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Plasmid Curing, Beta-Lactamase Production, Antibiogram and Metallo-β-lactamase (MBL) Detection in *Escherichia coli* and *Klebsiella* Species from Non-Hospital Sources of Abattoir and Poultry

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Abstract: Metallo-β-lactamases (MBLs) are carbapenem-hydrolyzing enzymes that give Gram-negative bacteria including *Escherichia coli* and *Klebsiella* species the exceptional ability to ward-off the antimicrobial onslaught of the carbapenems such as imipenem and meropenem. This study detected the possible occurrence of MBL-producing *E. coli* and *Klebsiella* species isolates from abattoir and poultry sources. A total of 370 samples comprising samples from abattoir benches/tables, cloacael of poultry birds and anal swabs of cow were used for this study. The isolation and identification of *E. coli* and *Klebsiella* species was bacteriologically carried out using standard microbiology techniques. MBL production and beta-lactamase production was carried out using the Hodges (Cloverleaf) test and nitrocefin sticks respectively. Antimicrobial susceptibility testing and plasmid curing analysis was correspondingly carried out by the Kirby-Bauer disk diffusion technique and 0.1 mg/ml of acridine orange. The results obtained showed that a total of 168 isolates of *E. coli* and 141 isolates of *Klebsiella* species were isolated from the abattoir and poultry samples. Beta-lactamase production was expressed more in *E. coli* isolates (38 %) than in isolates of *Klebsiella* species (29 %). More than 50 % of the isolates of *E. coli* and *Klebsiella* species showed high resistance to ceftriaxone, cefoxitin, imipenem, ceftazidime, ertapenem, gentamicin, amikacin, nitrofurantoin, cloxacillin and ciprofloxacin. MBL production was phenotypically detected in 8, 7 and 7 isolates of *E. coli* from abattoir table/bench, poultry birds and anal swabs of cow respectively. In isolates of *Klebsiella* species, MBL was phenotypically detected in a total of 6, 7 and 5 isolates from abattoir table/bench, poultry birds and anal swabs of cow respectively. Plasmid curing analysis showed that the MBL positive *E. coli* and *Klebsiella* species harboured their resistant genes on both their plasmids and chromosome. The occurrence of MBL-producing isolates of *E. coli* and *Klebsiella* species in abattoir and poultry samples as observed in this study calls for concern because of the clinical significance of MBLs. There is need for proper detection and reporting of multidrug resistant bacteria such as MBL-positive bacteria in order to forestall the emergence and spread of drug resistant bacteria in the non-hospital environment. Public health officials and the government should be aware of multidrug resistant bacteria in a non-hospital environment so that sustainable measures will be put in place to control their menace.

Key words: Plasmid · Antibiotic Resistance · *Enterobacteriaceae* · Metallo-Β-Lactamase · Abattoir · Poultry · Nigeria

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INTRODUCTION

Enterobacteriaceae is a large bacterial family that contains many bacterial organisms that are of medical importance including *Escherichia coli* and *Klebsiella* species. *E. coli* and *Klebsiella* species frequently cause some nosocomial and community-acquired infections including urinary tract infections (UTIs), bacteremia, sepsis and bacterial pneumonia [1- 3]. Most infections caused by *E. coli* and *Klebsiella* species are fast becoming resistant to some commonly used antibiotics because they possess genes that are responsible for the production of antibiotic-degrading enzymes such as metallo-β-lactamases (MBLs). Multidrug resistant bacteria that produce MBLs have the exceptional ability to resist the antimicrobial action of the carbapenems such as imipenem. These Gram-negative bacteria from abattoir and poultry sources are a constant source of the emergence and spread of antibiotic resistant bacteria in human population; and they can clonally spread to susceptible bacteria in their environment through genetic transfer means such as transformation and conjugation [4 - 10]. The non-hospital sources of MBL-producing bacteria including those that produce other antibiotic degrading enzymes such as extended spectrum β-lactamases (ESBLs) are of public health importance due to the possibility of transmission of resistant bacteria from these animals to humans either through the consumption of the meat and other products that come from them or through contact [1, 4,5]. Of particular concern is the fact that these resistant pathogens are responsible for some community-acquired infections that may lead to the hospitalization of the infected individuals [1, 4 - 7]. According to Tian *et al*. [7] the long-term exposure of humans and non-human hosts including livestock, poultry birds and bees to antibiotics could cause the accumulation of antibiotic resistance determinants in the gut microbiota of these animals. MBLs are beta-lactamase enzymes produced by pathogenic bacteria and which hydrolyzes the carbapenems and render them ineffective for treatment; and the long-term usage of the carbapenems as well as the continued and possibly irrational usage of other classes of antibiotics in hospital and non-hospital environment encourages the emergence and spread of drug resistant bacteria[7-11]. MBL production in bacteria is encoded by genes that have been procured by pathogenic bacteria either by mutation or by horizontal gene transfer from other resistant microbes [9]. MBLs have high affinity for zinc ions (Zn^{2+}); and thus the enzyme is largely inhibited by chelating agents such as EDTA *in vitro*. The environmental reservoirs of antibiotic resistant bacteria including those that produce MBLs are still ill-detected in this part of the world; and the detection of these organisms in clinically important samples in our hospitals is still wanting. The emergence and spread of resistant bacteria in the non-hospital environment especially amongst animals and poultry birds is very important to human health especially now that we experience an upsurge of zoonotic infections in our world. This study evaluated by phenotypic techniques the prevalence of MBL-producing *E. coli* and *Klebsiella* species from non-hospital sources of abattoir and poultry.

MATERIALS AND METHODS

Sampling: A total of 370 samples from abattoir tables (n=130), anal region of cow (n=120) and the cloacae of poultry birds (n=120) were collected from various abattoir and poultry farms in Abakaliki metropolis, Nigeria using sterile swab sticks socked in normal saline.

Isolation and Identification of Bacteria: *Escherichia coli* and *Klebsiella* species was bacteriologically isolated from the abattoir and poultry samples on eosin methylene blue (EMB) agar and MacConkey agar (Oxoid, UK) plates. The culture plates were incubated at 30°C for 18-24 hours. After incubation, suspected colonies of *E. coli* and *Klebsiella* species were aseptically subcultured onto freshly prepared EMB and MacConkey agar plate(s) for the isolation of pure cultures of *E. coli* and *Klebsiella* species. *E. coli* and *Klebsiella* species were microscopically identified using standard microbiology identification techniques [12].

Antimicrobial Susceptibility Testing (AST): AST was carried out as per the criteria of Clinical and Laboratory Standard Institute (CLSI) using standard antibiotic disks including imipenem (IPM, 10 µg), meropenem (MEM, 10 µg), ertapenem (ETP, 10 µg), cefoxitin (FOX, 30 µg), ceftazidime (CAZ, 30 µg), amikacin (AK, 10 µg), gentamicin (CN, 10 µg), cefotaxime (CTX, 30 µg), ceftriaxone (CRO, 30 µg), ciprofloxacin (CIP, 10 µg), ofloxacin (OFX, 10 µg), oxacillin (OX, 5 µg), ampicillin (AMP, 10 µg), aztreonam (ATM, 30 µg), nitrofurantoin (F, 10 µg) and cloxacillin (OB, 5 µg) [Oxoid, UK]. Susceptibility studies was carried out by the modified Kirby-Bauer disk diffusion method on Mueller-Hinton (MH) agar plates (Oxoid, UK) as was previously described [2,13,14]. All susceptibility plates were incubated at 30°C for 18-24 hours and AST results were recorded based on

their individual inhibition zone diameters (IZD) as susceptible (S) and resistant (R).

Beta-Lactamase Production: Beta-lactamase production was phenotypically evaluated using the Nitrocefin test sticks (Oxoid, UK) as described by the method of Akinduti *et al*. [15].

Screening Test for MBL Production: The screening of the bacterial isolates for the production of MBL enzymes was conducted 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 [14,16,17]. 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 [16 - 18].

Plasmid Curing: Plasmid curing experiment was undertaken to determine the location (plasmid or chromosomal) of the drug resistance determinants in the MBL-positive *E. coli* and *Klebsiella* species according to a previously described method using 0.1 mg/ml acridine orange [13].

Statistical Analysis: Statistical analysis was carried out with the Statistical Package for Social Sciences (SPSS) version 23.0. The significance of AmpC positive isolates and MBL positive isolates was determined using Chi square tests at p-value < 0.05 and at a confidence interval of 95 %. A p-value < 0.05 was considered statistically significant.

RESULTS

In this study, the prevalence of MBL-producing *E. coli* and *Klebsiella* species from non-hospital sources of abattoir and poultry was phenotypically evaluated. *E. coli* was isolated from 69/130 swab samples from abattoir tables, 51/120 swab samples from cloacal swabs of poultry birds and from 48/12 swab samples from the anal region of cows (Table 1). *E. coli* fermented lactose and produced pinkish colonies on MacConkey agar and metallic sheen colonies on eosin methylene blue (EMB) agar. The recovery rate of *Klebsiella* species isolates was 40/130 (28.4 %), 49/120 (34.8 %) and 52/120 (36.9 %) for samples from abattoir benches, poultry birds and rectal swabs of cow respectively (Table 1). *Klebsiella* species produced large and mucoid colonies on MacConkey agar and non-metallic mucoid colonies on EMB agar. Beta-lactamase production was phenotypically detected in 38 % *E. coli* and 29 % of the *Klebsiella* species isolates. The result of the susceptibility of the *E. coli* and *Klebsiella* species isolates to the tested antibiotics is shown in Table 2.

The *E. coli* isolates from abattoir and poultry sources showed reduced susceptibility to ceftriaxone (95.2 %), ceftazidime (96.4 %), cefotaxime (98.2 %), cefoxitin (74.4 %), oxacillin (81.5 %), ofloxacin (70.8 %), amikacin (64.9 %), ciprofloxacin (81.5 %) and aztreonam (93.5 %). However, the isolates of *Klebsiella* species showed similar levels of resistance to cefotaxime (96.5 %), aztreonam (96.5 %), ceftriaxone (89.4 %), oxacillin (87.2 %), ciprofloxacin (86.5 %), ceftazidime (82.3 %), cefoxitin (75.2 %) and cloxacillin (73.0 %).

There was no statistical difference in the percentage susceptibility of the *E. coli* isolates compared to the *Klebsiella* species isolates (p value > 0.05). Table 3 shows the result of the phenotypic screening of the *E. coli* and *Klebsiella* species isolates bacteriologically recovered from the various abattoir and poultry samples. MBL production was phenotypically detected in the *E. coli* and *Klebsiella* species isolates using the Hodges (Cloverleaf) technique. A total of 8 *E. coli* isolates from abattoir table/bench and 7 *E. coli* isolates from poultry and anal swabs of cow respectively was phenotypically confirmed to produce MBL by the Hodges test technique. The isolates of *Klebsiella* species produced MBL phenotypically in a total of 6 isolates from abattoir tables/benches, 7 isolates from poultry samples and 5 isolates from anal swabs of cow (Table 3). There was no statistical difference in the production of MBL in the *E. coli* and *Klebsiella* species isolates

Table 1: Isolation of *Escherichia coli* and *Klebsiella* species from abattoir and poultry samples

Organism	Swabs from abattoir benches (n = 130) n (%)	Cloacal swabs of poultry birds (n = 120) n (%)	Anal/rectal swabs of cow (n = 120) n (%)	Total
Escherichia coli	69 /130(41.1)	51 /120(30.4)	48/120 (28.6)	168
Klebsiella species	40/130 (28.4)	49 /120(34.8)	52/120 (36.9)	141

Keys: n = number of isolates; % = percentage prevalence= no, of isolates/no. of samples

Table 2: Percentage susceptibility of *Escherichia coli* and *Klebsiella* species

Antibiotics (µg)	*Escherichia coli* S	*Escherichia coli* R	*Klebsiella* species S	*Klebsiella* species R
CRO (30)	4.8	95.2	10.6	89.4
FOX (30)	25.6	74.4	24.8	75.2
IPM (10)	48.2	51.8	58.9	41.1
CAZ (30)	3.6	96.4	17.7	82.3
ETP (30)	13.1	86.9	15.6	84.4
OX (5)	18.5	81.5	12.8	87.2
OFX (10)	29.2	70.8	34.8	65.2
CN (10)	43.5	56.5	39.0	61.0
AK (10)	35.1	64.9	47.5	52.5
CIP (10)	18.5	81.5	13.5	86.5
CTX (30)	1.8	98.2	3.5	96.5
MEM (10)	44.6	55.4	56.7	43.3
AMP 10)	29.8	70.2	30.5	69.5
ATM (30)	6.5	93.5	3.5	96.5
F (10)	23.2	76.8	25.5	74.5
OB (500)	38.7	61.3	27.0	73.0

Non significance= (p value > 0.05)

Key: S = Susceptible, R = Resistant, IPM = imipenem, MEM = meropenem, ETP = ertapenem, FOX = cefoxitin, CAZ = ceftazidime, AK = amikacin, CN = gentamicin, CTX = cefotaxime, CRO = ceftriaxone, CIP = ciprofloxacin, OFX = ofloxacin, OX = oxacillin, AMP = ampicillin, ATM = aztreonam, F = nitrofurantoin, OB = cloxacillin

Table 3: Prevalence of *Escherichia coli* and *Klebsiella* species that produced MBL

Organism (n)	Source	Total n (%)
Escherichia coli (69)	Abattoir table/bench	8 (11.6)
Escherichia coli (51)	Poultry	7 (13.7)
Escherichia coli (48)	Anal swabs of cow	7 (14.6)
Klebsiella species (40)	Abattoir table/bench	6 (15.0)
Klebsiella species (49)	Poultry	7 (14.3)
Klebsiella species (52)	Anal swab of cow	5 (9.6)

p value > 0.05

Table 4: Plasmid curing analysis of selected *E. coli* and *Klebsiella* species isolates using 0.1 mg/ml of acridine orange

Organisms	Pre-curing (No. of isolates)	Cured isolates n	Cured isolates %	Not-cured n	Not-cured %
Klebsiella species	14	10	71.4	4	28.6
Escherichia coli	5	4	80	1	20

(p value > 0.05). The result of the plasmid curing analysis of the MBL-positive *E. coli* isolates and *Klebsiella* species using acridine orange (0.1 mg/ml) is shown in Table 4.

The plasmid curing experiment carried out in this study revealed that out of the 14 isolates of *Klebsiella* species selected for plasmid curing analysis, only 10 isolates harboured their resistance trait on their plasmid because their plasmid was successfully cured through acridine orange (0.1 mg/ml) treatment. However, out of the 5 isolates of *E. coli* treated with acridine orange (0.1 mg/ml), only 4 isolates of the *E. coli* harboured their resistance trait on their plasmid. Overall, while some of the *E. coli* and *Klebsiella* species isolates harboured their resistance traits on their plasmid, the other isolates which were not affected by acridine orange (0.1 mg/ml) treatment harboured their resistance trait on their chromosome (Table 4).

DISCUSSION

Abattoirs and poultry farms are good breeding grounds for the selection of antibiotic resistant microbes and also for the dissemination of resistant traits to other susceptible bacteria in the same environment. Antibiotics are usually added to the feed and water of livestock and poultry birds to encourage their growth and take care of possible infection. Such supplementation of antibiotics in the water and feed of food-producing animals poses health risk to the human population due to the selection of antibiotic resistance strains of bacteria and the possible spread of same in the food chain [1,16].

In this study, the occurrence of MBL-producing *E. coli* and *Klebsiella* species isolates from abattoir and poultry sources was phenotypically evaluated; and the production of beta-lactamase and the source of the resistance traits was also phenotypically evaluated using plasmid curing technique. A total of 168 *E. coli* isolates and 141 isolates of *Klebsiella* species was isolated from the abattoir and poultry samples bacteriological analyzed in this study. The rate of isolation of *E. coli* and *Klebsiella* species in this study is similar to the work of Leung *et al*. [19] and Akinduti *et al*. [15] that showed that *E. coli* and *Klebsiella* species were the most prevalent isolated members of the *Enterobacteriaceae* in their study conducted in Australia and Southwest Nigeria respectively. The production of beta-lactamase was largely detected in more *E. coli* isolates (38 %) than in the isolates of the *Klebsiella* species (29 %). According to Bush and Jacoby [8], the presence of beta-lactamase enzyme in bacteria provides opportunity for the horizontal transmission of resistance genes amongst bacteria in the same environment especially when they undergo mutation. Reduced susceptibility of the *E. coli* isolates was observed to the cephalosporins and carbapenems. However, very low levels of susceptibility of the *E. coli* isolates was also observed with cefoxitin, oxacillin, ofloxacin, amikacin, ciprofloxacin and aztreonam at resistance rate of 74.4, 81.5, 70.8, 64.9, 81.5 and 93.5 % respectively. This high level of *E. coli* resistance to some conventional antibiotics as obtainable in this study is in conformity to the work of Ogunleye *et al*. [20] who reported that *E. coli* from environmental samples are commonly resistant to some available antibiotics. Similarly, Bogaard *et al*. [4] and Majalija *et al*. [21] also showed in their respective works carried out in the Netherlands and Uganda that *E. coli* show high level resistance to the cephalosporins, carbapenems and some non-beta-lactams. The isolates of *Klebsiella* species recovered in this study was also found to show high level of resistance to the cephalosporins, carbapenems, other beta-lactams and some non-beta-lactams especially to cefotaxime (96.5 %), aztreonam (96.5 %), ceftriaxone (89.4 %), oxacillin (87.2 %), ciprofloxacin (86.5 %), ceftazidime (82.3 %), nitrofurantoin (74.5 %), amikacin (52.5 %), gentamicin (61.0 %), cloxacillin (73.0 %), imipenem (41.1 %) and ertapenem (84.4 %). Eze [22] noted in his work that isolates of *Klebsiella* species from environmental samples are notoriously resistant to some antibiotics. It could be observed in our study that the use of antibiotics as growth promoting agents in the production and rearing of animals contributes a great deal to the development and spread of antimicrobial resistance in abattoir and poultry environments. Out of the 168 isolates of *E. coli*, MBL production was phenotypically detected in only 22 isolates while 18 isolates of *Klebsiella* species produced MBL phenotypically out of the 141 isolates of *Klebsiella* species recovered in this study. Okazaki *et al*. [23] in their study showed that MBL-producing *Klebsiella* species exist in non-hospital environments. Out of the MBL-positive *E. coli* and *Klebsiella* species isolates selected for plasmid curing experiment, our result showed that 10 (71.4 %) isolates of *Klebsiella* species harboured their resistance traits in their plasmid while only 4 (80 %) isolates of *E. coli* harboured their resistance traits in their plasmids. According to the work of Akinjogunla and Enabulele [24], the genetic elements responsible for antimicrobial resistance in Gram-negative bacteria could be plasmid-borne or chromosomally-borne. As we have presumptively demonstrated in this study, *E. coli* and *Klebsiella* species from abattoir and poultry sources are

highly resistant to some available and potent antibiotics; and these organisms also produce metallo-β-lactamase (MBL) enzyme which give them the exceptional ability to be resistant to the carbapenems which are used to treat serious bacterial infections including those caused by bacteria that produce ESBLs. Also, the MBL-positive *E. coli* and *Klebsiella* species carried their resistance traits on their plasmids; and this provides an opportunity for the clonal spread of this resistance factor to susceptible bacteria in their environment. It is therefore important to control the use of antibiotics outside the hospital environment especially in animal husbandry and poultry practices in order to bring to a halt the proliferation of carbapenem-resistant strains of bacteria that produce MBLs.

CONCLUSIONS

The high prevalence and resistance of isolates of *E. coli* and *Klebsiella* species from poultry and abattoir sources to antibiotics as observed in this study points to the possible irrational misuse of antibiotics in the non-hospital environment. This study reported the prevalence of MBL-producing *E. coli* and *Klebsiella* species isolates from abattoir and poultry sources. Given the importance of the carbapenems in clinical medicine, it is important to always be on the lookout for MBL-producing bacteria in the community and hospital environment in order to preserve the efficacy of these important group of antibiotics which carbapenem-resistant strains hydrolyzes through the production of metallo-β-lactamases (MBLs).

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