Molecular detection of AmpC-beta lactamase CITM gene in gram-negative bacterial isolates obtained from clinical specimens in a tertiary hospital in Nigeria.

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Abstract

In most developing countries, the β-lactam antibiotics are still the most predominantly prescribed antibiotics to treat bacterial infections. Resistance to third-generation cephalosporins is typical of multidrug resistance (MDRs), being mainly due to the production of AmpC-type β-lactamases. The aim of the study was to detect *bla*$_{CITM}$ gene in Gram-negative bacterial isolates obtained from clinical specimens in a tertiary hospital.

A total of 220 Gram-negative bacterial isolates obtained from clinical specimens were involved in the study. The primary screening of the *AmpC* enzyme-producing strains was conducted by the Kirby-Bauer disk diffusion method. Isolates that yielded a zone diameter ≤18 mm (screen positive) to cefoxitin sensitivity disc were further subjected to confirmatory tests using the AmpC disc test technique. Genomic DNA was extracted using Quick-DNA^{TM} Fungal/Bacterial Miniprep Kit; Zymo Research). Conventional Polymerase Chain Reaction (PCR) analysis was carried out in order to detect the presence of *bla*$_{CITM}$.

Out of 220 isolates, 45 (20.5%) were found to be resistant to cefoxitin (Screening test for AmpC β-lactamase) of which 10 (4.5%) were positive for AmpC β-lactamase (confirmatory positive by AmpC Disk Test). However, polymerase chain reaction (PCR) analysis revealed that none of the isolates harboured the bla$_{CITM}$ gene. The results from this study implied that not all cefoxitin resistant isolates are AmpC β-lactamase producers. The absence of bla$_{CITM}$ gene may be as a result of other enzymatic mechanism such as extended spectrum beta lactamases (ESBLs) and metallo beta-lactamase (MBL) or because of the non-enzymatic mechanism such as porin channel mutation.

Keywords: Cefoxitin, cephalosporins, resistant, β-lactamase

Introduction

AmpC β-lactamase genes exist in most Gram-negative bacteria and represent the main mechanism of resistance to broad-spectrum β-lactam antibiotics. Two large groups of Gram-negative bacteria (GNB) are responsible for most clinical isolates which include the *Enterobacteriaceae* and the non-fermenters; nevertheless, other clinically concerning Gram-negative organisms exist, including *Neisseria spp.*, *Haemophilus spp*., *Helicobacter pylori*, and *Chlamydia trachomatis* (Oliveira *et al*., 2021). Cephalosporins are antibiotics prescribed regularly for a wide variety of infections in Nigerian hospitals. The production of bla-AmpC enzymes by many Enterobacteriaceae and other Gram-negative bacteria confers resistance to such class of antibiotics. These organisms have a range of mechanisms to prevent the action of many antimicrobials used in clinical medicine.

Antimicrobial resistance in GNB arises from the expression of antibiotic-inactivating enzymes and non-enzymatic mechanisms. Both may be intrinsically expressed by a given species (chromosomal genes), or acquired by a subset of strains as a consequence of two distinct albeit not mutually exclusive genetic events; they involve mutations in chromosomal genes (such as increase in the expression of intrinsic resistance mechanisms (either antibiotic-inactivating enzymes or efflux pumps), permeability alterations by loss of outer membrane porins, or target modifications (Ruppe *et al.*, 2015; Breijyeh *et al.*, 2020). Secondly, the horizontal transfers of mobile genetic elements (MGEs) carrying resistance genes, most notably plasmid-encoding beta-lactamases, aminoglycosides-modifying enzymes (AMEs), or non-enzymatic mechanisms such as Qnr (Plasmid-borne quinolone resistance gene) for fluoroquinolone (FQ) resistance in Enterobacteriaceae. Since these plasmids commonly bear multiple resistance determinants, a single plasmid conjugation may suffice to confer a multidrug resistance phenotype to the recipient strain (Ruppe *et al*., 2015; Breijyeh, *et al*., 2020). However, the most common mechanism of bacterial resistance among the GNB is the production of β-lactamases; enzymes that cleave the structural β-lactam ring of these drugs (Shivanna *et al*., 2014). In other words, resistance to β -lactams is attributable to the production of β-lactamases, those hydrolytic enzymes which are able to inactivate the antibiotics before they reach penicillin binding proteins (PBPs) located

at the cytoplasmic membrane of Gram-negative bacteria (Aryal *et al*., 2020).

Again, AmpC β-lactamase genes, especially *MOXM, CITM, DHAM, EBCM, FOXM* and *ACCM* are responsible for the development of broad-spectrum resistance to most of the β-lactams (other than cefepime and carbapenems). The genes encoding AmpC β-lactamase have spread extensively and are widely detected in bacterial plasmids. The first plasmid-encoded AmpC variant was first identified in 1989 from *Klebsiella pneumoniae* isolated in South Korea. It was named CMY-1 because of its phenotypic trait associated with cephamycinase and was notoriously resistant to cefoxitin (Philippon *et al*., 2002). In a short span of time many families of plasmid-mediated AmpC variants were detected, predominantly from the isolates of *K. pneumoniae* and *E. coli*. Bacteria possessing plasmid-mediated AmpC genotypes were attributed on the basis of homology in nucleic acid sequence, forming a larger number of bacterial genera which acted as a source of these plasmids and a number of AmpC families. To date, AmpC families have been reported globally (Jacoby, 2009).

The resistance of microbes to antimicrobial agents is a global phenomenon spanning from one country to another and antibiotic-resistant bacteria knows no border of any nation owing to the increased globalization and free movement of people today which conveys these microbes from one place to another. It represents a major and serious problem in the health sector, these makes the antibiotic selection for treatment difficult. Some of these organisms are multidrug resistant in nature – showing resistance to a wide variety of antibiotics used in clinical medicine (Iroha *et al*., 2008). In Nigeria, most antibiotics are available over the counter and can be dispensed without clinician's prescription. Excessive dispensing, abuse and irresponsible use of these antibiotics has resulted in the development of resistant strains. On the other hand, indiscriminate use of antibiotics, poor hygienic practices and lack of monitoring of microbial drug resistance in hospitals have created suitable conditions for the emergence and uncontrollable spread of the Amp C enzymes, thus making their detection complicated due to the variable affinity of these enzymes for different substrates (Yusuf *et al*., 2013).

Furthermore, Gram-negative bacteria have created an urgent and very important need. Although efforts are made to curb the growth and spread of drug-resistant pathogens, the studies on AmpC β-lactamases in resource-limited settings are

still inadequate. Several studies on the detection of AmpC beta-lactamases have been done worldwide including in Egypt (El-Hady and Adel, 2015;); Iran (Mirsalehian *et al*., 2014), There are limited numbers of studies on AmpC β-lactamases enzymes in Gram-negative bacteria in Nigeria - Ejikeugwu *et al*., 2017; Ogefere *et al*., 2016; Ejikeugwu *et al*., 2016; Yusuf *et al*., 2013 and so on, but most of them have concentrated on the phenotypic detection of AmpC beta-lactamases, with paucity of data in the detection of the genes from clinical specimens. One study conducted in Benin reported 15.23% of Gram-negative bacteria isolates as AmpC β-lactamases producers using phenotypic method (Ogefere *et al*., 2016). The prevalence of AmpC β-lactamases in this study was higher than that reported in Kano (10%) (Yusuf *et al*., 2013).

AmpC β-lactamases are difficult to identify by phenotypic tests alone and are often falsely detected as Extended Spectrum β-lactamases (ESBL) in clinical laboratories. Enterobacterales isolates which are positive in screening test of ESBL phenotype but negative on confirmatory assay are usually considered as potential AmpC β-lactamases-producers either conferred by chromosomal depression or plasmid transfer (Yu *et al*., 2006). Techniques to identify AmpC beta-lactamase-producing isolates are available but are still evolving and are not yet optimized for the clinical laboratory, which probably now underestimates this resistance mechanism. With the world-wide increase in the occurrence, types and rate of dissemination of these enzymes, their early detection is critical. Their accurate detection and characterization are important from epidemiological, clinical, laboratory, and infection control point of view.

Hence, the most important step in coping with increasing antimicrobial resistance (AMR) is the precise detection of pathogenic (and/or resistant) strains in diagnostic laboratories (Aryal *et al*., 2020) by isoelectric focusing and genotypic characterization which are considered gold standards as the results with the phenotypic tests can be ambiguous and unreliable (Akujobi *et al*., 2012). The detection of AmpC production in bacterial pathogens is important for ensuring effective antibiotic therapy. And the data obtained from this study will provide a sound epidemiological data to the clinicians in handling Cephalosporin resistance in order to improve the clinical management of patients suffering from infections.

Materials and methods

Study area

This study was carried out in Nnamdi Azikiwe University Teaching Hospital (NAUTH), Nnewi, a tertiary hospital in Anambra State, and a major referral center in South East Nigeria. Ethical approval was obtained from the Nnamdi Azikiwe University Teaching Hospital Research and Ethics Committee (NAUTH-REC) to allow for the collection of isolates from the NAUTH Medical Laboratory Department.

The approval number from NAUTH was NAUTH/CS/66/VOL.15/VER.3/120/2022/098.

Sample collection and identification of bacterial isolates

A total of 220 Gram-negative bacteria isolates was obtained from various clinical samples sent to the Medical Microbiology Laboratory of Nnamdi Azikiwe University Teaching Hospital (NAUTH). Discrete colonies of the isolates were sub-cultured onto freshly prepared MacConkey agar and incubated at 35^{o}C for 24 hours. After 24 hours, the sub-cultured isolates were identified on the basis of their colonial characteristics and morphology, oxidase test and other biochemical tests using the semi-automated Microbact 12A Gram negative bacilli identification system for both Enterobacteriaceae and other Gram-negative bacteria. This was done according to Manufacturer's (Oxoid) instruction. The emerging discrete colonies were then stored into cryotubes of brain heart infusion in glycerol broth (containing nutrients needed for their survival) before further processing.

These clinical isolates were collected from various clinical specimens and hospital wards of Nnamdi Azikiwe University Teaching Hospital (NAUTH), Anambra, Nigeria over a period of one month including; wound (22), HVS (14), sputum (2), urine (168), stool (10), ear swab (3) and semen (1).

Antimicrobial susceptibility testing

Antimicrobial susceptibility tests were performed on all Gram-negative isolates according to Clinical Laboratory Standard Institute guideline directives (CLSI, 2019). The results were expressed as sensitive, intermediate or resistant developed by CLSI. The commercially available antibiotic discs were used: ceftazidime (CAZ; 30 µg), gentamicin (CN; 10 µg), cefoxitin (FOX; 30 µg), ciprofloxacin (CIP; 5 µg), meropenem (MEM; 10 µg), ceftriaxone (CRO; 30 µg), cefotaxime (CTX; 30 µg), ofloxacin (OFX; 30 µg), and amikacin (AK; 10 µg), all from Oxoid Ltd

Basingstoke, UK. A lawn of a bacterial inoculum equivalent to 1.5 X 108 CFU/ml, was made on the surface of a Mueller-Hinton agar plate(s) using a sterile swab stick. Antibiotics were then placed on the lawn, and the plates incubated aerobically at 35-37ºC for 16-18 hours. The inhibition zone diameter (IZD) of each antibiotic produced by the Gram-negative bacteria isolates were measured in millimeters (mm), and were considered as sensitive, intermediate or resistant to the test antibiotics based on the document's breakpoint guidelines of the CLSI standard interpretive criteria (CLSI, 2019). The test was controlled using control strain of E. coli ATCC®* 25922.

Phenotypic determination and confirmation of ampc beta-lactamases

Isolates that yielded a zone diameter ≤18 mm (screen positive) to cefoxitin sensitivity disc were further subjected to confirmatory tests using the AmpC disc test technique as described by black *et al*., 2005. A lawn culture of E. coli ATCC 25922 was prepared on MHA plate. Sterile disks (6 mm) were moistened with sterile saline (20 µl) and inoculated with several colonies of test organism. The inoculated disk was then placed beside a cefoxitin disk (almost touching) on the inoculated plate. The plates were incubated overnight at 35°C. A positive test appeared as a flattening or indentation of the cefoxitin inhibition zone in the vicinity of the test disk. A negative test had an undistorted zone.

Molecular detection of *bla*$_{\text{CITM}}$

All the Gram-negative bacteria isolates that was positive for AmpC production phenotypically were subjected to Polymerase Chain Reaction (PCR) in order to detect the presence of the *bla*$_{\text{CITM}}$ in AmpC beta-lactamases-producing Gram-negative bacteria isolates. Each of the isolates was inoculated from nutrient agar plates (Oxoid, UK) into 5 mL of peptone water (Oxoid, UK). The inoculated broths were incubated at 30ºC for 18–24 hours with mild shaking. Bacterial cells from the overnight broth culture were aseptically harvested by centrifugation at 5000 rpm for 5 minutes, and the supernatant was decanted. The pellets collected at the bottom of the tube were resuspended in 100 µL of Tris-EDTA (TE) buffer. The cells in the TE buffer tubes were lysed by heating at 100 oC for 10 minutes in a water bath. Finally, the solution was centrifuged at 5000 rpm for 5 minutes (to remove cellular debris). The supernatant was used as the DNA template source for gene amplification. Conventional PCR amplification of AmpC β-lactamase genes from the test isolates was carried out using

primers synthesized and supplied by Inqaba Biotechnical Industries Ltd., South Africa, in an Eppendorf nexus gradient Mastercycler (Germany) with a final PCR mixture. Amplification included initial denaturation of DNA at 94^{o}C for 5 minutes, followed by 32 cycles of DNA denaturation at 95^{o}C for 30 seconds, annealing at 54^{o}C for 1 minute, and extension at 72^{o}C for 2 minutes.

To determine the isolates possessing the *bla*$_{CITM}$ AmpC β-lactamase genes, primers for CITM genes were used: CITM-Forward (TGG CCA GAA CTG ACA GGC AAA) and CITM-Reverse (TTT CTC CTG AAC GTG GCT GGC) (Inqaba Biotechnology Industry Ltd, South Africa). The amplified products were visualized by using gel-electrophoresis in 1.5% agarose gel stained with 0.1µL ethidium bromide. Gel electrophoresis of the PCR products was carried out using 1.5% agarose gel (Inqaba Biotechnical Industries Ltd, South Africa) for 2 hours at 80 V. The gel bands were observed under UV gel Transillunator at 280nm and the amplified bands were visualized alongside a 100bp DNA ladder which was used as molecular weight marker to confirm the size of the AmpC gene and photographed. (Wang *et al*., 2011). The expected amplicon size is 465bp (Aryal *et al*., 2020).

Quality control

A standard aseptic procedure was adopted for the procedures in this study. All batches of the culture media and chemical reagents were processed with aseptic techniques following CLSI guidelines.

Data presentation

The data obtained during the course of this study were computed properly using simple descriptive statistics, percentiles and qualitative analysis. Results were presented on tables and figures as appropriate.

Results

A total of 220 Gram-negative bacterial isolates were obtained from the NAUTH medical Microbiology Laboratory and *Escherichia coli* (41.8%) was the predominant organism followed by *Klebsiella pneumoniae* 18.2%, amongst others. Of the distribution of the organisms, 63/220 (28.6 %) of the specimens were received from the general outpatient department (GOPD), followed by 12.7% (28/220) from the Surgical outpatient unit (SOP), 11.8% (26/220) from the medical outpatient (MOP), 10% (22/220) from the male surgical ward etc.

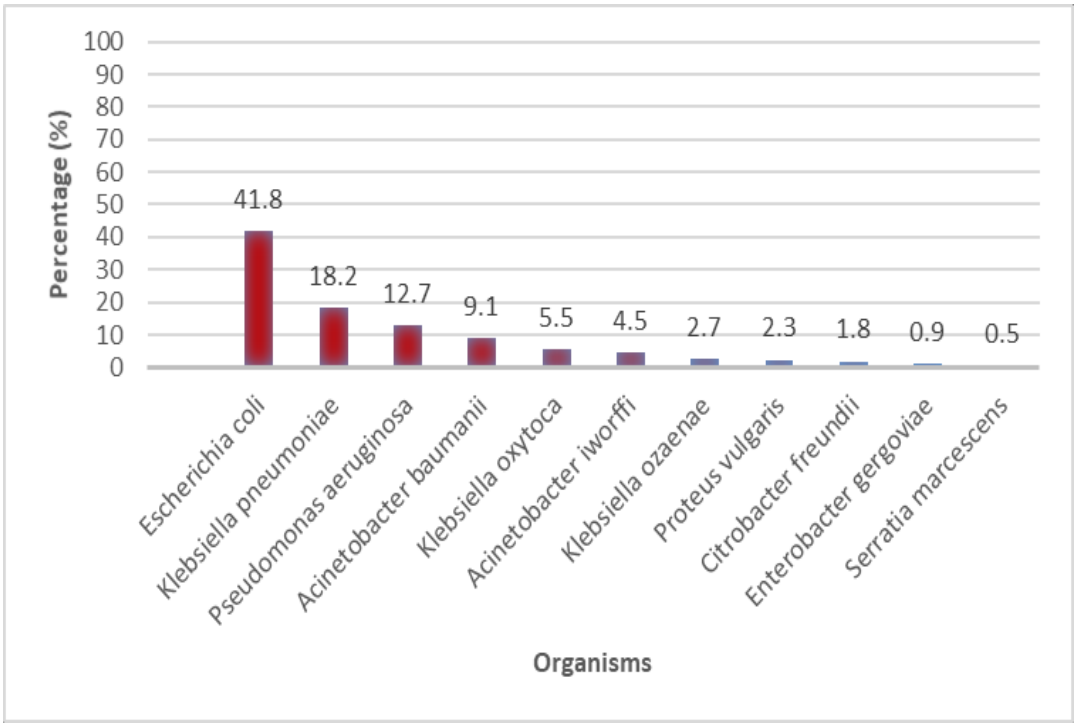

Figure 1: Distribution of the Gram-negative bacteria isolates from the total sample population.

Out of 220 Gram-negative bacteria isolates screened using Cefoxitin sensitivity disc (table 1), 45 isolates were resistant of which a confirmatory test was done using an AmpC disk. Table 2 showed that out of 45 isolates, only 10 showed positive AmpC i.e a flattening or indentation of the cefoxitin inhibition zone thus phenotypically confirming them AmpC Beta-lactamase producers. Therefore, the overall prevalence of AmpC β-lactamase was 4.5%. Highest prevalence was found among *Escherichia coli*.

Table 1: Cefoxitin sensitivity profile of the Gram-negative bacterial isolates tested

Class of Antibiotic used	**Generic name of antibiotic used and disc content**	**AST Profile (%)**		
		S	**I**	**R**
2nd Generation Cephalosporins	Cefoxitin 30ug	143(65.0)	32(14.5)	45(20.5)

S; Susceptible, I; Intermediate, R; Resistant

Table 2: Prevalence of phenotypically AmpC beta-lactamase producers at NAUTH.

Isolates	No. of isolates (%)	No. resistant to cefoxitin (%)	Positive AmpC (%) at phenotypic level
Escherichia coli	92(41.8)	12(26.7)	3(30.0)
Klebsiella pneumoniae	40(18.2)	15(33.3)	1(10.0)
Pseudomonas aeruginosa	28(12.7)	5(11.1)	1(10.0)
Acinetobacter baumanii	20(9.1)	2(4.4)	0(0.0)
Klebsiella oxytoca	12(5.5)	2(4.4)	1(10.0)
Acinetobacter iworffi	10(4.5)	3(6.7)	1(10.0)
Klebsiella ozaenae	6(2.7)	1(2.2)	0(0.0)
Citrobacter freundii	4(1.8)	2(4.4)	1(10.0)
Enterobacter gergoviae	2(0.9)	2(4.4)	1(10.0)
Proteus vulgaris	5(2.3)	0(0.0)	0(0.0)
Serratia marcescens	1(0.5)	1(0.0)	1(10.0)
Total	**220(100.0)**	**45(100.0)**	**10(100.0)**

All 10 isolates which were phenotypically positive for AmpC were negative for the CITM gene expression. This implies that they did not habour the CITM gene, and the resistance must have been attributed to other mechanisms of resistance.

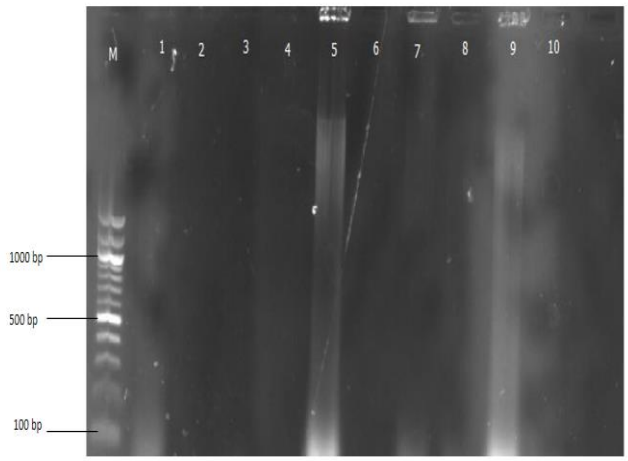

Figure 1: Agarose gel electrophoresis showing amplified CITM genes from the bacterial isolates (AMP1-AMP10)

Lanes 1 to 10 showed no bands. M represents the DNA Marker/Ladder.

Discussion

In the study, the predominant organism isolated was *E. coli* (41.8%) followed by *Klebsiella spp.* (23.7%). This was similar to findings observed in two tertiary hospitals in Southern Asia- (Akhter, 2015 and Upreti *et al*., 2018). This also agrees with a study done by Akujobi *et al*., (2010), who reported that. *E. coli* and *Klebsiella sp.* were the most frequently isolated Gram-negative organisms from clinical specimens especially from urogenital tract. However, it is evident that most authors found *E. coli* as the predominant organism. This could be attributed to the ubiquitous nature of the organism, being part of the human intestinal flora and having the ability to survive easily in community and hospital environment.

The antibiotic susceptibility patterns of the isolated organisms were interpreted using the Clinical and Laboratory Standard Institute (CLSI, 2017) interpretive criteria break points. Gram-negative bacteria showed high resistance rates to several classes of antibiotics. Based on cephalosporins resistance in this study, the isolates were highly resistant to the third (3rd) generation cephalosporins; ceftazidime 102 (46.4%) and cefotaxime 98 (44.5%). The high prevalence of cephalosporins resistance among clinical bacterial isolates reported by the present study agrees with the findings of Mofolorunsho et al. (2021). This high prevalence can be attributed to improper diagnosis and misuse of antibiotics in the study area. Also, it entails that there is emergence of cephalosporinases among resistant strains of these organisms as previously reported by Akujobi and Ewuru (2010). This calls for attention of the infection and control unit to review the antibiotics policy used in NAUTH, to avoid total resistance to the available antibiotics. The essence of epidemiological surveillance of bacterial infection and bacterial resistance to existing antibiotics is to create awareness and strengthen the implementation of infection prevention and control (IPC) strategies.

The prevalence of AmpC β-lactamase observed in this study was slightly higher than 3.3% reported by Ratna *et al*., (2003) and lower when compared to previous studies in Kano, Yusuf *et al*., (2013) at 10%. Molecular investigation and characterization for bla_{CITM} gene among the 10 phenotypic AmpC positive isolates revealed that none of the isolates produced

bla_{CITM} gene, which indicates that the resistance is due to other form of resistance or that there has not been dissemination of this gene in Nnamdi Azikiwe University Teaching Hospital, Nigeria. This is important for further studies by researchers (especially the public hospitals) to assess the degree of multi-drug resistances to our commonly prescribed antibiotics.

Conclusion

The results from this study implied that AmpC production is moderately on the increase in Nnamdi Azikiwe University Teaching Hospital (NAUTH), Nigeria and that the spread of these resistance conferring enzymes among bacterial isolates is an issue of public health concern. Health care practitioners are therefore advised to be careful on the use and abuse of antimicrobials to minimize the spread.

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Conflict of interest

The authors declare no conflict of interest.

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