DETECTION OF PLASMID-MEDIATED *AmpC* BETA-LACTAMASE ENZYME AMONG *Escherichia coli* ISOLATES IN LIVESTOCK, SOUTH NIGERIA.

ABSTRACT

AmpC beta-lactamases are bacterial enzymes that hydrolyse third generation extended spectrum cephalosporins and cephamycins engendering resistance to these categories of antibiotic and is a serious threat to the currently available antibiotic armory both in human and veterinary medicine. In this study, the detection of AmpC beta-lactamase-producing *E. coli* in some common livestock animals was studied. A total of 196 faecal samples were aseptically collected from cattle, chicken, goat and swine from different parts of Uyo Metropolis into sterile universal containers. Samples were processed by inoculating onto macConkey agar using streak plate technique and incubated at 37^{o}C for 18-24 hours after which growth were identified using standard identification procedures. Susceptibility profile of each of the identified *E. coli* isolate to some antibiotics was determined using the agar disk diffusion method. Resistant *E. coli* isolates to third and fourth generation cephalosporins were screened to detect ESBL producers using the modified double disk synergy test while AmpC beta-lactamase production was done by the modified disk test. The result shows that out of a total number of 123 *E. coli* isolates, 55.68% were potential ESBL producers while 30.68% were confirmed to be AmpC producers. The highest percentage of 5.37% came from Chicken, while the least percentage of 3.23% was from Pig and Goat respectively. The result of this study shows the presence of AmpC beta-lactamase producing *E. coli* in all the groups of animal tested. Therefore, improved surveillance of antibiotic use and antibiotic-resistant bacteria in farm animals should be given an urgent attention. Application of biosecurity and hygiene programs in the livestock breeding sector should be considered as a favorable effect on the restriction transfer of antibiotic resistance.

Keywords: Detection, ESBL, AmpC, *Escherichia coli*, Livestock

INTRODUCTION

Antibiotic resistance among microorganisms is a major problem, both in human and in the livestock industry. The persistent exposure of bacterial strains to a multitude of β-lactams antibiotics has induced dynamic and continuous production and mutation of certain enzymes in these bacteria, thereby expanding their activity against the newly developed β-lactam antibiotics. These enzymes are known as extended-spectrum β-lactamases (ESBL)[1,2]. This problem is further compounded by the over-expression of another type of enzyme that preferentially hydrolyzes narrow-, broad-, and expanded-spectrum cephalosporins and cephamycins. They are also capable of resisting inhibition by clavulanate, sulbactam, and tazobactam. These enzymes are referred to as AmpC β-lactamases. AmpC β-lactamases are clinically important cephalosporinases encoded on the chromosome of many

Enterobacteriaceae and a few other organisms where they mediate resistance to cephalothin, cefazolin, cefoxitin, most penicillins, and β-lactamase inhibitor/β-lactam combinations. In many bacteria, AmpC enzymes are inducible and can be expressed at high levels by mutation. Over-expression confers resistance to broad-spectrum cephalosporins including cefotaxime, ceftazidime, and ceftriaxone. Transmissible plasmids have acquired genes for AmpC enzymes, which consequently can now appear in bacteria lacking or poorly expressing a chromosomal *bla*AmpC gene, such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis.* AmpC enzymes encoded by both chromosomal and plasmid genes are also evolving to hydrolyze broad-spectrum cephalosporins more efficiently.

Many clinical laboratories currently test *Escherichia coli* for production of extended-spectrum β-lactamases (ESBLs) but do not attempt to detect plasmid-mediated AmpC β-lactamases probably because the available phenotypic tests are either inconvenient, subjective, or require reagents that are not readily available[3,4].

In Nigeria livestock industry, the occurrence of β-lactamase-producing *E. coli* has been broadly recognized in veterinary medicine, e.g. as causative agents for mastitis in dairy cattle[5]. This problem is becoming very rampart, because they are often encountered in routine diagnoses of disease conditions brought for confirmatory diagnosis in microbiology diagnostic units of some Tertiary Veterinary Teaching Hospitals[5]. There are only few studies in South-South Nigeria that investigated the prevalence of β-lactamase-producing bacteria in livestock. The risk of zoonotic transfer from livestock to people with close contact to these animals is still largely unknown, but some studies have implicated a transfer of ESBL-producing *E. coli* or ESBL genes from poultry or pigs to farm workers[6,7]. Besides this direct zoonotic transfer, other routes as foods of animal origin may be a risk factor for human colonization or infection[8]. It is therefore this potential transfer of extended spectrum β-lactamases from animal pathogens to strains that could pose a risk for human health that is among the most important challenges arising from the global problem with antimicrobial resistance.

Therefore, the aims and objectives of this study is to detect the presence of AmpC β-lactamase enzyme among *E. coli* resistant isolates obtained from Cattle, Goat, Poultry and Swine.

MATERIALS AND METHODS

Collection of Samples: A total of 196 fresh faecal samples from different parts of Uyo Metropolis in Akwa Ibom State, Nigeria were randomly and aseptically collected from cattle, chicken, goat and swine into sterile universal containers from January 2017 to March 2017. They were transported to Medical Microbiology and Parasitology laboratory of the University of Uyo Teaching Hospital and stored at 4^{o}C until when required for processing.

Processing of Samples: One gram of the faecal samples was emulsified in 5ml of sterile saline before inoculating by streak plate method on MacConkey agar (Oxoid, UK) and incubated at 37°C for 18–24 hours after which they were examined for growth. From the growth pattern of the organisms observed on the agar plate, distinct smooth, glossy rose-pink lactose fermenting colonies presumed to be *E.coli* were selected and subjected to identification procedures according to standard taxonomic identification schemes of Cowan[9].

Determination of Antimicrobial Susceptibility Profile

The susceptibility profile of each of the identified *E.coli* isolates to some selected antibiotics was determined using the agar disk diffusion method as per the recommendation of Clinical and Laboratory Standards Institute, CLSI[10]. The following antibiotics were used;
cefotaxime(30µg), cefpodoxime(30µg), ceftrioxone(30µg), ceftazidime(30µg),

cefepime(30µg), cephoxitin(30µg), Gentamicin(10µg), ciprofloxacin(5µg), norfloxacin(10µg), nitrofurantoin(100µg), cotrimoxazole(25µg), and imipenem(10µg). All the antibiotic discs were procured from Oxoid. The bacterial inoculum was prepared by suspending freshly grown bacteria in 5ml of sterile peptone water. The suspension was adjusted to achieve a turbidity equivalent to 0.5 McFarland turbidity standards after which the inoculated peptone water was poured onto Mueller Hinton (MH) agar plates and the excess drained out. The plates were allowed to dry and appropriate antibiotic disks were aseptically placed on the agar plate surface using sterile forceps. The plates were then incubated at 37°C for 18-24hours. Diameter of zone of inhibition was determined using the Kirby Bauer test method as described by Willey *et al*[11].

Chromogenic Agar culture: Few colonies of the *E. coli* isolates that exhibited resistance to third and fourth generation cephalosporins were homogenized in 1ml of sterile physiological saline (0.85%), and 50µl aliquots of the resulting suspension were inoculated onto chromogenic ESBL-Bx agar which was prepared from the dehydrated medium according to the manufacturer's instructions and incubated in ambient air at 37°C for 18 to 24h. After the optimal incubation period, specific coloration enhanced by the chromogenic media indicates the presence of ESBL production.

Screening for Extended Spectrum Beta-Lactamase Production

The *E. coli* isolates that exhibited ESBL production and resistance to third and fourth generation cephalosporins were further screened by the modified double disk synergy test to detect ESBL producers.

Modified Double Disc Synergy Test: This was performed by using amoxicillin-clavulanate (20/10µg) disc along with four cephalosporins; third generation-cefotaxime, ceftriaxone, cefpodoxime and fourth generation-cefepime. Briefly, the test isolates were cultured on a Mueller-Hinton agar plate. A disc which contained amoxicillin-clavulanate (20/10µg) was placed in the centre of the plate. The discs of cefotaxime, ceftriaxone, and cefpodoxime, were placed 15mm and that of cefepime, 20mm apart, centre to centre to that of the amoxicillin-clavulanate disc (Paterson and Bonomo, 2005). Any distortion or increase in the inhibition zone towards the disc of amoxicillin-clavulanate was considered positive for ESBL production.

AmpC Enzyme Production: Isolates that exhibited significant synergistic effect with only cefepime in the modified double disc synergy test (MDDST) were further tested for AmpC enzyme production using the modified disc test. The test is based on the use of Tris-EDTA to permeabilize a bacterial cell and release β-lactamases into the external environment. This was done as modified by Kaur *et al*[12]. Briefly, sterile plain 6mm disks were punched from Whatmann filter paper and AmpC disks prepared by applying 20µl of a 1:1 mixture of saline and Tris-EDTA to the disks. The disks were allowed to dry, and were stored at between 2 to 8°C. Suspension of standard *E. coli* ATCC 25922 equivalents to 0.5 McFarland turbidity standards was prepared and inoculated on a Mueller-Hinton agar plate. A 30µg cephoxitin disc was placed on the inoculated agar surface.

Prior to use, the prepared AmpC disks were rehydrated with 20µl of saline before being inoculated with several colonies of the test isolates. This was placed beside the cephoxitin disc and the plates incubated at 37°C for 18-24 hours. The plates were examined for either an indentation or a flattening of the zone of inhibition, which indicates the enzyme inactivation of cephoxitin as a positive result, or absence of distortion, indicating no significant inactivation of cephoxitin as negative result.

RESULTS

A total of 196 faecal samples obtained from 4 different livestock were collected and analyzed out of which 23.98% were obtained from cattle, 26.53% from chicken and goat respectively while 22.96% were from pig (Table 1). One hundred and twenty three *E. coli* isolates were harvested out of the total faecal samples analysed. Samples obtained from cattle and chicken each yielded 13.27% and 18.87% respectively, those obtained from goat yielded 16.33% while 14.29% were from samples obtained from Pig making a total of 62.76%. Of the 123 *E. coli* isolates obtained, 71.5% exhibited resistance to third and fourth generation cephalosporins. On further testing for ESBL production by the Modified Double Disc Synergy test (MDDST), 55.68% were ESBL producers out of which 12.5% were positive for AmpC co-production by the AmpC disc test while the remaining 43.18% were only ESBL producers. The highest percentage of 4.55% was however seen among isolates obtained from Pig followed by 3.41% from Chicken while Goat and Cattle each yielded 2.27% respectively (Table 2).

DISCUSSION

Extended Spectrum Beta-Lactamases (ESBLs) constitute a serious threat to the β-lactam therapy. Resistance against β-lactam antibiotics is increasingly being reported and is on the rise in *Enterobacteriaceae* from both humans and animals. Due to the difficulty in their detection by the current clinical methods available in most laboratories, many of these strains have been falsely reported to be susceptible to the widely used broad-spectrum β-lactams[13].

The result of this study shows the presence of ESBL producing *E. coli* in all the group of animals tested. A study conducted in Germany sought the presence of ESBL-producing *E. coli* in different dairy cattle, beef cattle and mixed farms (both dairy and beef). The results showed a high prevalence of different types of ESBLs[14]. The first reports of ESBL-producing bacteria from poultry were performed in Spain. *E. coli* strains isolated from faecal samples of healthy and sick poultry were found to harbor various types of ESBL genes[15]. Similarly, antimicrobial resistance in commensal Enterobacteriaceae from pigs were also confirmed in some Danish farms, where some ESBL producing *E. coli* strains were recovered from faeces of pigs[16]. Generally, animals and birds represent potential sources of spread of multidrug-resistant bacteria. This is as a result of the fact that the ESBL-encoding genes are often carried on plasmids, which can easily be transferred between isolates, bearing additional resistance determinants for other classes of antimicrobial agents, mainly fluoroquinolones, aminoglycosides and sulfonamides, contributing to the multidrug-resistant phenotype.
This study also indicates that out of the 123 *E. coli* isolates obtained, 55.68% were ESBL producers while 12.5% possess additional ability for AmpC production. This is in agreement with Kaur *et al*[12] who got 63.4% ESBL producers out of which 5.4% were AmpC producers. In this study, AmpC production has the highest prevalence of 4.55% among isolates obtained from Pig. This may be related to the use of antibiotics in pig production as previously shown by Jorgensen *et al*[16] who, in their earlier study, confirmed that the use of beta-lactam antibiotics, especially cephalosporins, might be one of the factors for the selection of ESBL/AmpC-producing bacteria in pigs. Also Carattoli *et al*[17] demonstrated that certain beta-lactams, including amoxicillin, used in pig production select for *bla*CTX-M-producing *E. coli* strains in the intestinal flora of pigs. In addition, non-beta-lactam antibiotics might play a role in the selection of beta-lactamase genes. The need for ESBL testing in the AmpC-producing species of *Enterobacteriacae* cannot be over-emphasized. In the presence of AmpC, along with ESBL in the gram negative organisms, the DDST may not show positivity, as the AmpC type of β-lactamase inhibits the action of clavulanate. Hence, it obscures the synergistic effect

of clavulanic acid and the third generation cephalosporins which are used. The possible approaches for overcoming the difficulty in the ESBL detection in the presence of AmpC include the use of tazobactam or sulbactam, which are much less likely to induce the AmpC β-lactamases and are therefore the preferable inhibitors of the ESBL detection tests with these organisms or testing cefepime as an ESBL detection agent[18].

Unusually high incidence of ESBLs should be a cause of concern to the regulators of the antibiotic policy. Nowadays, over reliance on third generation cephalosporins to treat gram negative infections is one of the prime factors responsible for increased resistance to this class of antibiotics.

Generally, intensive application of antibiotics in livestock husbandry increases the abundance of extended-spectrum β-lactamase-producing Enterobacteriaceae in animals and in consequence in their manure[19]. This is confirmed by the presence of ESBL- and AmpC-producing *E. coli* in manure from livestock husbandry that was reported by Hartmann *et al*[20] and Snow *et al*[21]. The problem remains that even though the occurrence of ESBL-producing bacteria has been broadly recognized in veterinary medicine, as causative agents for different infections in dairy cattle[15], only a few studies exist which investigates the prevalence of ESBL- and AmpC producing bacteria in Nigerian livestock, showing their existence in sick and healthy cattle, pig and poultry farms[5]. This confirms the fact that the risk of zoonotic transfer from livestock to people with close contact to these animals is still largely unknown. However, some studies have implicated a transfer of ESBL producing *E. coli* or ESBL genes from poultry or pigs to farm workers[6,7]. One of the greatest challenges in the routine susceptibility test done by clinical laboratories is that it normally fails to detect ESBL positive strains hence the phenotypic confirmatory test is highly indispensable in the detection ESBLs.

One important fact remains that normal but resistant bacterial microflora in animals and other zoonotic intestinal bacteria could infect humans more frequently through direct contact and also, through animal foodstuffs. These resistant bacteria species could also colonize humans and transfer genes of resistance to other members of the bacterial normal microflora. They could provoke infections and could also be regarded as a main reservoir of resistance genes. Therefore, the rapid dissemination of resistance genes via mobile gene elements increases the risk and creates prerequisites for more complications from a therapeutic point of view, with special emphasis on professionals groups associated with animal care, farmers, veterinarians at farms, slaughterhouse workers and other people engaged in animal foodstuff processing.

CONCLUSION

Improved surveillance of antibiotic use and antibiotic-resistant bacteria in farm animals is a serious issue that requires an urgent attention. Therefore, some important initiatives have to be taken in this regard especially in relation to antibiotic sales data. Countries should routinely monitor levels of antibiotic resistance in farm animals and on retail meat as most of them represents data for monitoring programs for antimicrobial resistance. International governments require cooperation to establish an international antimicrobial resistance surveillance monitoring program and monitor the antimicrobial resistance trends in human and animals for a long time. Both the benefit and risk outcomes of this exercise should be considered into the risk assessment and management. On the other hand, application of biosecurity and hygiene programs in intensive sector of livestock breeding would be a favorable effect on the restriction transfer of antibiotic resistance. Finally, to find a good strategy to control antimicrobial resistance, it is necessary to consider the chemotherapeutic medicine, microbiology and agricultural environment and fully understand molecular basis involved in the emergence of antimicrobial resistance.

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Table 1: Sources of *Escherichia coli* Isolates

Source of Sample	No. of Samples collected	No.(%) of *E. coli* isolated
Cattle	35	18(13.27)
Chicken	40	29(18.87)
Goat	40	25(16.33)
Pig	33	21(14.29)
TOTAL	**196**	**123(62.76)**

Table 2: ESBL and *AmpC* producing *E. coli* Isolates (n=88)

Source of Sample	No.(%) of potential ESBL producing isolates	No.(%) of ESBL & AmpC producing isolates	No.(%) of only producing isolates
Cattle	10(11.36)	2(2.27)	8(9.09)
Chicken	13(14.78)	3(3.41)	10(11.36)
Goat	10(11.36)	2(2.27)	6(6.82)
Pig	16(18.18)	4(4.55)	14(15.91)
TOTAL	**49 (55.68)**	**11(12.5)**	**38(43.18)**