ministry of Health and Human Services with reference number MOH/STA/204/VOL.1/31. During the period of study, these samples were collected between 10 March and 3 June, 2016. Clinical isolates (Stock culture) were obtained from the laboratory benches of the Medical Microbiology Department and samples of wastewater and wastewater sediments were collected from sewers (gutters) from the two tertiary hospitals. Sites selected for the study were drains from various wards which includes; The theatre, Female surgical ward, Pediatric ward, Male and female medical ward, Amenity ward (ward block), Resident doctors hostel and cafeteria, Laboratory (Chemical pathology, Microbiology, Hematology and Histopathology), Administrative block, and Accident and Emergency ward (A and E) BSUTH, FMC samples site include Laboratory, A ward (Male ward 18 yrs and above), Gynecology ward, Theatre and Female ward. Samples were subcultured routinely onto slants prepared from nutrient agar.

2.2 Wastewater and Sediment Samples

Wastewater and sediment samples were collected in the month of September, 2016. The water samples were collected into sterile bottles from the various units aseptically by using disposable micro pipette at each collection unit. Samples were safely transported by road to the laboratory, and immediately analysed.

Sediments were collected by wearing gloves and using clean hand trowel from different wastewater sampling sites to scoop sediments from the bottom of the sewers and introduced into sterile Bijou bottles. The trowel was properly cleaned using alcohol (ethanol 75%) before using in another site to avoid contamination.

2.3 Clinical Bacterial Isolates

Clinical bacterial (Gram-negative multidrug resistant stock culture) isolates were collected from stocks from the laboratory benches of the Department of Medical Microbiology of the hospitals listed above. Collection of clinical isolates was done between 10 March and 3 June, 2016. Samples identities were confirmed using different laboratory synthetic media and biochemical tests. The clinical samples were from samples of body fluid (urine and blood samples), swab (high vaginal, endocervical, wound, ear and sputum samples) and stool samples.

2.4 Isolation of β-lactam Resistant Gram-negative Bacteria from Environmental Sources

Beta lactam resistant Gram negative bacteria were isolated from wastewater and wastewater sediments. This was done by supplementing Peptone water with Ampicillin antimicrobial susceptibility test disc 10 µg (Oxoid) as described by Liu et al. (2010). Stock solution of peptone water was prepared according to manufacturer instructions. 5 ml each was dispensed into an incubating bottle and sterilized at 121°C for 15 minutes and allowed to cool. The sterile ampicillin discs 10 µg was aseptically introduced into the sterile peptone water at 50°C to a final concentration of 60 µg/ml.
Water: For the wastewater samples, 1 ml was introduced into the sterile incubating bottles containing the sterile peptone water supplemented with ampicillin discs 60 µ/ml and incubated for 18-24 h at 37ºC.

Sediments: Serial dilutions were carried out with the sediment samples and 1 ml of 10⁻¹ diluent was introduced into the sterile peptone water supplemented with ampicillin discs 60 µ/ml and also incubated for 18-24 hours at 37ºC.

The 18-24 h incubated water and sediments samples above were subsequently streaked on MaConkey agar with the aid of sterile wire loop and incubated at 37ºC for 18-24 h. This was done for all the wastewater and sediments samples.

2.5 Selection for Carbapenems Resistant Bacteria Isolates Using Disc Diffusion Method

Antibiotic testing was carried out on both β-lactam resistant organisms selected on MaConkey agar and all the Gram negative confirmed clinical bacteria isolates collected from the two hospitals, Benue State University Teaching Hospital and Federal Medical Center Makurdi using the Kirby-Bauer method [22].

The 18-24 hours old culture of each isolate was introduced into a sterile test tube containing normal saline (5 ml) and its turbidity adjusted to match 0.5 MacFarland standards. Sterile cotton swab stick was dipped into the standardized bacterial test suspension and used to evenly inoculate the entire surface of a Mueller-Hinton agar plates (HIMEDIA, INDIA). After the agar surface had dried for about 5 minutes, Imipenem 10 µg, Ertapenem 10 µg, Ceftazidine 30 µg, Ceftriaxone 30 µg, and Ciprofloxacin 5 µg antibiotic discs (Oxoid) were placed on the inoculated plate using sterile forceps. The plates were allowed to remain on the bench for 1 hour for a period of pre-incubation diffusion and incubated at 35ºC. After 16-18 h of incubation, the diameters of the zones of inhibition were measured with meter rule and recorded in millimeter (mm). This was done for all the isolates selected.

The CLSI (2014) standard was used for the interpretation of the zone of inhibition of the selected antibiotic discs used.

2.6 Detection of Carbapenemase Producers Using Modified Hodge Test (MHT)

A typed culture E. coli ATCC 25922 of 0.5 McFarland was prepared. A dilution 1:10 was made by adding 0.5 ml of 0.5 McFarland E. coli suspension to 4.5 ml of sterile normal saline. Mueller-Hinton agar was prepared by dissolving 9.5 g in 250 ml of water and sterilized at 121ºC for 15 minutes. This was then poured into Petri dishes after cooling to about 50ºC. A sterile cotton swab stick was dipped into the 1: 10 dilution of E. coli ATCC 25922 suspension and used to evenly inoculate the entire surface of the Mueller-Hinton agar plate to make a lawn. A 10 µg ertapenem susceptible disc (Oxoid) was placed in the centre of the plate. In a straight line, 18-24 h old cultures of the test isolates (intermediate/total imipenem resistant from 3.3.3 isolates) were streaked from the edge of the disc to the edge of the plates. Four organisms were streaked on a plate containing one ertapenem disc.

The inoculated plates were then incubated overnight at 35ºC in an incubator for 16-24 h after which the plates were brought out and observed for clover leaf shape.

2.7 Statistical Analysis

Data obtained were subjected to frequencies and Chi-square analysis using IBM Statistical Package and Service Solution (SPSS) version 20. The level of significant was defined as p ≤ 0.05.

3. RESULTS

A total of 403 clinical isolates reportedly, multidrug Gram-negative bacteria were collected from two (2) tertiary hospitals; Federal Medical Centre (FMC), and Benue State Teaching Hospital (BSUTH). Two hundred and seventy one (271) Gram-negative bacteria from FMC, and 132 from BSUTH. Two hundred and ninety nine (299) isolates were confirmed to be Gram negative while 104 isolates were Gram positive. Thirty-nine Gram negative bacteria were isolated from the environmental sample.

Out of a total of 338 clinical and environmental Gram negative bacteria from the two hospitals, 56 isolates shown resistance/intermediate resistance to imipenem. Of these 13(23.21%) were positive for carbapenemase production.
Out of a total of 56 isolates comprising of both environmental and clinical isolates (showing resistance/intermediate resistance to imipenem) tested, a total of 13 (23.21%) were positive for carbapenemase production, 5/39 (12.82%) from environmental for both FMC and BSUTH and 8/299 (2.68%) from clinical samples also for both FMC and BSUTH. Percentage of carbapenemase producers is higher in the environmental samples compared to the clinical isolates. From the environmental samples, carbapenemase producers were isolated from both water and sediments samples but more from sediments while from the clinical samples, carbapenemase producers were from urine, ear swab, high virginal swab, and stool specimen.

Table 1. Source and place of isolation of the environmental bacteria isolates from FMC

| S/N | Place of isolation          | Isolates code | Sources | Gram negative bacterial isolated                     |
|-----|-----------------------------|---------------|---------|------------------------------------------------------|
| 1   | Laboratory department       | S1NLF         | Sediment| Providencia stuartii                                 |
| 2   | Male Ward (A Ward)          | SILF          | Sediment| Citrobacter diversus                                 |
| 3   | Gynaecology Ward            | WINLF         | Water   | Shigella sonnei                                      |
| 4   |                             | WILF          | Water   | Citrobacter diversus                                 |
| 5   |                             | S2NLF         | Sediment| Proteus mirabilis                                    |
| 6   |                             | S2LF          | Sediment| Escherichia coli                                     |
| 7   |                             | W2NLF         | Water   | Proteus vulgaris                                     |
| 8   |                             | W2LF          | Water   | Citrobacter diversus                                 |
| 9   |                             | S3NLF         | Sediment| Proteus mirabilis                                    |
| 10  |                             | S3LF          | Sediment| Citrobacter diversus                                 |
| 11  |                             | W3NLF         | Water   | Providencia stuartii                                 |
| 12  |                             | W3LF          | Water   | Klebsiella pneumoniae                                |
| 13  |                             | S4NLF         | Sediment| Proteus vulgaris                                     |
| 14  |                             | S4LF          | Sediment| Klebsiella pneumoniae                                |
| 15  |                             | W4NLF         | Water   | Serratia liquefaciens                                |
| 16  |                             | W4LF          | Water   | E. coli                                              |
| 17  |                             | S5NLF         | Sediment| Providencia stuartii                                 |
| 18  |                             | S5LF          | Sediment| Citrobacter koseri                                   |
| 19  |                             | W5NLF         | Water   | Proteus mirabilis                                    |
| 20  |                             | W5LF          | Water   | Erwinia chrysanthemi                                 |

Table 2. Source and place of isolation of the environmental bacteria isolates from BSUTH

| S/N | Place of isolation         | Isolates code | Sources | Gram negative bacterial isolated                     |
|-----|----------------------------|---------------|---------|------------------------------------------------------|
| 1   | Wards Block                | S1NLTH        | Sediment| Proteus vulgaris                                     |
| 2   |                            | S2LTH         | Sediment| E. coli                                              |
| 3   |                            | W2NLTH        | Water   | Proteus vulgaris                                     |
| 4   |                            | W2LTH         | Water   | Citrobacter diversus                                 |
| 5   | Residence Doctors Hostel/ Cafeteria | S2NLTH | Sediment | Proteus vulgaris                                     |
| 6   |                            | S2LTH         | Sediment| Citrobacter diversus                                 |
| 7   |                            | W2NLTH        | Water   | Proteus vulgaris                                     |
| 8   | Laboratories               | S3NLTH        | Sediment| Shigella sonnei                                      |
| 9   |                            | S3LTH         | Sediment| Citrobacter diversus                                 |
| 10  |                            | W3NLTH        | Water   | Proteus vulgaris                                     |
| 11  |                            | W3LTH         | Water   | Citrobacter diversus                                 |
| 12  | Administrative Block       | S4NLTH        | Sediment| Proteus vulgaris                                     |
| 13  |                            | S4LTH         | Sediment| Citrobacter diversus                                 |
| 14  |                            | W4NLTH        | Water   | Klebsiella pneumoniae                                |
| 15  | Accident And Emergency Ward | W4LTH | Water   | Proteus mirabilis                                    |
| 16  |                            | S5NLTH        | Sediment| Proteus vulgaris                                     |
| 17  |                            | S5LTH         | Sediment| Citrobacter freundii                                 |
| 18  |                            | W5NLTH        | Water   | Proteus vulgaris                                     |
| 19  |                            | W5LTH         | Water   | Citrobacter koseri                                   |
Table 3. Number/percentage of carbapenemase-producing gram negative bacteria from Environmental and clinical samples

| Total No of isolates | Antibiotics tested | No resistant | Carbapenemase producers No. (%) |
|----------------------|-------------------|--------------|--------------------------------|
| 338                  | Imipenem          | 56           | 13 (3.85)                      |

Table 4. Antibiogram of clinical carbapenemase positive isolates

| S/No. | Isolates no | Specimen types | IMP | ETP | CIP | CAZ | CRO | Organisms       |
|-------|-------------|----------------|-----|-----|-----|-----|-----|-----------------|
| 1     | 861         | Blood          | I   | I   | R   | R   | R   | E. coli         |
| 2     | 423         | Stool          | I   | R   | R   | R   | R   | E. coli         |
| 3     | Pr4         | Stool          | I   | R   | S   | R   | S   | Salmonella sp.  |
| 4     | 508         | HVS            | I   | I   | R   | R   | R   | E. coli         |
| 5     | MB50        | Urine          | R   | S   | R   | R   | I   | E. coli         |
| 6     | 467         | Urine          | I   | S   | S   | R   | S   | Klebsiella sp.  |
| 7     | 352(2)      | E/S            | I   | R   | S   | S   | R   | Pseudomonas sp. |

Key: CAZ (Ceftazidime), CRO (Ceftriaxone), CIP (Ciprofloxacin), ETP (Ertapenem), IMP (Imipenem), HVS (High Vagina Swab), E/S (Ear Swab)

Table 5. Antibiogram of environmental carbapenemase positive isolates

| S/No. | Isolates code | Specimen types | IMP | ETP | CIP | CAZ | CRO | Organisms          |
|-------|---------------|----------------|-----|-----|-----|-----|-----|-------------------|
| 1     | 1LF           | Sediment       | I   | R   | R   | R   | R   | Citrobacter diversus |
| 2     | 3NLF          | Sediment       | I   | R   | S   | S   | R   | Proteus mirabilis   |
| 3     | 5LF           | Sediment       | R   | R   | R   | R   | R   | Citrobacter koseri  |
| 4     | 5NLF          | Sediment       | I   | R   | R   | R   | R   | Proteus vulgaris    |
| 5     | 5NLT          | Water          | I   | R   | S   | S   | R   | Proteus vulgaris    |

Key: CAZ (Ceftazidime), CRO (Ceftriaxone), CIP (Ciprofloxacin), ETP (Ertapenem), IMP (Imipenem)

Table 6. Frequency and susceptibility profile of carbapenemase positive bacterial

| Carbapenemase positive gram negative bacteria | Susceptibility profile | Total |
|---------------------------------------------|------------------------|-------|
|                                             | S             | I     | R     |     |
| Citrobacter diversus                       | 0             | 1     | 4     | 5   |
| Citrobacter koseri                         | 0             | 0     | 5     | 5   |
| E. coli                                    | 2             | 6     | 17    | 25  |
| Klesiella pneumoniae                       | 3             | 1     | 1     | 5   |
| Proteus mirabilis                          | 2             | 1     | 2     | 5   |
| Pseudomonas sp.                            | 2             | 1     | 2     | 5   |
| Proteus vulgaris                           | 1             | 2     | 7     | 10  |
| Salmonella sp.                             | 2             | 1     | 2     | 5   |
| Total                                      | 12            | 13    | 40    | 65  |

\[ \chi^2 = 17.656, df = 14, p = 0.22 \] Key: S – Sensitive, I – Intermediate, R – Resistant

4. DISCUSSION

The overall percentage resistance of carbapenemase producers was 3.85%. This was close to the prevalence rate in USA and Lebanon with 4.20% and 2.50 respectively. But at variance with prevalence rate obtained from Germany, Jordan, Nepal and Colombia with 13.80%, 5.60%, 7.40% and 8.80% respectively [23,24,25,26,27,28]. This proportional variance could be attributed to the restrictions imposed on antibiotic use and the time each country started using carbapenems. Antimicrobial therapeutic protocols and practices vary from one hospital to the other, making comparisons and interpretations of carbapenem resistant variations a difficult task. Sample size, sample sources, time when the study took place, laboratory techniques used and other factors may contribute to the variable rates.
Table 7. Frequency of carbapenemase positive gram-negative bacterial to specific antibiotics

| Carbapenemase positive Gram negative bacterial | Ceftazi-dime | Ceftriazone | Ciprofloxacin | Ertapenem | Imipe-nem | Total |
|----------------------------------------------|-------------|-------------|--------------|-----------|-----------|-------|
| Citrobacter diversus                         | 1           | 1           | 1            | 1         | 1         | 5     |
| Citrobacter koseri                           | 1           | 1           | 1            | 1         | 1         | 5     |
| E. coli                                      | 5           | 5           | 5            | 5         | 5         | 25    |
| Klebsiella pneumoniae                        | 1           | 1           | 1            | 1         | 1         | 5     |
| Proteus mirabilis                            | 1           | 1           | 1            | 1         | 1         | 5     |
| Pseudomonas sp.                              | 1           | 1           | 1            | 1         | 1         | 5     |
| Proteus vulgaris                             | 2           | 2           | 2            | 2         | 2         | 10    |
| Salmonella sp.                               | 1           | 1           | 1            | 1         | 1         | 5     |
| **Total**                                    | **13**      | **13**      | **13**       | **13**    | **13**    | **65**|

\[ \chi^2 = 0.000, df = 28, p = 1.00 \]
Table 8. Multiple antibiotic resistant pattern of carbapenemase positive bacterial

| Number of antibiotic classes | MAR patterns         | No. (%)  |
|------------------------------|----------------------|----------|
| 2                            | CAZ, IMP             | 1 (7.69) |
| 3                            | CAZ, ETP, IMP        | 1 (7.69) |
|                              | CRO, ETP, IMP        | 2 (15.38)|
| 4                            | CAZ, CIP, CRO, IMP   | 1 (7.69) |
|                              | CAZ, CRO, ETP, IMP   | 2 (15.38)|
| 5                            | CAZ, CIP, CRO, ETP, IMP | 6 (46.15)|

Key: CAZ (Ceftazidime), CRO (Ceftriaxone), CIP (Ciprofloxacin), ETP (Ertapenem), IMP (Imipenem), MAR (Multiple Antibiotic Resistance)

E. coli was found to be the most resistant to carbapenems and other antibiotics. This varies from the finding of Rawan et al. (2018) who reported that Klebsiella sp. was found to be more resistant to carbapenems. Carbapenemase producing organisms display higher level of resistance to almost all antibiotics and multiple level of resistance to almost all antibiotics. This is also reported by Li and Nikaido. (2009). Multiple resistance increases patient treatment failures and mortality, and health care cost [29].

5. CONCLUSION

Any levels of resistance to carbapenems calls for the need to reduce the indiscriminate use of this group of antibiotics, and monitoring both in the hospitals and environment should be encouraged. Further work should be done using molecular techniques to check for specific resistant genes of the bacteria.

CONSENT

As per international standard or university standard, patient’s written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

Ethical approval was obtained from the government of Benue State of Nigeria. Ministry of Health and Human Services with reference number MOH/STA/204/VOL.1/31 Not applicable (Submission is not randomized controlled trial).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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