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# DETECTION OF AMP C BETA LACTAMASES IN CLINICAL ISOLATES OF *ESCHERICHIA COLI* AND *KLEBSIELLA*

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**ABSTRACT**
**Detection of AmpC-mediated resistance in Gram negative organisms poses a problem due to misleading results in phenotypic tests. There are no recommended guidelines for detection of this resistance mechanism and there is a need to address this issue as much as the detection of extended spectrum beta lactamases (ESBLs) since both may co-exist and mask each other. Several methods have been used to detect the presence of AmpC β-lactamase production in some isolates but most of these methods are not reliable. There is a need for a reliable method of evaluating the presence of AmpC β-lactamases in clinical isolates. A total of 81 consecutive non repetitive clinical isolates of *Escherichia coli* (n=40) and *Klebsiella* spp. (n=41) were screened for AmpC production by disc diffusion method using cefoxitin (30 µg) disc and confirmed by inhibitor based test using boronic acid as inhibitor. A total of 16 *E.coli* isolates (40%) and 16 *Klebsiella* isolates (39.02%) screened harbored AmpC enzymes, of which 43.75% of *E.coli* and 56.25% of *Klebsiella* isolates co-produced ESBL enzymes. Pure AmpC production was observed in 56.25% of *E.coli* and 43.75% of *Klebsiella* isolates. The inhibitor based test was useful in identifying cefoxitin susceptible AmpC producers and could also effectively differentiate ESBL from AmpC producing isolates.**

**KEY WORDS: ESBL, antibiotic susceptibility, clinical samples, β-lactam disks.**

## INTRODUCTION

AmpC β-lactamases have gained importance since the late 1970s as one of the mediators of antimicrobial resistance in Gram negative bacilli. These enzymes are cephalosporinases capable of hydrolyzing all β-lactams to some extent (1). AmpC β-lactamases are two types – plasmid-mediated and chromosomal or inducible AmpC. Chromosomal AmpC enzymes are seen in organisms such as *Citrobacter freundii*, *Enterobacter cloaca*, *Morganella morgani*i, *Hafnia alvei* and *Serratia marcescens* and are typically inducible by β-lactam antibiotics such as cefoxitin and imipenem but poorly induced (if at all) by the third or fourth generation cephalosporins (2). The most common cause of AmpC over expression in clinical isolates is a mutation in *ampD* leading to AmpC hyperinducibility or constitutive hyperproduction (3).

Plasmid-mediated AmpC β-lactamases have been found worldwide but are less common than extended-spectrum β-lactamases (ESBLs), and in *E.coli*, they appear to be less often a cause of cefoxitin resistance than an increased production of chromosomal AmpC β-lactamase (4). Most strains with plasmid-mediated AmpC enzymes have been isolated from patients after several days of hospitalization, but recently, AmpC-producing isolates in cultures from long-term care facilities, rehabilitation centers, and outpatient clinics have been reported (5;6). Risk factors for bloodstream infections caused by AmpC-producing strains of *Klebsiella pneumoniae* include long hospital stay, care in an intensive care unit (ICU), central venous catheterization, need for an indwelling urinary catheter, and prior administration of antibiotics, especially broad-spectrum cephalosporins and β-lactamase inhibitor combinations, and are thus similar to risk factors for infection by ESBL-producing *K.pneumoniae* strains (7;8).

For clinical microbiologists, detection of AmpC-mediated resistance in Gram negative organisms poses a problem because the phenotypic tests may be misleading resulting in misreporting and treatment failures. There are presently no CLSI or other approved criteria for AmpC detection (9). Organisms producing enough AmpC β-lactamase will typically give a positive ESBL screening test but fail for the confirmatory test involving increased sensitivity with clavulanic acid (10). Screening with cefoxitin disc is recommended for initial detection. However, it does not reliably indicate AmpC production. Some of the phenotypic tests include the three-dimensional tests (11), double-disk test (12) and Etest strips with gradient of cefotetan or cefoxitin on one half and the same combined with a constant concentration of cloxacillin on the other half (13).

This study was undertaken to detect the presence of AmpC β-lactamases in clinical Gram negative isolates by inhibitor based method using boronic acid (BA) (14) and also assess if this test could be used to differentiate between ESBLs and AmpC producers.

## MATERIALS AND METHOD

A total of 40 *E.coli* isolates and 41 *Klebsiella* isolates were obtained from clinical samples from Federal Medical center, Owerri, Nigeria. Of the 40 *E.coli* isolates, 10 isolates each were obtained from urine samples, wound, stool and high vaginal swab (HVS). Of the 41 *Klebsiella* isolates, 16 were obtained from urine, 12 from wound while 13 isolates were from stool samples.

## ANTIBIOTIC SUSCEPTIBILITY TEST

Antibiotic susceptibility testing was performed using Kirby Bauer method on Mueller-Hinton agar according to CLSI guidelines (15). The antibiotics tested (in µg) were cefoxitin (30), ceftazidime (30), gentamicin (5), ciprofloxacin (5), ampicillin (10), amikacin (30), cefepime (30), cefotaxime (30) and imipenem (10). *E.coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as control strains.

## ESBL SCREENING

Isolates were tested for ESBL production using the method described previously (16). Briefly, disks containing standard 30 µg of aztreonam, ceftazidime and cefriaxone were placed 15 mm (edge to edge) from an amoxicillin-clavulanic acid disk (20 and 10 µg, respectively) on inoculated Mueller-Hinton agar. Inoculated media were incubated overnight at 35 °C. An enhanced zone of inhibition between any one of the β-lactam disks and the disk containing clavulanic acid was interpreted as evidence for the presence of an ESBL.

## AMP C β-LACTAMASE PRODUCTION TEST

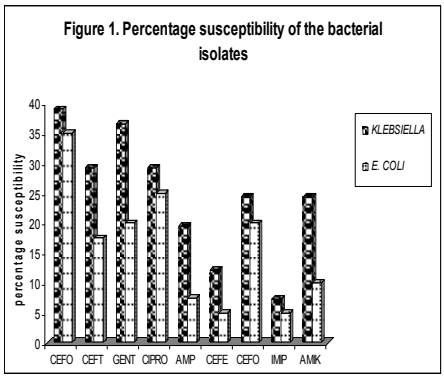
All isolates were tested for AmpC β-lactamase production on disks containing boronic acid (14). A disk containing 30 µg of cefoxitin and another containing 30 µg of cefoxitin with 400 µg of boronic acid were placed on Mueller-Hinton agar. An organism demonstrating a zone diameter around the disk containing cefoxitin and boronic acid ≥5 mm than the zone diameter around the disk containing cefoxitin alone was considered an AmpC producer.

## STATISTICAL ANALYSIS

Data obtained from this study were analyzed using a two-way analysis of variance (ANOVA) and values for P≤0.05 were considered statistically significant.

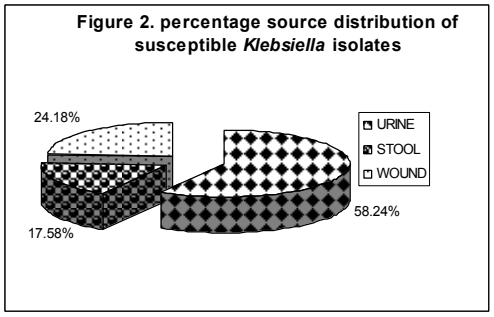
## RESULTS

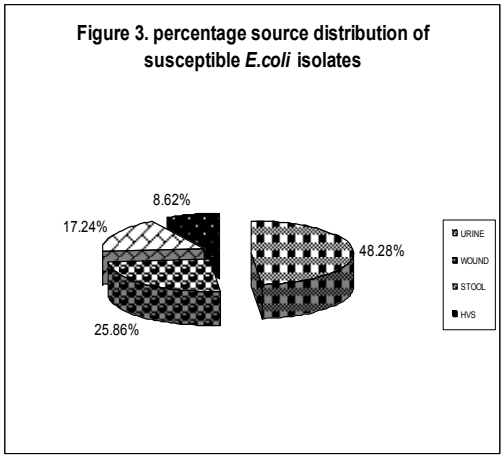
All the isolates were susceptible to at least one of the antibiotics. Most of the *E.coli* isolates were susceptible to cefoxitin, gentamicin, ceftazidin, ciprofloxacin, cefotaxime and amikacin, while majority of them were resistant to imipenem (figure 1). The *Klebsiella* species were less susceptible to ampicillin, cefepine, imipenem and amikacin but the majority were susceptible to cefoxitin, ciprofloxacin and cefotaxime. *Klebsiella* species were more susceptible to the antibiotics tested than the *E.coli* isolates.




**KEY:** CEFO- cefoxitin, CEFT- ceftazidin, GENT- gentamicin, CIPRO- ciprofloxacin AMP- ampicillin, CEFE- cefepine, CEFO- cefotaxime IMIP- imipenem , AMIK- amikacin

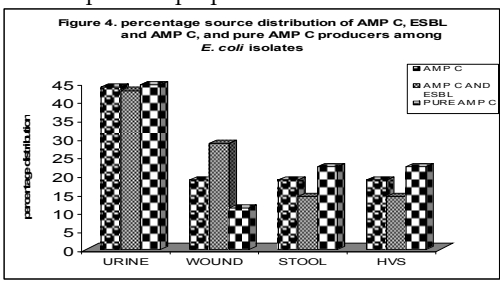
It was observed that most of the susceptible isolates were from urine samples (58.24% and 48.28% for *Klebsiella* and *E.coli*, respectively), while the least were from stool (17.58%) and HVS (8.62%) samples for *Klebsiella* and *E.coli* isolates respectively (figures 2 and 3).The number of *Klebsiella* isolates got from urine samples were more than *E.coli* isolates. The percentage prevalence of susceptible isolates from urine samples was significantly higher than those of the other samples (p≤0.05).



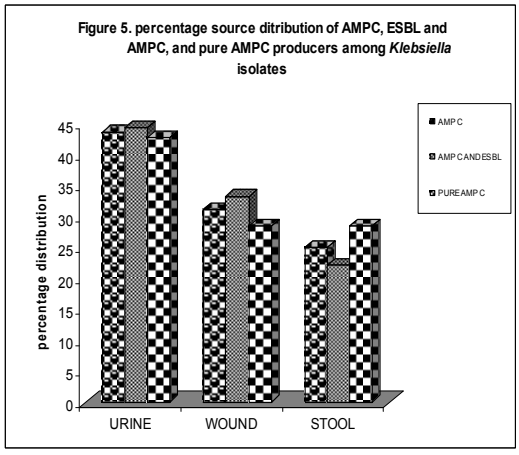




AmpC beta lactamase was detected in 16(40%) of the *E.coli* isolates. Of these isolates, 7(43.75%) were in combination with ESBL while 9(56.25%) were

pure AmpC producers. The percentage prevalence of these isolates from urine samples was significantly higher than those from other samples ($p \leq 0.05$). Wound samples had the least percentage distribution of the isolates which are pure AmpC producers (11.11%) (Figure 4). In each case, the number of pure AmpC producers was more than those with combined ESBL and AmpC production except in the wound sample where the number of combined ESBL and AmpC producers was more than the pure AmpC producers.




Sixteen (39.02%) out of 41 *Klebsiella* isolates were AmpC producers. Of this number, 9(56.25%) were both ESBL and AmpC producers while 7(43.75) were pure AmpC producers. Most of these isolates were also from urine samples (figure 5).The number of isolates with combined ESBL and AmpC production were more than the number of pure AmpC producers in both urine and wound samples but in the stool sample, the number of pure AmpC producers was more than those with combined ESBL and AmpC production.




**DISCUSSION**

Currently, CLSI documents do not indicate the screening and confirmatory tests that are optimal for detection of AMP C beta lactamases (17). However, several studies have been done on various test methods namely, the three dimensional test (11), modified double disk test (18), AmpC disk test (19), inhibitor based method employing inhibitors like boronic acids (14), broth microdilution method (14) and cefoxitin agar method (20). In spite of many phenotypic tests, isoelectric focusing (21) and genotypic characterization (22) are considered gold standards as the results with the phenotypic tests can be ambiguous and unreliable. In the present study, inhibitor based method with boronic acid was employed for AMP C β-lactamase screening.

A study by Manchanda and Singh, (23) discovered that few of their isolates were susceptible to cefoxitime, all of the cefoxitin-susceptible isolates that harboured an AmpC β-lactamase had MICs of cefoxitin <2 mg/L using the broth dilution method. Bauernfeind et al. (33) isolated a clinically significant strain of *K. pneumoniae* that harboured a novel type of AmpC β-lactamase and that also demonstrated a low level of activity against cephamycins (cefoxitin MIC 4 mg/L). This is at variance with the present study where majority of the isolates were found to be susceptible to cefoxitime. In 2002, Barlow and Hall found *ampC* alleles from the chromosomes of two β-lactam-sensitive *C. freundii* strains isolated in the 1920s, before the clinical use of antibiotics (24). Cefoxitin resistance in AmpC non-producers could be due to some other resistance mechanism(s). Lack of permeation of porins as one of the resistance mechanisms has been reported (25). Hernandez-Alles et al. (26) have demonstrated that interruption of a porin gene by insertion sequences is a common type of mutation that causes loss of porin expression and increased cefoxitin resistance in *K. pneumoniae*. AmpC production in cefoxitin-susceptible isolates may have a mechanism similar to that of ESBLproducing organisms that appear susceptible to ceftazidime by the disc diffusion method. These data indicate that although screening methods that use cefoxitin in standardized methods to detect AmpC-harbouring isolates are useful, they are not perfect. The results in the present study showed that screening should include all the clinical isolates showing resistance to any of the cephalosporins and/or aztreonam, irrespective of their cefoxitin susceptibility status.

AmpC beta lactamase was detected in 16(40%) of the *E.coli* isolates. Of these isolates, 7(43.75%) were in combination with ESBL while 9(56.25%) were pure AmpC producers. The percentage prevalence of these isolates from urine samples was significantly higher than those from other samples ($p \leq 0.05$). Wound samples had the least percentage of susceptible isolates (11.11%). Sixteen (39.02%) out of 41 *Klebsiella* isolates were AmpC producers. Of this number, 9(56.25%) were both ESBL and AmpC producers while 7(43.75) were pure AmpC producers. This prevalence was higher when compared with the reports from other parts of the world (27; 28; 29; 30).

It has been stated that the AmpC beta lactamases when present along with ESBLs can mask the phenotype of the later (31). In this study, it was found that both of these enzymes were equally expressed suggesting a possible low level expression of AmpC enzymes. However, in all

these AmpC producers, chromosomal derepressed and plasmid mediated enzymes were not distinguished as this requires genotypic confirmatory tests. Sixteen of the forty-one *Klebsiella* isolates screened were AmpC producers suggesting the presence of plasmid mediated mechanism as these species do not harbor chromosomal AmpC genes (31). The susceptibility of ceftazidime and cefotaxime serves as a poor marker for the identification of AmpC enzymes (14). It is known that plasmid mediated AmpC enzymes can sometimes appear falsely susceptible to these drugs (32). There are newer Ambler class C (ACC) type of enzymes which have relatively a lower activity to cefoxitin (33) and hence appear susceptible. In the present study, some of the AmpC producing isolates were susceptible to the third generation cephalosporins and cefoxitin by the disc diffusion method. This indicates the probable presence of such ACC enzymes. This needs to be confirmed by molecular methods.

The inhibitor based confirmatory method appears promising for AmpC detection as it increased the sensitivity of the test by picking up additional AmpC producers. Boronic acid has been reported to be effective inhibitor of class C beta lactamases (34; 35; 36). In addition, it can also differentiate ESBL enzymes from the AmpC enzymes.

To conclude, a mixed type of drug resistance mechanisms seem to operate in the isolates tested. There is need for a correct and reliable phenotypic test to identify AMP C beta lactamases and ESBL producers. Inhibitor based method using boronic acid appears to be effective in discriminating this type of resistant isolates.

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