Trends in Medicine

Research Article

Phenotypic detection of metallo-β-lactamase (MBL) and AmpC enzymes among abattoir isolates of *Escherichia coli* and *Klebsiella* species in Abakaliki, Nigeria

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Abstract

The surveillance of antibiotic resistance and antibiotic usage in livestock and other agricultural practices is still relatively poor in Nigeria; and this allow drug resistant bacteria to emerge and spread in the community almost undetected. Bacteria that produce metallo-β-lactamases (MBLs) and AmpC enzymes are notably resistant to the carbapenems and cephamycins respectively. And these antibiotics are important antimicrobial agents used clinically for the treatment of infectious diseases. In this study, the frequency of MBL- and AmpC-producing *Klebsiella* species and *Escherichia coli* isolates was phenotypically investigated. A total of 50 anal swab samples were bacteriologically analyzed for the selective isolation of *Klebsiella* species and *E. coli* isolates which were identified by standard microbiology techniques. Antimicrobial susceptibility testing was carried out using the modified Kirby-Bauer disk diffusion technique. The production of MBLs and AmpC enzymes was phenotypically detected using Hodges (Cloverleaf) test and ceftazidime-imipenem antagonism test (CIAT) respectively. The multidrug resistant nature of the MBL- and AmpC-producing *Klebsiella* species and *E. coli* isolates was calculated using the multiple antibiotic resistance index (MARI) formular. A total of 5 *E. coli* isolates and 12 isolates of *Klebsiella* species were bacteriologically recovered from the anal swab samples. The *Klebsiella* species were resistant or intermediately resistant to cefoxitin (66.7%), ceftazidime (58.3%), cloxacillin (100%), ertapenem (83.3%), amikacin (16.7%) and ofloxacin (16.7%). *E. coli* isolates were also found to be resistant to cefoxitin (40%), ceftazidime (40%), cloxacillin (80%) and ofloxacin (20%). MBL was phenotypically detected in one (1) isolate of *E. coli* while AmpC enzyme production was detected in 9 isolates of *Klebsiella* species phenotypically. On average, the MBL-producing *E. coli* isolate and AmpC-producing *Klebsiella* species were multiply resistant to 7 antibiotics. This study has presumptively shown that bacteria from abattoir origin including *Klebsiella* species and *E. coli* isolates are notable MBL and AmpC enzyme producers, and they are multidrug resistant in nature. The use of antibiotics in agriculture and animal husbandry is a major driving force in the emergence and spread of resistant bacteria in the non-hospital environment. This should be discouraged at all levels to preserve the efficacy of available antibiotics especially in this environment where the detection of MBL- and AmpC-producing bacteria from clinical and non-clinical samples is still pitiable.

Introduction

Food-producing animals habouring multidrug resistance genes together with genes that mediate the production of some high-profile antibiotic hydrolyzing enzymes such as metallo-beta-lactamases (MBLs), extended spectrum beta-lactamases (ESBLs) and AmpC enzymes possess health risks to the human population particularly due to their potential contribution to the spread of drug resistant microorganisms in the community. And one of the biggest current challenges facing the health sector across the globe especially in the area of infection control and prevention is in the adequate containment of multidrug resistant Gram-negative organisms (MDRGNOs) such as *Escherichia coli* and *Klebsiella* species which are known to be notable for their antibiotic resistance prowess. The recurrent and irrational use of antibiotics in animal husbandry allows bacteria to develop and acquire drug resistant genes over time through selective pressure. This phenomenon accounts for the singular reason there is for the emergence and dissemination of drug resistant bacteria in the community especially in non-hospital environment [1]. Antibiotic resistant bacteria pose a significant health problem that could be degenerated through the continued irrational use of antibiotics [2]. The increase in the rates of antibiotic resistance is becoming a major cause for concern in isolates of the *Enterobacteriaceae* family [3]. Metallo-β-lactamases (MBLs) are group of β-lactamases that hydrolyze carbapenems, and have potent but variable enzymatic activity against other beta-lactam antibiotics except the monobactams [4,5]. MBLs, which are a type of carbapenemases, are an emerging public health problem among clinically important Gram negative organisms and bacteria isolated from non-clinical sources [6,7]. The presence of MBL-producing bacteria in the hospital and non-hospital environment puts the use of the carbapenems under threat [6,8-11]. AmpC enzymes are cephalosporinases encoded on the chromosomes of many *Enterobacteriaceae* and a few other bacteria where they mediate resistance to cephalothin, cefazolin, cefoxitin and most penicillin [12]. These enzymes are active on cephamycins such as cefoxitin; and they

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are encoded by bacterial chromosomes and plasmids in Gram negative bacilli. According to Jacoby [13], AmpC enzyme production in most *Enterobacteriaceae* is low but the hyper-production of AmpC enzymes in these organisms is usually induced by the exposure of the bacteria to beta-lactam drugs. The long-term and undue use of antibiotics in livestock, poultry and other agricultural activities selects for drug resistance in bacteria [1,13,14]. *Enterobacteriaceae* producing AmpC beta-lactamases have become a major therapeutic challenge; and their detection is of significant clinical relevance since AmpC producers may appear susceptible to expanded-spectrum cephalosporins when initially tested [6,13,15]. This may lead to inappropriate antimicrobial regimens and therapeutic failure since organisms expressing AmpC beta-lactamase are resistant to the antimicrobial actions of these drugs. The plethora of reports on MBL- and AmpC-producing bacteria from different geographical locations calls for need to always be on the lookout for these multidrug resistance enzymes [1,7,8,15,16]. In this study, the occurrence of MBL and AmpC-producing *Klebsiella* species and *E. coli* was phenotypically characterized.

Materials and methods

Collection and processing of samples

Fifty (50) anal swab samples were collected from the anal region of cows from a local abattoir in Abakaliki metropolis, Ebonyi State, Nigeria using sterile swab sticks soaked in normal saline. Each of the swab sticks was returned to their respective containers and labeled. All samples were transported to the Microbiology Laboratory Unit of Ebonyi State University, Abakaliki within one hour of collection for bacteriological analysis. Each of the collected samples was inserted into 5 ml of freshly prepared nutrient broth (Oxoid, UK) and the tubes were loosely covered with cotton wool, and incubated at 30°C for 18-24 h. Bacterial growth was identified by the presence of turbidity or cloudiness in the broth culture after incubation [17].

Isolation and identification of *Escherichia coli* and *Klebsiella* species

A loopful of the turbid solution from the overnight broth culture was plated aseptically onto MacConkey agar (MAC) and eosin methylene blue (EMB) agar (Oxoid, UK) plates, and these were incubated at 30°C for 18-24 h. Suspect colonies of *E. coli* and *Klebsiella* species were subcultured onto freshly prepared MAC and EMB agar plates for the isolation of pure cultures of *E. coli* and *Klebsiella species. E. coli* produces colonies with metallic green sheen on EMB agar and lactose-fermenting colonies on MAC; and *Klebsiella* species produce small, circular, elevated and mucoid colony on MAC and non-metallic green sheen mucoid colonies on EMB agar [17].

Antimicrobial susceptibility testing

This was performed using seven (7) different single antibiotic disks comprising: amikacin (AK, 30 µg), cefoxitin (FOX, 30 µg), cloxacillin (OB, 10 µg), ceftazidime (CAZ, 30 µg), ofloxacin (OFX, 5 µg), ertapenem (ETP, 10 µg) and imipenem (IPM, 10 µg) as per the guidelines of the Clinical and Laboratory Standard Institute (CLSI). All antibiotic disks were in the single disk format, and they were procured from Oxoid limited (Oxoid, UK). The antimicrobial susceptibility testing was performed on Mueller-Hinton (MH) agar plates (Oxoid, UK) using the modified Kirby-Bauer disk diffusion technique as was previously described [18,19].

Screening test for MBL production

All bacterial isolates were phenotypically screened for the production of MBL enzymes by the Kirby-Bauer disk diffusion technique using imipenem (IPM, 10 µg), meropenem (MEM, 10 µg) and ertapenem (ETP, 10 µg) [Oxoid, UK] as was previously described [4,19,20]. Isolates showing inhibition zone diameter (IZD) of ≤ 23 mm were considered and suspected to produce MBL phenotypically.

Phenotypic confirmation of MBL production

The Hodges (Cloverleaf) test was used to phenotypically confirm MBL production in the bacterial isolates. This was performed by aseptically swabbing Mueller-Hinton (MH) agar plates with *Escherichia coli* ATCC 25922 strain. The inoculated MH agar plates were allowed for about 5 min; and imipenem (10 µg) disk was aseptically placed at the center of the MH agar plates. The test bacteria (adjusted to 0.5 McFarland turbidity standards) were heavily streaked from the imipenem (10 µg) disk to the edge of the MH agar plates. Susceptibility plates were incubated for 18-24 hrs at 30°C. The plates were macroscopically observed for indentation, and the growth of the test bacteria towards the imipenem (10 µg) susceptibility disk. Presence of indentation and growth of test bacteria towards the carbapenem disk is indicative of metallo-β-lactamase (MBL) production phenotypically [4,20].

Screening test for AmpC enzyme production

Bacterial strains that produce AmpC beta-lactamase enzymes are resistant to the cephamycins but susceptible to the fourth-generation cephalosporin, cefepime [18,21]. The susceptibility of the test isolates to cefoxitin disk (30 µg) was used as the primary screening test to screen all the isolates for possible production of AmpC enzymes. AmpC enzyme production was suspected in those test isolates that showed reduced susceptibility to cefoxitin disk as per the breakpoints recommended by the CLSI [18,19]. Test isolates showing inhibition zone diameter (IZD) < 18 mm were suspected for the production of AmpC beta-lactamase enzyme.

Detection of AmpC enzyme production

AmpC enzyme production was phenotypically confirmed by the ceftazidime-imipenem antagonism test (CIAT) using ceftazidime (30 µg), cefoxitin (30 µg) and imipenem (10 µg) disk. Ceftazidime disc and imipenem disk were placed at a distance of 20 mm apart on MH agar plate previously inoculated with a suspension of the test bacteria (adjusted to 0.5 McFarland turbidity standards). Cefoxitin disk (30 µg) was also placed at a distance of 20 mm from the ceftazidime disk for comparison. The entire susceptibility test plates were incubated at 30°C for 18-24 h. Antagonism indicated by a visible reduction in the inhibition zone around the ceftazidime disk adjacent to the imipenem or cefoxitin disk was inferred as a positive inducible AmpC beta-lactamase production [1].

Determination of Multiple Antibiotic Resistance Index (MARI)

Multiple antibiotic resistance index (MARI) was calculated to determine the multiple antibiotic resistance profile of the isolated *Klebsiella* species and *E. coli* isolates that were positive for MBL and AmpC enzyme production. This was done according to the method of Akinjogunla and Enabulele [22]. MARI was calculated using the formular: **MARI = a/b**; where '**a**' represents the number of antibiotics which the resistant bacteria was resistant to; and '**b**' represents the total number of antibiotics to which the resistant bacteria has been evaluated for.

Results

In this study, a total of 50 anal swab samples were bacteriologically analyzed for the selective isolation of *Escherichia coli* and *Klebsiella*

species isolates. Table 1 shows the rate of isolation of *Klebsiella* species and *E. coli* isolates from the anal/rectal swab samples, as well as their Gram reaction, biochemical reaction and colonial features on culture media.

The result of the antimicrobial susceptibility pattern of the isolated *Klebsiella* species is shown in Table 2. The *Klebsiella* species were resistant or intermediately resistant to cefoxitin (66.7%), ceftazidime (58.3%), and (83.3%). None of the isolates of *Klebsiella* species were susceptible to cloxacillin (100%). Table 3 shows the result of the antimicrobial susceptibility profile of the 5 *E. coli* isolates bacteriologically recovered on this study from the anal swab samples. The antimicrobial susceptibility test revealed that the 5 (100%) isolates of *E. coli* were completely susceptible to imipenem (IPM), ertapenem (ETP) and amikacin (AK). However, 3 (60%) isolates of the *E. coli* were susceptible to cefoxitin (FOX) and ceftazidime while only 1 (20%) isolate was susceptible to cloxacillin (OB).

Table 4 shows the prevalence of *Klebsiella* species and *E. coli* isolates that are positive for metallo-β-lactamase (MBL) production and AmpC enzymes in this study. In this study, only one (1) isolate of *E. coli* was phenotypically confirmed to produce MBL by the Hodges (Cloverleaf) test while none of the *Klebsiella* species isolates produced MBL phenotypically. AmpC enzyme production was phenotypically confirmed in 9 isolates of *Klebsiella* species by the ceftazidime-imipenem antagonism test (CIAT) method used in thus study. None of the *E. coli* isolates produced AmpC enzyme (Table 4). Table 5 shows the result of the multiple antibiotic resistance index of the *E. coli* and *Klebsiella* species that produced MBL and AmpC enzymes respectively.

Table 1. Isolation of *Escherichia coli* and *Klebsiella* species

Bacteria	Sample (n)	Number (%)	Morphological appearance	Gram reaction	Biochemical reaction
Escherichia coli	Anal swabs (25)	5	Non-mucoid pinkish colonies on MAC; metallic green sheen colonies on EMB	Gram negative	Indole positive MR positive
Klebsiella species	Anal swabs (25)	12	Mucoid colonies on MAC; non-metallic green sheen colonies on EMB	Gram negative	Citrate positive Urease positive

n-Number**;** %-Percentage; EMB-Eosin methylene blue; MAC-MacConkey; MR-Methyl red

Table 2. Percentage antimicrobial susceptibility profile of 12 isolates of *Klebsiella* species

Antibiotics	% Resistant	% Susceptible
IPM (10 µg)	0	100
FOX (30 µg)	66.7	33.3
AK (10 µg)	16.7	83.3
OFX (10 µg)	16.7	83.3
CAZ (30 µg)	58.3	41.7
OB (200 µg)	100	0
ETP (10 µg)	83.3	16.7

FOX-Cefoxitin; IPM-Imipenem; CAZ-Ceftazidime; ETP-Ertapenem; OFX-Ofloxacin; AK-Amikacin; OB-Cloxacillin

Table 3. Percentage antimicrobial susceptibility profile of 5 isolates of *E. coli*

Antibiotics (µg)	Susceptible n (%)	Resistant n (%)
IPM (10)	5 (100)	0 (0)
ETP (10)	5 (100)	0 (0)
FOX (30)	3 (60)	2 (40)
CAZ (30)	3 (60)	2 (40)
OB (200)	1 (20)	4 (80)
OFX (10)	4 (80)	1 (20)
AK (10)	5 (100)	0 (0)

IPM-Imipenem; ETP-Ertapenem; FOX-Cefoxitin; CAZ-Ceftazidime; OB-Cloxacillin; OFX-Ofloxacin; AK-Amikacin

Table 4. Frequency of MBL and AmpC positive *Klebsiella* species and *E. coli* isolates

Resistance mechanism	*Klebsiella* species	*Escherichia coli*
MBL	Nil	1
AmpC	9	Nil

MBL-Metallo-β-lactamase

Table 5. Result of multiple antibiotic resistance index

Isolate no.	MARI
E4	0.3
K2	0.1
K3	0.6
K4	0.6
K9	0.4
K10	0.7
K11	0.6
K13	0.4
K17	0.6
K23	0.6

E-*Escherichia coli*; K-*Klebsiella*

Discussion

Metallo-β-lactamase (MBL) and AmpC enzyme production in Gram negative bacteria is one of the major resistance mechanisms that allow bacterial isolates including members of *Enterobacteriaceae* and other non-enteric bacteria to resist the antimicrobial onslaught of some available potent antimicrobial agents. This mechanism of resistance has tremendous public health implications as they limit treatment options for some bacterial-related infections caused by MBL- and AmpC-producing bacteria. In this study, the occurrence of metallo-β-lactamase (MBL) and AmpC-producing *Escherichia coli* and *Klebsiella* species of abattoir origin was bacteriologically and phenotypically investigated. *Klebsiella* species and *E. coli* was appreciably isolated from the anal/rectal swabs of cows that were used in this study. The rate of isolation of *Klebsiella* species and *E. coli* in this study does not correspond to our earlier report and that of others in which *Klebsiella* species and *E. coli* were recovered at a high rate from both clinical and non-clinical sources [16,18,23]. The result obtained from the susceptibility studies showed that *E. coli* exhibited the highest susceptibility to imipenem (100%), ertapenem (100%) and amikacin (100%). More than 50% of the *E. coli* isolates were resistant to cloxacillin. All the *Klebsiella* isolates were particularly resistant to cloxacillin (100%), ertapenem (83%), cefoxitin (67%) and ceftazidime (58%). Most of the *Klebsiella* isolates were susceptible to some of the antibiotics such as imipenem (100%), amikacin (83%) and ofloxacin (83%). The high level of resistance of the *Klebsiella* species and *E. coli* isolates used in this study to some commonly available antibiotics correspond to our previous reports and that of others in which *E. coli* and *Klebsiella* species were found to be highly resistant [4,18,24]. The resistance profile of the *Klebsiella* species and *E. coli* isolates recovered in this study connote to the usage of antibiotics in animals as growth promoting agents; and this practice contributes a great deal to the emergence and spread of resistant bacteria in the community [25]. In this study, MBL was phenotypically confirmed in one (1) isolate of *E. coli* while AmpC enzymes were detected in 9 isolates of *Klebsiella* species. The detection of MBL in the *E. coli* isolates was only phenotypically confirmed in one (1) isolate of *E. coli*, and this is very low when compared to our previous report and that of others on the frequency of MBL-producing *Enterobacteriaceae*

from both community and hospital samples [1,3-5]. Our result on the prevalence of MBL- and AmpC-producing bacteria does not correspond to previous study in which *E. coli* and *Klebsiella* species that produced MBL and AmpC enzymes was reported at a higher rate [1,15,23,26,27]. The MBL-producing *E. coli* isolate and AmpC-producing *Klebsiella* species were multiply resistant to 7 antibiotics. This shows that MBL- and AmpC-producing bacteria are multidrug resistant in nature. The presence of MBL-producing bacteria in the community portend serious health risk because organisms producing MBL are notably resistant to carbapenems including imipenem and meropenem, which are antibiotics reserved for serious bacterial infections including those caused by bacteria that produce extended spectrum beta-lactamases [1,9]. On the other hand, bacteria that produce AmpC enzymes are remarkably resistant to the cephamycins including cefoxitin and cefotetan, which are second generation cephalosporins that are used for the treatment of bacterial-related infections [13,18,28-30]. Conclusively, the early and accurate detection of MBL- and AmpC-producing *Enterobacteriaceae* from abattoir and clinical samples is of utmost public health importance due to the multidrug resistant nature of these organisms. Our study shows that *Klebsiella* species and *E. coli* from abattoir sources produce MBL- and AmpC enzymes that allow them to be resistant to the antimicrobial onslaught of carbapenems and cephamycins respectively; and these organisms are multidrug resistant in nature. The rapid spread of resistance among bacteria may be attributed to the widespread and inappropriate use of antibiotics in animal husbandry and for other non-clinical purposes. Appropriate preventive measures are advocated to prevent the emergence of newer resistance mechanisms among bacteria species in non-clinical environments. We recommend proper detection of resistant bacteria from community samples, as well as the discouragement or stoppage of the use of antibiotic as growth promoting agents in animal husbandry and other agricultural practices.

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