

## Preliminary Antibiogram Studies and Phenotypic Detection of Metallo- $\beta$ -lactamase (MBL) in klebsiella species and p Aeruginosa from a non-hospital Milieu

### Abstract

The rational use of antibiotics in the non-hospital environments including poultry and abattoir is vital to the sustainable containment of the emergence and spread of antibiotic resistant bacteria, as well as the preservation of the efficacy of available antimicrobials. Antibiotic usage in animal husbandry should be curtailed and possibly replaced with sustainable measures such as proper sanitation, vaccination and other acceptable practices that are devoid of antibiotics, since the singular use of antibiotics promotes antibiotic resistance in bacteria. This preliminary study investigated isolates of Klebsiella species and Pseudomonas aeruginosa from abattoir samples for metallo- $\beta$ -lactamase (MBL) production using the modified Hodges (Cloverleaf) test. A total of 25 rectal/anal swab samples (comprising 50 samples in all) collected at various times were bacteriologically analyzed for the isolation and identification of Klebsiella species and P. aeruginosa isolates using standard microbiology techniques. Antimicrobial susceptibility testing was carried out using the modified Kirby-Bauer disk diffusion technique. The MBL-producing isolates of Klebsiella species and P. aeruginosa were also evaluated for their multiple antibiotic resistance profile. The Klebsiella species were resistant to imipenem (100%), ceftazidime (83.3%) and cloxacillin (100%) while P. aeruginosa isolates were found to be resistant to ceftazidime (100%), cloxacillin (100%), ertapenem (65%) and amikacin (40%). All the 12 isolates of Klebsiella species recovered in this study were phenotypically positive for MBL production by the Modified Hodges test. However, a total of 14 (70%) isolates out of 20 P. aeruginosa isolates were phenotypically confirmed to be MBL positive by the modified Hodges test. All isolates of Klebsiella species and P. aeruginosa that were positive for MBL production were multiply resistant to 5 antibiotics out of the 7 antibiotics used for this study. Rational use of available antimicrobials and proper detection of MBL-producing bacteria and other drug resistance phenotypes is warranted to forestall the spread of antibiotic resistant bacteria in the non-hospital environment such as abattoir milieu.

**Keywords:** Gram-negative bacteria; Antibiotics; Metallo-beta-lactamase (MBL); Antibiotic resistance; Nigeria.

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Research Article

Chika Ejikeugwu<sup>1\*</sup>, Chika Ezeador<sup>2</sup>, Blessing Okereke<sup>1</sup>, Nkemdilim Eze<sup>1</sup>, Happiness Abani<sup>1</sup>, Chijioke Edeh<sup>1</sup>

<sup>1</sup>Department of Applied Microbiology, Faculty of Science, Nigeria

<sup>2</sup>Department of Medical Microbiology and Parasitology, Nigeria

### \*Address for Correspondence

Chika Ejikeugwu, Department of Applied Microbiology, Faculty of Science, Nigeria

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### Introduction

The increase in antibiotic resistance reports among Gram negative bacteria, particularly Klebsiella species and Pseudomonas aeruginosa from non-hospital sources, and which are capable of producing metallo- $\beta$ -lactamases (MBLs) puts the use of the carbapenems for therapeutic purposes at risk. MBLs are a type of carbapenemases cum beta-lactamases that hydrolyze the carbapenems including imipenem, and render same inefficacious for treatment (Walsh et al. [1], Ejikeugwu et al. [2]). Beta-lactamases are important components of the antimicrobial resistance mechanism found in Gram negative bacteria including P. aeruginosa and Klebsiella species (Walsh et al. [1], Franco et al. [3], Varaiya et al. [4], Zavasci et al. [5], Samah et al. [6]), but some bacteria have gone further to express and/or produce newer beta-lactamases such as MBLs - which give these organisms the exceptional ability to resist the antimicrobial onslaught of some potent and available antimicrobials like imipenem, a carbapenem. However, mutation in these earlier beta-lactamases (such as TEM and SHV enzymes) has led to the emergence of organisms that express multidrug resistance enzymes such as extended spectrum beta-lactamases (ESBLs), not to mention the least, MBLs. According to Bush and Jacoby (2010), the production  $\beta$ -lactamase is most frequently suspected in a Gram-negative bacteria isolates that demonstrates resistance to a  $\beta$ -lactam antibiotic. As previously reported, Gram negative bacteria that produce MBLs are typically resistant to carbapenems, aminoglycosides and fluoroquinolones, and this further compromises therapeutic option in the face of an infection or disease (Zavasci et al. [5]; Jacoby et al. [7]; Uma Karthika et al. [8]; Samah et al. [6]). MBLs as aforementioned are  $\beta$ -lactamases that belong to Ambler's class B type of enzymes, and they degrade a wide variety of  $\beta$ -lactams including penicillins, cephalosporins as well as carbapenems through hydrolysis reaction (Bush et al. [9]; Walsh et al. [1]). Though

they show remarkable resistance to carbapenems (which are used to treat ESBL infections), bacteria that produce MBLs are usually susceptible to aztreonam, a monobactam. However, MBL-producing bacteria and other multidrug resistant bacteria such as ESBL-producing bacteria are a threat to public health, and they are also of clinical importance since these organisms are resistant to a wide variety of antibiotics especially the  $\beta$ -lactams and some non- $\beta$ -lactams which are important antimicrobials used clinically for the treatment of infectious diseases. MBL production in Gram negative bacteria is mainly mediated by some plasmid-encoded genes such as blaIMP-1 and blaVIM-1 (Ejikeugwu et al. [10]; Walsh et al. [1]; Varaiya et al. [4]; Bora et al. [11]). These genes are usually located on genetic elements such as integrons that also carry genes encoding for resistance to other antibiotics including aminoglycoside; and this warrants for the multidrug resistant nature of MBL-producing Gram negative bacteria (Walsh [12]; Walsh et al. [1]; Franco et al. [3]). However, organisms producing MBLs in non-clinical isolates especially those from poultry and abattoir sources are of immense public health importance since they could serve as repertoires for the preservation and dissemination of MBL-producing organisms in the non-hospital environment. The emergence of multidrug resistant bacteria in both the hospital and non-hospital environments poses a serious public health challenge since these organisms defy the antimicrobial onslaught of some current antibiotics. Studies have shown that zoonoses from abattoir wastes are yet to be fully controlled in more than 80 % public abattoirs in developing countries inclusive of Nigeria (Cadmus et al. [13]; Bello and Oyedemi [14]; Yousuf et al. [15]; Ejikeugwu et al. [16]). And this could serve as route for the onward transmission of infectious diseases and antibiotic resistant pathogens in human populations. Owing to this, the antimicrobial susceptibility and phenotypic detection of metallo- $\beta$ -lactamase (MBL) was presumptively investigated in Klebsiella species and P. aeruginosa isolates recovered from a non-hospital milieu (abattoir).

## Materials and methods Keywords

### Sample Collection and Processing

Rectal/anal swab samples (n=50) were collected from the anal region of cows (ready for slaughter) from a local abattoir in Abakaliki metropolis, Ebonyi State, Nigeria using sterile swab sticks soaked in normal saline. The samples were collected with minimal or no hurt to the animals. All samples were labeled and transported (according to all relevant national and international guidelines) to the Microbiology Laboratory Unit of Ebonyi State University, Abakaliki within one hour of collection; and they were each inserted into 5 ml of freshly prepared nutrient broth (Oxoid, UK) and incubated at 30°C for overnight. Bacterial growth was identified by the presence of turbidity or cloudiness in the broth culture after incubation.

### Bacterial Isolation and Identification

Suspensions of the turbid solution from the broth culture was

plated aseptically onto MacConkey (MAC) and cetrimide selective agar (CSA) (Oxoid, UK) plates, and incubated at 30°C for overnight. Suspect colonies of P. aeruginosa and Klebsiella species were subcultured onto freshly prepared MAC and CSA plates for the isolation of pure cultures of Klebsiella species and P. aeruginosa respectively. Klebsiella species produce small, circular, elevated and mucoid colonies on MAC while P. aeruginosa produces colonies with greenish pigmentation on cetrimide selective agar. All isolates of Klebsiella species and P. aeruginosa were further characterized using standard microbiology identification techniques (Cheesbrough [17]).

### Susceptibility testing

Antimicrobial susceptibility testing (AST) was carried out on Mueller-Hinton agar plates using the modified Kirby-Bauer disk diffusion technique as per the guidelines of the Clinical and Laboratory Standard Institute (CLSI). A total of seven (7) single antibiotic disks comprising: amikacin (AK, 10  $\mu$ g), cefoxitin (FOX, 30  $\mu$ g), cloxacillin (OB, 10  $\mu$ g), ceftazidime (CAZ, 30  $\mu$ g), ofloxacin (OFX, 5  $\mu$ g), ertapenem (ETP, 10  $\mu$ g) and imipenem (IPM, 10  $\mu$ g) (Oxoid, UK) were used for AST. All susceptibility plates were incubated at 30°C for 18-24 hrs; and inhibition zone diameters (IZDs) were recorded and interpreted using the standard antibiotic breakpoint of CLSI (Ejikeugwu et al., 2016; CLSI, 2011).

### Screening and Phenotypic Confirmation of 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  $\mu$ g), meropenem (MEM, 10  $\mu$ g) and ertapenem (ETP, 10  $\mu$ g) [Oxoid, UK] as was previously described (Ejikeugwu et al. 2014; CLSI 2011; Varaiya et al. 2008). Isolates showing inhibition zone diameter (IZD) of  $\leq 23$  mm were considered and suspected to produce MBL phenotypically. The Cloverleaf (Hodges) 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  $\mu$ g) 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  $\mu$ g) 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  $\mu$ g) susceptibility disk. Presence of indentation and growth of test bacteria towards the carbapenem disk is indicative of metallo- $\beta$ -lactamase (MBL) production phenotypically (Ejikeugwu et al., 2014; Varaiya et al. 2008).

### 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 *P. aeruginosa* isolates that were positive for MBL production (Ejikeugwu et al. 2017a). MARI was calculated using the formula:  $MARI = a/b$ ; 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

(Table 1) show the distribution of the isolated *Klebsiella* species and *Pseudomonas aeruginosa* isolates that was bacteriologically recovered from the rectal/anal swab samples. There were more isolates of *P. aeruginosa* recovered from the anal swab samples than isolates of *Klebsiella* species. The result of the antimicrobial susceptibility of the isolates of *Klebsiella* species is shown in Table 2. Amikacin and ofloxacin were the best performing antibiotics in terms of their antimicrobial onslaught against the isolates of *Klebsiella* species used in this study. The result of the antimicrobial susceptibility profile of the isolated *P. aeruginosa* isolates is shown in Table 3. The *P. aeruginosa* isolates were resistant or intermediately resistant to ertapenem (65%), ofloxacin (35%) and amikacin (40%). However, none of the *P. aeruginosa* isolates were susceptible to ceftazidime and cloxacillin (Table 3). Interestingly, *P. aeruginosa* isolates acted differently in terms of its susceptibility to ceftazidime, cloxacillin, ertapenem and ofloxacin - to which they showed reduced susceptibility (Figure 1).

Bacteria	Sample (n)	Number (%)	Morphological appearance	Gram reaction	Biochemical reaction
<i>Pseudomonas aeruginosa</i>	Anal swabs (25)	20 (80)	Colonies with greenish pigmentation on cetrimide selective agar	Gram negative	Oxidase positive
<i>Klebsiella</i> species	Anal swabs (25)	12 (48)	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 1:** Isolation and morphological features of *Klebsiella* species and *Pseudomonas aeruginosa*

Antibiotics (µg)	Resistance n (%)	Susceptible n (%)
Imipenem (10)	12 (100)	0 (0)
Ceftazidime (30)	12 (100)	0 (0)
Amikacin (10)	0 (0)	12 (100)
Ofloxacin (10)	2 (16.7)	10 (83.3)
Ertapenem (10)	9 (75)	3 (25)
Cloxacillin (10)	12 (100)	0 (0)
Ceftazidime (30)	10 (83.3)	2 (16.7)

**Table 2:** Antibigram of isolates of *Klebsiella* species



**Figure 1:** Susceptibility of a *P. aeruginosa* strain to tested antibiotics. The *P. aeruginosa* isolate was susceptible to imipenem (IPM), ceftazidime (CAZ) and amikacin (AK), but resistant to ceftazidime (FOX), ofloxacin (OFX), ertapenem (ETP) and cloxacillin (OB).

Antibiotics (µg)	Resistance n (%)	Susceptible n (%)
ETP (10)	13 (65)	7 (35)
FOX (30)	20 (100)	0 (0)
IPM (10)	0 (0)	20 (100)
CAZ (30)	1 (5)	19 (95)
OFX (10)	7 (35)	13 (65)
OB (200)	20 (100)	0 (0)
AK (10)	8 (40)	12 (60)

**Table 3:** Antibigram of isolates of *P. aeruginosa*

(Table 4) shows the prevalence of MBL-producing isolates of *P. aeruginosa* and *Klebsiella* species recovered in this study. All the 12 isolates of *Klebsiella* species recovered in this study were positive for MBL production. A total of 14 isolates of *P. aeruginosa* out of the 20 *P. aeruginosa* isolates were phenotypically confirmed to be MBL positive by the Hodges test method. On average, the result of the multiple antibiotic resistance profile of the MBL-producing isolates of *Klebsiella* species and *P. aeruginosa* bacteriologically recovered in this study revealed that the bacterial isolates were found to be resistant to about 5 antibiotics out of the 7 antibiotics that were tested in this study.

Phenotype	<i>Klebsiella</i> species n (%)	<i>Pseudomonas aeruginosa</i> n (%)
MBL positive	12 (100)	14 (70)
MBL negative	0 (0)	6 (30)

**Table 4:** Occurrence of MBL-producing isolates

## Discussion

Metallo-beta-lactamases (MBLs) are expanded spectrum enzymes that gives Gram negative bacteria the exceptional ability to resist the antimicrobial onslaught of some potent and available antimicrobial agents especially the carbapenems such as imipenem and meropenem. Carbapenems are used for the treatment of serious bacterial related infections including those caused by pathogenic bacteria that produces extended spectrum beta-lactamase (ESBL) enzymes. However, some Gram negative bacterial strain including *Klebsiella* species and *P. aeruginosa* now possess the ability to resist the antimicrobial prowess and potentials of the carbapenems especially in treatment of infectious bacterial diseases. In this study, the antibiogram and occurrence of MBL-producing isolates of *Klebsiella* species and *P. aeruginosa* was bacteriologically and phenotypically investigated in rectal/anal swab samples obtained from ready-to-be slaughtered cows in an abattoir in Abakaliki, Nigeria. The *Klebsiella* specie and *P. aeruginosa* isolates recovered in this study showed varying levels of susceptibility to the tested antibiotics. Interestingly, all the *Klebsiella* species showed reduced susceptibility to imipenem (100%), ceftazidime (83.3%) and cloxacillin (100%), which are respectively a carbapenem, 2nd-generation cephalosporin, 3rd-generation cephalosporin and a penicillin-family antibiotic that are used for the treatment and management of infections caused by both Gram positive and Gram negative bacteria. According to literature, Gram negative bacteria with potential to produce MBL are usually resistant to imipenem, and this is should draw attention of possible MBL-producing strain (Yong et al. 2009; Walsh et al. 2005; Clare et al. 2006; Dahiya et al. 2015). Nonetheless, the *Klebsiella* species were found to show some appreciable level of susceptibility to the antimicrobial onslaught of amikacin (100%), ofloxacin (83.3%) and ertapenem (25%). On the other hand, *P. aeruginosa* isolates were found to be resistant to ceftazidime (100%), cloxacillin (100%), ertapenem (65%) and amikacin (40%). Contrarily, the *P. aeruginosa* isolates were completely susceptible to imipenem (100%) while showing some level of resistance to ertapenem (65%), a member of the carbapenem family to which imipenem also belongs to. Nonetheless, ceftazidime, ofloxacin and amikacin had some inhibitory activity against the *P. aeruginosa* isolates at the rates of 95 %, 65 % and 60 % respectively. Previous reports have clearly shown that Gram negative bacteria including *Klebsiella* species and *P. aeruginosa* from non-hospital samples possess traits that allow them to resist the antimicrobial onslaught of some available antibiotics (Tortola et al, 2005; Iyobe et al. 2001; Hyun-Ho et al. 2013); and the data from these studies are also in line with our report in which the *Klebsiella* species and *P. aeruginosa* isolates from abattoir had remarkable reduced level of susceptibility to the tested antibiotics. In our study, MBL production was phenotypically confirmed in 12 isolates of *Klebsiella* species and 14 (70%) isolates of *P. aeruginosa*. Other studies also reported the production of MBL in *Klebsiella* and *P. aeruginosa* isolates from non-hospital sources (Enwuru et al. 2011; Okazaki et al. 2016; Won et al. 2011). Our previous data in line with other reports have shown that abattoir environment may be a potential reservoir of antibiotic resistant bacteria (including those that produce MBL), and these data are

suggestive of possible transmission of resistant phenotypes different from the hospital environment (Ejikeugwu et al. 2017a; Ejikeugwu et al. 2016; Hyun-Ho et al. 2013; Yong et al. 2009). The use of antibiotics as a supplement in the feed and water sources of livestock as well as in poultry and other agricultural practices may be a major contributing factor for the emergence and spread of multidrug resistant bacteria such as MBL-producing gram negative bacteria in the non-hospital environment (Wegener, 2013). Taken together, the data from this study suggest that Gram negative bacteria, particularly *Klebsiella* species and *P. aeruginosa* from abattoir origin have the potential to resist the antimicrobial action of some potent antibiotics. This is not too good for public health since humans depends on these animals for their source of protein. More so, antibiotic resistant bacteria including those harbouring phenotypes or traits for the production of MBL may enter the food chain and spread to human population, thereby causing community acquired infection undetected. Since the detection of MBL-producing bacteria and other multidrug resistant organisms is not a routine in our hospitals in this part of the world; this study suggest and clarifies the need to make the detection of these organisms part of the normal antimicrobial susceptibility testing in order to forestall any disease outbreak due to them. Conclusively, our preliminary data gives credence to the possible emergence and spread of MBL-producing bacteria in non-hospital environments such as abattoir. However, further molecular characterization of our isolates is required to type our MBL strains and see if they are comparable to that obtainable elsewhere. Therefore efficient detection protocol for MBL-producing bacteria is required in this part of the world in order to curtail the spread of drug resistant bacteria through abattoir.

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