Original Article

Detection of Metallo-β-lactamase (MBL) among Carbapenem-Resistant Gram-Negative Bacteria from Rectal Swabs of Cow and Cloacae Swabs of Poultry Birds

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Abstract

Background: Metallo-β-lactamases (MBLs) are carbapenem-hydrolyzing enzymes that give Gram-negative bacteria the exceptional ability to resist the antimicrobial onslaught of the carbapenems such as imipenem and meropenem. MBL-producing Gram-negative bacteria exist in the community and hospital environment and they put their use of the carbapenems at risk. **Aim:** This study phenotypically evaluated the prevalence of MBL-positive bacteria from carbapenem resistant Gram-negative bacteria of abattoir and poultry origin. **Materials and methods:** A total of 370 environmental samples comprising samples from abattoir tables, anal swabs of cow and cloacae swab samples of poultry birds were bacteriologically analyzed for the isolation of carbapenem Gram-negative bacteria. Antibiogram was determined by the modified Kirby-Bauer disk diffusion method and MBL production was confirmed using the modified Hodges test (MHT) technique. Nitrocefin sticks were used to screen the bacterial isolates for β-lactamase production. **Results:** A total of 168 *Escherichia coli*, 141 *Klebsiella* species and 147 *Pseudomonas aeruginosa* isolates were recovered from the samples. More than 50% of the isolated Gram-negative bacteria were highly resistant to carbapenems, cephalosporins, aminoglycosides and fluoroquinolones. β-lactamase production was detected in *E. coli* (38%), *P. aeruginosa* (33%) and *Klebsiella* species isolates (29%). The *E. coli* isolates was resistant to imipenem (51%), meropenem (55.4%) and ertapenem (86.9%). *Klebsiella* species and *P. aeruginosa* showed resistance to imipenem, meropenem and ertapenem at the rates of 41.1%, 43.3%, 84.4%; and 66.7%, 60.5%, 61.2% respectively. MBL was phenotypically detected in 22 (39.9%) carbapenem-resistant *E. coli* isolates, 21 (45.7%) *Klebsiella* species isolates and 20 (38.9%) *P. aeruginosa* isolates. **Conclusion:** Conclusively, this study reported the occurrence of MBL-producing *E. coli*, *Klebsiella* species and *P. aeruginosa* isolates from abattoir and poultry sources. The occurrence of MBL-producing bacteria in abattoir and poultry samples portends serious health implication for humans who depend on these animals for source of food; and this is due in part to the transmission of drug-resistant bacteria to human population.

Keywords: Carbapenem-resistant bacteria; Metallo-β-lactamase; Community acquired infection; Carbapenemases

Introduction

Food-producing animal's habouring Gram-negative bacteria possessing multidrug resistant genes together with genes that provoke the production of metallo-beta-lactamases (MBLs) possess health risks to the human population. This is because bacterial pathogens positive for MBL production are resistant to the carbapenems, which are widely used to treat serious Gram-negative infections including those caused be extended spectrum β-lactamase (ESBL)-producing bacteria. 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), especially those that have been previously reported to produce MBLs. [1-4]. MBLs are carbapenem-hydrolyzing beta-lactamases which belong to molecular Class B of Ambler beta-lactamase classification, and which have the ability to hydrolyze and confer resistance to carbapenems such as imipenem, meropenem, ertapenem and doripenem. [5-7]. MBLs, which are a type of carbapenemases, are an emerging public health problem among clinically important Gram-negative organisms and environmental isolates including *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and the *Enterobacteriaceae*. [2,3,7]. The broad spectrum activity and stability of the carbapenems to most beta-lactamase enzymes

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such as ESBLs makes the carbapenems an effective tool for the treatment of severe Gram-negative infections. [3,8,9]. Nevertheless, there are plethora of reports on the prevalence of MBL-producing Gram-negative bacteria from both clinical and environmental isolates around the world. [4,10-14]. The MBLs are known to confer variable range of high resistance to all beta-lactam antibiotics except the monobactams and their presence in clinically important Gram-negative bacteria have put the use of the carbapenems under threat. [5,7]. The MBLs belong to a group of beta-lactamases which requires divalent cations such as zinc ions as cofactors for their enzyme activity. [9]. The production of MBLs as a carbapenem resistance factors is a top arsenal of pathogenic bacteria used to make less-efficacious the therapeutic effects of the carbapenems and cephalosporins. [4,9,15]. MDRGNOs that produce MBLs in the community especially amongst animals and poultry birds are a constant source of the emergence and spread of antibiotic resistant bacteria in human population. [9,15]. These non-hospital sources of MBL-producing bacteria are of public health importance due to the possibility of transmission to humans through contact or consumption of contaminated animal and poultry products. This study evaluated phenotypically the detection of MBL among carbapenem-resistant Gram-negative bacteria from abattoir and poultry sources.

Methods

Determination of sample size

Sample size determination for this study was determined by the Cochran's formular; and a total of three hundred and seventy (370) environmental samples comprising samples from abattoir tables (n=130), anal region of cow (n=120) and the cloacae of poultry birds particularly broilers (n=120) were used in this study. The samples were collected from abattoirs and poultry farms in Abakaliki metropolis, Nigeria over a one year period (July, 2015 – June, 2016).

Isolation of bacteria

The environmental swab samples were each cultured in 5 ml double strength of nutrient broth (Oxoid, UK) and incubated overnight at 30°C. A loopful of the specimen or turbid solution was plated aseptically onto cetrimide selective agar plate(s), MacConkey agar (MAC) plates and eosin methylene blue (EMB) agar for the selective isolation of *P. aeruginosa*, *Klebsiella* species and *Escherichia coli* respectively. The culture plates were incubated at 30°C for 18-24 hours. *P. aeruginosa* isolates produces greenish pigmentation on cetrimide selective agar; *E. coli* also 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 sheen mucoid colonies on EMB agar. [16].

Quality control test

Quality control test to determine and confirm the isolated organisms as *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella* species was done with the reference strains: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 10145 and *Klebsiella* species ATCC 700603 (Oxoid, UK); and this was based on the microscopical and morphological and/or colonial characteristics of these control strains on culture media plates of MAC, cetrimide selective agar and EMB agar. The morphological appearances of the quality control organisms under the microscope were also used to evaluate the organisms isolated. These quality control strains were also used as standards for performing antimicrobial susceptibility testing.

Antimicrobial susceptibility testing (AST)

AST was carried out on all the recovered test bacterial isolates using the modified Kirby-Bauer disk diffusion method on Mueller-Hinton (MH) agar plates (Oxoid, UK) as was previously described, and based on the guideline of the Clinical and Laboratory Standard Institute, CLSI. [4,12,17]. This was done using single disks of: imipenem (IPM, 10 µg), meropenem (MEM, 10 µg), ertapenem (ETP, 10 µg), cefoxitin (FOX, 30 µg), ceftazidime (CAZ, 30 µg), cefotaxime (CTX, 30 µg), ceftriaxone (CRO, 30 µg), cloxacillin (OB, 500 µg), ciprofloxacin (CIP, 10 µg), aztreonam (ATM, 30 µg), ampicillin (AMP, 10 µg), nitrofurantoin (F, 10 µg), oxacillin (OX, 10 µg), ofloxacin (OFX, 10 µg), amikacin (AK, 10 µg) and gentamicin (CN, 10 µg). Antimicrobial susceptibility test results were recorded as susceptible (S), intermediate (I) and resistant (R) according to CLSI criteria. [17].

Nitrocefin test for beta-lactamase production

Beta-lactamase production by resistant test isolates was evaluated using the Nitrocefin test sticks (Oxoid, UK) as described by the method of Akinduti et al. [18].

Screening for metallo-beta-lactamase (MBL) production

To phenotypically screen for the production of MBL in the test isolates, the susceptibility of test organisms to imipenem (IPM), meropenem (MEM), and ertapenem (ETP) was evaluated as per the CLSI criteria (CLSI, 2011). Test isolates showing inhibition zone diameter (IZD) of ≤ 23 mm were considered and suspected to produce MBL enzyme; and these isolates were further tested using a phenotypic confirmation test. [6,11].

Modified Hodges Test for phenotypic detection of MBL

The modified Hodges or Cloverleaf test was performed by aseptically swabbing MH agar plates with *Escherichia coli* ATCC 25922 strain. The inoculated MH agar plates were allowed for about 5 min; and imipenem (10 µg) disks were aseptically placed at the center of the MH agar plates. The test bacteria (that showed reduced susceptibility to any of the carbapenems, and as adjusted to 0.5 McFarland turbidity standards) were heavily streaked from the edge of the IPM disk to the circumference of the MH agar plates. Susceptibility plates were incubated for 18-24 hrs at 30oC. The plates were macroscopically observed for indentation, and the growth of the test bacteria towards the imipenem disk. Growth of test bacteria towards the carbapenem disk is indicative of MBL production phenotypically. [6,11,13,14].

Multiple antibiotic resistance index (MARI)

Multiple antibiotic resistance indexes were determined for MBL-producing Gram-negative bacteria. The multiple antibiotic resistance index (MARI) was determined by the method of Akinjogunla and Enabulele [19]. using the formular: MARI = a/b; where 'a' represents the number of antibiotics to

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, environmental samples including cloacal swabs of poultry birds, anal swabs of cow and swab samples from abattoir and poultry sources were bacteriologically analyzed for the isolation of carbapenem Gram-negative bacteria that produce MBL phenotypically. Overall, *E. coli* was isolated from 69 swab samples out of 130 swab samples from slaughter/abattoir tables, 51 swab samples out of 120 cloacal swab samples of poultry birds, and from 48 swab samples out of 120 swab samples from the anal region of cows [Table 1]. The recovery rate of *Klebsiella* species isolates from the environmental swab samples including swab samples from abattoir tables, cloacal swab samples from poultry birds and anal swab samples from cows was 28.4%, 34.8%, and 36.9% respectively [Table 1]. *P. aeruginosa* was isolated from 56 swab samples out of 130 swab samples from abattoir/slaughter tables, 48 swab samples out of 120 cloacal swab samples, and from 43 swab samples out of 120 anal swab samples from cows. Abattoirs/slaughter houses and poultry farms are good grounds for the breeding, development and spread of antibiotic resistant bacteria including *E. coli*, *Klebsiella* species and *P. aeruginosa*.

Table 1: Recovery rate of gram negative bacteria on bacteriological media.

Organisms	Swabs from slaughter/ abattoir benches (n = 130) n (%)	Cloacal swabs of poultry birds	Anal/rectal swabs of cow (n = 120) n (%)	Total
Escherichia coli	69 (41.1)	51 (30.4)	48 (28.6)	168
Pseudomonas aeruginosa	56 (38.1)	48 (32.7)	43 (29.3)	147
Klebsiella species	40 (28.4)	49 (34.8)	52 (36.9)	141

Keys: n = number of isolates; % = percentage

The percentage susceptibility of the *Enterobacteriaceae* isolates in this study including *E. coli* and *Klebsiella* species isolates to the test antibiotics is shown in Figure 1. Figure 2 shows the percentage susceptibility of the *P. aeruginosa* isolates to the tested antibiotics.

Very low levels of susceptibility of the *E. coli* isolates was observed in cefoxitin, oxacillin, ofloxacin, amikacin, ciprofloxacin, and aztreonam at a rate of 74.4%, 81.5%, 70.8%, 64.9%, 81.5% and 93.5% respectively [Figure 1]. The resistance pattern most commonly observed amongst the *Klebsiella* species isolates was resistance to cefotaxime (96.5%), aztreonam (96.5%), ceftriaxone (89.4%), oxacillin (87.2%), ciprofloxacin (86.5%), ceftazidime (82.3%) and cloxacillin (73.0%). The antibiotic resistance pattern of the organisms isolated in this study confirmed that more than 50% of the *P. aeruginosa* isolates showed high level resistance to the carbapenems including imipenem (66.7%), ertapenem (61.2%) and meropenem (60.5%). Reduced susceptibility of the *P. aeruginosa* isolates was also observed in cefoxitin (80.3%), cefotaxime (79.6%), ceftriaxone (64.6%) and ceftazidime (54.4%) [Figure 3].

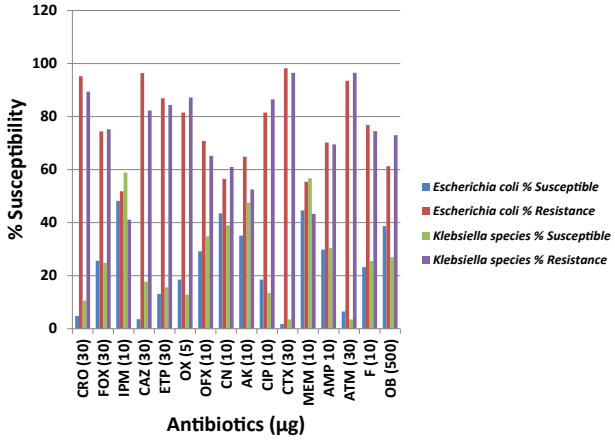

Figure 1: Percentage susceptibility profile of *E. coli* and *Klebsiella* species isolates.

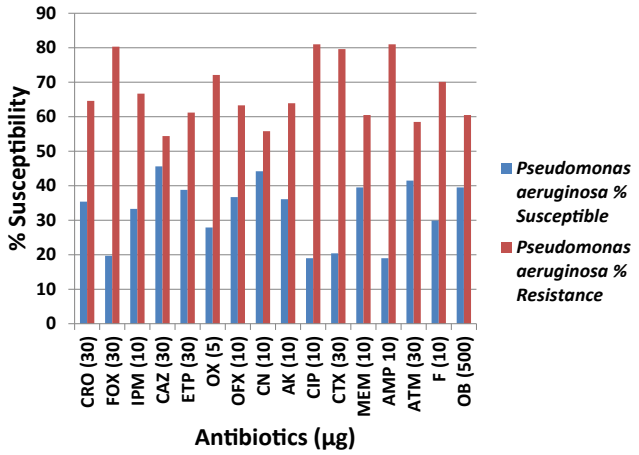

Figure 2: Susceptibility of the *P. aeruginosa* isolates.

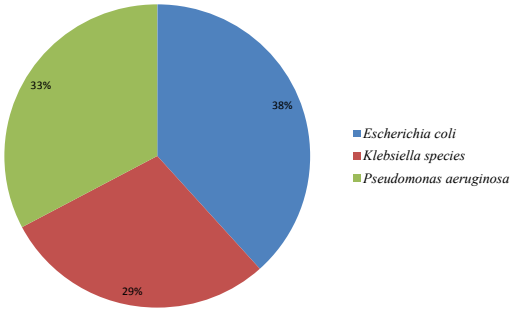

Figure 3: Beta-lactamase production by the test gram negative bacteria.

Figure 3 shows the occurrence of beta-lactamase production in the Gram-negative bacteria at varying rates. *E. coli* produced beta-lactamase enzyme at the rate of 38% while *P. aeruginosa* produced the enzyme at the rate of 33%. The rate of beta-lactamase production in *Klebsiella* species isolates was 29%.

Table 2 shows the result of the susceptibility of the bacterial isolates to the carbapenems including imipenem, meropenem and ertapenem. Out of the 168 *E. coli* isolates recovered in this study, a total of 87 isolates (51.8%), 93 isolates (55.4%), and 146 (86.9%) *E. coli* isolates were resistant to imipenem, meropenem and ertapenem respectively. A total of 58 isolates of *Klebsiella* species (41.1%), 61 (43.3%) *Klebsiella* species, and 119 (61.2%) *Klebsiella* species isolates were resistant to imipenem, meropenem and ertapenem respectively [Table 2]. The *P. aeruginosa* isolates were also resistant to imipenem

Table 2: Number of carbapenem producing gram-negative bacteria.

Bacteria	Imipenem (10 µg) n (%)	Meropenem (10 µg) n (%)	Ertapenem (10 µg) n (%)
Escherichia coli (n=168)	87 (51.8)	93 (55.4)	146 (86.9)
Klebsiella species (n=141)	58 (41.1)	61 (43.3)	119 (84.4)
Pseudomonas aeruginosa (n=147)	98 (66.7)	89 (60.5)	90 (61.2)

(n=98, 66.7%), meropenem (n=89, 60.5%), and ertapenem (n=90, 61.5%). Notably, the *P. aeruginosa* isolates were more resistant to the carbapenems used in this study (imipenem, meropenem and ertapenem) than the *Klebsiella* species and *E. coli* isolates. The prevalence of MBL-producing Gram-negative bacteria is shown in Table 3. Metallo-β-lactamase (MBL) production was phenotypically detected by the modified Hodges test technique in a total of 22 *E. coli* isolates, 21 *Klebsiella* species isolates and 20 *P. aeruginosa* isolates. The MBL-producing *E. coli*, *Klebsiella* species and *P. aeruginosa* isolates had multiple antibiotics resistance index in the range of 0.6 to 0.8; and this implies that the carbapenem-resistant Gram-negative bacteria confirmed phenotypically to produce MBL in this study are multiple resistant in nature. The MBL positive *E. coli*, *Klebsiella* species and *P. aeruginosa* isolates were resistant to more than six (6) antibiotics out of the 16 antibiotics used in this study.

Table 3: Prevalence of metallo-β-lactamase (MBL) producing gram-negative bacteria.

Organism	Source	Number of MBL positive isolates	Percentage (%)
Escherichia coli	Abattoir	8	11.6
Escherichia coli	Poultry	7	13.7
Escherichia coli	Anal swabs of cow	7	14.6
Total		**22**	**39.9**
Pseudomonas aeruginosa	Abattoir	7	12.5
Pseudomonas aeruginosa	Poultry	8	14.6
Pseudomonas aeruginosa	Anal swabs of cow	6	18.6
Total		**21**	**45.7**
Klebsiella species	Abattoir	7	15
Klebsiella species	Poultry	5	14.3
Klebsiella species	Anal swab of cow	8	9.6
Total		**20**	**38.9**

Discussion

In efforts to limit the export of multidrug resistant bacteria pathogens including those that produce metallo-β-lactamase (MBL) from the hospital to the community and vice-versa, it is needful to implement sustainable monitoring and detection techniques that will enable laboratory personnel to effectively detect and report such occurrences. The effective monitoring of the development and spread of antimicrobial resistance in zoonotic pathogens including *E. coli*, *Klebsiella* species and *P. aeruginosa* is critical to the containment of any disease outbreak due to these drug-resistant microbes. In this present study, the production of metallo-β-lactamase (MBL) was phenotypically detected from carbapenem-resistant Gram-negative bacteria of abattoir and poultry origin. *E. coli* isolates, followed by *P. aeruginosa* isolates was the most prevalent Gram-negative bacteria isolated in this study. The increasing reports of the development and spread of Gram-negative bacteria in abattoir and poultry samples portend public health risk due in part to their possible antibiotic resistant nature and their ability to cause several bacterial infections in human populace. Akinduti et al. [18] reported in their study that *E. coli*, *Klebsiella* species and *P. aeruginosa* were the most prevalent organisms isolated from environmental samples including samples from poultry farms. Beta-lactamase enzymes were phenotypically detected in the Gram-negative bacteria at varying rates. The *E. coli* isolates in this study produced beta-lactamase enzyme at the rate of 38% while *P. aeruginosa* produced the enzyme at the rate of 33%. The rate of beta-lactamase production in *Klebsiella* species isolates was 29%. A previous study has shown that the presence of beta-lactamase enzyme in bacteria provides opportunity for the horizontal transmission of these enzymes from one organism to another. [15] Out of the 168 isolates of *E. coli* recovered from the environmental samples in this study, a total of 160 (95.2%) isolates was resistant to ceftriaxone. It was also found that 162 (96.4%) isolates of the *E. coli* isolates and 165 (98.2%) *E. coli* isolates were resistant to ceftazidime and cefotaxime. Very low levels of susceptibility of the *E. coli* isolates was also observed with cefoxitin, oxacillin, ofloxacin, amikacin, ciprofloxacin, and aztreonam at a rate of 74.4%, 81.5%, 70.8%, 64.9%, 81.5% and 93.5% respectively. The high levels of resistance of *E. coli* isolates from environmental samples (as obtainable in this study) have been reported in the Netherlands, Nigeria, and Uganda. [20-22] Bergenholtz et al. [23] also reported high resistance of *E. coli* isolates from environmental samples to antibiotics as reported in this study. Olutayo and Abimbola [24] showed in their study that 100 *E. coli* isolates recovered from abattoir effluents were resistant to imipenem and meropenem [24]. Rossolini et al. [25] reported that *Enterobacteriaceae* from environmental samples were highly resistant to imipenem and meropenem. In Southwest Nigeria, Ogunleye et al. [22] reported that *E. coli* isolates recovered from poultry were highly resistant to imipenem and meropenem. Also in a previous study of ours, the resistance of *E. coli* to the carbapenems (as obtainable in this study) has also been reported [6]. *Klebsiella* species isolates were resistant to imipenem (41.1%), meropenem (43.3%) and ertapenem (84.4%). The resistance pattern most commonly observed amongst the *Klebsiella* species isolates was resistance to cefotaxime (96.5%), aztreonam (96.5%), ceftriaxone (89.4%), oxacillin (87.2%), ciprofloxacin (86.5%), ceftazidime (82.3%) and cloxacillin (73.0%). In Switzerland, the resistance of *Klebsiella* species to the carbapenems has also been reported as obtained in this study. [26] The *P. aeruginosa* isolates recovered in this study also showed high level resistance to the carbapenems including imipenem (66.7%), ertapenem (61.2%) and meropenem (60.5%). Reduced susceptibility of the *P. aeruginosa* isolates was also observed in cefoxitin (80.3%), cefotaxime (79.6%), ceftriaxone (64.6%) and ceftazidime (54.4%). All the *P. aeruginosa* isolates also showed reduced susceptibility to oxacillin, ofloxacin, ciprofloxacin, ampicillin, aztreonam, nitrofurantoin and cloxacillin. This result is similar to results obtained by Aibinu et al. [11] and Olutayo and Abimbola [24] who both reported similar levels of reduced susceptibility of *P. aeruginosa* isolates to antibiotics in southwest Nigeria. In a recent study in southeast Nigeria, similar level of resistance amongst *P. aeruginosa* isolates from abattoir has also been

reported.[27] The occurrence of MBL-producing *E. coli* isolates in this study was 39.9%. This is similar to the work of Leung et al.[28] who reported in Australia 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.[30] who recorded higher prevalence of MBL-producing *E. coli* isolates in their study carried out in Kashmir. The prevalence of MBL-producing *E. coli* isolates in this study also agreed with the work of Chouchani et al.[31] who reported the occurrence of MBL-producing *E. coli* isolates (13%) in their study. The occurrence of MBL-producing *Klebsiella* species in this study was 38.9%. Similar rates of MBL-producing *Klebsiella* species have been reported in Nigeria, Asia, Europe and other parts of Africa.[6,18,29,32] However, a similar work done in Australia showed that none of the *Klebsiella* species isolates recovered from environmental samples produced metallo beta-lactamase enzyme.[28] In this study, *P. aeruginosa* isolates that produce MBL enzymes were phenotypically detected in a total of 21 (45.7%) isolates. This result is not in agreement with those reported by Abd El-Baky et al.[33] in which 31 isolates of *P. aeruginosa* was phenotypically detected to produce MBL enzymes in Asia. Akinduti et al.[18] also reported a lower rate of MBL-positive *P. aeruginosa* isolates (3.3%) in their study carried out in Southwest Nigeria. Shibata et al.[34] also reported a higher occurrence rate of MBL-producing *P. aeruginosa* isolates in their study in which 116 *P. aeruginosa* isolates were discovered phenotypically to produce MBL enzymes in Japan. However, the result of MBL enzyme production amongst the *P. aeruginosa* isolates screened in this study is in agreement with the report of Saderi et al.[14] who reported similar prevalence of MBL-producing *P. aeruginosa* isolates in their study carried out in Iran. There was no statistical difference (p value > 0.05) in the phenotypic detection of MBLs in the *E. coli*, *Klebsiella* species and *P. aeruginosa* isolates evaluated in this study. The effective monitoring of the development and spread of antimicrobial resistance in zoonotic pathogens especially those that produce high profile antibiotic degrading enzymes such as MBLs is critical to the containment of any disease outbreak due to these microbes. Proper sanitization, better hygienic practices and immunization should be employed in the rearing and production of food-producing animals instead of using antibiotics in order to forestall the emergence and spread of drug resistant bacteria.

Conclusion

This study reported the prevalence of MBL-producing Gram-negative bacteria from abattoir and poultry sources in Abakaliki metropolis of Ebonyi state, Nigeria. The MBL producing bacteria in this study showed high level of resistance to some commonly available antibiotics especially to those in the class carbapenems and cephalosporins. Our study indicates a possible emergence and spread of MBL-producing bacteria in abattoir and poultry sources; and this could be connoted to the irrational use of antibiotics in animal husbandry and poultry practices.

Limitations

This study was only conducted in a limited number of abattoirs and poultry farms in Abakaliki metropolis, Nigeria; and thus the findings cannot be generalized to the emergence and spread of MBL-producing bacteria in Abakaliki or Nigeria as a whole. However, a further all-ecompassing study that incorporates molecular techniques is required to obtain sound epidemiological data as to the actual prevalence of MBL-producing bacteria in Abakaliki and Nigeria.

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Conflict of Interest

All authors disclose that there was no conflict of interest.

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