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Research Article
Molecular Identification of MBL Genes ^{bla}IMP-1 and ^{bla}VIM-1 in *Escherichia coli* Strains Isolated from Abattoir by Multiplex PCR Technique

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Abstract

Background and Objective: Globally, infections caused by antibiotic resistant bacteria still pose a threat to public health. The widespread use of antibiotics in food-animal production allows resistant strains of microbes to evolve. The contamination of the environment with animal wastes (containing resistant bacteria) is a major route via which human populations become infected by these microbes. Metallo-beta-lactamase (MBL) is one of the resistance mechanism at the disposal of Gram-negative bacteria including *Escherichia coli* that is chiefly responsible for bacteria resistance to the carbapenems. This study evaluated the antibiogram and occurrence of MBL genes from *E. coli* isolates recovered from abattoir. **Materials and Methods:** A total of 120 rectal swab samples from cows in a local abattoir were used for this study. Each of the rectal swab samples were bacteriologically analyzed for the presence of MBL-producing *E. coli* using the inhibition based assay and multiplex PCR technique. Specific primers for ^{bla}IMP-1 and ^{bla}VIM-1 MBL genes were used for the multiplex PCR analysis. The data were analyzed using SPSS with chi-square test and one-way analysis of variance. **Results:** A total of 48 (40%) isolates of *E. coli* was recovered from the 120 rectal swab samples. The *E. coli* isolates were highly resistant to ceftriaxone (72.9%), cefoxitin (75%), ceftazidime (100%), ertapenem (83.3%), oxacillin (81.3%), ciprofloxacin (70.8%), cefotaxime (93.8%), aztreonam (97.9%) and nitrofurantoin (75%). Of the 48 *E. coli* isolates from abattoir, 15 (31%) *E. coli* isolates were phenotypically confirmed to produce MBLs. However, only 8 *E. coli* isolates were genotypically confirmed to harbour ^{bla}IMP-1 gene by the multiplex PCR used in this study. None of the *E. coli* phenotypes harboured the ^{bla}VIM-1 MBL gene. **Conclusion:** This study reported the first multiplex PCR detection of ^{bla}IMP-1 MBL gene in *E. coli* isolates from rectal swab of cows in Abakaliki, Nigeria. The molecular identification of the genes encoding MBL production in Gram-negative bacteria from community samples is vital for a reliable epidemiological investigation, surveillance and the forestalling of the emergence and spread of these organisms through the food chain and food-producing animals.

Key words: MBL genes, bacterial characterization, antibiogram, PCR technique, Gram-negative bacteria

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The use of antibiotics in animal husbandry allows resistant microbes to evolve through the process of natural selection or selective pressure. These resistant bacteria can persist in meat, poultry/animal products and animal wastes and serve as route via which the environment becomes contaminated with them. Antibiotic resistant microbes from animals represent the most relevant reservoir of resistance to antibiotics and other antimicrobial agents in the community and this is due in part to their ability to acquire and incorporate into their genome, antibiotic resistance genes from their environment. Metallo-beta-lactamases (MBLs) are beta-lactamase enzymes produced by pathogenic bacteria and which hydrolyzes the carbapenems such as imipenem, meropenem and ertapenem and render them ineffective for treatment[1,2]. MBLs efficiently hydrolyze all beta-lactam drugs except aztreonam, a monobactam. They confer variable range of high levels of resistance to all beta-lactam antibiotics and some non-beta-lactams such as fluoroquinolones and aminoglycosides. Their presence in clinically important Gram-negative bacteria have put the use of the carbapenems under threat[1,3]. The MBLs have high affinity for zinc ions (Zn^{2+}). The enzyme is largely inhibited by chelating agents such as EDTA and dipicolinic acid *in vitro*. This informed the basis upon which the expression of MBLs can be detected phenotypically in pathogenic bacteria[1,3]. Genes responsible for the expression of MBLs can be either chromosomally-mediated or plasmid-mediated[1,2]. The carbapenems are often the last line of treatment option for a variety of infectious diseases including those caused by multidrug resistant organisms. The occurrence of MBL-producing bacteria in a hospital or non-hospital setting poses not only a therapeutic problem but also serious concern for infection control management in the health system[4,5]. This is due to the fact that organisms producing MBLs are multidrug resistant in nature. They limit therapeutic options for treatment. MBLs may be disseminated in hospital environment through genetic transfer elements such as transposons, plasmids and integrons amongst clinically important bacteria and the extensive use of the carbapenems especially irrationally has given impetus to the spread of organisms that produce the enzymes[1,4,6]. Antimicrobial resistance is a natural biological phenomenon but it can be contained and averted through proper drug prescription, rational use of antibiotics and through timely and appropriate detection of antibiotic resistant bacteria. The purported continual usage of antimicrobial agents as growth promoting agents in the production of food-producing animals selects for antimicrobial resistant bacteria that give rise to other resistant microbes. The detection of MBL-producing Gram-negative bacteria from community samples including samples from rectal swabs of cow is important because of the possible transfer of antibiotic resistant bacteria through the food chain and food-producing animals to humans. More so, determining the genetic factors responsible for these resistance determinants is vital to the provision of sound epidemiological data that will assist in the surveillance and control of the emergence and spread of drug resistant bacteria in the community. This study investigated the antibiogram and occurrence of MBL-producing *Escherichia coli* isolates from rectal swabs of cow using both phenotypic technique and multiplex PCR technique.

MATERIALS AND METHODS

Sample collection and processing: One hundred and twenty (120) rectal swab samples were collected from the anal region of cows using sterile swab stick(s) soaked in normal saline. The samples were collected from January, 2016-May, 2016. All the samples were transported to the Microbiology Laboratory Unit of Ebonyi State University, Abakaliki, Nigeria for bacteriological analysis according to all relevant national and international guidelines. Samples were aseptically inoculated in nutrient broth (Oxoid, UK) and incubated at 30°C for 18-24 h prior to bacterial isolation[7]. Following overnight incubation at 30°C, the test tubes were examined for visible bacterial growth as evidenced by turbidity. Tubes showing turbidity were each sub-cultured onto freshly prepared solid culture media plates for the isolation of the test bacterium.

Isolation and identification of *Escherichia coli*: A loopful of the turbid solution from the overnight nutrient broth culture were aseptically plated onto eosin methylene blue (EMB) agar (Oxoid, UK) and MacConkey agar (Oxoid, UK) plates and incubated at 30°C for 18-24 h. After incubation, suspected colonies of *E. coli* growing on the culture media plates was aseptically subcultured onto freshly prepared EMB and MacConkey agar (MAC) plate(s) for the isolation of pure cultures of *E. coli*. The following tests were carried out: Indole test, methyl red (MR) test and Gram stain/morphology examination[7].

Antimicrobial susceptibility testing: Antimicrobial susceptibility testing was carried out on all *E. coli* isolates based on the guideline of the Clinical and Laboratory Standard Institute (CLSI) using the modified Kirby-Bauer disk diffusion technique. Single antibiotic disks comprising imipenem

Table 1: Multiplex PCR conditions for PCR amplification of MBL genes

Gene target(s)	Primer sequence (5'-3', as synthesized)	Expected amplicon size (bp)
*bla*IMP-1	F1 (5'-ACC GCA GCA GAG TCT TTG CC-3')	587
	R1 (5'-ACA ACC AGT TTT GCC TTA CC-3')	
*bla*VIM-1	F3 (5'-AGT GGT GAG TAT CCG ACA G-3')	261
	R3 (5'-ATG AAA GTG CGT GGA GAC-3')	

F: Forward primer, R: Reverse primer

(IPM, 10 µg), meropenem (MEM, 10 µg), ertapenem (ETP, 10 µg), cefoxitin (FOX, 30 µg), ceftazidime (CAZ, 30 µg), sulphamethoxazole-trimethoprim (SXT, 25 µg), gentamicin (CN, 10 µg), cefotaxime (CTX, 30 µg), ceftriaxone (CRO, 30 µg), ciprofloxacin (CIP, 10 µg), ofloxacin (OFX, 10 µg), oxacillin (OX, 10 µg), ampicillin (AMP, 10 µg), cefepime (FEP, 30 µg), aztreonam (ATM, 30 µg), nitrofurantoin (F, 10 µg) and cloxacillin (OB, 200 µg) was used for susceptibility testing. Antimicrobial susceptibility testing was carried out on Mueller-Hinton (MH) agar plates (Oxoid, UK) as was previously described[8-10]. The test antibiotic disks were each aseptically placed at a distance of 25 mm apart on MH agar plates already swabbed with the test isolates (adjusted to 0.5 McFarland turbidity standards). All culture plates were incubated at 30°C for 18-24 h. The zones of inhibition was recorded and interpreted as susceptible or resistant based on the standard antibiotic breakpoints of CLSI[10].

Screening and phenotypic detection of metallo-β-lactamase (MBL): To phenotypically screen for the production of MBL in the test *E. coli* isolates, the susceptibility of the isolates to the carbapenems including imipenem and meropenem was evaluated as per the CLSI criteria[10]. Isolates with inhibition zone diameter $\leq$23 mm was phenotypically evaluated for MBL production using the inhibition based assay technique. *E. coli* isolates (adjusted to 0.5 McFarland turbidity standards) were aseptically swabbed on MH agar plates. Imipenem (10 µg) and meropenem (10 µg) disks impregnated with EDTA (1 µL) was aseptically placed on the MH agar plates. Supplementary imipenem (10 µg) and meropenem (10 µg) disks without EDTA was also placed adjacent to the carbapenem disks encumbered with EDTA. All plates were incubated at 30°C for 18-24 h. A difference of $\geq$7 mm between the zones of inhibition of any of the carbapenem disks with and without the chelating agent (EDTA) infers MBL production phenotypically[3,11-13].

Multiplex PCR amplification of MBL genes: Multiplex PCR technique was performed to amplify the gene sequence of MBL genes, including *bla*IMP-1 and *bla*VIM-1 genes, as described previously (Table 1)[14]. The genotypes of all 15 *E. coli* isolates were analyzed for the presence of *bla*IMP-1 and *bla*VIM-1 genes in a thermal cycler (Lumex instruments, Canada) with a final volume of 26.5 µL master mix comprising 0.2 µL of *taq* polymerase enzyme U/µL, 2.5 µL of 10X PCR buffer along with 2.5 µL $MgCl_2$, 1 µL of 10 pM from each of the forward and reverse primers, 2.5 µL of dNTPs MIX (2 mM), 3 µL of DNA template (from the test isolates), 14.8 µL of nuclease-free water. The PCR conditions used for gene amplification were as described by Shibata *et al.*[14]. A 100 bp DNA molecular marker was used as the positive control (marker) while the negative control was nuclease-free water. Amplified PCR products were separated according to their individual sizes using 1.5% agarose gel electrophoresis which was run for 2 h at 80 V.

Statistical analysis: The data were analyzed using SPSS version 23.0 (SPSS, Chicago, IL, USA) with chi-square test and one-way analysis of variance. The differences in data were considered statistically significant at $p<0.05$.

RESULTS

Table 2 shows the rate of isolation and identification of *Escherichia coli*. *E. coli* was isolated from 48 swab samples out of 120 swab samples from the anal region of cows. The recovery rate of *E. coli* from the rectal swab samples analyzed in this study was 40% (Table 2). Based on the data obtained in this preliminary result, *E. coli* ferments lactose and produces pinkish colonies on MAC agar. On EMB agar, *E. coli* produces colonies with metallic sheen (Table 2). Table 3 shows the result of antimicrobial susceptibility testing of 48 *E. coli* isolates. The antimicrobial susceptibility test reveals a high rate of resistance among the 48 *E. coli* isolates. Resistance was noted in the *E. coli* isolates especially to carbapenems, penicillins, aminoglycosides, fluoroquinolones and cephalosporins used in this study.

A total of 47 (97.9%) *E. coli* isolates and 48 (100%) *E. coli* isolates was resistant to aztreonam and ceftazidime. This marked the highest resistant rate of *E. coli* in this study. It was also found that 36 (75%) isolates of the *E. coli*, 39 (81.3%) *E. coli* isolates, 45 (93.8%) *E. coli* isolates, 34 (70.8%) *E. coli* isolates and 40 (83.3%) *E. coli* isolates were resistant to nitrofurantoin, oxacillin, cefotaxime, ciprofloxacin and ertapenem, respectively (Table 3).

Table 2: Isolation and characterization of *Escherichia coli*

Organism	Sample source	Indole test	Methyl red test	Gram staining	Colonial features of *E. coli* on culture media	n (%) isolation of *E. coli*
Escherichia coli	Anal swabs of cow	+ve	+ve	-ve	Metallic sheen colonies on EMB and pinkish colonies on MAC	48 (40 %)

Keys: n: Number of isolates, %: Percentage, -ve: Negative, +ve: Positive, EMB: Eosin methylene blue agar, MAC: MacConkey agar

Table 3: Susceptibility test results of *Escherichia coli*

Antibiotics (µg)	*Escherichia coli*	
	S n (%)	R n (%)
CRO (30)	13 (27.1)	35 (72.9)
FOX (30)	12 (25)	36 (75)
IPM (10)	37 (77.1)	11 (22.9)
CAZ (30)	0 (0)	48 (100)
ETP (30)	8 (16.7)	40 (83.3)
OX (5)	9 (18.8)	39 (81.3)
OFX (10)	24 (50)	24 (50)
CN (10)	34 (70.8)	10 (20.8)
SXT (25)	25 (52.1)	23 (47.9)
CIP (10)	14 (29.2)	34 (70.8)
CTX (30)	3 (6.3)	45 (93.8)
MEM (10)	26 (54.2)	22 (45.8)
AMP 10)	18 (37.5)	30 (62.5)
ATM (30)	1 (2.1)	47 (97.9)
F (10)	12 (25)	36 (75)
OB (500)	15 (31.3)	33 (68.8)

Key: S: Susceptible, R: Resistant, IPM: Imipenem, MEM: Meropenem, ETP: Ertapenem, FOX: Cefoxitin, CAZ: Ceftazidime, SXT: Sulphamethoxazole-trimethoprim, CN: Gentamicin, CTX: Cefotaxime, CRO: Ceftriaxone, CIP: Ciprofloxacin, OFX: Ofloxacin, OX: Oxacillin, AMP: Ampicillin, ATM: Aztreonam, F: Nitrofurantoin and OB: Cloxacillin

Table 4: Frequency of MBL-producing *E. coli* detected by inhibition-based assay

Organism	Source	MBL positive n (%)	MBL negative n (%)
Escherichia coli	Abattoir (Anal swabs of cow)	15 (31)	33 (68.8)

Table 5: Occurrence of MBL genes in the *Escherichia coli* phenotypes

MBL genes	Number of isolates (%)	Antibiogram[a] (Number of isolates)
*bla*IMP-1	8 (16.7)	IPM (5), ETP (3), FOX (4), CAZ (3), CN (6), CTX (7)
*bla*VIM-1	0 (0)	

[a]Antibiogram, IPM: Imipenem (10 µg), ETP: Ertapenem (10 µg), FOX: Cefoxitin (30 µg), CAZ: Ceftazidime (30 µg), CN: Gentamicin (10 µg) and CTX: Cefotaxime (30 µg)

Table 4 shows the result of the phenotypic detection of MBL-producing *E. coli* isolates using the inhibition-based assay. The MBL production was phenotypically detected in only 15 (31%) *E. coli* isolates out of the 48 isolates of *E. coli* that was phenotypically screened for the production of MBL using the inhibition-based assay (Table 4). The production of MBL is phenotypically inhibited by EDTA *in vitro* since this carbapenem-hydrolyzing enzyme requires zinc ion (Zn^{2+}) for enzyme activity. Figure 1 shows a positive test plate

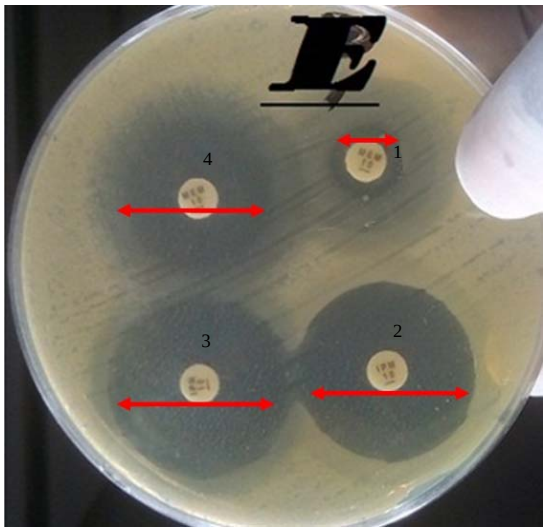

Fig. 1: A strain of *Escherichia coli* tested using the inhibition based assay, and showing a positive result for the production of metallo-β-lactamase (MBL) phenotypically. The difference in zone size between imipenem and meropenem disks used alone and imipenem and meropenem impregnated with EDTA differ by ≥7 mm, Key: 1: Meropenem (10 µg) without EDTA, 2: Imipenem (µg) with EDTA, 3: Meropenem (µg) with EDTA and 4: Imipenem (10 µg) without EDTA

of the inhibition based assay in which the production of MBL in the test *E. coli* isolate was inhibited by the presence of EDTA (as shown in the lower panel of the plate) while the upper panel of the plate lacked EDTA and thus the test isolated showed reduced susceptibility to the test carbapenem. Figure 2 shows a negative test plate in which the production of MBL was not phenotypically detected by the inhibition based assay.

Table 5 shows the frequency of MBL genes detected in the 15 *Escherichia coli* phenotypes by multiplex PCR technique. Out of the 15 MBL *E. coli* phenotypes analyzed by multiplex PCR technique for the detection of specific MBL genes including *bla*IMP-1 and *bla*VIM-1 MBL genes, only the *bla*IMP-1 MBL genes was detected in the *E. coli* phenotypes (Table 5). The *bla*VIM-1 MBL genes were not detected by the multiplex PCR technique used in this study. The prevalence of

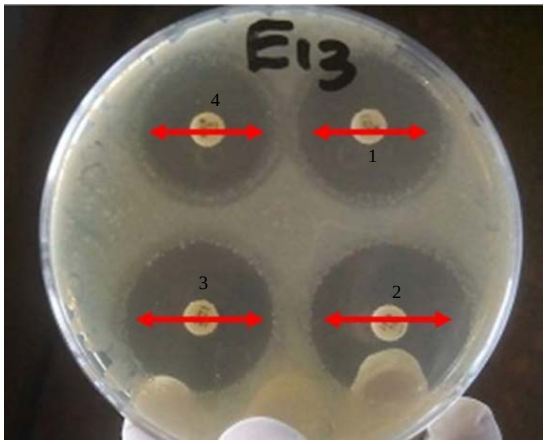

Fig. 2: *Escherichia coli* isolate susceptible to imipenem and meropenem with and without EDTA. This isolate of *E. coli* did not produce MBL phenotypically, Key: 1: Meropenem (10 µg) without EDTA, 2: Imipenem (µg) with EDTA, 3: Meropenem (µg) with EDTA and 4: Imipenem (10 µg) without EDTA

*bla*IMP-1 gene in the MBL *E. coli* phenotypes was 36.4%. This implies that only 8 isolates out of the 15 MBL *E. coli* phenotypes investigated by multiplex PCR harboured the *bla*IMP-1 MBL genes (Table 5). The *E. coli* phenotypes that harboured the *bla*IMP-1 MBL genes were resistant to imipenem, ertapenem, cefoxitin, ceftazidime, gentamicin and cefotaxime (Table 5). Electrophoretic analysis of the amplified PCR product of the MBL phenotypes after gene amplification using multiplex PCR technique revealed bands in the electrophoretogram. The electrophoretic analysis was positive for *bla*IMP-1 MBL genes with a base pair size of 587 bp (Fig. 3).

DISCUSSION

Metallo-β-lactamase (MBL) is a carbapenem-hydrolyzing enzyme produced by Gram-negative bacteria and which gives bacteria the exceptional ability to resist the antimicrobial onslaught of carbapenems such as imipenem. The increasing reports of the development and spread of multidrug resistance genes amongst pathogenic bacteria in both the community and hospital environments is alarming[11,15-18]. A total of 48 *E. coli* isolates was bacteriologically recovered from the rectal swab samples analyzed in this study. *E. coli* has been noted as a causative agent in diarrheal and gastrointestinal infections[18-22]. The prevalence of *E. coli* (40%) in this study is similar to a previous report by Iroha *et al.*[21] who reported the prevalence of antibiotic resistant *E. coli* isolates and other Gram-negative bacteria from abattoir effluent samples. Akinniyi *et al.*[22] also reported in their study that *E. coli* was among the most prevalent bacteria isolated from environmental samples including samples from poultry farms. In Australia, Leung *et al.*[23] also reported similar prevalence of *E. coli* from environmental samples. The irrational use of antibiotics in the rearing and production of food-producing animals is very strategic for the development and dissemination of antibiotic resistant bacteria in the community. The occurrence of *E. coli* in environmental samples have been previously reported as important causative factor in some community acquired infections[22,16,17,23]. The *E. coli* isolates showed varying levels of susceptibility and resistance to the antimicrobial activity of the antibiotics used in this study. Most importantly, the isolated *E. coli* was highly resistant to ceftriaxone (72.9%), cefoxitin (75%), ceftazidime (100%) and cefotaxime (93.8%). Reduced susceptibility to cloxacillin (68.8%), nitrofurantoin (75%), aztreonam (97.9%), ampicillin (62.5%), ciprofloxacin (70.8%), oxacillin (81.3%) and ertapenem (83.3%) was also recorded. There was no statistical difference in the antibiogram data of *E. coli* to the tested antibiotics ($p>0.05$). The high levels of resistance of *E. coli* isolates in this study is similar to previous studies reported in the Netherlands, Nigeria and Uganda[24-26]. Bergenholtz *et al.*[17] also reported high resistance of *E. coli* isolates from environmental samples to some conventional antibiotics. In Uganda, it was also reported that 168 *E. coli* isolates out of 182 *E. coli* isolates were resistant to several antibiotic classes including penicillins, fluoroquinolones, aminoglycosides and tetracyclines[25]. The negative impact of antimicrobial usage in animals on humans has been elucidated by Hamer and Gill[27]. *E. coli* have been previously noted as producers of MBLs and such phenotypes confers on the bacteria the exceptional ability to be resistant to a wide variety of antibiotics[28,29]. The MBL production was significantly detected in only 15 (31%) isolates of *E. coli* out of the 48 isolates of *E. coli* that was phenotypically screened ($p<0.05$). Multiplex PCR analysis using *bla*IMP-1 and *bla*VIM-1 specific primers revealed that the MBL phenotypes in this study harboured only the *bla*IMP-1 MBL genes. The *bla*VIM-1 MBL gene was not detected in the *E. coli* phenotypes confirmed by multiplex PCR in this study. The prevalence of MBL-producing *E. coli* that were found to harbour MBL genes in this study is 16.7%. This rate is much higher than the result of Adwan *et al.*[30] who reported 87.4% carriage rate of *bla*IMP-1 genes in *E. coli* isolates in their study. Similar rates of *bla*IMP-1 gene prevalence have also been reported by Chouchani *et al.*[31] in Tunisia. Enwuru *et al.*[32] in Lagos, Nigeria reported that the prevalence of MBL positive bacteria harbouring the *bla*IMP-1 and *bla*IMP-2 genes in their study was between 40-50%.

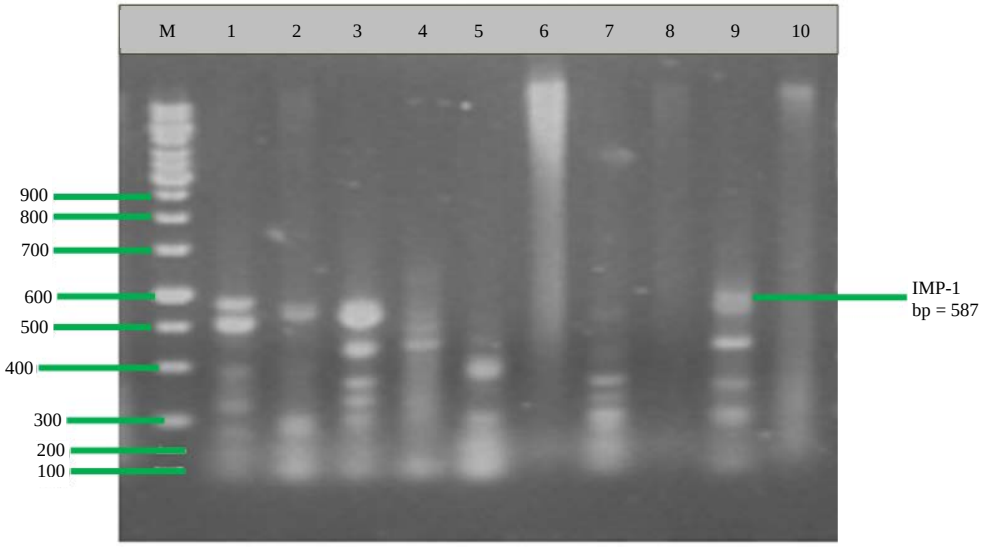

Fig. 3: Electrophoretogram of amplification of *bla*IMP-1 MBL genes amplified from different DNA samples from MBL positive *E. coli* phenotypes, Key: Lane M: DNA markers/ladder, Lanes 1, 2, 3 and 9: Amplified products of blaIMP-1 MBL genes with a base pair size of 587 bp, Lanes 4, 5, 6, 7 and 8: Lanes without gene amplification, Lane 10: Negative control which contains nuclease free water

Mansouri *et al.*[33] in Iran also reported lesser prevalence of MBL genes in their study. The occurrence of MBL-producing *E. coli* isolates in this study is similar to the work of Leung *et al.*[23] in Australia who reported the occurrence of MBL-producing *E. coli* from environmental samples. Chakraborty *et al.*[29] also reported similar prevalence of *E. coli* isolates positive for MBL production in India. This result is also similar to the work of Bashir *et al.*[28] who recorded higher prevalence of MBL-producing *E. coli* isolates in their study carried out in Kashmir. To our knowledge this is the first description of *bla*IMP-1 MBL-producing *Escherichia coli* from rectal swabs of cow in Abakaliki, Nigeria. Our results draw attention to the importance of MBL genes as a key mediator of carbapenem resistance in *E. coli* isolates from the non-hospital environment. The most interesting finding in the present study was the first multiplex PCR detection of *bla*IMP-1 genes from *E. coli* isolates emanating from abattoir samples. *bla*IMP-1 Genes represents the most common resistance traits in carbapenemase-producing *Enterobacteriaceae* and this MBL gene is known to be distributed globally[1].

CONCLUSION AND RECOMMENDATION

From this study, it was observed for the first time that *E. coli* from rectal swabs of cow in Abakaliki, Nigeria, harbour *bla*IMP-1 MBL genes. This gene is responsible for bacterial resistance to the carbapenems. The occurrence of *E. coli* isolates that harbour genes for metallo β-lactamase (MBL) production in abattoir samples is implicative for carbapenem resistance in the non-hospital environment.

Authors recommend improved personal and environmental hygiene coupled with better alternative growth promoting agents that excludes the use of antibiotics in animal husbandry. A well-organized detection protocol, antibiotic surveillance system and intervention measure is paramount to contain the emergence and spread of Gram-negative bacteria harbouring genes for MBL production in the community.

SIGNIFICANCE STATEMENTS

This study identified the presence of *bla*IMP-1 MBL genes that is responsible for the resistance of Gram-negative bacteria to carbapenems such as imipenem and meropenem. Carbapenems are used to treat serious bacterial infections including those caused by organisms that produce extended spectrum β-lactamases (ESBLs). This study provides some epidemiological data necessary to take action towards containing the emergence and spread of MBL-positive bacteria in the non-hospital environment.

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