

First Report Occurrence of *CIT* and *DHA*AmpC β -lactamase gene in *Escherichia coli* and *Klebsiella pneumoniae* from clinical sample in south eastern, Nigeria.

ABSTRACT

Background and Objectives:

AmpC β -lactamases have gained importance over the years, since their discovery as one of the enzymes responsible for antimicrobial resistance in Gram-negative bacilli especially in *Escherichia coli* and *Klebsiella pneumoniae*, it has led to a significant challenge to patient care. Hence, this study was designed to assess the Occurrence of *CIT* and *DHA*AmpC β -lactamase gene in *Escherichia coli* and *Klebsiella pneumoniae* from clinical sample in south eastern, Nigeria

Methodology:

This cross-sectional study was performed during period of 8-months on sixteen (16) consecutive non-repetitive isolates of *Escherichia coli* and *Klebsiella pneumoniae* obtained from diagnostic laboratories and medical unit of Alex Ekweume Federal University Teaching Hospital, Abakaliki in southeastern Nigeria. Isolates were accurately identified through routine microbiological diagnostic tests. The bacteria were first tested for cefoxitin resistance using a disc diffusion test, followed by phenotypic confirmation of AmpC production using phenyl boronic acid assays. *Escherichia coli* and *Klebsiella pneumoniae* strains were further screen for AmpC β -lactamase *CIT* and *DHA* genotype by polymerase chain reaction.

Result: Of the sixteen (16) confirmed phenotypic AmpC β -lactamase producing bacteria, 100 % of the following AmpC β -lactamase genes (*blaDHA*, *blaCIT*) were detected in *E. coli* from urine and wound samples of both male and female patients. The overall proportion of AmpC β -lactamases gene in *Klebsiella pneumoniae* were *blaDHA*(100 %) and *blaCIT*(100 %), in both male and female.

Conclusion: This study indicate the occurrence of *CIT* and *DHA*AmpC genotype. The AmpC β -lactamases found in this study are clinically relevant β -lactamases because they can confer a MDR phenotype. Thus awareness of the occurrence of AmpC β -lactamase-producing bacteria could be very valuable for achieving more accurate epidemiological results, as well as controlling their spread while surveillance is needed to follow any further emergence of other AmpC β -lactamase genotype

Keyword: AmpC beta-lactamase, *Escherichia coli*, *Klebsiella pneumoniae*, *CIT*, *DHA*

1. INTRODUCTION

Escherichia coli and *Klebsiella pneumoniae* are one of the bacteria causing infections in the *Enterobacteriaceae* family. This family's two critical genera are associated with both opportunistic nosocomial pathogens. These two genera, especially *E. coli* and *Klebsiella pneumoniae*, are opportunistic pathogens that cause septicemia, bacteremia, infantile enteritis, meningitis, and urinary tract infections of soft tissues. These human pathogens are responsible for infections and diseases in hospitalized patients with immunodeficiency and underlying conditions such as diabetes mellitus and chronic pulmonary disorders [1, 2]. However, the widespread use of antibiotics poses a selective pressure leading to the selection of resistant bacteria and has dramatically increased over the years endangering the lives of many patients. A recent systematic review identified an increase in the worldwide prevalence of AmpC β -lactamase in the Gram negative organisms *Escherichia coli* and *Klebsiella pneumoniae* over the past ten years [3]. Some *Escherichia coli* and *Klebsiella pneumoniae* are capable of producing enzymes called AmpC β -lactamase, characterized by their ability to inactivate cephalosporins in addition to other extended-spectrum cephalosporins and being resistant to clavulanic acid [4]. Infections caused by AmpC-positive bacteria are of particular clinical and epidemiological importance and cause higher patient morbidity and mortality [2, 5, 6]. According to previous reports in Bahrain, India, Pakistan, Korea, and Spain, the majority of the strains with AmpC genotype are generally multidrug resistant [7, 8, 9, 10]. AmpC β -lactamase gene are encoded by chromosomes or plasmids and affect a wide range of β -lactam drugs. Based on the sequence similarities with species-specific AmpC enzymes, plasmid AmpC variants are classified into five evolutionary groups: the CIT variants (CMY-2 types) originating in *Citrobacter freundii*, the *Enterobacter* sp. EBC variants (ACT-1 type, MIR-1), the *Morganella morganii* DHA variants, the *Hafnia alvei* ACC variants, and the *Aeromonas* sp. FOX and MOX variants, one of

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which is widely expressed in each geographical region [2, 6, 10, 11, 12] but the knowledge of some AmpC beta-lactamase genotype circulating this area remain unknown. Among these, ACC, FOX, MOX, DHA, CMY, CIT, and EBC genotypes are most commonly reported elsewhere [2, 610]. To date, no clinical research on the occurrence of AmpC beta-lactamase genotype have been conducted in Abakaliki, South eastern, Nigeria. Accurate detection of AmpC genotype (*CIT* and *DHA*) is not only critical for proper health management of patients suffering from *Escherichia coli* and *Klebsiella pneumoniae* infections but also useful for epidemiological analyses of the geographical distribution of AmpC genes.

2. MATERIALS AND METHOD

2.1 Bacterial Isolation

The study received Ethical review board approval (number SMOH/ERC/042/21) from the ethics committee of the Ebonyi State Ministry of Health, Abakaliki and conducted in accordance with the Declaration of Helsinki [13, 14].

This cross-sectional study was performed during period of 8-months on sixteen (16) consecutive non-repetitive isolates of *Escherichia coli* and *Klebsiella pneumoniae* obtained from diagnostic laboratories and medical unit of Alex Ekwueme Federal University Teaching Hospital, Abakaliki in southeastern Nigeria and it located in 6.32°N latitude and 8.12°E and longitude and is situated at an elevation of 117 meters above sea level. Isolates were accurately identified through routine microbiological diagnostic tests [15, 16]. The confirmed isolates were stored in a brain-heart infusion broth (Merck Co. Germany) containing 20% glycerol at -70°C until further test [15].

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2.2 Screening and Confirmatory Testing of AmpC Production

All isolates were first screened for the probable production of AmpC beta-lactamases by placing a cefoxitin disk (30 µg) on Mueller-Hinton agar (Merck Co., Germany) [17]. Isolates that conferred an inhibition zone diameter of <18 mm were considered potential producers of AmpC and subjected to the confirmatory phenotypic test. Screening-positive AmpC producers were confirmed by an inhibitor-based method on a disk containing boronic acid [18]. Briefly, a lawn culture of the tested isolates was made on a Mueller-Hinton agar plate using 0.5 McFarland solutions. Two disks of cefoxitin (30 µg) with and without phenylboronic acid (400 µg) was placed onto the agar surface and the results were interpreted. If the growth inhibition zone around the antibiotic with phenylboronic acid was 5 mm or greater when compared to the disk containing only cefoxitin, the isolate was considered an AmpC producer [17, 18].

2.3 Bacterial DNA extraction and polymerase chain reaction

The bacterial DNA of phenotypic confirmed AmpC beta-lactamase producing *E. coli* and *K. pneumoniae* were extracted from pure overnight cultures using the ZR fungal/bacterial DNA MiniPrep kit [14, 19]. The AmpC-beta-lactamase encoding genes (*CIT*, and *DHA*) were amplified individually on a SimpliAmp™ thermal cycler (Applied Biosystems, Foster City, CA) using the specific primers (Invitrogen, U.S. A) for *CIT* gene F-TGGCCAGAACTGACAGGCAAA; R-TTTCTCCTGAACGTGGCTGGC; *DHA* gene; F-AACTTTCACAGGTGTGCTGGGT; R-CCGTACGCATACTGGCTTTC [20]. The amplification reaction was performed in a final volume of 20 µL containing Master Mix (Bioneer, South Korea), primers at concentrations of 10 pM, 50–100 ng of extracted DNA templates, and ddH₂O. The PCR conditions for the amplifications were as follows, 5 min at 95°C for the initial denaturation step; 30 cycles of 30 sec at 95°C for DNA denaturation, 30 sec for primer annealing). The temperature depended on the sequences of primers (primer extension at 72 °C for 1 min and a final extension of 5 min at 72°C. The PCR products were separated on 1.5% agarose gel prepared in 1X TBE (Tris/Boric/EDTA) buffer and visualized under UV transilluminator using a gel documentation system.

Comment [13]: Authors should use controls while PCR. Without controls results obtained cannot be considered accurate. Since you are telling 100% results have been obtained with Amp and CIT genes.

3. RESULT

3.1 Occurrence of AmpC beta-lactamase genes in isolates of *E. coli* and *K. pneumoniae*

Of the sixteen (16) confirmed phenotypic AmpC beta-lactamase producing bacteria, 100 % of the following AmpC beta-lactamase genes (*blaDHA*, *blaCIT*) were detected in *E. coli* from urine and wound samples of both male and female patients as shown in Table 1. The overall proportion of AmpC beta-lactamases gene were *blaDHA* (100 %) and *blaCIT* (100 %), in both male and female as shown in Table 2.

Table 1: Occurrence of AmpC beta-lactamase genes in isolates of *E. coli*

Clinical Sample	Gender	Bacteria coding	<i>blaDHA</i> (%)	<i>blaCIT</i> (%)
Urine	Male	E1	1(12.5)	1(12.5)
		E2	1(12.5)	1(12.5)

	Female	E3	1(12.5)	1(12.5)
		E4	1(12.5)	1(12.5)
Wound swab	Male	E5	1(12.5)	1(12.5)
		E6	1(12.5)	1(12.5)
	Female	E7	1(12.5)	1(12.5)
		E8	1(12.5)	1(12.5)
Total		(n=8)	8(100)	8(100)

Table 2: Occurrence of AmpC β -lactamase genes in *K. pneumoniae* isolates

Clinical Sample	Gender	Bacteria coding	DHA (%)	blaCIT (%)
Urine	Male	K9	1(12.5)	1(12.5)
		K10	1(12.5)	1(12.5)
	Female	K11	1(12.5)	1(12.5)
		K12	1(12.5)	1(12.5)
Wound swab	Male	K13	1(12.5)	1(12.5)
		K14	1(12.5)	1(12.5)
	Female	K15	1(12.5)	1(12.5)
		K16	1(12.5)	1(12.5)
Total		(n=8)	8(100)	8(100)

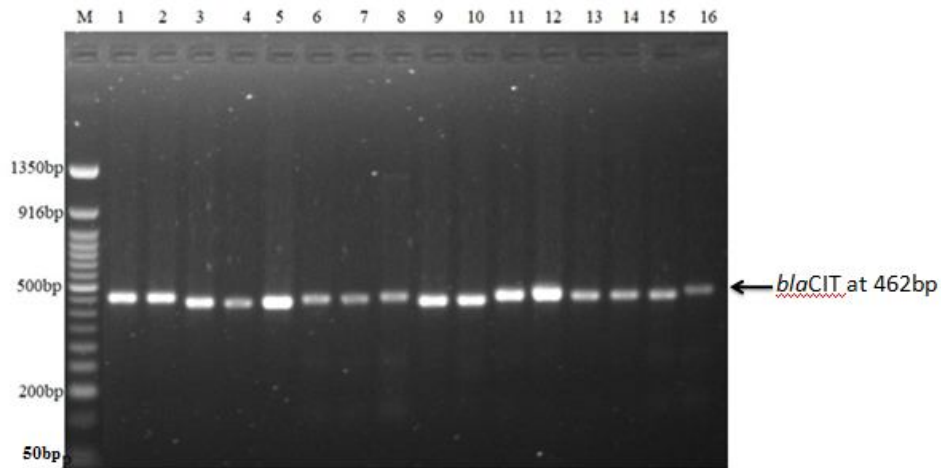


Plate 1: Gel image amplification of *blaCIT* at about 462bp. A 50bp ladder was used to estimate the base pair size of the amplicons. Lane M – 50bp Molecular marker, Lane 1 – 8 = *E. coli*, Lane 9 – 16 = *K. pneumoniae* isolates.

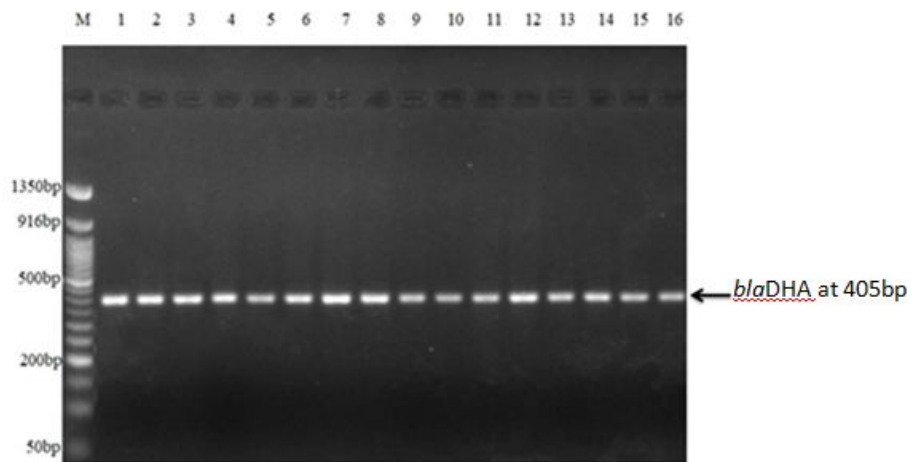


Plate 2: Gel image amplification of *blaDHA* at about 302bp. A 50bp ladder was used to estimate the base pair size of the amplicons. Lane M – 50bp Molecular marker, Lane 1 – 8 = *E. coli*, Lane 9 – 16 = *K. pneumoniae* isolates.

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4. DISCUSSIONS

AmpC β -lactamases was identified by PCR which revealed the predominance of *bla_{DHA}*(100 %), and *bla_{CIT}*(100 %). Our findings reiterate with report from other studies; Malekiet al. [21] in Ilam (West of Iran) reported *CIT* and *DHA* as the most frequent AmpC genes in *E. coli* isolates. Ghanavati et al.[22] in Tehran (North of Iran) also reported the clusters of *CIT*, and *DHA* genes as the most abundant genes in *Klebsiella* isolates, respectively. In Bahrain plasmid-derived AmpC β -lactamase among clinical strains of *Klebsiella pneumoniae* and *E. coli* 10.3% and 31% of cefoxitin-resistant isolates harbored the *DHA* and *CIT* gene, respectively [10]. Geographic diversity in the molecular subtypes of AmpC genes has been discovered through studies conducted in various parts of the world. In this regard, the *CIT* AmpC subtype has been widely identified in the United States and Canada [23]. Woodford et al. [24] also described the prevalence of *CIT* positive, AmpC producing *E. coli* in the United Kingdom and Ireland.

In a study by Mohamudha et al. [25], *DHA* was more common in both *Klebsiella* species and *E. coli* isolates (46.7% and 38%, respectively). *DHA*-1 is a plasmid-mediated AmpC-lactamase derived from *M. Morganii*'s chromosomal AmpC gene [26]. In a study conducted by Pai et al. [27] and Moland et al.[28], it was discovered that mortality of patients infected with organisms that produce *DHA*-1 was higher than that of patients infected with organisms that produce CMY-1, raising concerns about the spread of this inducible plasmid-mediated AmpC-lactamase.

In another report, all *E. coli* isolates were positive for the *CIT* family [29, 30]. In this regard, *bla_{DHA}*, *bla_{CIT}*, type of AmpC has been widely detected in this study showing rapid plasmid expression and dissemination of the gene in the area in recent time. Although they seem to be a variation in the occurrence of *bla_{DHA}* and *bla_{CIT}* reported in this study. Such discrepancies could be linked to geographical area, number of samples, the species studied, and the period of study influence the prevalence, and type of acquired AmpCs detected. As a result, comparing the prevalence of acquired AmpCs across studies is difficult.

According to the findings, the *CIT* and *DHA* gene appears to be the most important factor in the plasmid dissemination of AmpC producing isolates. The AmpC β -lactamases found in this study are clinically relevant β -lactamases because they can confer a MDR phenotype and also expressed resistance towards β -lactamase inhibitors, broader cephalosporin because resistance can arise in such strains by further mutations, resulting in reduced porin expression.

5. CONCLUSION

The study provides data of first report occurrence of *DHA* and *CIT* types AmpC isolates which is alarming. The spread of these AmpC β -lactamases resistance genes within the hospital or across the country via conjugation may become a major public health concern. As a result of identifying *DHA* and *CIT* types of AmpC may assist physicians in prescribing the most appropriate antibiotic, reducing the selective pressure that leads to antibiotic resistance.

LIMITATIONS

The lack of data on the sequencing of AmpC cluster genes was one of the study's limitations. Furthermore, due to a lack of funding, only the presence of *CIT* and *DHA* AmpC genes was investigated, while other AmpC genes, chromosomal hyperproduction or purine loss mutations, were not investigated

CONSENT

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

ETHICAL APPROVAL

In compliance with international standard or university standard written ethical approval has been collected and preserved by the author (s).

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Comment [15]: First report can be considered only after controls are also being tested.

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