Molecular Characterization of Extended Spectrum Beta lactamase Producing Enterobacteriaceae Isolated from Duck Droppings

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Abstract: The transmission of the members of the Enterobacteriaceae family with extended spectrum has become a cause for concern. Food animals have been researched to be reservoirs of antimicrobial resistance especially in developing part of the world where there is indiscriminate prophylactic use of antibiotics in animal farming. This study investigated molecular identification and antibiotics resistance of extended spectrum beta lactamase producing Enterobactericeae isolated from duck droppings. A total of twenty-two (22) isolates from forty (40) duck dropping samples in this study were Gram negative bacteria. The isolation of associated Enterobacteriaceae from the duck droppings was carried out using the standard microbiological methods, ESBL phenotypic detection was carried out using combination disc test and double disc synergism test. The major findings were the presence of multidrug resistant *Citrobacter freundii* 7(31.82%), *Klebsiella pneumoniae* 7(31.82%), *Proteus* spp. 3(13.64%), and *Serratia marcescens* 5(22.72%) to commonly used antibiotics such as ceftazidime, cefixime, nitrofurantoin, ofloxacin, augmentin and cefuroxime. Double disk synergy test showed 5(22.72%) were ESBL producers which include: *Citrobacter freundi* 3(42.86%), *Klebsiella pneumoniae* 1(14.29%) and *Proteus* spp. 1(33.33%). The PCR amplification of the ESBL genes in the five isolates to bla_{TEM} ESBL gene revealed a negative result bands corresponding to bla_{TEM} ESBL though positive for ESBL production. This could be as a result of expression of other genes like CTX, SHV, OXA and other types. This research shows that ducks are also reservoir of ESBL producing Enterobacteriaceae which calls for concern. There is, therefore need for more stringent measures and policies to be put in place by public health regulatory bodies to check misuse of antibiotics in food-animal farming in Nigeria.
Key word: ESBLs, Enterobacteriaceae, duck droppings and bla_{TEM} gene

INTRODUCTION

In veterinary medicine, broad spectrum antibiotics have been widely used as registered and off-label prophylactic treatment in livestock which resulted to the spread of Enterobactericeae resistance to 3rd and 4th generation cephalosporin. This has been highlighted as an emerging problem and cause for concern in public health, as it raises potential risk of transfer of β- lactam resistant pathogens from livestock to human through food (Geser, 2012; Trott, 2013).

The use of antibiotics in food animals has been widely researched and subjected to criticism since contamination of meat and milk by residues of antibiotics has been documented in Europe, Japan and China (Vragovic *et al*., 2011). The extensive use of colistin in animal production including swine and poultry has also been reported to increase the risk of transferring resistant bacteria to human (Abiola, 2021). Transmission from food producing animals to human can occur through contaminated animal retail products (Lazarus *et al.*, 2015); although the risk of this might be low if animal products are adequately prepared and cooked. Resistance can also be transmitted through environment, animal fecal matter/ land application of poultry fecal litters, waste water and contaminated produce (Aarestrup, 2015; Yang *et al*., 2019).

Extended spectrum beta lactamase (ESBL) are enzymes capable of hydrolysing penicillins, broad-spectrum cephalosporins and monobactams, and are generally derived from TEM and SHV-type enzymes (Rupp, 2003). Despite extensive studies surveying ESBLs in pig, chicken, and cattle (Jian-Hua *et al.,* 2007; Horton *et al.,* 2011), the diversity of ESBLs in duck and its living environments have seldom been investigated. Antimicrobial are used as prophylaxis to prevent and control the spread of infectious diseases on duck farms,

which could facilitate the selection of antimicrobial-resistant bacteria. Hence, domestic ducks can act as potential vessels for resistant bacteria and may play an important role in the dissemination of resistant genes (Chong *et al.*, 2009). In view of these, this study is aimed at investigating the molecular identification and antibiotics resistance patterns of extended spectrum beta lactamase producing Enterobactericeae isolated from duck droppings.

MATERIALS AND METHODS

Sample collection and analysis: A total of 40 dropping samples of duck were collected from three major duck farms, Onipanu (Aladiye), Ketu (poultry) and Eruwen, Ikorodu, all in Lagos State, Nigeria. The fecal samples were collected very early in the morning (7 am) with the aid of plastic sterile swab, quickly placed in peptone broth as transport media and taken immediately to the laboratory of Microbiology University of Lagos, Akoka for analysis. All culture media for analysis were prepared according to the manufacturer's instruction manual. These were autoclaved at 121℃ for 15 minutes. All glass ware were washed thoroughly, sterilized and air dried in the oven at 160℃ for 1 h.

Microbiology analysis of the samples: Samples collected were first cultured on MacConkey agar (Lab M, UK) plates and Salmonella-shigella agar (Lab M, UK) after which incubation was done at 37℃ for 18-24 hrs. The plates were examined for the presence of lactose fermenters characterized by the appearance of pink colonies and non-lactose fermenter characterized by off-white opaque colonies. Some plates exhibited distinct features of 'swarming growth', which indicated probable presence of *Proteus* spp. (Cheesebrough, 2006). The lactose fermenters were repeatedly sub-cultured on nutrient agar to obtain pure cultures. Biochemical tests and phenotypical identification were carried out which includes: Kligler Iron test (Rapid Lab, UK), motility-indole-urease test (Rapid lab UK) and Triple sugar iron test (Rapid Lab UK) (CLSI, 2022).

Antimicrobial sensitivity testing of bacterial isolates: Antimicrobial sensitivity test was performed on all the isolates according to Clinical and laboratory standard institute (CLSI, 2022) using the Kirby-Bauer disc diffusion method to evaluate sensitivity of the isolates. Antibiotics discs used include: gentamycin (GEN) 10 µg, cefuroxime (CRX) 30 µg, ofloxacin (OFX) 5 µg, ceftazidime (CAZ) 30 µg, augmentin (AUG) 30 µg, cefixime(CXM)5 µg, nitrofurantoin (NIT) 30 µg and ciprofloxacin (CIP) 5 µg. All the discs were manufactured by Abtek Biological limited. The results were interpreted using the standard chart according to Clinical laboratory standard institute (CLSI, 2022).

Testing for ESBLs production in bacterial isolates: The ESBLs production was examined by using the double disc synergy test which involves placing amoxicillin (20 µg)/clavulanic acid (10 µg) combination disc at the center of each inoculated Muller-Hinton agar plate containing the test organism. Cefotaxime (30 µg) and ceftazidime (30 µg) (Oxoid, UK) single disc were then placed center to center (25 mm apart) from the amoxicillin/clavulanic acid disc and incubated at 37℃ for 18-24 hrs. Appearance of a key hole like effect due to enhanced activity of ceftazidime and cefotaxime with clavulanic acid (Hasan *et al.*, 2013) with zone size of more than 5 mm was considered positive for ESBLs production (CLSI, 2022).

Characterization of ESBLs producing bacterial isolates: Bacterial lysate was prepared from all the isolates found to be positive for ESBLs production phenotypically and were tested for the presence of bla_{TEM} genes by Polymerase chain reaction assay using specific primers (Table 1). The PCR was carried out in a 0.2 ml thin wall PCR tubes using the bacterial lysate as template DNA with a final volume of 25 µl containing 10 × buffer, 1.5 mM $MgCl_2$, 200 pM of each oligonucleotide primers, 200 µM of each

dNTPS, 1 U of *Taq* polymerase and 4.0 µl DNA lysate. The PCR was carried out in a thermal cycler and the cycling condition for *bla$_{TEM}$* was: initial denaturation at 94℃ for 7 min followed by 30 cycles of amplification with denaturation at 94℃ for 30 s, annealing at 53℃ for 30 s, and extension at 72℃ for 30 s, ending with a final extension at 72℃ for 5 min (Weill *et al*., 2004).

RESULTS

Citrobacter freundii and *Klebsiella pneumoniae* recorded the highest percentage of occurrence which is 31.8% each while *Serratia marcescens* recorded 22.7% and *Proteus* spp. 13.6% being the lowest percentage of occurrence as shown in Table 2.

Citrobacter freundii, *Klebsiella pneumoniae*, *Serratia marcescens* and *Proteus* spp., exhibited high resistance to most of the antibiotics (cefuroxime, ceftazidime, cefixime, augmentin and ofloxacin). *Klebsiella puemoniae* and *Proteus spp*., showed 100% resistant to cefuroxime except *Serratia marcescens* and *Citrobacter freundii* with 80% and 71.4% respectively (Table 2). Only *Proteus* spp. exhibited a high resistant to gentamicin at 66.7% while *Citrobacter freundii* and *Klebsiella pneumoniae* showed 42.9%. *Serratia marcescens* exhibited the lowest resistant at the percentage of 40 as shown in Table 2. Among the isolates screened using the double disc synergy test, only five (5) isolates were positive to ESBL production. These included *Citrobacter freundii* 3(60%), *Klebsiella pneumoniae* 1(20%) and *Proteus* spp. 1(20%).

PCR amplification of ESBL gene in the five isolates to bla$_{TEM}$ gene revealed a negative finding of gene bands corresponding to bla$_{TEM}$ ESBL type though positive for ESBL production (Figure 1).

Table 1: Detail of oligonucleotide primers

Gene	Primer sequence	Expected amplicon size(bp)	Reference
bla$_{TEM}$	Forward: 5′-ATAAAATTCTTGAAGACGAAA-3′ Reverse: 5′-GACAGTTACCAATGCTTAATC-3′	1080	Weill *et al*, 2004

Table 2: Percentage occurrence of bacterial isolates from duck droppings

Organisms	Number of isolates	Percentage occurrence (%)
Citrobacter freundii	7	31.82
Serratia marcescens	5	22.72
Klebsiella pneumonia	7	31.82
Proteus spp.	3	13.64
Total	22	100

Table 3: Antibiotic susceptibility pattern of the bacterial isolates

Antibiotics (Disc potency µg)	No. and (%) of *Citrobacter freundii*			No. and (%) of *Klebsiella pneumonia*			No. and (%) of *Proteus* spp.			No. and (%) of *Serratia marcescens*		
	S	I	R	S	I	R	S	I	R	S	I	R
AUG(30)	0(0)	0(0)	7(100)	0(0)	0(0)	7(100)	0(0)	0(0)	3(100)	1(20)	0(0)	4(80)
CAZ (30)	1(14.3)	1(14.3)	5(71.4)	1(14.3)	1(14.3)	5(71.4)	1(33.3)	0(0)	2(66.7)	1(20)	0(0)	4(80)
CRX(30)	2(28.6)	0(0)	5(71.4)	0(0)	0(0)	7(100)	0(0)	0(0)	3(100)	0(0)	0(0)	5(100)
CXM (5)	1(14.3)	1(14.3)	5(71.4)	0(0)	0(0)	7(100)	0(0)	0(0)	3(100)	1(20)	0(0)	4(80)
CIP (5)	2(28.6)	1(14.3)	4(57.1)	2(28.6)	2(28.6)	3(42.9)	1(33.3)	0(0)	2(66.7)	3(60)	0(0)	2(40)
GEN (10)	3(42.9)	1(14.3)	3(42.9)	2(28.6)	2(28.6)	3(42.9)	1(33.3)	0(0)	2(66.7)	3(60)	0(0)	2(40)
NIT (300)	1(14.3)	0(0)	6(85.7)	1(14.3)	0(0)	6(85.7)	0(0)	0(0)	3(100)	1(20)	0(0)	4(80)
OFL (5)	0(0)	0(0)	7(100)	0(0)	0(0)	7(100)	0(0)	0(0)	3(100)	1(20)	0(0)	4(80)

Key: AUG- augmentin, CAZ- ceptazidime, CRX-cefuroxime, CXM-cefixime , CIP-ciprofloxacin, GEN-gentamicine, NIT- nitrofurantoin, OFL-ofloxacin

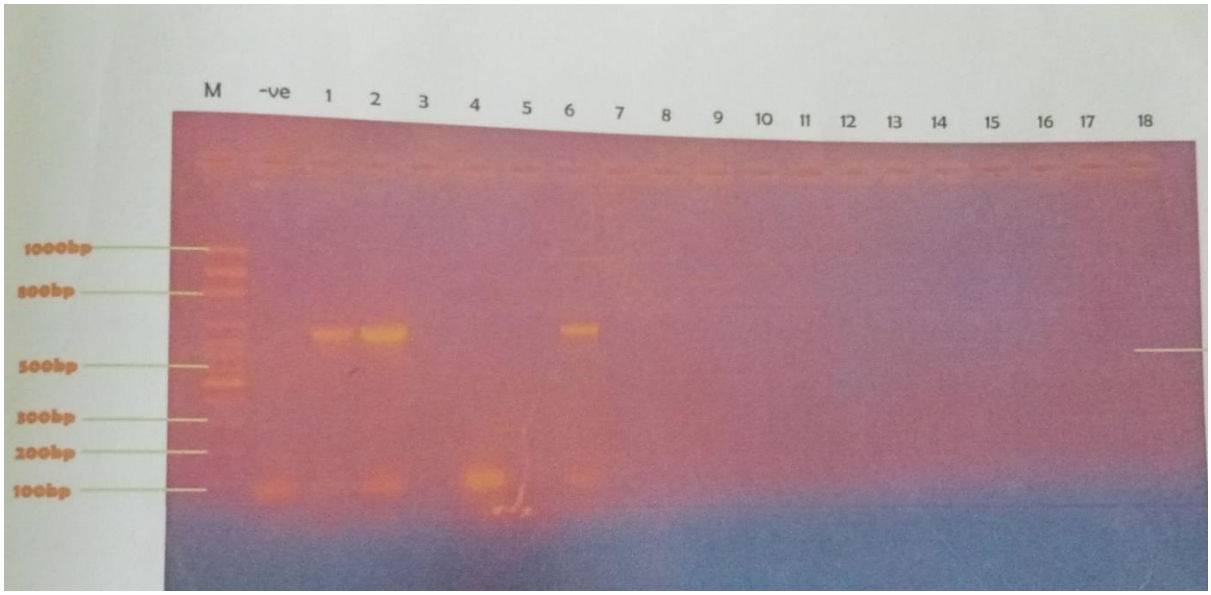

Figure 1: PCR amplification of bla$_{TEM}$ gene (Lane M= molecular marker, lane –ve= negative control)

DISCUSSION

In this part of the world where there is little or no restriction to purchase of antibiotic off counter, hence encourages an average livestock farmer access to antibiotics for treatment of livestock especially as preventive or curative measures, calls for concern. The use of gentamicin and ciprofloxacin in livestock production in South west Nigeria, was reported to increase from year 2010 to 2012 (Adesokan *et al*., 2015). The ESBL genes in Enterobactericeae from chicken had emerged and spread rapidly worldwide and reported to pose treat to human health through food chain (Blanc *et al.,* 2006). However, there are no adequate data published on incidence of resistance to different antibiotics or prevalence of ESBL genes in Enterobactericeae from duck in Lagos. Hence, farm ducks were selected to investigate antibiotics resistance in this region.

The major findings in this study is the presence of multi drug resistant *Citrobacter fruendii* 7(31.8%), *Klebsiella pneumoniae* 7(31.8%), *Proteus* spp. 3(13.6%) and *Serratia marcescens* 5(22.7%) which was similar to the one found in clinical isolates reported in Kano: *Citrobacter* spp. 47 (5.7%), *Klebsiella pneumoniae* 118 (14.4%) and *Proteus* spp. 139 (17.1%) (Yusha'u *et al*., 2007). These resistances reiterate the need for public health concern as resistant strains are gradually being transmitted from animal to human through consumption of contaminated food products, faecal-oral route and poor hygiene. The rate of multi drug resistant bacteria observed in this study 55% (22/40) is close to the findings of multi drug resistant *Escherichia coli* 63% (75/114) isolated from cattle faeces in Ado-Ekiti, Nigeria (Ajayi *et al.,* 2011). Also, *Escherichia coli* 98.9% (87/88), 100% (4/4) and 96.4% (53/55) isolated from egret, whistling ducks and cattle faecal samples in Ibadan, Nigeria (Kayode *et al.,* 2021). This calls for public health concerns as food animals are consistently reported as reservoirs of multi drug resistant bacteria. Resistance of isolates to Gentamicin in this study is low which is similar to the report of resistance of *Escherichia coli* isolated from whistling duck droppings sample reported in Ibadan, Nigeria (Kayode *et al.,* 2021). However, high gentamicin resistance of *Escherichia coli* isolated from cattle faeces was reported by Ajayi *et al.* (2011). This could probably be as a result of very poor

hygiene in cattle farming which allows for use of high antibiotics to prevent infection or illness in cattle than duck. Out of 22 multi drug samples isolated, 22.7 % were ESBL producers: *Citrobacter freundii* 3(13.6%), *Klebsiella pneumoniae* 1(4.54%) and *Serratia marcescens* 1(4.54%) which is higher than 2/24(8.3%) reported in whistling ducks in Ibadan, Nigeria (Kayode *et al.,* 2021). However, these values are lower than the report in Ado-Ekiti where eighty-one (71%) isolates were positive for ESBL by combination disc test, 90 (78.9%) were positive for double disc synergism test, and 93 (81.6%) were positive for ESBL brilliance agar (Olugbenga *et al*., 2015). The low occurrence of multi drug resistant isolates in this study could be as a result of presence of other antibiotic such as carbepenems and AmpC -β- lactams other than ESBLs which can affect the detection of ESBLs, hence leading to false negative results (Rice, 2000). In another report, *Klebsiella pneumoniae* or *Escherichia coli* have been documented to harbor plasmid mediated AmpC -type β-lactamases, while some of these organisms co-express both AmpC -β-lactamases and ESBLs (Thompson, 2001). In addition, the presence of AmpC -β-lactamase has been reported to resist inhibition by clavulanate, hence mask the synergistic effect of clavulanate and cephalosporin against ESBLs resulting in false negative test for detection of ESBLs (Phillippon *et al.,* 2002).

In another report, some antibiotics such as class A carbapenemases have been documented not to be easily detected by clavulanate-based methods, but with more sensitive methods such as Modified Hodge test(MHT) and Tris/EDTA-based and boronic acid based test (Anderson, 2007). The low occurrence of ESBL could also probably be as a result of animal sample used as ESBL occurrence has been reported to be high in cattle, chicken and pig in Nigeria (Ajayi *et al.,* 2011; Olugbenga *et al.,* 2015). This study showed that the proportion of ESBL-producing isolates among Enterobacteriaceae may not be high in duck farm, but certainly not negligible.

However, PCR amplification of ESBL genes in the five (5) isolates in this study revealed a negative result of gene band corresponding to bla_{TEM} ESBL type although positive for ESBL production. This could be due to expression of other genes like CTX, SHV and OXA types. Unlike the study in Ado-Ekiti, in which TEM and CTX-M genes were detected in 48(42.1%) and 51(44.7%) isolates respectively, while SHV gene was not detected in any of the cattle isolates (Ajayi *et al.,* 2011). Another report shows bla_{TEM} in 48 (42.1%), $bla_{CTX\text{-}M}$ in 51 (44.7%) and bla_{SHV1} was not detected in any of the isolates in cattle and pig isolates in Ado-Ekiti (Olugbenga *et al.,* 2015). This further reiterates multi drug resistant ESBL producing bacterial isolates from cattle and pigs dropping samples are high.

CONCLUSION

The findings from this study showed that there is a significant occurrence of ESBL-producing organisms in duck droppings samples, isolated from three different farms in Lagos, Nigeria. Majority of the ESBL enzyme producers in this study were *Citrobacter freundii, Klebsiella pneumoniae* and *Proteus* spp. strains. Most of the organisms isolated were multidrug resistant strains, while the ESBL producers exhibited resistance to cephalosporin. There is therefore need, for polices and enforcement of regulations guiding prophylactic use of antimicrobial in food animal farming in Nigeria. There should be more awareness campaign to cut across literate and illiterate livestock farmers, as this will help reduce transmission of resistant strains to human through food chain, faecal contamination of surface water and close interaction with duck which is common in this part of the World and can pose a threat to human medicine.

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