Occurrence of Metallo-beta-lactamase in Multidrug Resistant *Escherichia coli* Isolated from Donkey Slaughter Market

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Abstract: Animals used in food production have been identified as one of the major primary sources of antibiotic resistant pathogenic bacteria within the Enterobacteriaceae family. The *Escherichia coli* harboring metallo-β-lactamases (MBLs) is a serious threat to global health. This study was aimed at the occurrences of metallo-beta-lactamase in multidrug resistance *Escherichia coli* isolated from donkey slaughter market. A total of 75 swab samples were collected from equipment used in donkey slaughters and *E. coli* was identified using Eosin Methylene Blue Agar (EMBA), MacConkey Agar (MCA) media and other microbiological standard techniques. The *E. coli* isolates were tested for multidrug resistance (MDR) using disk diffusion method and multiple antimicrobial resistances index (MARI) were calculated. The presence of metallo-beta-lactamase (MBL) in multidrug resistance *E. coli* was confirmed using modified Hodge test method. Thirty (40.0%) swab samples were positive for *E. coli*, knife had 8(32.0%), table had 12(48.0%) and slab harbored 10(40.0%). The isolates were resistance to tetracycline (40.0%-80.0%), gentamicin (25.0%-30.0%), erythromycin (40.0%-50.0%), ampicillin (70.0%-75.0%) and ciprofloxacin (20.0%-37.5%). Three major antibiotics resistance pattern were revealed from the 14 (46.7%) isolate that were MDR-*E. coli* which includes; TE-CN-E-AMP-CIP, TE-E-AMP-CIP and TE-CN-E-AMP. This study revealed the occurrences of MBLs in MDR- *E. coli* to be 5(35.7%). The presence of MBLs in MDR *E. coli* isolated from donkey creates strong threat to the treatment of such infection in clinical setting and it calls for an urgent veterinary surveillance program to monitor antibiotics used as growth enhancers in animal production.

Key word: Metallo-beta-lactamase, MDR *E. coli*, donkey slaughter equipment, public health.

INTRODUCTION

The livestock industry especially donkey plays a vital role in various regions such as in Asia, Middle-east, Africa like Nigeria, where it is a significant source of meat and milk consumption (FAO, 2019). Despite its importance, concerns about food safety and potential zoonotic diseases have been raised some years ago (Eyitayo *et al*., 2018). One of the potential risks associated with donkey meat is the presence of pathogens, such as *Escherichia coli* which can lead to gastrointestinal illnesses in humans when the contaminated product is consumed (USDA-FSIS, 2021). The transmission of *E. coli* from animals to humans has been a subject of increasing public health concern, particularly when it involves the food production chain (CDC, 2021). The improper handling and processing of donkey meat can potentially introduce pathogenic *E. coli* and other bacterial contamination (Wang *et al*., 2017). The donkey scientifically known as *Equus africanus asinus* is a domesticated member of the horse family, Equidae (Orhan *et al.,* 2012). Donkeys have been used as a work animal for at least 5000 years. They are mostly found in under developed countries where they are used principally as draught or pack animals.

In developing countries donkeys are valued in particular for their ability to survive under harsh conditions (Swai and Bwanga, 2008). Interestingly, donkey faeces is sometimes used in rural communities to rub/coat the inner walls of mud buildings where human beings live, as local fertilizers and this creates a strong potential for contamination and/or infection of those persons who perform this work and people who live in

such houses, touch these surfaces and farmers who use it for crop productions (Pritchard *et al*., 2019). Furthermore, it has been established that donkeys shed *E. coli* (Jesse *et al*., 2015) which poses a great risk to the people handling these animals directly or indirectly, it could also expose these people to diseases caused by this organism. In developing countries, including Nigeria, Ethiopia, animals are commonly slaughtered and processed under unhygienic conditions and these further compromises the microbiological quality and safety of the meat obtained from the animals (Bello *et al*., 2015; Dulo *et al*., 2015).

Food-producing animals such as donkey harboring multidrug resistance genes together with genes that mediate the production of some high-profile antibiotic hydrolyzing enzymes such as metallo-beta-lactamases (MBLs),extended spectrum beta-lactamases (ESBLs) and AmpC enzymes possess health risks to the human population particularly due to their potential contribution to the spread of multidrug resistant microorganisms in the community (Ejikeugwu *et al.,* 2018). MBL-encoding genes are usually carried by mobile genetic elements that facilitate horizontal gene transfer (HGT) between bacteria and harbor a great ability to spread (Pierce *et al.,* 2017). MBL-producing bacteria are regarded as the most important environmental pathogens, and further spread of them in the healthcare settings will pose a serious global threat in the future. Therefore, active surveillance is needed to detect the prevalence and incidence of MBL-producing bacteria in the environment and help prevent the spread of these organisms (Beresford and Maley, 2019).

The worldwide distribution of *E. coli* harboring metallo-β-lactamases (MBLs) and AmpC β-lactamases (AmpC) is a serious threat, and due to MBL production, carbapenem resistance is progressively spreading among clinical isolates of *E. coli* (Tewari *et al.,* 2018).Treatment of infections has been complicated by the emergence of multidrug-resistant (MDR) strains of *E. coli* (Aghil *et al.,* 2021).Food-producing animals have been identified as the primary source of antibiotic-resistant pathogenic bacteria within the Enterobacteriaceae family across multiple countries (Dey *et al.,* 2023; Sobur *et al.,* 2019; Ejikeugwu *et al.,* 2016). Among the most encountered members of Enterobacteriaceae in this category are *Escherichia coli, Klebsiella* species, *Shigella* species, and *Salmonella* species, owing to their role in causing diseases in livestock (de Souza *et al.,* 2023; Ugbo *et al.,* 2023). Limited information is available on the prevalence of MBLs in MDR *Escherichia coli* from animal at Ebonyi State, knowing that Ebonyi people depend much on donkey meat as their source of meat and protein. If not checkmated periodically, this could lead to the spread of MDR *Escherichia coli* harboring MBLs in the environment. Thus, investigating metallo-beta-lactamases (MBLs) in this bacterium is of utmost importance to address, manage the emergence and spread of antibiotic-resistant strains among food-producing animals, it is also a strong critical measure for safeguarding both animal and human health. Thus, the need for studying the occurrences of metallo-beta-lactamase in multidrug resistance *Escherichia coli* isolated from donkey slaughter market.

MATERIALS AND METHODS

Study area: This study was conducted in a popular donkey market (Nkwo Jaki) at Ezzamgbo in Ohaukwu Local Government Area of Ebonyi State. Ohaukwu Local Government Area has an estimated population of 196,000 (NPC 2006) with three major clans namely; Ezzangbo, Ngbo, Effium and covers an estimated area of 252 km^2. The area lies within latitudes 6^0 3' N to 6^0 50' N and longitudes 7^0 80' E to 8^0 00' E with climatic conditions such as rainy season (March-October) and dry season (October-February).

Sample collection and processing: Exactly 75 samples (25 each from knifes, tables and slabs) were taken aseptically from donkey abattoir at Ezzamgbo, Ohaukwu Ebonyi

State using sterile swab stick. The sterile swab sticks were used to collect the samples from the donkeys slaughter equipment by rotating at an angle of 180^{o}C. The swab sticks were returned to their respective containers and labeled properly. The samples were immediately transported to the Applied Microbiology Laboratory of Ebonyi State University, Abakaliki for bacteriological analysis. Briefly, each of the collected (knifes, tables and slabs) samples were inserted into 5 ml of freshly prepared nutrient broth and the tubes were loosely covered with cotton wool. The tubes were arranged on test tube rack and were incubated at 37^{o}C for 18-24 hours. Bacterial growth was suspected by the presence of turbidity or cloudiness in the tubes after incubation. Tubes that showed turbidity were further sub-cultured onto solid culture media plates for the isolation of the primary bacterium (Ejikeugwu *et al*., 2018).

Isolation and identification of Escherichia coli: The bacterial colonies obtained were further inoculated on freshly prepared Eosin Methylene Blue Agar (EMBA) (HiMedia M317) and MacConkey Agar (MCA) (HiMedia MH081) media and incubated at 37°C for 18-24 hours for the purpose of isolation of *E. coli.* Further identification of *E. coli* was done using Gram staining and other standard microbiological methods which includes the biochemical test; Triple Sugar Iron Agar (TSIA) (HiMedia M021): Simmons Citrate Agar (SCA) (HiMedia M099), IMVIC media, such as Sulfide Indole Motility (SIM) (HiMedia M181): Methyl red (MR): Voges-Proskauer (VP) (Merck; 105712) (Yanestria *et al*., 2022).

Antibiotic susceptibility testing: Susceptibility testing was done on Mueller Hinton agar plates (Oxoid, UK) using the Kirby-Bauer disk diffusion method as per the criteria of Clinical Laboratory Standard Institute (CLSI, 2020). The different classes of antibiotics disk which includes Fluoroqinolone (CIP) ciprofloxacin; 5µg, Macrolides (E) erythromycin; 30µg, Tetracycline (TE) tetracycline; 30 µg, Beta-lactams (AMP) ampicillins; 30µg, and Aminoglycosides (CN) gentamycin; 500µg. All the antibiotic disks were procured from Oxoid limited (Oxoid, UK). A loopful of the test organism (adjusted to 0.5 McFarland turbidity standards) was streaked on freshly prepared Muller-Hinton agar plates; and the plates were allowed to stand for 15 minutes. The antibiotic disks were placed at a distance of 30mm apart from each other and 15mm away from the edge of the plate and the susceptibility plates were incubated at 37^{o}C for 24 hours (Ugbo *et al*., 2023; CLSI, 2020). The zones of inhibition diameter were measured according to the CLSI criteria.

Multiple antimicrobial resistance index (MARI): The MAR index for a single isolate was calculated as the number of antibiotics to which an isolate is resistant to (**a**) divided by the total number of antibiotics tested against the isolate (**b**) (Ejikeugwu *et al*., 2017).

Screening for the presence of metallo-betalactamase (MBL): All the multidrug resistance *Escherichia coli* isolates were screened for the production of MBL by determining their susceptibility to any of the carbapenems including imipenem (IPM) (10 µg), meropenem (MEM) (10 µg), ertapenem (ETP) (10 µg) (Dey *et al*., 2023). The Kirby-Bauer disk diffusion technique were used, and each of the antibiotics disk were placed at a distance of 20 mm apart and the plates were incubated at 37^{o}C for 18-24hours. MBL enzyme-producing isolates were suspected when the test organism(s) showed reduced susceptibility to any of the tested antibiotics. The isolates showing inhibition zone diameter (IZD) of ≤ 23 mm were suspected to produce MBL and these isolates were subjected to phenotypic confirmation test according to the method of (Ejikeugwu *et al*., 2016).

Phenotypic detection of metallo β-lactamase (MBLs): The multidrug resistance *Escherichia coli* isolates found to be resistant to imipenem or meropenem as identified in the screening test were subjected to phenotypic studies for the presence of metallo β-lactamase (MBL)

using modified Hodge test method. The pure culture of the multidrug resistance *Escherichia coli* isolates were adjusted to 0.5 McFarland turbidity standards and aseptically swabbed on Mueller-Hinton (MH) agar plates. The standard antibiotic disks of imipenem (10 µg) and meropenem (10 µg) impregnated with EDTA (1 µg) were aseptically placed on MH agar plates. Additionally, supplementary imipenem (10 µg) and meropenem (10 µg) disks without EDTA were also placed alongside with the antibiotic disks impregnated with the chelating agent (EDTA) at a distance of 20 mm away from each other. The chelating agents were initially tested on the test bacteria prior to the phenotypic assay to confirm there were no inhibitory effect on the test organisms. All the plates were incubated at 37^{o}C for 18-24 hours and zone of inhibition were recorded after incubation. A difference of ≥ 7 mm between the zones of inhibition of any of the carbapenem disks with or without the chelating agents infers metallo-betalactamase production according to (Bajracharya *et al*., 2023).

RESULTS

E. coli isolates were isolated from 30(40.0%) samples out of the 75 donkey slaughter equipment samples analyzed. The result revealed that slab harbored highest number of *E. coli* isolates 10(40.0%), followed by table 12(48.0%) and knife had the least occurrence of *E. coli* 8(32.0%) table (Table 1). The *E. coli* isolated from the slaughter equipment presented different kinds of resistance to the tested antibiotics; tetracycline (40.0%-80.0%), gentamicin (25.0%-30.0%), erythromycin (40.0%-50.0%), ampicillin (70.0%-75.0%) and ciprofloxacin (20.0%-37.5%). The most effective antibiotics against the tested *E. coli* isolates were ciprofloxacin (80.0%) and gentamicin (75.0%) (Table 2). The test revealed that 46.7% of *E. coli* isolates were MDR. Some of the *E. coli* isolates were approximately totally resistant to ampicillin (AMP), gentamycin (CN), ciprofloxacin (CIP), tetracycline (TE), erythromycin (E) and presented three different MDR patterns which includes; TE-CN-E-AMP-CIP, TE-E-AMP-CIP and TE-CN-E-AMP (Table 3). Out of the 14 MDR *E. coli* isolates, knife had 3 (37.5%), table had 6 (50.0%) and slab harbored 5 (50.0%). However, five (35.7%) MDR *E. coli* isolates were confirmed to produce metallo β-lactamase (MBLs), knife 1 (33.3%), table 2 (33.3%) and slab 2 (40.0%) (Table 4).

Table 1: Distribution of *E. coli* isolates from donkey slaughter equipment

Sample/Source	No of Samples	No Positive for *E. coli*	Percentage (%)
Knife	25	8	32.0
Table	25	12	48.0
Slab	25	10	40.0
Total	75	30	40.0

Table 2: Antimicrobial susceptibility profile of *E. coli* isolated from donkey slaughter equipment

SOURCE	TE		CN		E		AMP		CIP	
	S(%)	R(%)	S(%)	R(%)	S(%)	R(%)	S(%)	R(%)	S(%)	R(%)
Knife	4(50.0)	4(50.0)	6(75.0)	2(25.0)	4(50.0)	4(50.0)	2(25.0)	6(75.0)	5(62.5)	3(37.5)
Table	7(58.3)	5(41.7)	9(75.0)	3(25.0)	7(58.3)	5(41.7)	3(25.0)	9(75.0)	8(66.7)	4(33.3)
Slab	2(20.0)	8(80.0)	7(70.0)	3(30.0)	6(60.0)	4(40.0)	3(30.0)	7(70.0)	8(80.0)	2(20.0)

Key: E = erythromycin, CIP = ciprofloxacin, AMP = ampicillin, CN = gentamicin, TE = tetracycline

Table 3: Multidrug resistance patterns of *E. coli* isolates from donkey slaughter equipment

Isolates Code	Isolates	Number of antibiotics that isolates were resistant to (a)	Total number of antibiotics tested (b)	MAR index (a/b)
K 13	*E. coli*	TE-E-AMP-CIP	5	0.80
K 24	*E. coli*	TE-CN-E-AMP-CIP	5	1.00
T 4	*E. coli*	TE-CIP-E-AMP	5	0.80
T 16	*E. coli*	TE-E-AMP-CN-CIP	5	1.00
T 21	*E. coli*	TE-E-AMP-CIP	5	0.80
T23	*E. coli*	TE-CN-E-AMP	5	0.80
S 2	*E. coli*	TE-E-AMP-CN-CIP	5	1.00
S 9	*E. coli*	TE-CN-E-AMP-CIP	5	1.00
S 14	*E. coli*	TE-E-AMP-CIP	5	0.80
Average MDR index				8/9 (0.88)

Key: K = Knife; T = Table; S = Slab; CIP = ciprofloxacin; E = erythromycin; TE = tetracycline; AMP = ampicillin; and CN = gentamycin

Table 4: Prevalence of MDR-*Escherichia coli* producing metallo β-lactamase (MBLs)

Sample/Source	No of *E. coli* screened	No of MDR-*E. coli* suspected for MBL (%)	No of MDR-*E. coli* +ve for MBL (%)	No of MDR-*E. coli* -ve for MBL (%)
Knife	8	3 (37.5)	1 (33.3)	2 (66.7)
Table	12	6 (50.0)	2 (33.3)	4 (66.7)
Slab	10	5 (50.0)	2 (40.0)	3 (60.0)
Total	30	14 (46.7)	5 (35.7)	9 (64.3)

DISCUSSION

The global emergence and fast spread of pathogenic microorganisms showing multidrug resistance to antimicrobial agents is a serious public health threat. *Enterobacteriaceae* especially *Escherichia coli* producing metallo beta-lactamase (MBLs) is one of the mechanisms associated with severe bacterial infections in clinical setting. Multidrug resistant properties of these microorganisms have limited and complicated treatment options. This study recorded presence of *E. coli* in donkey slaughter equipment with high level of prevalence at percentage of 40.0. *E. coli* isolates (5.2%) has been reported by previous researcher on slaughtered donkey from slaughter house in Kaduna State, Nigeria (Esonu *et al*., 2022), but their report was very low when compared to the observation of the current study and this could be attributed to the differences in the study areas. Another study revealed a significant prevalence of *E. coli* strains (cattle- 88.7%), (chicken- 81%), (swine- 89.5%) and raising concerns about potential transmission to humans through contaminated food animals (Eyitayo *et al*., 2018). A total prevalence of 12.0% *E. coli* was reported from animal harvested for human consumption in Ethiopia. The detection of *E. coli* from animal origin raises concerns about the potential for contamination of meat products and the transmission of this pathogen to consumers (Adanech and Temesgen, 2018). *E. coli* is a diverse group of bacteria which is a normal flora of in the gastrointestinal tract of animals which are harmless, but can be pathogenic in case of disease condition in the animals. Thus, in the context of food safety, the presence of pathogenic *E. coli* on slaughter equipment is a critical issue that requires attention to prevent food-borne illnesses (Yanestria *et al*., 2022; Adanech and Temesgen, 2018). Another study on the microbial contamination of cattle carcasses and slaughter observed the presence of *Enterobacteriaceae*, emphasizing the importance of maintaining hygienic practices to prevent contamination (Pierluigi

et al., 2016). Several other studies have also investigated the presence of *E. coli* in animal such as goat, sheep, cattle, donkey, chickens slaughter environments and reported a great level of prevalence (Eltai *et al*., 2020; Esonu *et al*., 2022; Ugbo *et al*., 2023). The identification of *E. coli* on animals slaughter equipment underscores the importance of implementing rigorous hygiene practices and sanitation measures in abattoirs to minimize the risk of contamination.

The *E. coli* isolates recovered from the slaughter equipment presented different degrees of resistance to the tested antibiotic classes; tetracycline (40.0% to 80.0%), gentamicin (25.0% to 30.0%), erythromycin (40.0% to 50.0%), ampicillin (70.0% to 75.0%) and ciprofloxacin (20.0% to 37.5%). High level of resistance to tetracycline, ampicillin as reported in this study is in accordance with the observation of Mahmoodi *et al.* (2020) who reported *E. coli* resistance to tetracycline as (17.5% to 42.3%), ampicillin (24.6% to 64.7%), low level of resistance to ciprofloxacin (5.2% to 25.4%) and gentamicin (1.9% to 6.5%). Ejikeugwu *et al.* (2016) reported similar level of resistance on *E. coli* isolates from abattoir environment, where they observed that ciprofloxacin showed resistance of 28.1% and gentamicin (18.8%).High level of resistance has been reported on *E. coli* isolates by previous researcher to ciprofloxacin (66.1%) (Nazmul *et al*., 2019). A study on pathogenic *E. coli* isolates from chicken meat in Bharatpur, Chitwan reported lower resistance level to ciprofloxacin 16.67% and gentamicin (33.3%) (Bajracharya *et al*., 2023) and is in line the findings of this study which reported resistance to ciprofloxacin to be (20.0%) and gentamicin (25.0%).Out of the thirty *E. coli* isolates identified from donkey slaughters equipment, 14 (46.7%) were recorded to present multidrug resistance. Multidrug resistance *E. coli* (MDR *E. coli*) isolates in this study showed resistance to four and five different classes of antibiotics with three major different patterns which includes; TE-CN-E-AMP-CIP, TE-E-AMP-CIP and TE-CN-E-AMP. Similar findings of antibiotic resistance pattern includes; ATM-CIP-CN-E, ATM-CIP-C-E, ATM-CN-E, ATM-CIP-E, CIP-C-E were reported on multidrug resistance avian pathogenic *E. coli* (MDR-APEC) isolated from ducks on wet markets in Surabaya (Kendek *et al*., 2024). Multidrug resistance *E. coli* isolates was reported to show resistance to three (CTX, TZP, IMP (19.0%)); four (ATM, MEM, CIP, CN (23.0%)); five (MEM, AMC, FEP, AMP, CEX (15.0%)) and six (AMC, CTX, ATM, IMP, CIP, CEX (5.0%)) different classes of antibiotics (Nazmul *et al.*, 2019. Previous study on multidrug resistance *E. coli* from animal source observed that isolates were resistance to three and five different classes of antibiotic; amoxicillin (AMOX), doxycycline (DO), cotrimoxazole (COT) (41.61%); amoxicillin (AMOX), doxycycline, (DO), cotrimoxazole (COT), azithromycin (AZM) (25.0%) and amoxicillin (AMOX), doxycycline (DO), cotrimoxazole (COT), azithromycin (AZM), gentamicin (GEN) (16.6%) (Bajracharya *et al*., 2023). The detection of MDR *E. coli* in slaughter equipment showing resistance to three and five different classes of antibiotics suggests that those classes of antibiotics may have be abused as growth promoters, and during treatment of infections in the animals. This study revealed the occurrences of metallo β-lactamase (MBLs) in MDR- *E. coli* to be 5 (35.7%); knife had 1 (33.3%), table harbored 2 (33.3%) and slab had2 (40.0%). The study done on *E. coli* isolated from animals which includes cow and cloacae swabs of poultry birds revealed the presence of MBLs phenotypically to be 39.9% and 45.7% respectively (Ejikeugwu *et al.*, 2017) and is in agreement with 35.7% of MBLs report in this present study. The prevalence of MBL-producing *E. coli* isolates in this study is in accordance with the observation of the occurrence of MBL-producing *E. coli* isolates (Chouchani *et al*., 2011). Metallo β-lactamase (MBLs) producing *E. coli* (5.0%) was reported from animal (chicken) (Dey *et al*., 2023), and 66.0% in clinical (Mahmoodi *et al*., 2020).

Another study from animal reported similar prevalence of *E. coli* isolates positive for MBL production in India (Chakraborty *et al*., 2010). The presence of MBLs in MDR *E. coli* is a significant concern as it has been linked to numerous outbreaks of foodborne illnesses in humans and this observation highlights a potential foodborne public health hazard (Madec *et al*., 2017) thus, the need for regular screening of MBLs from animal. The MBL-producing *E. coli* isolates can carry mobile genetic elements with great ability to spread in the environment (Mahmoodi *et al*., 2020). This study establishes early detection of MBL-producing *E. coli* isolates particularly their reservoir to help in maintaining suitable antimicrobial therapies.

CONCLUSION

Occurrence of MBLs in MDR *E. coli* was reported in donkey slaughter equipment to be 35.7% which is alarming and these suggest serious public health threat if not controlled. The identification of MDR *E. coli* on slaughter equipment revealed the need for stringent hygiene practices in abattoirs to mitigate contamination risks and ensure consumer safety. However, the encouragement of periodical research and accurate detection of MBL- production in MDR *E. coli* isolates from animal source, abattoir environment and clinical samples is of utmost public health importance due to its multidrug resistant properties harbored by these organisms to safeguarding both animal and public health.

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