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Detection of chromosomal *bla*$_{\text{CTX-M-2}}$ in diverse *Escherichia coli* isolates from healthy broiler chickens

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ABSTRACT

The rise of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* in food-producing animals is a growing concern for public health. We investigated ESBL producers isolated from broiler chickens in Brazil and characterized 19 CTX-M-2-producing *E. coli*. The *bla*$_{\text{CTX-M-2}}$ gene was detected downstream to IS*CR1* element, associated with sul-1 type integron, chromosome-located. CTX-M-2-producing *E. coli* exhibited different PFGE-types and phylogenetic groups, showing a non-clonal dissemination. The sequence types found (ST93, ST155 and ST2309) have been associated to humans and animals worldwide. Herein, we report the chromosomal location of *bla*$_{\text{CTX-M-2}}$ on *E. coli*, alerting for the risks of multi-drug resistant bacteria in food-producing animals.

Prophylactic antibiotics are used in poultry production to prevent gastrointestinal infections and thus improve the performance of commercial broilers. The prevalence of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* colonizing the intestinal tract of healthy food-producing animals, including poultry, has increased worldwide [1]. There is a risk of transmission of poultry-associated ESBL-producing Enterobacteriaceae to humans [2]. Many genetic platforms associated with mobilization and support of *bla*$_{\text{CTX-M}}$ were identified, specially IS*Ecp1* and IS*CR1*elements, where the latter is particularly important for *bla*$_{\text{CTX-M-2}}$ dissemination [3]. In this study, we report different clones of CTX-M-2-producing *E. coli*, with chromosomal-located gene.

From 2011 to 2012, two-hundred cloacal swabs were harvested from healthy broilers in two poultry farms from São Paulo State, Brazil. Swabs were seeded onto MacConkey agar plates containing 1mg/L of cefotaxime and also on plates containing 1mg/L of ceftazidime, and incubated for 24h/37ºC. From each sample, one colony per morphotype was selected and identified by the API 20E test (BioMérieux, France). ESBL-producing *E. coli* were screened by double disk synergism using cefotaxime and ceftazidime plus amoxicillin/clavulanic acid, which showed 19 (9,5%) isolates with ESBL phenotype. The bla_{TEM}, bla_{SHV}, bla_{CTX} and bla_{OXA} genes were searched by PCR and the sequencing confirmed the presence of $bla_{\text{CTX-M-2}}$ gene in these isolates using primers described previously [4]. The genetic environment of $bla_{\text{CTX-M-2}}$ was identified following previously described methods [5]. The IS*CR1* was found upstream of the $bla_{\text{CTX-M-2}}$ and the resistance gene was associated with sul-1 type integron structure. The susceptibility test was performed by agar disk diffusion method using beta-lactams amoxillin/clavulanic acid, piperacillin/tazobactam, cefotaxime, ceftazidime, cefepime, cefoxitin, aztreonam, ertapenem and non-beta-lactams, nalidixic acid (NAL), ciprofloxacin (CIP), levofloxacin (LVX), tetracycline (TET), gentamicin (GEN), trimethoprim-sulfamethoxazole (SXT) and chloramphenicol (CHL). The results were evaluated according to the Clinical Laboratory Standards Institute recommendations [6]. Most isolates showed resistance to all beta-lactams tested, with 100% susceptibility to ertapenem. All isolates showed resistance to the non-beta-lactam antibiotics CIP, LVX, NAL and TET, 73.7% (14/19) were resistant to GEN and SXT and 31.5% (6/19) were resistant to CHL (Fig. 1).

Plasmid-mediated resistance to quinolone antibiotics associated to the CTX-M-2-producing *E. coli* was checked by PCR based on the presence of *qnr*A, *qnr*B, *qnr*S, and *acc(6')-Ib-cr* genes, as described elsewhere [7]. Two isolates presented *qnr*B19 gene (Fig. 1).

The phylogenetic group characterization, following the methods previously described [8], identified 68.5% (13/19) from phylogenetic group A, 26.3% (5/19) isolates from group D and 5.2% (1/19) from group B1 (Fig. 1). Moreover, genomic relationship was analysed by pulsed-field gel electrophoresis (PFGE) using CHEF DRIII PFGE System (Biorad, USA) after enzymatic digestion with *Xba*I. Results were evaluated using the Bionumerics Software version 5.01 (Applied Maths, USA) and the isolates were considered from the same PFGE-type when the genomic similarity was greater than 85% (Fig. 1). Therefore, according to the criteria used, the 19 CTX-M-2-producing *E. coli* isolates belong to 6 PFGE-types. Four different PFGE-types (A, B, C and D) were found among the 6 isolates from farm 1. In farm 2, two PFGE-types (E and F) were found among the 13 isolates. The relatedness between PFGE-types from both farms was at most 75% (Fig. 1). The MLST, carried out according to the 'Achtman scheme' (http://mlst.ucc.ie/dbs/Ecoli), showed three sequence types (STs). ST2309 was not assigned to any clonal complex (CC) to date, furthermore it was only described in broilers. ST93 was ascribed to CC168 and ST155 to CC155 (Fig. 1). ST93 was dominant among isolates and together with ST155, has been associated to humans and animals worldwide [9-11].

Plasmids were searched and characterized following the PCR-based replicon typing method [12]. All 19 ESBL-producing isolates presented replicons F, B/O, K, I1 and FIB. The PFGE after *S1*-nuclease digestion, the Southern blot and hybridization with specific probes were performed to search for $bla_{\text{CTX-M-2}}$ gene in plasmids, however it was not found. The method was repeated after I-*Ceu*-I-PFGE using probes for *E. coli* 16S rRNA gene and for $bla_{\text{CTX-M-2}}$ to evaluate chromosomal localization. These probes hybridized in the same position, confirming the insertion of $bla_{\text{CTX-M-2}}$ in the chromosome (Fig. 2) of all 19 ESBL isolates. Moreover, to check the possibility of chromosomal integration of the plasmids along

with the resistance gene, probes for each replicon (F, B/O, K, I1 and FIB) were used in the same membrane. Nevertheless, no plasmids were integrated in the chromosome.

All isolates were considered multidrug-resistant (non-susceptible) to at least one agent in three or more antimicrobial categories) [13], showing an important reservoir of resistance genes in these food-producing animals. The *bla*$_{\text{CTX-M-2}}$ gene is the most prevalent ESBL-encoding gene in human *E. coli* isolates in Brazil [14] and is frequently reported in animals and environmental sources [1]. Although *bla*$_{\text{CTX-M}}$ genes are usually harboured in plasmids, the chromosomal insertion of these genes was found in *E. coli* [15-17]. Herein, we showed the chromosomal *bla*$_{\text{CTX-M-2}}$ in diverse *E. coli* isolates. The IS*CR1* found in these CTX-M-2 producing *E. coli* isolates has been associated with the mobilization of CTX-M-encoding genes in both Gram-negative and Gram-positive pathogens [18].

The quinolone resistance detected in all *E. coli* isolates might be related to chromosomal mutations in quinolone-resistance-determining region or associated with other resistance mechanisms (*e.g.* porin deficiencies, overexpressing of efflux). Nevertheless, the presence of *qnr* gene may represent risk of dissemination of co-resistance in bacteria [19].

Our findings suggest that the continuous use of subinhibitory antibiotic concentrations, in the poultry environment, may promote the genetic recombination in *E. coli*, as shown elsewhere [20]. This hypothesis is supported due to different PFGE-types and phylogenetic groups detected, pointing to non-clonal dissemination of chromosome-encoding CTX-M-2 in *E. coli*. The stability of the chromosomal *bla*$_{\text{CTX-M-2}}$ in bacterial strains is still to be elucidated.

In summary, this study identified 19 CTX-M-2-producing *E. coli* isolated in Brazil from commercial broilers for human consumption, and characterised the insertion of the *bla*$_{\text{CTX-M-2}}$ into the bacterial chromosome.

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Transparency Declarations

None to declare.

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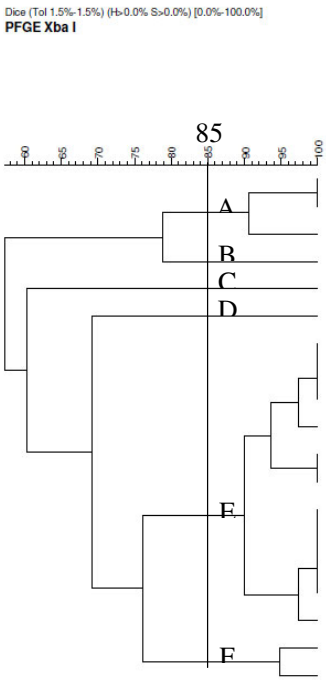
FIG. 1. Dendrogram of genomic similarity obtained by *Xba*I-PFGE of 19 ESBL-producing *E. coli* isolates from broiler chickens. NAL, nalidixic acid; CIP, ciprofloxacin; LVX, levofloxacin; TET, tetracycline; GEN, gentamicin; SXT, trimethoprim/sulfamethoxazole; CHL, chloramphenicol.

FIG. 2. Digested fragments of the genomic DNA of CTX-M-2-producing *E. coli* isolates representing the diverse PFGE-types detected. (A) PFGE profiles using I-*Ceu*-I enzyme. (B) Southern blot and hybridization with $bla_{\text{CTX-M-2}}$ probe after I-*Ceu*-I-PFGE. (C) Southern blot and hybridization with probe for *E. coli* 16S rRNA gene after I-*Ceu*-I-PFGE.



	Resistance	Chicken Farm	Resistance non-beta-	Phylogenetic	MLST Sequence	MLST Clonal
47	CTX-M-2	1	NAL,CIP,LVX,TET CHL	D	2309	none
88	CTX-M-2	1	NAL,CIP,LVX,TET,CHL	D	2309	none
96	CTX-M-2	1	NAL,CIP,LVX,TET,CHL	D	2309	none
04	CTX-M-2	1	NAL,CIP,LVX,TET,CHL	D	2309	none
9b	CTX-M-2	1	NAL,CIP,LVX,TET,GEN,SXT	A	93	168
89	CTX-M-2	1	NAL,CIP,LVX,TET,CHL	B1	155	155
163a	CTX-M-2	2	NAL,CIP,LVX,TET,GEN,SXT	A	93	168
164	CTX-M-2	2	NAL,CIP,LVX,TET,GEN,SXT	A	93	168
173	CTX-M-2	2	NAL,CIP,LVX,TET,GEN,SXT	A	93	168
151a	CTX-M-2	2	NAL,CIP,LVX,TET,GEN,SXT	A	93	168
186	CTX-M-2	2	NAL,CIP,LVX,TET,GEN,SXT	A	93	168
192	CTX-M-2	2	NAL,CIP,LVX,TET,GEN,SXT	A	93	168
118	CTX-M-2	2	NAL,CIP,LVX,TET,GEN,SXT	A	93	168
119	CTX-M-2	2	NAL,CIP,LVX,TET,GEN,SXT	A	93	168
120	CTX-M-2	2	NAL,CIP,LVX,TET,GEN,SXT	A	93	168
145b	CTX-M-2	2	NAL,CIP,LVX,TET,GEN,SXT	A	93	168
150	CTX-M-2	2	NAL,CIP,LVX,TET,GEN,SXT	A	93	168
129	CTX-M-2/qnrB 1	2	NAL,CIP,LVX,TET,GEN,SXT	A	93	168
132	CTX-M-2/qnrB 1	2	NAL,CIP,LVX,TET,GEN,SXT,CHL	D	93	168

FIG. 1.

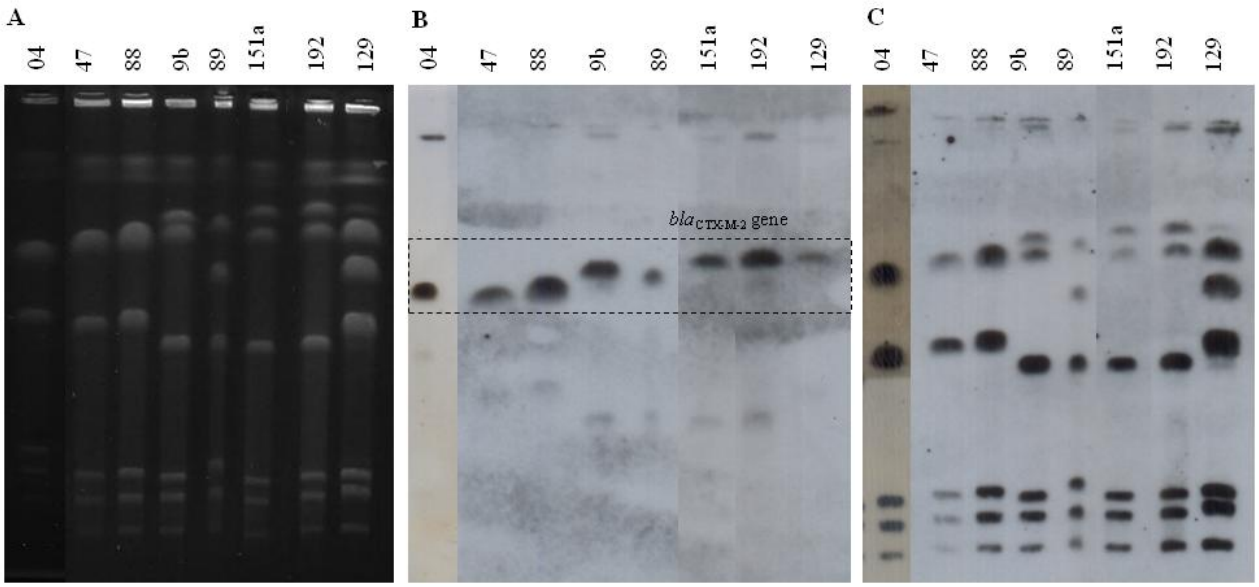


FIG. 2.