Antibiotic resistance profile of extended spectrum β-Lactamase producing Escherichia coli *i*solated from cattle dung

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Abstract

Animal dung are potential reservoir for maintenance and dissemination of antibiotic resistant bacteria into other animals, human and the environment. The frequent discharge of animal dung containing residual antibiotics into the environment without proper or adequate treatment could trigger the onset of antibiotics resistance in environmental bacteria. Infection caused by extended-spectrum beta-lactamases (ESBL) producing organism is a major problem regarding antibiotic resistance. ESBL – producing organisms frequently exhibit resistance to other antimicrobial classes such as fluoroquinolones, trimethoprim-sulfamethoxazole, due to associated resistance mechanisms, which may be either chromosomally- or plsmid- encoded. Therefore, this study was carry out to investigates the antibiotic resistance profile of ESBL producing E.coli isolated from cattle dung. This cross-sectional study was carried out in the Department of Microbiology University of Ibadan, Nigeria. A total of twenty nine (29) Gram-negative E.coli were isolated from the cattle dung and molecular characterization was carried out using uidA gene detection. ESBL producers were identified using the double-disc synergy test. Out of the 29 isolates, 93.1% (n=27) were ESBL producers. Most of the ESBL producers were resistant to commonly used antibiotics. Carbapenems especially imipenem (IMI) was the most effective drug showing excellent sensitivity; Amoxicillin (AMC) also had better sensitivity result. Most of the ESBL producers were highly resistance to Cefpdoxime (100%), Penicillin G (100%), Cefotaxime (100%), Trimethoprime-sulphamethaxazole (86.8%) and Oxytetracycline (82.6%).ESBL production should be detected routinely in Agriculture and Animal husbandry. Rational use of antibiotics in Animal feeds and treatments must be done promptly to prevent the development and spread of ESBL producing microorganisms.

Keyword: Antibiotic resistance; ESBL producers; *Escherichia coli;* Cattle dung; Animal husbandary

profil de résistance aux antibiotiques d'escherichia coli producteur de β-lactamase à spectre étendu isolé à partir de fumée de bovins

Résumé

Les excréments d'animaux sont un réservoir potentiel pour le maintien et la dissémination de bactéries résistantes aux antibiotiques dans d'autres animaux, l'homme et l'environnement. Le rejet fréquent de déjections animales contenant des antibiotiques résiduels dans l'environnement sans traitement approprié ou adéquat pourrait déclencher l'apparition d'une résistance aux antibiotiques chez les bactéries environnementales. L'infection causée par un organisme producteur de bêta-lactamases à spectre étendu (BLSE) est un problème majeur concernant la résistance aux antibiotiques. Les organismes producteurs de BLSE présentent fréquemment une résistance à d'autres classes d'antimicrobiens telles que les fluoroquinolones, le triméthoprime-sulfaméthoxazole, en raison de mécanismes de résistance associés, qui peuvent être codés par les chromosomes ou les plasmides. Par conséquent, cette étude a été menée

pour étudier le profil de résistance aux antibiotiques des E. coli producteurs de BLSE isolés à partir de bouses de bovins. Cette étude transversale a été réalisée au Département de microbiologie de l'Université d'Ibadan, au Nigeria. Au total, vingt-neuf (29) E. coli Gram négatifs ont été isolés de la bouse de bovin et la caractérisation moléculaire a été réalisée à l'aide de la détection du gène uidA. Les producteurs de BLSE ont été identifiés à l'aide du test de synergie à double disque. Sur les 29 isolats, 93,1 % (n=27) étaient producteurs de BLSE. La plupart des producteurs de BLSE étaient résistants aux antibiotiques couramment utilisés. Les carbapénèmes, en particulier l'imipénème (IMI), étaient le médicament le plus efficace et présentaient une excellente sensibilité ; L'amoxicilline (AMC) avait également un meilleur résultat de sensibilité. La plupart des producteurs de BLSE étaient hautement résistants au Cefpdoxime (100%), à la Pénicilline G (100%), au Céfotaxime (100%), au Triméthoprime-sulfaméthaxazole (86,8%) et à l'Oxytétracycline (82,6%). La production de BLSE devrait être détectée systématiquement en Agriculture et élevage. L'utilisation rationnelle des antibiotiques dans l'alimentation et les traitements des animaux doit être faite rapidement pour prévenir le développement et la propagation des microorganismes producteurs de BLSE.

Mot-clé : Résistance aux antibiotiques ; producteurs de BLSE ; Escherichia coli; Fumier de bétail; Élevage d'animaux

روث الحيوانات هو خزان محتمل لصيانة ونشر المضادات الحيوية المقاومة في بقية الحيوانات، والإنسان والبيئة، الإفرازات المتكررة بدون علاج مناسب يمكن أن يؤدي إلى ظهور مقاومة المضادات .لروث الحيوانات الذي يحتوي على مضادات حيوية متبقية في البيئة التي تنتج الكائن الحي هي مشكلة كبيرة فيما يتعلق (ESBL)العدوى الناجمة عن امتداد الطيف البيتالاكتاماز .الحيوية في البكتريا البيئية غالبًا ما تظهر مقاومة للفصول الأخرى المضادة للميكروبات مثل ESBLالكائنات الحية المنتجة لـ .بمقاومة المضادات الحيوية الفليوروكينولونات والترايميثوبريم وسلفاميثوكسازوا بسبب آليات المقاومة المرتبطة بها، التي قد تكون إما كروموسومية البلازميد إنتاج الإشريكية القولونية المعزولة من ESBLالمشفرة لذلك، تم إجراء هذه الدراسة للتحقيق في ملف مقاومة المضادات الحيوية لـ تم إجراء هذه الدراسة المتقاطعة في قسم علم الأحياء الدقيقة جامعة إبادن نيجيريا، تم عزل ما مجموعه تسعة وعشرون .روث الماشية ، تم تحديد uidAجرامًا من الإشريكية القولونية السلبية من روث الماشيه وتم إجراء التوصيف الجزيئي باستخدام اكتشاف جينات كان معظم ESBLنتيجة (n=27) 93.1%من بين تسعة وعشرين عزلة كان .باستخدام اختبار تآزر القرص المزدوج ESBLمنتجي هو الدواء الأكثر imipenemخاصة Carbapenemsكان ,كاربابينيم .مقاومين للمضادات الحيوية الشائعة الاستخدام ESBLمنتجي يقاومون بشدة ESBLكان معظم منتجي .فعالية الذي يظهر حساسية ممتازة أموكسيسللينكان لها أيضا نتائج حساسية أفضل الأوكسيتتراسيكلين 86.8%تريميثوبريم سلفميتاساسول (100٪)سيفوتاكسيم %بنسلين جي ١٠٠(100٪) Cefpdoxime يجب القيام بالاستخدام الرشيد للمضادات .بشكل روتيني في الزراعة وتربية الحيوانات ESBLيجب الكشف عن إنتاج (82.6%) الحيوية في الأعلاف والعلاجات الحيوانية على الفور لمنع تطور وانتشار الكائنات الحية الدقيقة المنتجة لـ ESBL.

Introduction

The use of antibiotics in agriculture and animal husbandry is increasingly being considered a global health issue, both from the animal health and welfare aspect and because of the development of antibiotic resistance in animal pathogens (Davies 2013; O'Neill 2015). Animal manure is a major source of antibiotic resistant bacteria entering the environment, especially the soil as animals do not utilize all the antibiotics in feeds and a large proportion is excreted in the urine or feaces (Binh*et al.* 2007; Ghosh and LaPara 2007).

Antibiotic resistance (AR) is defined as resistance of a microorganism to an antibiotic medicine to which it was originally sensitive. AR is a natural phenomenon, which is amplified by continuous and unnecessary exposure to antibiotics (WHO 2014).

A specific type of antibiotic resistance that currently represents a major public health concern is the 3rd generation cephalosporin

resistance induced by extended spectrum beta-lactamase (ESBL)-production (Canton *et* al*.,* 2008). ESBL-producing bacteria are resistant to almost all beta-lactam antibiotics, and often to other classes of antibiotics as well. ESBLs represent a major group of β-lactamases which have the ability to hydrolyze and cause resistant to various type of newer β-lactam antibiotics including the extended-spectrum (or third-generation) cephalosporins and monobactams (aztreonam) but not the cephamycins (cefoxitin and cefotetan) and carbapenems (Vinodhini*et al.,* 2014).

The emergence and spread of extended spectrum β-lactamase (ESBL)-producing *E. coli* associated with cattle has been of a great concern (Bush and Jacoby 2010). Cephalosporin antibiotics have been reported as a commonly used antibiotic in dairy veterinary medicine because they are effective in treating mastitis caused largely by *E. coli* strains which explains the increased resistance of *E. coli* to third generation cephalosporins (Toyotaka *et al*., 2014). *Escherichia coli* serves as a sentinel organism for antimicrobial resistance development in different types of animals, because it is a common enteric commensal, can be a pathogen, and easily acquires resistance and therefore can act as a reservoir that can transfer resistance to other species/pathogens (Ashbolt *et al.* 2013).

Antibiotic resistance profile and reporting of drug resistant strain especially ESBL producing strains in animal wastes would give an insight to the appropriate antibiotic therapy and would also help in awareness towards misuse and overuse of antibiotics in animal husbandry (Paterson and Bonomo, 2005). This study was performed to isolate and screen ESBL producing *E. coli* from cattle waste.

Materials and Method

Sample site

Cattle dungs were collected weekly for 6 months from dairy cattle units of the University of Ibadan teaching and research farm located at Abadina area of the University metropolis, Ibadan, Nigeria. All collected samples were transported to the Environmental Microbiology and Biotechnology laboratory, Department of Microbiology, University of Ibadan.

Isolation, Identification and Confirmation of ESBL production

Isolation of ESBL producing Bacteria was done using CHROMagar ESBL. ESBL presumptive colonies of *Escherichia Coli* were identified based on color and morphology, according to manufacturer's instructions. ESBL production in the isolates was confirmed using the double disc synergy test (DDST) following the guidelines of CLSI (2018), by using a disc of amoxicillin-clavulanate (20/10 µg) along with three 3rd generation cephalosporins; cefotaxime, ceftazidime, and cefpodoxime (Randegger*et al.,* 2001). The antibiotic disks were placed 20mm apart, centre-to-centre.

Antibiotic Susceptibility testing

Antibiotic susceptibility testing was done using the Kirk Bauer disk diffusion method as recommended by the Clinical laboratory standards institute (CLSI) guidelines.10 commercially available antibiotic disks were used for antibiotic susceptibility testing. The zones of inhibition were measured and interpreted using the CLSI guidelines (CLSI, 2018).

Table 1: ***Panel of antibiotics used.***

Antibiotics	Symbol	Concentration
Penicillin G	PEN	24µg
Ceftazidime	CAZ	30µg
Cefpodoxime	CPD	30µg
Cefotaxime	CTX	30µg
Imipinem	IMI	10µg
Amoxicillin	AMC	30µg
Streptomycin	STR	50µg
Enrofloxacin	ENR	10µg
Oxytetracycline	OXT	30µg
Trimethoprim-Sulphamethaxazole	SXT	25µg

Detection of uidA gene in ESBL producing E. coli

Purified cultures of the isolates were characterized using molecular methods, by targeting *uidA* gene, which encodes β-D glucuronidase production in *E. coli,* using the methods reported by Janezic*et al.* (2013).This is based on amplifying regions of the *uidA* gene that code for 1,-glucuronidase, expression of which forms the basis for fecal coliform detection. Amplification of the *uidA*genes specific to *E. coli* species was performed following previous protocols (Anastasi *et al.,* 2010; Anbazhagan, *et al.,* 2011), with minor modifications. PCR reaction mixtures was prepared as standard 15 µL volume constituting 3 µL of PCR Master Mix, 10 µLnuclease-free PCR water, 0.5 µL mixture each of the forward and reverse primers and 1 µL of template DNA, making a final volume of 15µL. Amplifications was carried out using DNA thermal cycler and oligonucleotide primer sequences.The amplification conditions were as follows:5min at 95°C,followed by 40 cycles of 30s at 95°C, 45 sat 50°C, 45 sat 72°C,and a finale longation step of 10min at 72°C. PCR amplicons were stored at 4°C until electrophoresis.

DNA Extraction

Total genomic DNA was extracted from all presumptive isolates using Zymo Research Genomic DNA^{TM}–Tissue MiniPrep Kit following the manufacturer's instructions (Inqaba Biotechnical Industry Ltd, Pretoria, South Africa). The quality and quantity of the extracted DNA was determined using the agarose gel electrophoresis.

PCR amplification

The mixture consisted of 1× PCR buffer (10 mmol l^{-1} Tris HCl pH 8·8, 1·5 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} KCl, 0·1% Triton X 100), 1 U of Taq DNA polymerase (Finnzymes, Espoo, Finland), 0·5µmol l^{-1} of each primer, 200µmol l^{-1} of each dNTPs (Invitek, Berlin, Germany) and 2·5µl of template DNA. PCR reaction was performed in total volume of 25µl. Conditions of PCR amplification were as follows: initial denaturation at 94°C for 90s, and 30 cycles withdenaturation at 94°C for 30s, annealing at 58°C for 25s and extension at 72°C for 30s.

Detection of PCR products

The amplified products were loaded onto a 2% agarose gel containing ethidium bromide (0·25µg ml^{-1}) and run in 1× TBE buffer (tris-borate buffer) for 30 minutes at 100V. PCR fragments were visualized with UV transilluminator. A 100bp DNA ladder (Fermentas, Burlington, Canada) was loaded on each gel as a DNA size standard.

Results

A total of 29 *E. coli* strains were isolated and identified phenotypically, they were also screened for ESBL production. 27 (93.75%) *E. coli* strains were confirmed ESBL positive (ESBL producers) and 2 (6.25%) were non ESBL producers.27 (93.75%)*E.*

*coli*strainspossessed the *uidA* gene and 2 (6.25%) does not possess the *uidA* gene.

The *E. coli* strains were tested against 10 antibiotics and ESBL production was confirmed by testing *E.coli* strains against third generation cephalosporins Ceftazidime, Cefpdoxime, Cefotaxime.

The antibiotics susceptibility test result is shown in Fig 1a and 1b. It revealed that 86% of the isolates were resistant to sulphamethaxazole; 83% were resistant to oxytetracycline. All the isolates were resistant to cefpodoxime,penicillin-G and cefotaxime (100% respectively). Streptomycin showed 38% resistance; 21% were resistant to enrofloxacin; 10% were resistant to amoxicillin-clavulanate. Ceftazidime showed 10% resistance. All the isolates were susceptible toimipenem.

The *E.coli* strains are 100% susceptible to imipenem. Susceptibility of the *E. coli*strains to third generation cephalosporins is very low with ceftazidime having 50% susceptibility and Cefpdoxime and Cefotaxime having 0% susceptibility (100% resistant). Penicillin also showed no susceptibility to the *E.coli* strains (100% resistant). Other antibiotics showed varied susceptibility and resistance, Amoxicillin showed (86.2% susceptibility and 10.3% resistance), Streptomycin (31.0% susceptibility and 37.9% resistance), Oxytetracycline (13.8% susceptibility and 82.8% resistance), Sulphamethaxazole (13.8% susceptibility and 86.2% resistance) and Enrofloxacin (10.4% susceptibility and 20.7% resistance. Intermediate resistance was recorded in 5 out of 10 antibiotics; Enrofloxacin (69.0%), Caftazidime (37.9%), Streptomycin (31.0%), Oxytetracycline (6.9%) and Amoxicillin (3.5%) Fig 1.

Table 2 shows the Antibiotic susceptibility pattern of the isolated *E.coli* strains to the selected antibiotics. Figs. 1a and 1b, shows the percentage antibiotic susceptibility and resistance pattern of the *E.coli* strains against the selected antibiotics used.

Table 2: Antibiotic susceptibility pattern of the isolates

Antibiotic	Sensitivity (%)	Intermediate (%)	Resistance (%)
SXT	13.79(n=4)	0(n=0)	86.21(n=25)
OXT	10.34(n=3)	6.90(n=2)	82.76(n=24)
ENR	10.34(n=3)	68.97(n=20)	20.69(n=6)
STR	31.03(n=9)	31.03(n=9)	37.94(n=11)
AMC	93.10(n=27)	3.45(n=1)	3.45(n=1)
PEN	0(n=0)	0(n=0)	100(n=29)
CAZ	44.83(n=13)	44.83 (n=13)	10.34(n=3)
CPD	0(n=0)	0(n=0)	100(n=29)
CTX	0(n=0)	0(n=0)	100(n=29)
IMI	100(n=29)	0(n=0)	0(n=0)

Discussion

Escherichia coli is a common enteric commensal, specific strains of which can cause human and animal disease. It is one of the groups of seven species that the WHO has highlighted as of key AMR concern and serves as a sentinel organism for antimicrobial resistance development. Of concern has been the emergence and spread of ESBL-producing *E. coli* associated with cattle and other farm animals (Liebana *et al.,*2013).

In this study we determined the antibiotic resistance pattern of *E. coli* strains isolated from cattle dung in a dairy farm unit, with a focus on ESBL producers. On this farm,different types of antibiotics are being given to treat the cattle, and this will contribute to antibiotic resistance gene selection and gene horizontal transfer among different species of bacteria.

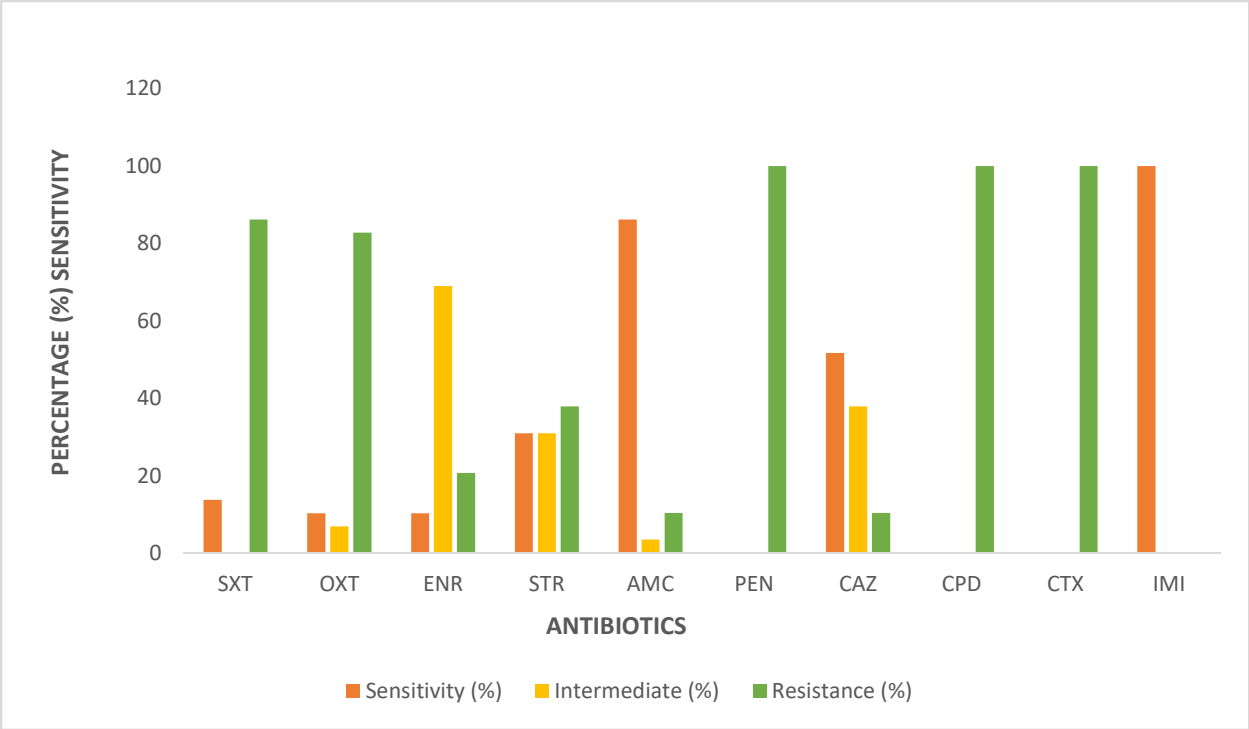

Fig 1: Percentage antibiotic susceptibility pattern

This study showed that MDR *E. coli* were present in all samples. The total percentage of MDR strains resistant to three or more antibiotics was 57.9% (73 isolates) (including ESBL resistance and resistance to other antibiotics). This was in line with Schmid *et al.* 2013 who found out that ESBL-producing *E. coli* can be isolated from cattle farms more than twice as often using selective enrichment procedures.

The presence of ESBL-mediated resistance in the isolates was not surprising given the use of cephalosporins for mastitis treatment in the herd. What was more unexpected was the level of resistance carriage to older antibiotics, with resistance to oxytetracycline, streptomycin and sulphonamide seen in over a third of strains. Co-acquisition of these resistance genes with ampicillin resistance was strongly indicated and may indicate the presence of a multi-resistance genetic element present in these isolates. Schmid *et al.* (2013) found ESBL *E. coli* from some farms that did not use β-lactam antibiotics and they suggested that the presence of such isolates is due to using other classes of antibiotics that can select for ESBLs as well. In a previous study of *E. coli* strains isolated from cattle, most strains were resistant to ampicillin (64%), tetracycline (74%), streptomycin (60%) and sulphonamide (76%) with low occurrence (1%) of enrofloxacin resistance (EMEA 1999). In a later study by Novakov´ a´ *et al.* (2009), all the *E. coli* isolates from dairy calves and lambs showed multi-resistance to tetracycline, streptomycin and compound sulphonamides with less resistance to enrofloxacin (Novakov´ a´ *et al.* 2009). The resistance patterns observed in the current study mirror these findings and resemble a historical record of antibiotic use and development of resistance to them (Toleman and Walsh), and may represent a genetic archaeology of the use of veterinary antimicrobials.

According to Jacoby and Sutton (1991), resistance determinants against aminoglycosides, tetracycline, sulphonamides and cephalosporins are often situated on the same plasmid. Metagenomics, PCR and exogenous plasmid isolation studies of cow manure have also detected diverse resistance genes (Jacoby and Sutton 1991; van Overbeek*et al.* 2002; Wichmann *et al.* 2014).

Conclusion

It was concluded that ESBL production should be detected routinely in Agriculture and Animal husbandry also, rational use of antibiotics in animal feeds and treatments must be done promptly to prevent the development and spread of ESBL producing microorganisms in their dungs.

Animal dungs should be adequately treated before disposal into the environment.

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