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A novel plasmid carrying $bla_{CTX\text{-}M\text{-}15}$ identified in commensal *Escherichia coli* from healthy pregnant women in Ibadan, Nigeria

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ABSTRACT

The aim of this study was to investigate the molecular characteristics of commensal *Escherichia coli* producing extended-spectrum β-lactamases and showing fluoroquinolone resistance circulating in a healthy population in Ibadan, Nigeria. In total, 101 faecal samples from healthy pregnant women on the day of admission to hospital were collected and plated on eosin–methylene blue agar supplemented with cefotaxime. Genotyping demonstrated the presence of the $bla_{CTX\text{-}M\text{-}15}$ gene in all of the cefotaxime-resistant isolates ($n = 32$), and there was circulation of prevalent clones. The *aac(6′)-Ib*-cr, *qnrS1*, *qepA1* and *qnrB1* genes were identified in several strains. A novel plasmid supporting the spread of the $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1}$ and *qnrS1* genes was identified in these isolates by complete DNA sequencing.

1. Introduction

In the last decade, a variety of plasmid-mediated extended-spectrum β-lactamases (ESBLs) and fluoroquinolone resistance genes have emerged worldwide in Enterobacteriaceae both from clinical and community settings. The increasing incidence of community-acquired infection due to ESBL-producing bacteria represents a great concern [1–8].

Spread of particular *Escherichia coli* clones and epidemic plasmid types has been observed in many countries, which supported the abrupt worldwide spread of particular ESBL variants. In particular, *E. coli* sequence type 131 (ST131) is a globally disseminated, multidrug-resistant clone responsible for a high proportion of urinary tract and bloodstream infections and is associated with production of the CTX-M-15 ESBL and resistance to fluoroquinolones [9]. The $bla_{CTX\text{-}M\text{-}15}$ gene in this clone is often identified on IncF plasmids but also on other plasmid types [5,10,11]. IncF is one of the most frequent plasmid families detected in Enterobacteriaceae. They are usually low-copy-number plasmids, >100 kb in size and carry more than one replicon promoting the initiation of replication (FII, FIA and FIB) [11,12].

Despite the global success of the ST131 *E. coli* clone, scarce information is available on the prevalence and types of multidrug-resistant *E. coli* clones and plasmids circulating in developing countries. Recent studies reported CTX-M-type ESBLs in *E. coli* clinical isolates from hospitals, the community and healthy food-producing animals in Nigeria often associated with fluoroquinolone resistance [3,4,13]. In *E. coli* isolates from outpatients from Southeastern Nigeria, the association of $bla_{CTX\text{-}M\text{-}1}$ ESBL genes with bla_{TEM}, $bla_{OXA\text{-}1}$ and *aac(6′)-Ib*-cr genes were observed [14].

In this study, CTX-M-15-positive commensal *E. coli* isolated from healthy pregnant women on the day of admission to hospital in Ibadan, Nigeria, were analysed.

2. Materials and methods

2.1. Bacterial strains

From September to November 2011, 101 random faecal samples were collected from healthy pregnant women on the day of admission to hospital in Ibadan (Nigeria). Samples were plated on eosin–methylene blue agar containing 8 mg/L cefotaxime (Sigma, Steinheim, Germany). Thirty-two samples were positive for *E. coli* growing on cefotaxime. One isolate per patient was selected for further study and was sent to the Istituto Superiore di Sanità (Rome, Italy) for phenotypic characterisation and molecular typing.

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2.2. Antimicrobial susceptibility

Escherichia coli strains were tested for antimicrobial susceptibility by the disk diffusion method following European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (http://www.eucast.org) for the following drugs: cefotaxime; ciprofloxacin; gentamicin; tetracycline; and chloramphenicol.

2.3. bla$_{CTX\text{-}M\text{-}15}$ and plasmid-mediated quinolone resistance (PMQR) genes

All strains were screened for the following PMQR and β-lactamase genes using previously published primers and conditions: *qnrA*; *qnrB*; *qnrS*; *qnrC*; *qnrD*; *aac(6′)-Ib*-cr; *qepA*; *oqxAB*; *bla*$_{OXA}$; *bla*$_{SHV}$; *bla*$_{TEM}$; *bla*$_{LAP}$; *bla*$_{CTX\text{-}M\text{-}1g}$ and *bla*$_{CMY}$ [15–18]. Amplicons were screened for identification of the variant gene.

2.4. Bacterial typing

Genetic relatedness was initially determined by enterobacterial repetitive intergenic consensus (ERIC) PCR. Strains differing by more than two bands were classified as different ERIC types; strains differing by one band were classified as ERIC subtypes (i.e. A2), following previously described procedures [19]. Multilocus sequence typing (MLST) was performed for the major ERIC types as previously described [20]. Sequence types (STs) were assigned at the http://mlst.warwick.ac.uk/mlst/dbs/Ecoli website.

2.5. bla$_{CTX\text{-}M\text{-}15}$-carrying plasmids

Plasmid content was determined by the PCR-based replicon typing (PBRT) method using the PBRT kit (Diatheva s.r.l., Fano, Italy).

Five prototypic plasmids were transferred by transformation in *E. coli* TOP10 chemically competent cells (Invitrogen, Milan, Italy). Plasmid DNA was purified using a QIAGEN Plasmid Midi Kit (QIAGEN Inc., Milan, Italy). Transformants were selected on Luria–Bertani agar plates (Sigma) containing 8 mg/L cefotaxime. Replicons in the transformants were determined by PBRT.

2.6. Whole plasmid sequencing

The complete DNA sequence of plasmid pPGRT46 was determined by applying the 454-Genome Sequencer FLX^{TM} procedure (Roche Diagnostics, Monza, Milan) on a library obtained using plasmid DNA purified from the PgR46 strain using a PureLink^{TM} HiPure Plasmid Filter Midiprep Kit (Invitrogen). Contigs with 130-fold coverage were generated by the gsAssembler software v.2.6 (Roche Diagnostics, Monza, Milan). Gene prediction was performed for the complete plasmid sequence with Artemis Software v.8 (Sanger Institute). Pairwise alignment was performed by a BLASTN and BLASTP homology search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). PCR-based gap closure was performed to obtain the complete circular plasmid sequence.

The plasmid sequence has been deposited at the NCBI GenBank under accession no. **KM023153**.

2.7. Screening for the pPGRT46 plasmid

Two simplex PCR were designed to screen for the presence of the pPGRT46 plasmid: one PCR targets the FIB-like replicon (FIB-pKPN3-FW, 5′-GAAATCAACAGGGAAGAGCAAGGA-3′; and FIB-pKPN3-RV, 5′-GCTGCTCCATCGCCTTACGC-3′); and the second targets a specific locus identified on the pPGRT46 plasmid (BCCT-FW, 5′-ATCGACTTCAAGGCGAATAC-3′; and BCCT-RV, 5′-TGAAAGCCTTAGCATGGCG-3′).

3. Results and discussion

3.1. Escherichia coli producing CTX-M-15 from Nigeria

Thirty-two cefotaxime-resistant *E. coli* strains were isolated from 101 faecal samples obtained from pregnant women on the day of admission to the hospital in the city of Ibadan. All strains were positive for the *bla*$_{CTX\text{-}M\text{-}15}$ gene; 17 of them were also positive for *bla*$_{TEM\text{-}1}$, 12 for *bla*$_{OXA\text{-}1}$ and 10 for *bla*$_{CMY\text{-}2}$ (Table 1).

Of the 32 CTX-M-15-positive strains, 26 (81%) also showed resistance to ciprofloxacin, likely due to mutations in the *gyrA* and *parC* genes, which were not further investigated. However, 22 of these isolates were positive for one or more PMQR genes: *aac(6′)-Ib*-cr was detected in 17 strains; 7 strains carried *qnrS1*; 2 strains carried *qnrB1*; and 3 strains carried *qepA1*; one strain carried both *qnrS1* and *qnrB1*. None of the isolates carried *qnrA*, *qnrC*, *qnrD* or *oqxAB* genes.

Nine ERIC patterns were observed in this collection and strains were classified in ERIC types A–I. One strain representative of ERIC types A, B, C and D, respectively, was chosen and typed by MLST (Table 1). Molecular typing of this collection clearly highlighted the presence of certain more frequent ERIC types, suggesting that some *E. coli* clones were circulating in the community of healthy pregnant women in Ibadan. However, none of these strains was related to the ST131 clone.

3.2. Plasmid analysis

High variability of plasmid replicons was observed in these strains, also including rare plasmid types such as IncI2 and IncX2. Only three (9%) of the 32 strains were untypeable by the PBRT method.

Five prototypic strains representative of the major ERIC and MLST types were chosen for plasmid analysis (underlined strains in Table 1). The *bla*$_{CTX\text{-}M\text{-}15}$ gene was successfully transferred from all of them. This gene was located on an IncF plasmid carrying the FIA, FIB and FII replicons in the PX1 (ST617) strain and on an IncX2 plasmid in the PX53 strain. Differently, the *bla*$_{CTX\text{-}M\text{-}15}$ plasmids transferred from strains PgR46 (ST10), PX4 (ST3147) and PX42 types were not typeable by the PBRT method.

The complete sequence of one of these untypeable plasmids, named pPGRT46, identified in the PgR46 transformant and carrying the *bla*$_{CTX\text{-}M\text{-}15}$, *bla*$_{TEM\text{-}1}$ and *qnrS1* genes was obtained and compared with other plasmids in GenBank (Fig. 1). The largest portion of the pPGRT46 matched with plasmid pCK41, identified in the *Edwardsiella tarda* fish pathogen in South Korea (EMBL accession no. **HQ332785**) [21]. Both harbour the same functional replicon, named FIB-pKPN3, previously identified in the pKPN3 plasmid in clinical isolates of *Klebsiella pneumoniae* [22]. The *bla*$_{CTX\text{-}M\text{-}15}$ gene environment was similar to that previously described on the *bla*$_{CTX\text{-}M\text{-}15}$-carrying plasmid pKDO1 (**JX42442**) identified in *K. pneumoniae* from paediatric patients in the Czech Republic [22] and consisted of the IS*Ecp1* inserted into a Tn*3* transposase gene *tnpA*, *strA*, *strB*, *sul2* and an In*4*-type class 1 integron with the *dfrA14* gene cassette, flanked by the *uvp1* resolvase gene that is typical of the class 1 integrons identified on IncN plasmids, followed by the relict of the IncN replicase gene interrupted by an IS*26* insertion. pPGRT46 carried *qnrS1* in the same environment previously described in plasmid pINF5 (**AM234722**).

A completely novel region that did not match with any nucleotide sequence in GenBank was identified in pPGRT46. In this region, a putative remnant phage was identified, associated with a novel transporter of the betaine/carnitine/choline transporter (BCCT) family, flanked by the *omp* gene encoding for a novel putative outer membrane protein. A PCR screening was devised to recognise the pPGRT46 plasmid in the other CTX-M-15-producing

Table 1
Characteristics of the *Escherichia coli* strains analysed in this study.

Strain name[a]	Resistance	ERIC (MLST)	β-Lactamase and PMQR genes[a]	Replicon typing[a]	pPGRT46[a]
PX19	CTX, CIP, GEN, TET	A	*bla*$_{CTX-M-15}$, *qepA1*	FIA, FIB, FII	neg.
PX1	CTX, CIP, GEN, TET, CHL	A (ST617)	*bla*$_{CTX-M-15}$	FIA, FIB, FII	neg.
PX63	CTX, CIP, GEN, TET, CHL	A	*bla*$_{CTX-M-15}$, *qepA1*, *aac(6′)-Ib*-cr	FIA, FIB, FII	neg.
PX53	CTX, CIP, GEN, TET	A	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$ *qnrS1*, *aac(6′)-Ib*-cr	X2	neg.
PX66	CTX, CIP, GEN, TET, CHL	A	*bla*$_{CTX-M-15}$, *qnrS1*, *qnrB1*, *aac(6′)-Ib*-cr	HI2, Y	neg.
PX18	CTX, CIP, GEN, TET, CHL	A	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1}$	FIA	neg.
PX14	CTX, CIP, GEN, TET, CHL	A	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{CMY-2}$, *bla*$_{TEM-1}$, *aac(6′)-Ib*-cr	FIB, FII	neg.
PX7	CTX, CIP, GEN, TET, CHL	A2	*bla*$_{CTX-M-15}$, *bla*$_{CMY-2}$, *bla*$_{TEM-1}$, *qnrS1*	FII	neg.
PX40	CTX, CIP, GEN, TET	B	*bla*$_{CTX-M-15}$	FIA, FIB, FII	neg.
PX55	CTX, CIP, GEN, TET	B	*bla*$_{CTX-M-15}$	FIA, FIB	neg.
PX5	CTX, CIP, GEN, TET	B	*bla*$_{CTX-M-15}$, *aac(6′)-Ib*-cr	FIA, FIB	neg.
PgR46	CTX, GEN, TET	B (ST10)	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$, *qnrS1*, *aac(6′)-Ib*-cr	Y	FIB-pKPN3; BCCT
PgR61	CTX, GEN, TET	B	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$, *qnrS1*, *aac(6′)-Ib*-cr	FII	FIB-pKPN3; BCCT
PX60	CTX, CIP, GEN, TET, CHL	B	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1}$	FIB, FII	neg.
PX61	CTX, CIP, GEN, TET, CHL	B	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1}$, *aac(6′)-Ib*-cr	FII	neg.
PX58	CTX, GEN, TET	B	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{CMY-2}$, *bla*$_{TEM-1}$, *aac(6′)-Ib*-cr	N/T	neg.
PX64	CTX, GEN, TET	B	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{CMY-2}$, *bla*$_{TEM-1}$	FIA, FIB	neg.
PgR58	CTX, CIP, GEN, TET, CHL	B	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{CMY-2}$, *bla*$_{TEM-1}$, *aac(6′)-Ib*-cr	FIA, FIB, FII	neg.
PX4	CTX, CIP	C (ST3147)	*bla*$_{CTX-M-15}$, *qnrS1*, *bla*$_{CMY-2}$	N/T	neg.
PX8	CTX, CIP, GEN, TET, CHL	C	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1}$, *aac(6′)-Ib*-cr	FIA, FIB	neg.
PX9	CTX, GEN, TET	D	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$	FIA, FIB, FII	neg.
PX68	CTX, CIP, TET, CHL	D (ST156)	*bla*$_{CTX-M-15}$, *qepA1*	N/T	neg.
PX48	CTX, CIP, GEN, TET, CHL	D	*bla*$_{CTX-M-15}$, *bla*$_{CMY-2}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1}$, *aac(6′)-Ib*-cr	N, FIB	FIB-pKPN3; BCCT
PX52	CTX, TET, CHL	E	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$ *bla*$_{TEM-1}$ *qnrS1*, *aac(6′)-Ib*-cr	I2	neg.
PgR10	CTX, CIP, GEN, TET, CHL	E	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *qnrB1*	HI2	neg.
PX49	CTX, CIP, GEN, TET, CHL	F	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{CMY-2}$	FIA, FIB, FII	neg.
PX10	CTX, CIP, GEN, TET, CHL	G	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$, *aac(6′)-Ib*-cr	HI2, I1	FIB-pKPN3; BCCT
PX51	CTX, CIP, GEN, TET, CHL	G	*bla*$_{CTX-M-15}$, *bla*$_{CMY-2}$, *aac(6′)-Ib*-cr	FIB, X1, FII	neg.
PX42	CTX, CIP, GEN, TET, CHL	G	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$	FIB, FII, Y	FIB-pKPN3; BCCT
PX46	CTX, CIP, GEN, TET, CHL	H	*bla*$_{CTX-M-15}$, *aac(6′)-Ib-cr*	FIA, FIB, X1	neg.
PX44	CTX, CIP, GEN, TET, CHL	I	*bla*$_{CTX-M-15}$	FIA, FIB, HI2, X1,	neg.
PX56	CTX, CIP, TET, CHL	I	*bla*$_{CTX-M-15}$, *bla*$_{CMY-2}$, *aac(6′)-Ib-cr*	FII	neg.

N/T, not typeable; neg., negative for the FIB-pKPN3 and BCCT PCRs used as markers for the presence of the pPGRT46 plasmid.
[a] Prototypic strains selected for transformation experiments and their resistance genes and replicons transferred by transformation are underlined.

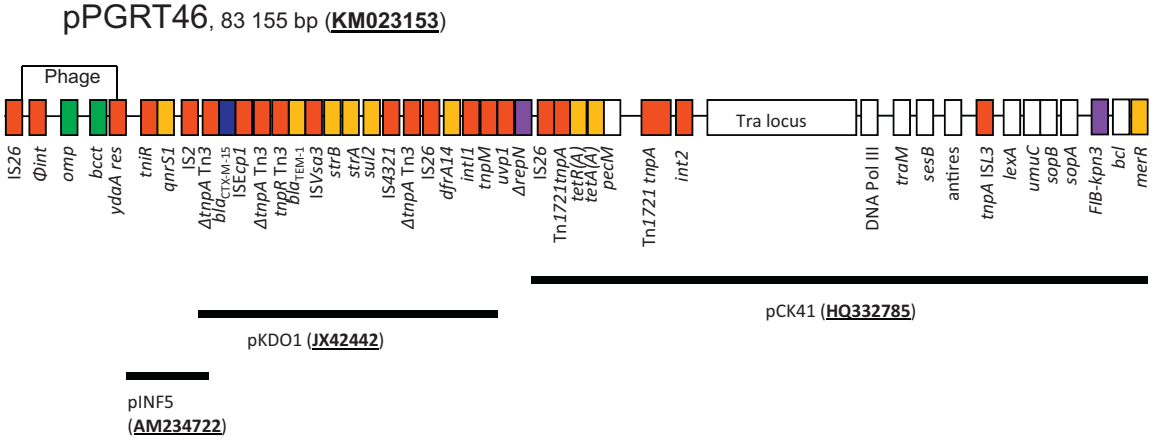

Fig. 1. Major structural features of plasmid pPGRT46 identified in *Escherichia coli* PgR46 strain. Coloured boxes represent different functions/loci: white, plasmid scaffold regions (i.e. the *tra* locus); yellow, resistance determinants; red, transposon-related genes (*tnpA*, *tnpR*, *tnpM*), class 1 integrase and insertion sequences; blue, *bla*$_{CTX-M-15}$; violet, replicase genes; and green, the novel betaine/carnitine/choline transporter (BCCT) flanked by an *omp* gene. The black lines indicate the region of the most closely related plasmids pKDO1 (**JX424423**), pINF5 (**AM234722**) and pCK41 (**HQ332785**), showing ≥99% nucleotide identity by BLASTN. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

strains from Nigeria. A PCR for the FIB-pKPN3 replicon and a PCR devised on the gene of the new BCCT transporter identified plasmids relative to the pPGRT46 in another four strains of this collection, belonging to different STs and ERIC types (Table 1).

Further evidence is required to determine whether this putative phage carrying a novel transporter may have an influence on virulence and resistance adaptation of the *E. coli* recipient host.

4. Conclusions

In the present study, ca. 30% of the commensal *E. coli* strains obtained from pregnant women in the city of Ibadan, Nigeria, were cefotaxime-resistant and positive for the *bla*$_{CTX-M-15}$ gene. Most of these strains also showed resistance to ciprofloxacin. Bacterial typing suggested a multifocal distribution of the

CTX-M-15-producing *E. coli* strains. IncF plasmids have been involved in the intercontinental spread of CTX-M-15 [11], and in this study a variety of heterogeneous IncF plasmids were identified. A novel IncF plasmid was identified at the origin of dissemination of $bla_{CTX\text{-}M\text{-}15}$ and *qnrS1* in some of these isolates. The biological characteristics of the colonising strains suggest that the predominant CTX-M gene is carried by plasmids that are exchanged between strains.

These results are in agreement with what has been recently reported by Woerther et al. [8]: it has been estimated that over 110 million ESBL-positive *E. coli* carriers appear to be present in the community populations of Africa, and CTX-M enzymes are practically exclusively of allele 15. Antibiotic use, poor access to drinking water, poverty, and hygiene failures in the community and in hospitals, further dramatically increased ESBL-positive *E. coli* transmission and dissemination in this continent [8].

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Competing interests

AC has been a consultant for Diatheva s.r.l. (Fano, Italy) for research and development of the PBRT kit; AC does not receive royalties from the company for commercialisation of the product but the Istituto Superiore di Sanità (Rome, Italy) does. All other authors declare no competing interests.

Ethical approval

Not required.

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