

Occurrence of extended-spectrum beta-lactamases in human and bovine isolates of *Escherichia coli* from Oyo state, Nigeria

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Abstract

Introduction: The main objective of the study was the molecular characterization of extended spectrum β -lactamases (ESBL) in *Escherichia coli* isolates collected from human and bovine samples in Oyo state, Nigeria.

Methodology: Between August 2010-2011, 114 *E. coli* isolates were collected from hospitals (n = 57) and bovine (n = 57). PCR and sequencing were used for identification of ESBLs, upstream sequences, plasmid-mediated quinolone resistance (PMQR) genes and class 1 integrons. Plasmid incompatibility groups were identified among ESBL-positive isolates by PCR. Genetic relatedness was assessed by rep-PCR and MLST. Transfer of ESBL determinants to the recipient strain *E. coli* J53 was performed by broth mating assays.

Results: CTX-M15 was the unique ESBL found in eight human isolates. Six CTX-M-15 producers also carry the *aac(6')-Ib-cr* gene and/or *qnrB* gene, and class 1 integrons. FIA, FIB, H11, H12, F, Y and K were the plasmid replicon types found. CTX-M-15 and PMQR determinants were transferred by conjugation in two *E. coli* assigned by MLST to ST131 and ST2695, a new allele.

Conclusions: The study highlights the dissemination ability of CTX-M-15 associated with PMQR, and the presence of class 1 integrons, able to capture additional genes, justifying the urgent need of antimicrobial resistance surveillance in Nigeria.

Key words: *Escherichia coli*; extended-spectrum beta-lactamases; plasmid-mediated quinolone resistance; genetic mobile elements

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Introduction

Extended-spectrum β -lactamases (ESBLs) are plasmid borne, and are harbored by Gram-negative bacteria. They can be divided into three major groups: TEM, SHV, and CTX-M types [1]. ESBL-producing strains can increase morbidity and mortality rates, in part as a result of associated resistance to other antibiotic families, which limits therapeutic options and raises healthcare costs [2]. Plasmid-mediated quinolone resistance genes (PMQRs) have been reported to be associated with ESBLs (and other β -lactamases) [3]. Many reports have described and characterized ESBLs in *Klebsiella* spp. and *Escherichia coli*, including reports from African countries [4,5]. However, few reports on the prevalence of ESBL and PMQR determinants in both human and bovine isolates in Nigeria have been documented. A recent study reported high rates of resistance to quinolones in association with β -lactams among *E. coli* isolates from healthy animals in Nigeria

[6]. Thus, the objectives of this study were to determine the prevalence of ESBL in *E. coli* clinical isolates from human and bovine origins collected in Oyo state, Nigeria, to perform their molecular characterizations, and to assess their potential dissemination.

Methodology

Bacterial isolates

Between August 2010 and August 2011, 57 *E. coli* isolates were collected from different inpatients from four hospitals located in Oyo state, Nigeria: two tertiary care hospitals (University College Hospital Ibadan and Bowen Teaching Hospital Ogbomosho), and two secondary hospitals (Oluyoro Catholic Hospital Ibadan and General Hospital Adeoyo Ibadan). During the same period, 57 *E. coli* isolates were also isolated from fecal samples of different healthy bovine animals from diverse farms at slaughter in Oyo state, Nigeria.

Farms from three different locations (Ibadan, Ogbomoso, and Iseyin) were included in the study.

Susceptibility testing and phenotypic ESBL detection

Susceptibility testing for 12 antibiotics was performed using the Kirby-Bauer method. Results were interpreted using Clinical Laboratory and Standards Institute criteria [7]. The double disk synergy test was used to screen the production of ESBLs in all isolates [8].

Identification of ESBL genes and upstream sequences, PMQR genes, and class 1 integrons

PCR was used to screen for *bla*_{TEM}, *bla*_{CTX-M}, and *bla*_{SHV} types of β -lactamases using specific primers [9] in presumptive ESBL producers. *E. coli* 39FFC (*bla*_{TEM}), *E. coli* 144FFC (*bla*_{CTX-M}), and *Klebsiella pneumoniae* 339FFC (*bla*_{SHV}) were used as positive controls. PCR products were purified with Exosap IT (Affymetrix, Santa Clara, USA), and sequenced in both strands (Macrogen, Seoul, Korea). Nucleotide sequences were analyzed with BioEdit software, and database searches were performed using the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

All isolates were screened by multiplex PCR for the most common *qnr* genes: *qnrA*, *qnrB*, and *qnrS* [10]. Two simplex PCRs were carried out to detect *aac(6')-lb* and *qepA* genes [12-13]. Human isolates positive for the *aac(6')-lb* gene were further analyzed by digestion with a *BtsCI* enzyme (New England Biolabs Ipswich, USA) to identify *aac(6')-lb-cr*, which lacks the *BtsCI* restriction site present in the wild type gene [11]. *ISEcp*, *Is26*, and *Is903* elements were investigated in the ESBL-positive isolates [9].

Integrase gene, *intI1*, was screened by PCR in ESBL-positive and Qnr-positive isolates [12]. Isolates positive for *intI1* were subjected to amplification with 5'-CS and 3'-CS primers [13] to amplify the variable region of class 1 integrons. PCR products were purified and sequenced as previously described.

Plasmid replicon typing

Plasmid incompatibility groups were identified among positive ESBL isolates. The PCR-based Inc/rep typing method consists of five multiplex-PCRs recognizing three different replicon types and three simplex PCRs for K, F, and B/O replicon types [14].

Conjugation experiments

The transfer of ESBL determinants to the recipient strain *E. coli* J53 (sodium azide resistant) was

performed by broth mating assays, at a bacterial cell ratio of 1:10. Overnight cultures were centrifuged, the sediment suspended in 50 μ L of PBS buffer, and 20 μ L were plated in duplicates of Luria-Bertani agar containing sodium azide (100 mg/L) and cefotaxime (1 mg/L). The antibiotic susceptibility profile was determined for transconjugants by the disk diffusion method. PCR amplification of the ESBL determinants and plasmid replicon types was also performed to confirm the transfer of resistance determinants and to identify the plasmid replicon type involved in the transfer of resistance, using the methodology described.

Genetic relatedness and sequence typing by MLST

The rep-PCR genomic fingerprinting method was performed on the ESBL producers to assess their genetic relatedness [15]. Multilocus sequence typing (MLST) analysis was performed [16] on the two ESBL-positive strains that were successful on conjugation assays.

Results

Origin of bacterial isolates, antibiotic susceptibility, and phenotypic detection of ESBLs

Of the isolates from human clinical samples, 34 were from urine, 10 were from high vaginal swabs, 9 were from stool, 3 were from sputum, and 1 was from an endocervical swab. The majority of the human samples (54%) were isolated from the University College Hospital (UCH), the first teaching hospital in Nigeria. Seventy-four percent of the bovine samples ($n = 42$) were obtained from farms in Ibadan, which is the capital city of Oyo state and the third-largest metropolitan area in Nigeria.

Table 1 shows the susceptibility profiles obtained for the isolates. As shown, the bovine isolates were more susceptible to the antimicrobials tested than were the human isolates. ESBLs were only detected in the human *E. coli* isolates. ESBL-positive strains were also resistant to gentamicin (87.5%), nalidixic acid (62.5%), and ciprofloxacin (50%), but susceptible to cefoxitin and imipenem (100%). The ESBL-producing isolates were predominantly isolated from urine samples, followed by high vaginal swabs.

Characterization of ESBL and PMQR

Table 2 summarizes the molecular characterization of ESBL producers and a bovine isolate. CTX-M-15 enzyme was identified in eight human *E. coli* isolates, confirming the resistance phenotype. All isolates also carried the non-ESBL *bla*_{TEM-1}. Two and six *E. coli*

*bla*_{CTX-M-15} producers showed the *qnrB* and *aac(6')-lb-cr*-variant genes, respectively. Two isolates carried both the *qnrB* and *aac(6')-lb-cr* genes. A single bovine strain carried the *qnrS* gene. ESBLs and other PMQRs were not found in the bovine isolates.

An *ISEcpI* element was detected upstream of the *bla*_{CTX-M-15} gene. The *IS26* element was located in three of the ESBL producers, and one isolate had both the *IS26* and *IS903* elements.

Rep-PCR identified three DNA fingerprint patterns among the *bla*_{CTX-M-15} producers. They were from two hospitals in Oyo state; UCH had the highest number of isolates (n = 7). Most of the isolates shared a common fingerprinting pattern (data not shown).

Plasmid identification

Plasmids carrying the CTX-M-15 β-lactamase and the PMQRs determinants were assigned to the FIA, FIB, HI2, F, and K replicon types (Table 2). In the bovine strain, plasmids were assigned to the HII, FIB, and Y incompatibility groups.

Conjugation assays

Transfer by conjugation of the ESBL and PMQR phenotypes to *E. coli* J53 was successful for two out of the eight *bla*_{CTX-M-5}-positive strains. The transconjugants showed resistance profiles identical to the donors. The *bla*_{TEM-1}, *bla*_{CTX-M-15}, *aac(6')-lb-cr*-variant, and *qnrB* genes were confirmed to be present in transconjugants. The transferred plasmids were assigned to the FIB and HI2 replicon types (Table 2). *E. coli* with conjugative plasmids belonged to different sequence types; HI5, carrying *aac(6')-lb-cr*, *bla*_{TEM-1}, and *bla*_{CTX-M-15}, was identified as ST131, while *E. coli* H1, harboring *qnrB*, *aac(6')-lb-cr*-variant, *bla*_{TEM-1}, and *bla*_{CTX-M-15}, was assigned to ST2695, a new allele.

Characterization of class 1 integrons

The *intI1* gene was present in the *qnrS*-borne bovine strain (no gene cassettes inserted in the variable region) and in 87.5% of the human ESBL-positive isolates. Two different gene cassette arrays were identified in six ESBL-positive isolates: *aadA1*, encoding resistance to streptomycin and spectinomycin, and *dfrA17-aadA5*, encoding resistance to streptomycin/spectinomycin and trimethoprim (Table 2).

Discussion

ESBLs, especially CTX-M-15 β-lactamase, usually located in conjugative plasmids, are disseminated worldwide. This study confirmed the presence of CTX-M-15 in human clinical isolates in Nigeria, though at a moderate prevalence. A strong association between ESBL production and quinolone resistance has been reported in *Enterobacteriaceae* [2]. In this study, the resistance to nalidixic acid and ciprofloxacin shown by the CTX-M-15 producers could be linked partially to the simultaneous carriage of PMQRs. The *aac(6')-lb-cr*-variant was shown to be prevalent; *qnrB* was found in two isolates. Five different plasmid replicon types (HI2, FIA, FIB, F, and K) were identified, but only FIB, HI2, and K were shown to conjugate. Both CTX-M-15 and TEM-1 determinants, as well as the *qnrB* and *aac(6')-lb-cr*-variant, could be transferred (Table 2), demonstrating the potential of co-dissemination by horizontal gene transfer of resistance to β-lactams and quinolones. Recently, a study reported high rates of resistance to β-lactams and quinolones among Gram-negative isolates from different hospitals in Nigeria [17], which may support our findings at a molecular level.

Table 1. Antibiotic susceptibility pattern of the human and bovine *E. coli* isolates

Antibiotics	Human (%)			Bovine (%)		
	R	I	S	R	I	S
Amoxicillin	88	0	12	18	5	77
Amoxicillin plus clavulanic acid	30	47	23	0	0	100
Ceftazidime	9	4	88	0	2	98
Cefotaxime	14	5	81	0	0	100
Aztreonam	5	9	81	0	0	100
Cefepime	0	4	97	0	0	100
Imipenem	0	2	98	0	0	100
Cefoxitine	0	0	100	0	0	100
Nalidixic acid	47	4	49	4	0	97
Ciprofloxacin	40	0	60	0	0	100
Trimethoprim	83	0	18	25	0	75
Gentamicin	46	0	54	0	0	100

R: resistance, I: intermediate, S: susceptible

Table 2. Origin, antimicrobial resistance pattern, and genetic characteristics of human and bovine *E. coli* isolates

Strain	Hospital/farm location ^a	Specimen ^b	Antimicrobial resistance pattern ^c	PMQR gene	β-lactamase gene	Transferred genes ^d	Plasmid replicon	Is elements	Gene cassette(s) on integrons	ST ^e
BV36	Ibadan	Feces	W/AML	<i>QnrS</i>	-	-	HII, Y, FIB	-	-	-
H1	UCH	Urine	CZ/CT/AT/W/CN/AML	<i>QnrB, aac(6')-lb-cr</i>	<i>bla</i> _{TEM-1} , <i>bla</i> _{CTX-M 15}	<i>QnrB, aac(6')-lb-cr, bla</i> _{TEM} , <i>bla</i> _{CTX-M 15}	FIB, H12, K	<i>IsEcp1</i>	<i>aadA1</i>	2695
H6	UCH	Urine	CZ/CT/NA/W/AML	-	<i>bla</i> _{TEM-1} , <i>bla</i> _{CTX-M 15}	-	FIB	<i>IsEcp1, IS26</i>	<i>aadA5, dfrA17</i>	-
H12	UCH	HVS	CZ/CT/AT/CIP/NA/W/CN/AML	<i>aac(6')-lb-cr</i>	<i>bla</i> _{TEM-1} , <i>bla</i> _{CTX-M 15}	-	FIA, FIB	<i>IsEcp1</i>	<i>aadA5, dfrA17</i>	-
H13	UCH	HVS	CZ/CT/CIP/NA/W/CN/AML	-	<i>bla</i> _{TEM-1} , <i>bla</i> _{CTX-M 15}	-	FIA, FIB	<i>IsEcp1, IS26</i>	-	-
H15	GHA	HVS	CZ/CT/AMC/AT/CIP/NA/W/CN/AML	<i>aac(6')-lb-cr</i>	<i>bla</i> _{TEM-1} , <i>bla</i> _{CTX-M 15}	<i>aac(6')-lb-cr, bla</i> _{TEM} , <i>bla</i> _{CTX-M 15}	FIB	<i>IsEcp1</i>	-	131
H22	UCH	Urine	CZ/CT/AMC/CIP/NA/W/CN/AML	<i>aac(6')-lb-cr</i>	<i>bla</i> _{TEM-1} , <i>bla</i> _{CTX-M 15}	-	FIA, FIB	<i>IsEcp1</i>	<i>aadA5, dfrA17</i>	-
H45	UCH	Urine	CT/W/CN/AML	<i>aac(6')-lb-cr</i>	<i>bla</i> _{TEM-1} , <i>bla</i> _{CTX-M 15}	-	H12, K, FREPB	<i>IsEcp1, IS26</i>	<i>aadA1</i>	-
H47	UCH	Urine	CT/AMC/W/CN/AML	<i>QnrB, aac(6')-lb-cr</i>	<i>bla</i> _{TEM-1} , <i>bla</i> _{CTX-M 15}	-	H12, FREPB	<i>IsEcp1, IS26, Is903</i>	<i>aadA1</i>	-

^aUCH: University College Hospital, Ibadan; GHA: General Hospital Adeoyo, Ibadan^bHVS: high vaginal swabs^cCZ: ceftazidime; CT: cefotaxime; AT: aztreonam; W: trimethoprim; CN: gentamicin; AML: amoxicillin; NA: nalidixic acid; CIP: ciprofloxacin; AMC: amoxicillin plus clavulanic acid^d- No transferred gene. Conjugation not successful.^e, Sequence type

Nevertheless, only two *E. coli* were able to conjugate; these belonged to distinct sequence types, as determined by MLST. ST131 was identified as carrying *bla*_{CTX-M-15} and *aac(6')-lb-cr*. The first report recently made in West Africa of *E. coli* ST131 harboring *bla*_{CTX-M-15} and *aac(6')-lb-cr*, found predominantly among hospital isolates, was from Lagos, Nigeria [18]. Our results support the dissemination of this strain in the country, since they were collected in a different state. The other *bla*_{CTX-M-15} *E. coli*, harboring the *qnrB* and *aac(6')-lb-cr* genes, was assigned to ST2695, a new allele, not previously reported to be associated with CTX-M-15.

The IncF plasmidic family was the most common found in this study; it includes the F1A and F1B groups frequently reported to be associated with CTX-M and *aac(6')-lb-cr* resistance genes [14]. However, reports about the association of the *qnrB* with the plasmid replicon types found in this study are limited, as are reports about the *qnrS* association with HII, Y, and/or FIB plasmid types detected in bovine isolates. The lack of conjugative transfer of the CTX-M-15 determinant (and PMQR associated genes) may indicate that in the other *E. coli* isolates, the resistance determinants were encoded on non-conjugative plasmids.

Class 1 integrons coding for resistance to other antibiotic families were also associated with CTX-M-15 producers, though their genetic location was not investigated. However, this finding shows the higher ability of the strains to capture additional resistance genes.

E. coli is commonly associated with urinary tract infections, and the majority of CTX-M-15 strains were found in urine, followed by high vaginal swab samples. Samples were mainly collected at the UCH. This may be due to the fact that most patients in this tertiary care hospital are referred from other hospitals, where they have been started on antibiotherapy. Also, in developing countries like Nigeria, resources are limited and antibiotics are indiscriminately used both in humans and animals, which could result in dissemination of ESBL producers.

In contrast with other Nigerian reports [6,19,20], bovine *E. coli* isolates did not show considerable resistance, which may be attributed to less bovine antibiotic dosing in this geographic location. The predominant resistance rate was seen for tetracycline (44%), followed by trimethoprim (25%). Resistance to amoxicillin was detected in 23% of the strains, correlating with data obtained from reports on bacteria

isolated from meat tables in the geographical location where the study was conducted [20].

The use of cephalosporins and fluoroquinolones for prophylaxis and treatment of bovine animals is rare in Nigeria, which supports the high rates of susceptibility of *E. coli* bovine isolates. However, these resistance determinants may be selected by other drugs frequently used in food animals. Chah *et al.* [19] reported the wide use of ampicillin in poultry production in Nigeria, which may provide a selective pressure favoring the emergence of *E. coli* strains that carry plasmids with TEM-1/2 and ESBL determinants. These other food animals may serve as reservoirs of ESBL-producing *E. coli* strains that could be transferred to humans and other animals. However, in this study, we could not find an association between *E. coli* from human and bovine origins.

The study reports the prevalence of CTX-M-15 in human clinical Nigerian *E. coli* strains carrying genetic mobile elements such as conjugative plasmids and class 1 integrons that can amplify the spread of resistance in the country, reinforcing the crucial need for antimicrobial surveillance in a country where over-the-counter antibiotic sales and indiscriminate use of antibiotic are common.

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