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Original Article

Detection of Extended Spectrum Beta-Lactamases Among Gram Negative Bacilli Recovered from Cattle Feces in Benin City, Nigeria

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Abstract

This study was carried out to determine the prevalence of extended spectrum beta-lactamase (ESBL) among Gram negative bacteria isolated from cattle feces in Benin City, Nigeria. A total of 250 Gram negative bacteria isolates were recovered from cattle feces and were processed microbiologically using standard techniques. Emergent colonies were identified and antibacterial susceptibility tests were determined using Kirby-Bauer disk diffusion method. All bacterial isolates were screened for the presence of ESBL using the double-disc synergy method. A total of 37 (14.8%) isolates were positive for ESBL, with 33 (13.2%) indicated by ceftazidime, while only 4 (1.6%) were indicated by both ceftazidime and cefotaxime ($P < 0.0001$). Of the Gram negative bacterial isolates recovered, *Salmonella* species was the most prevalent ESBL-producer with 55.0% prevalence ($P = 0.0092$), while no isolate of *Pseudomonas aeruginosa* produced ESBL. ESBL-positive isolates showed poor susceptibility to the tested antibacterial agents in comparison with non-ESBL-producers and imipenem was the most active antibiotic. The prevalence of ESBL among Gram negative bacilli recovered from cattle feces was 14.8%. The study advises prudent use of antibiotics in the treatment of cattle and harps on improved hygiene in managing cattle, as they are potential reservoirs of ESBL-producing organisms.

Keywords: antibiotics, bacterial isolates, cattle, extended spectrum beta-lactamase, feces, resistance

Introduction

Antimicrobial resistance continues to plague healthcare delivery worldwide and has become of serious public health concern in developing nations (WHO, 2014). Animals being reared either as pets or for consumption have been identified as potential reservoirs of resistant fecal flora (Schmid *et al*., 2013). Fecal flora is comprised largely of the Enterobacteriaceae family and one major mechanism of resistance among these Gram negative rods is the production of extended spectrum beta-lactamase (ESBL) (dos Santos et al., 2013). Extended spectrum beta-lactamases (ESBLs) are enzymes that inactivate a large number of β-lactam antibiotics including extended-spectrum very-broad-spectrum cephalosporins and monobactams (Paterson and Bonomo, 2005). The genetic determinants of ESBL enzyme are largely plasmid borne, being of TEM, SHV, OXA and CTX-M genes predominantly with the propensity for transfer between species (Paterson and Bonomo, 2005; Schmid *et al*., 2013). They are inhibited by clavulanic acid, sulbactam and tazobactam, and have been reported to coexist with resistance to other antimicrobial classes like aminoglycosides and quinolones (Paterson and Bonomo, 2005).

As commensal organisms in the intestinal tract, ESBL-producing enterobacteria harbored by food-producing animals could be discharged into the environment, thereby serving as disseminators of genes encoding for β-lactam resistance (Geser *et al*., 2011). A clear correlation has been shown between antibiotic use and resistance gene prevalence in food animals among seven European countries for which data on antibiotic use and antibiotic resistance were available (Chantziaras *et al*., 2014). For a developing nation like Nigeria, where there are no strict policies on antibiotic use both for humans and in veterinary practice, the distribution of resistance genes in food animals can best be imagined.

Various reports have discussed in recent times the dissemination of ESBL-producing Enterobacteriaceae in healthy food-producing animals in several countries in Europe and Asia or in food products like meat, fish and raw milk (dos Santos *et al*., 2013; Ohnishi et al., 2013). In Nigeria, a previous study in Ibadan, South-Western Nigeria, detected no ESBL-producing isolate in the feces of healthy cattle (Inwezerua *et al*., 2014). ESBL-producing isolates have however been isolated from pigs (24.2%) and chicken

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(22.2%) in South-East Nigeria (Duru *et al*., 2013; Ugwu *et al*., 2015). These discrepancies, in addition to paucity of data on the prevalence of ESBL among cattle in South-South Nigeria necessitated the current study. The aim of the hereby study was to determine the prevalence of ESBL-producing Gram negative bacteria among cattle in Benin City, Nigeria, using a phenotypic method.

Materials and Methods

Sample collection

The study was a cross-sectional study, carried out at the University of Benin cattle farm and Aduwawa cattle market in Benin City, Nigeria. A total of 120 cattle of Bokolo breed were used for this study. Sterile wide-mouth containers were used to collect fecal specimens from these cattle. The containers were labeled and transported immediately to the laboratory.

Isolation of gram negative bacteria

The stool specimens were inoculated on MacConkey, Deoxycholate citrate agar and Selenite-F broth. They were thereafter incubated aerobically at 37 °C overnight. The Selenite-F broth was subcultured onto MacConkey and Deoxychonate agar after 24 hours incubation and inoculated plates incubated aerobically at 37 °C overnight. The emergent isolates were identified using standard techniques as previously described (Barrow and Feltham, 2003). Colonies of isolates which were gram negative were further identified using biochemical tests to include: indole, citrate, glucose, urease, lysine, oxidase, hydrogen sulphide, lactose, nitrate, ornithine, mannitol, xylose, ONPG, Voges proskauer, Tryptophan deaminase, inositol, gelatin, malonate, sucrose, adonitol, raffinose, salicin and preserved on nutrient agar slants for further analysis.

Detection of extended spectrum beta lactamase

The presence of ESBL was detected in bacterial isolates using the double-disc synergy test (Livermore and Brown, 2001). Briefly, test organisms were emulsified in sterile water and the turbidity matched with 0.5 McFarland standards (equivalent to 1 x 108 colony forming units per ml). Once matched, a sterile cotton wool swab was dipped in the organism suspension and excess liquid was removed by turning the swab on side of the test tube. The entire surface of Mueller-Hinton agar plate was seeded by swabbing in three directions with the swab. A disc containing amoxicillin–clavulanate (30 µg) (Oxoid, England) was placed at the centre of the agar plate. A ceftazidime disc (30 µg) was placed 25 mm from the amoxicillin–clavulanate disc and another disc containing cefotaxime (30 µg) was placed on the opposite side of the amoxicillin–clavulanate disc. The plates were incubated at 37 °C overnight and ESBL production was inferred as positive if there was an expansion of the zone of inhibition between the ceftazidime and amoxicillin–clavulanate disc, cefotaxime and amoxicillin–clavulanate disc or both.

Determination of antibiotic susceptibility/resistance of the isolates: disc susceptibility tests were performed on all bacterial isolates using the British Society for Antimicrobial Chemotherapy (BSAC) method (Andrew, 2009).

Data analysis

The data obtained were analyzed with Chi square X2 using the statistical software INSTAT® (Graph Pad Software Inc, La Jolla, CA, USA).

Results

In the current study, 250 Gram negative bacterial isolates were recovered from fecal specimens of 120 cattle. A total of 37 (14.8%) isolates were positive for ESBL with 33 (13.2%) positive for ceftazidime only, while 4 (1.6%) were positive using ceftazidime + ceftriaxone. The difference was statistically significant ($P < 0.0001$) (Table 1).

The Gram negative bacterial isolates recovered included *E. coli* (98), *Klebsiella* spp. (19), *Proteus mirabilis* (36), *Proteus vulgaris* (39), *Providencia* spp. (18), *Salmonella* spp. (10), *Shigella* spp. (15) and *Pseudomonas aeruginosa* (15). In relation to bacterial isolates, the highest prevalence of ESBL-production was observed for *Salmonella* species with 55% prevalence, while no isolate of Pseudomonas aeruginosa produced ESBL. The differences observed were statistically significant ($P = 0.0092$) (Table 2).

Antibacterial drugs used in susceptibility testing showed poor efficacy to ESBL-producing organisms with nitrofurantoin (2.7%) and cefuroxime (5.4%) showing the least activity. Imipenem was the most active against ESBL-producers (Table 3). Non-ESBL-producing isolates showed remarkable susceptibility to ofloxacin (72.8%), ciprofloxacin (74.7%) and imipenem (84.0%), the least susceptibility was observed for nitrofurantoin (5.2%) and cefixime (12.7%) (Table 4).

Discussion

In the hereby study, 14.8% of Gram negative bacilli were ESBL-producers. This prevalence rate was slightly higher than a study in Switzerland which reported 8.4% among calves in a slaughter house, but in contrast to a study in South-West Nigeria which reported an absence of ESBL genes among isolates from bovine fecal specimens from slaughter houses (Reist *et al*., 2013; Inwezerua *et al*., 2014). The observation shows that the prevalence of ESBL producers in animals is on the rise and may pose risk to humans (Chantziaras *et al.,* 2014). ESBL production was also more likely to be detected using ceftazidime antibiotic alone.

Various studies have remarked that ceftazidime antibiotic alone is the best indicator for TEM and SHV–derived ESBL, while the use of ceftazidime and cefotaxime enhances the detection of TEM, SHV and CTX-M ESBL types (Paterson and Bonomo, 2005; Ogbolu *et al*., 2011). We can therefore infer that TEM and SHV genes are the predominant genes causing ESBL-production among bacterial isolates from cattle in Benin City, South Nigeria. This observation is in stark contrast to a previous study on human specimens in Benin City in which 95.1% of ESBL positive isolates were detected by both ceftazidme and cefotaxime (Ogefere *et al*., 2015). The finding however aligns with another study in South-Western Nigeria on human specimens which showed TEM and SHV as the dominant ESBL types using molecular techniques (Ogbolu *et al*., 2011).

Table 1. Type of ESBL-production by ESBL-producing isolates recovered from cattle feces

Antibiotics	Number of recovered bacterial isolates	Number positive for ESBL (%)
CAZ	250	33 (13.2)
CTX	250	0 (0)
CAZ + CTX	250	4 (1.6)

CAZ- Ceftazidime, CTX- cefotaxime, ESBL- Extended spectrum betalactamase P<0.0001

Table 2. Distribution of ESBL among Gram negative bacterial isolates recovered from cattle feces

Bacterial isolates	Number of isolates recovered	Number positive for ESBL (%)
Escherichia coli	98	16 (16.3)
Klebsiella species	19	1 (5.3)
Proteus mirabilis	36	6 (16.7)
Proteus vulgaris	39	2 (5.1)
Providencia species	18	3 (16.7)
Salmonella species	10	5 (50.0)
Shigella species	15	4 (26.7)
Pseudomonas aeruginosa	15	0 (0)
TOTAL	**250**	**37 (14.8)**

ESBL- Extended spectrum betalactamase, Bacterial isolates vs ESBL: P = 0.0092

Table 3. Susceptibility profile of ESBL producing organisms recovered from cattle feces

Bacterial isolates	CIP (5 µg)	CAZ (30 µg)	CRX (30 µg)	GEN (10 µg)	CXM (5 µg)	OFX (5 µg)	AUG (30 µg)	NIT (30 µg)	IMP (10 µg)
Escherichia coli (n =16)	10 (62.5)	3 (18.8)	2 (12.5)	6 (37.5)	1 (6.3)	9 (56.3)	3 (18.8)	1 (6.3)	12 (75.0)
Klebsiella spp . (n = 1)	1(100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	1 (100)
Proteus mirabilis (n = 6)	1 (16.7)	0 (0.0)	0 (0.0)	3 (50.0)	0 (0)	1 (16.7)	2 (33.3)	0 (0)	4 (66.6)
Proteus vulgaris (n = 2)	0 (0)	0 (0)	0 (0)	1 (50.0)	0 (0)	1 (50.0)	0 (0)	0 (0)	1 (50.0)
Providencia species (n= 3)	2 (66.7)	0 (0)	0 (0)	1 (33.3)	0 (0)	1 (33.3)	0 (0)	0 (0)	1 (33.3)
Salmonella species (n = 5)	4 (80.0)	2 (40.0)	0 (0)	3 (60.0)	0 (0)	4 (80.0)	0 (0)	0 (0)	4 (80.0)
Shigella species (n= 4)	1 (25.5)	0 (0)	0 (0)	2 (50.0)	1 (25.0)	3 (75.0)	0 (0)	0 (0)	2 (50)
TOTAL (n = 37)	**19 (51.35)**	**5 (13.5)**	**2 (5.4)**	**15 (40.5)**	**2 (5.4)**	**20 (50.1)**	**5 (13.5)**	**2 (5.4)**	**25 (67.6)**

N= number of isolates, number in brackets = value in percentage, CIP- ciprofloxacin, CAZ- ceftazidime, CRX- cefuroxime, GEN- gentamicin, CXM-cefixime, OFX-ofloxacin, AUG- amoxicillin-clavulanate NIT- nitrofurantoin, IMP- imipenem.

Table 4. Susceptibility profile of non-ESBL producing bacterial isolates recovered from cattle feces

Bacterial isolates	CIP (5 µg)	CAZ (30 µg)	CRX (30 µg)	GEN (10 µg)	CXM (5 µg)	OFX (5 µg)	AUG (30 µg)	NIT (30 µg)	IPM (30 µg)
Escherichia coli (n= 82)	64 (78.0)	31(37.8)	18 (22.0)	60 (73.2)	12 (14.6)	63 (76.8)	31 (37.8)	7 (8.5)	70 (85.4)
Klebsiella species (n= 18)	14 (77.7)	13 (72.2)	4 (22.2)	15 83.3)	2 (11.1)	13 (72.2)	10 (55.6)	1 (5.5)	14 (77.8)
Proteus mirabilis (n= 30)	23 (76.7)	16 (53.3)	1 (3.3)	16 (53.3)	1 (3.3)	23 (76.7)	18 (60.0)	0 (0)	22 (73.3)
Proteus vulgaris (n= 37)	23 (62.2)	4 (10.8)	1 (2.7)	26 (70.1)	4 (10.8)	21 (56.8)	9 (24.3)	0 (0)	33 (89.2)
Providencia species (n= 15)	13 (86.7)	9 (60.0)	2 (13.3)	14 (93.3)	2 (13.3)	12 (80.0)	4 (26.7)	1 (6.7)	13 (86.7)
Salmonella species (n= 5)	3 (60.0)	3 (60.0)	1 (20.0)	5 (100)	0 (0)	4 (80.0)	0 (0)	(0)	5 (100)
Shigella species (n= 11)	8 (72.2)	4 (36.4)	0 (0)	8 (72.2)	0 (0)	9 (81.8)	0 (0)	0 (0)	10 (90.9)
Pseudomonas aeruginosa (n= 15)	11 (73.3)	5 (33.3)	5 (33.3)	4 (26.7)	5 (33.3)	12 (80.0)	0 (0)	2 (13.3)	12 (80.0)
TOTAL	**159 (74.7)**	**85 (39.9)**	**33 (15.5)**	**148 (69.5)**	**27 (12.7)**	**155 (72.8)**	**70 (32.9)**	**11 (5.2)**	**179 (84.0)**

N= number of isolates, number in brackets = value in percentage, CIP- ciprofloxacin, CAZ- ceftazidime, CRX- cefuroxime, GEN- gentamicin, CXM-cefixime, OFX-ofloxacin, AUG- amoxicillin-clavulanate NIT- nitrofurantoin, IMP- imipenem.

The genus *Salmonella* were the highest producers of ESBL in the current study. In a previous study evaluating rectal swabs of pigs in Nigeria, *Salmonella* species ranked next to *Klebsiella* species in ESBL production (Ugwu *et al*., 2015). The finding is also in contrast to previous studies on human specimens in South, North-West and South-West Nigeria which observed *Enterobacter* species and *Klebsiella* species as the most prevalent producers of ESBL (Ogefere *et al*., 2015; Ogbolu *et al*., 2011; Yusuf *et al*., 2013). Salmonella species, which could be either diarrheagenic (non-typhoidal) or septicemic (typhoidal), can under stress, accept genetic materials from other bacteria, notably E. coli in the gastrointestinal tract (Aibinu *et al*., 2007). Plasmids harboring ESBL genes are capable of horizontal transfer between species and the gut may therefore be an ecological niche for this interspecies transfer.

In Nigeria, there are no strict laws on grazing and it is commonplace to find cattle in streets, farm lands and river banks being led by a herdsman. They may therefore be potential reservoirs of ESBL-producing *Salmonella* species and other organisms when they defecate in such open grazing places.

As it has been highlighted by various authors, ESBLs mediate resistance to the thirdgeneration cephalosporins (Paterson and Bonomo, 2005; Ogbolu *et al*., 2011; Snow *et al*, 2012). The antimicrobial susceptibility pattern of ESBL-producing organisms in the hereby study was congruent with the fact as the cephalosporins- ceftazidime, cefixime and cefuroxime showed poor efficacy against these organisms *in vitro*.

In Nigeria, there are no strict policies on use of antibiotic both for humans and veterinary care (Eucast, 2013; Ugwu *et al*., 2015). Indiscriminate use of β-lactams (particularly 3rd and 4th generation cephalosporins) has led to selection pressure and development of resistance to these drugs by Enterobacteriaceae (Geser *et al*., 2011). Equally worthy of note is the resistance to the fluoroquinolones (ciprofloxacin and ofloxacin) and aminoglycoside (gentamicin) which was marked among these organisms.

Previous studies on ESBL-producing bacterial isolates from pigs and cattle in Switzerland and Turkey showed 42.9% and 15.4% resistance respectively to ciprofloxacin (Geser *et al*., 2011; Schmid *et al*., 2013). ESBL-producing isolates in the studies cited also showed 42.9% and 15.4% resistance respectively to gentamicin (Geser *et al*., 2011; Schmid *et al*., 2013). The current findings were therefore not too surprising as plasmids bearing the genes encoding ESBLs have been shown to also carry genes encoding resistance to aminoglycosides, quinolones and trimethioprim/sulfamethoxazole (Paterson and Bonomo, 2005).

The poor susceptibility of non-ESBL-producing organisms to cephalosporin antibiotics suggests that some other mechanism of resistance may be at play. One such mechanism is the production of AmpC.

AmpCs are able to hydrolyze cephalosporins, cephamycins (e.g. cefoxitin and cefotetan), aminopenicillins and monobactams (Livermore and Brown, 2001). Recent studies on human specimens in South-South and South-West Nigeria have documented the role of AmpC beta-lactamase in resistance to this class of antibiotics (Ogbolu *et al*., 2011; Ogefere *et al*., 2016). Though imipenem showed the highest activity, prudent use is advocated as they are drugs of last resort.

Conclusions

The prevalence of ESBL among Gram negative bacilli recovered from cattle feces was 14.8%. Prudent use of antibiotics in the treatment of cattle is advocated, as well as improved hygiene in managing cattle, as they are potential reservoirs of ESBL-producing organisms.

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