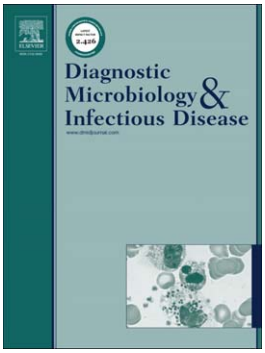

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Evaluation and characterization of plasmids carrying CTX-M genes in a non-clonal population of multidrug-resistant *Enterobacteriaceae* isolated from poultry in Brazil

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Abstract

The increasing presence of ESBL-producing bacteria in food-producing animals might impact on public health. In this study, ESBL-producing enterobacteria were investigated in the microbiota of chickens produced in Brazil. We detected *bla*$_{\text{CTX-M-2}}$, *bla*$_{\text{CTX-M-8}}$ and *bla*$_{\text{CTX-M-15}}$ in 13 *Escherichia coli* isolates, within 9 different PFGE-types. *Escherichia fergusonii* and *Klebsiella pneumoniae* were found carrying *bla*$_{\text{CTX-M-2}}$. Plasmid Inc groups found included repF, FIB, FIC, I1, Y, B/O, A/C, K and HI1. F plasmids were present in 87.5% of the isolates, however, no resistance gene was harbored in this replicon. The pMLST for IncI1 showed ST113 and the novel ST130, ST131 and ST132 harboring *bla*$_{\text{CTX-M-8}}$. IncK plasmids carried *bla*$_{\text{CTX-M-2}}$ in one *E. coli* isolate. Non-typeable plasmids carrying *bla*$_{\text{CTX-M-2}}$ or *bla*$_{\text{CTX-M-15}}$ had up to 260kb. *bla*$_{\text{CTX-M-2}}$ was also associated with class I integron and IS*CR1* and *bla*$_{\text{CTX-M-8}}$ with IS*10*. Overall, similar resistance elements were disseminated among a diverse population of ESBL-producing enterobacteria.

1. Introduction

Extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* are a concern for contemporary public health worldwide. These bacteria have been identified with increased prevalence in the community and are associated with mortality in hospitals (Hawkey, 2015). An increasing number of studies report resistance genes and ESBL-producing enterobacteria in food-producing animals, especially poultry (Liebana, et al., 2013).

Many ESBL-producing enterobacteria carry other resistance genes associated in the same ESBL-carrying plasmid. In such cases the use of antimicrobial agents, other than cephalosporins, can contribute for the maintenance and dissemination of ESBL encoding-genes (Persoons, et al., 2011). The wide prevalence of resistance genes in food-producing animals occurs essentially for two reasons, the selection of resistant isolates by indiscriminate use of antimicrobial agents in the fields and presence of mobile genetic elements (MGE) disseminating resistance genes to different bacterial hosts (Liebana, et al., 2013).

Currently, studies involving ESBL-producing *E. coli* isolated from poultry and from humans have demonstrated similar characteristics shared among isolates, especially those related to MGEs, which are closely related among animal and human isolates. These studies have also suggested that the spread of resistant isolates through the food chain may contribute for the increasing number of infections or colonization by ESBL-producing *E. coli* in humans (Carattoli, 2013; Kluytmans, et al., 2013).

The main mechanism of dissemination and acquisition of antimicrobial resistance genes in *Enterobacteriaceae* has been attributed to the horizontal transfer of MGE, especially plasmids carrying these genes, hindering the efficacy of treatments for infections caused by ESBL-producers (Carattoli, 2013).

Thus, the present study assessed the presence of β-lactamase-encoding genes and evaluated the population structure of resistant enterobacteria isolated from apparently healthy broiler chickens. The genetic relationship among ESBL-producing isolates was compared within different farms to evaluate the occurrence of clonal dissemination in the fields. Moreover, the study aimed to characterize plasmids carrying ESBL genes to investigate the MGE such as integrons and insertion elements that may facilitate the maintenance of resistance genes and exchange of genetic material in the animal environment.

2. Material and methods

2.1. Isolates

From 2011 to 2012, two-hundred cloacal swabs were harvested from 40 days-old commercial broilers in two poultry farms (1 and 2 with distance around 250Km between their) from São Paulo State, Brazil (100 swabs per farm). Cloacal swab samples were streaked on MacConkey (MC) agar containing cefotaxime (1µg/mL) and on MC agar with ceftazidime (1µg/mL), incubated at 37°C for 24h. One colony per plate was selected to conduct the present study. The bacterial colonies were identified by classical biochemical methods and confirmed by API 20E system (bioMérieux, France).

2.2. Antimicrobial susceptibility testing

The antimicrobial susceptibility of the isolates were determined by using the disk diffusion methods (CLSI, 2012), and the results were interpreted according to recommendations of the Clinical and Laboratory Standards Institute (CLSI) 2013, document M100-S23. Fifteen antimicrobial agents were tested, including β-lactam

antibiotics: amoxicillin-clavulanic acid (AMC), piperacillin/tazobactam (TZP), cefotaxime (CTX), ceftazidime (CAZ), cefoxitin (FOX), cefepime (FEP), aztreonam (ATM), ertapenem (ETP) and non β-lactam antibiotics: nalidixic acid (NAL), ciprofloxacin (CIP), levofloxacin (LEV), tetracycline (TET), gentamicin (GEN), trimethoprim-sulfamethoxazole (SXT) and chloramphenicol (CHL). ESBL-producing isolates were screened by double disk synergism (DDS) using cefotaxime and ceftazidime plus amoxicillin/clavulanic acid (Jarlier, et al., 1988).

2.3. Determination of population structure

Genetic relationship among isolates was determined by analysis of *Xba*I-digested genomic DNA on pulsed field gel electrophoresis (PFGE), performed in CHEF DRIII System (Bio-Rad, USA) (CDC, 2004). Gels were analyzed with the BioNumerics fingerprinting software (Applied Maths, Belgium) and the normalized profiles were compared using the Dice similarity index. The dendrogram was constructed using the unweighted-pair group method using average linkage algorithm (UPGMA). The homology cutoff value of 85% was used to group the related isolates within the same PFGE-type.

The determination of phylogenetic groups was assigned according to previously described method (Clermont, et al., 2000). Briefly, this method designates one out of four phylogenetic groups (A, B1, B2, or D) to each *E. coli* isolate based on the presence of *chuA*, *yjaA* genes and TSPE4.C2 DNA fragment.

2.4. Detection of β-lactamase genes

The investigation of $bla_{\text{CTX-M}}$ (groups 1, 2, 9, 8 and 25) was carried out by PCR (Saladin, et al., 2002). Purified PCR amplicons (illustra™ GFX™ PCR DNA and Gel

Band Purification Kit, GE Healthcare, USA) were directly sequenced using the ABI 3730 DNA Analyser (Life Technologies-Applied Biosystems). The DNA sequences and translated amino acid sequences obtained were compared with references found in the LAHEY home page (http://www.lahey.org/Studies/).

2.5. Genetic environment of β-lactamase genes

The genetic environment of β-lactamase genes was determined by PCR of upstream and downstream regions, as described previously (Dhanji, et al., 2010). Briefly if IS*CR1* was found upstream or downstream of β-lactamase genes, two additional PCRs were performed using the Long PCR Enzyme Mix (Thermo-Scientific, USA) with forward primers Sul1-F (5'GCC CTG TCC GAT CAG ATG CA 3') or Int1-F (5′-TCC AGAACC TTG ACC GAA CG-3′) combined with a reverse primer for the β-lactamase genes to search for class 1 integron structures (Dhanji, et al., 2010).

2.6. PCR-based Replicon Typing (PBRT) and resistance gene location

After identification of β-lactamase genes in resistant isolates by PCR and confirmation by sequencing, plasmids were investigated and characterized by the PBRT scheme, as previously described (Carattoli, et al., 2005). Southern blot and hybridization methods were performed to locate the replicon type carrying the ESBL-encoding gene. Briefly, plasmid DNA was digested with *S1* nuclease and analyzed on PFGE gels (*S1*-PFGE). The PFGE gel was submitted to a southern blot in the nylon membrane (Hybond-N+, GE Healthcare Life Sciences, USA) and hybridization with specific probes for detection of the resistance gene and the incompatibility (Inc) groups. The identification of the replicon type harboring the resistance gene was determined by

binding of probes for resistance gene in the same position of the replicon in the membrane.

2.7. Conjugation experiments

Transferability of plasmids carrying β-lactamase genes was determined by conjugation with recipient *Escherichia coli* strains assisted by antimicrobial resistance markers and lactose fermentation characteristics of the recipient strain. The lactose positive transconjugants obtained in azide-resistant *E. coli* J53 were selected on MacConkey agar containing 2 µg/mL of cefotaxime and 100 µg/mL of sodium azide and the lactose negative obtained in streptomycin-resistant *E. coli* C600 were selected with 300 µg/mL of streptomycin. The presence of β-lactamase genes in the transconjugants was confirmed by PCR. Inc groups of the resistance plasmids from transconjugants were assigned using the PBRT method, as previously described (Carattoli, et al., 2005).

2.8. Plasmid multilocus sequence typing (pMLST)

The primers and PCR conditions for the pMLST of IncI plasmids followed the instructions described in http://pubmlst.org/plasmid/primers/. The amplicons were purified with illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, USA) and both strands were sequenced using an ABI 3730 DNA Analyser (Life Technologies-Applied Biosystems, USA). The nucleotide sequences were aligned using ChromasPro Software version 1.41 (Technelysium, Australia). The allele types were analyzed in the database at http://www.pubmlst.org/plasmid/.

3. RESULTS

3.1. Antimicrobial susceptibility

Among the studied isolates, DDS test detected 16 ESBL-producing enterobacteria isolates (8%), which were 13 *E. coli*, 2 *Escherichia fergusonii* and 1 *Klebsiella pneumoniae*. The ESBL-producing isolates showed resistance to most β-lactam antibiotics tested (resistance to broad-spectrum cephalosporins and aztreonam), remaining susceptible only to carbapenens (ETP) (Table 1). Co-resistance phenotypes to non β-lactam antibiotics were also observed, including to GEN (6/16), SXT (5/16), CHL (3/16), TET (15/16) and to quinolones NAL (15/16), CIP (15/16) and LEV (14/16). The 16 ESBL-producing isolates were multidrug-resistant (MDR), which are not susceptible to at least one agent in three or more antimicrobial categories (Magiorakos, et al., 2012).

3.2. Characterization of β-lactamase genes and genetic environment

Investigation of β-lactamase groups by PCR revealed 16 isolates (8%) carrying $bla_{\text{CTX-M}}$ type gene. The 16 CTX-M-producing isolates consisted of 9 *E. coli* isolates carrying $bla_{\text{CTX-M-8}}$, 3 carrying $bla_{\text{CTX-M-2}}$ and 1 carrying $bla_{\text{CTX-M-15}}$ and also 2 *E. fergusonii* and 1 *K. pneumoniae* isolate carrying $bla_{\text{CTX-M-2}}$. The CTX-M-2-producing *E. fergusonii* and *E. coli* and the CTX-M-15-producing *E. coli* were isolated in farm 1, the CTX-M-2-producing *K. pneumoniae* was isolated in farm 2, whilst CTX-M-8-producing *E. coli* isolates were found on both farms (Table 1).

The genetic environment characterized in CTX-M-producing isolates showed the insertion sequence IS*CR1* and a class 1 integron structure with 7kb upstream (5' region) from the resistance genes in 6 $bla_{\text{CTX-M-2}}$ isolates (3 *E. coli*, 2 *E. fergusonii* and 1

K. pneumoniae). The IS*10* was found upstream from $bla_{\text{CTX-M-8}}$ in 8 *E. coli* isolates (Table 1).

3.3. Clonal analysis by PFGE and phylogroup determination

Clonal diversity was showed among 13 *E. coli* isolates, classified in 9 PFGE-types. The 2 *E. fergusonii* isolates belonged to the same PFGE-type with 100% similarity. There were no common clones shared among farms studied (Figure 1).

The phylogenetic groups A, B1, and D were found among *E. coli* isolates. Seven isolates belonged to the phylogenetic group A, four were assigned to the phylogenetic group D and two isolates belonged to phylogenetic group B1. Phylogenetic group B2 was not found (Table 1).

3.4. Characterization of replicon types, hybridization and conjugation

A wide variety of plasmids replicons were identified from groups IncF (14 F, 11 FIB, 1 FIC), IncI1 (13), IncY(4), and one from each group A/C, K and HI1. Furthermore, the southern blot and hybridization showed 9 *E. coli* isolates harbored $bla_{\text{CTX-M-8}}$ gene in plasmids IncI1 with approximately 50kb. One *E. coli* isolate (MA41) showed $bla_{\text{CTX-M-2}}$ harbored in the IncK plasmid (35kb). *K. pneumoniae* and *E. fergusonii* isolates harbored $bla_{\text{CTX-M-2}}$ gene in plasmids that were not typeable by PBRT, yet the hybridization identified the resistance plasmids with long lengths with 250kb and 260kb, respectively (Table 1). The resistance genes and replicon types associated identified by hybridization were subsequently confirmed by PCR using the transconjugants obtained. *E. coli* isolates resulted in transconjugants with *E. coli* C600 and *E. fergusonii* isolates with *E. coli* J53. The replicons carrying the resistance gene are underlined in Table 1. The *E. coli* isolates MA86, MA93 and *K. pneumoniae* did not

result in transconjugants with the recipient strain *E. coli* C600, however the hybridization showed that the resistance genes were harbored in large plasmids ranging from 200kb to 260kb, not typeable by PBRT.

3.5. pMLST

The pMLST for IncI1 plasmid replicons was performed with transconjugants and identified ST113 (allelic profile 1/2/5/10/10). Furthermore, three novel STs were identified in the present work and deposited in the pMLST database as ST130 (allelic profile 1/2/8/10/19), ST131 (allelic profile 1/9/23/4/3) and ST132 (allelic profile 1/2/23/4/3) (Table 1).

4. Discussion

Antimicrobial resistance in commensal and pathogenic enterobacteria causes high impact on public health. Animal reservoirs may be involved in the dissemination of resistant bacteria, which may occur by contact with animals, contaminated food of animal origin, raw meat, water and environment. Plasmids carrying resistance genes in commensal *E. coli* were shown to be successfully transferred *in vivo* to pathogenic bacteria, including *Salmonella* (Canton, et al., 2011). Considering the use of antimicrobial agents in production animals, we investigated resistance genes in commensal enterobacteria isolated from broiler chickens, furthermore the epidemiological features were deeply analyzed to elucidate the mechanisms involved in the dissemination of the resistance genes.

In the present work, ESBL-producing *E. coli, E. fergusonii* and *K. pneumoniae* were isolated from the intestinal microbiota of apparently healthy broilers. Among 16 ESBL-producing isolates, 93.75% (15/16) were also resistant to NAL, CIP and TET;

87.5% (14/16) were also resistant to LEV; 37.5% (6/16) were also resistant to GEN and 31.25% (5/16) were also resistant to SXT. As noticed, a high level of co-resistance to quinolones and fluoroquinolones were showed in the ESBL-producing isolates. Together with β-lactams, these important classes of antimicrobial agents are the first choice for treatments of infections caused by enterobacteria. Considering that all 16 ESBL-producing isolates were MDR, the frequent usage of these antimicrobial agents acts as selective pressure on these bacteria contributing to increase its prevalence in the environment (Canton, et al., 2011) and to the successful colonization of the poultry intestinal tract.

Total prevalence of ESBL-producing *E. coli* isolates in different animal species and meat in 2009 ranged from 2% to 9%, depending on the source, according to the European Food Safety Authority (Liebana, et al., 2013). In the present study, the prevalence of ESBL-producing enterobacteria was 8%. Currently in Brazil, one-day old chicks may receive third-generation cephalosporin to prevent omphalitis and gastrointestinal infections, which may contribute to select resistant microbiota and consequently increase the reservoirs of MDR enterobacteria in the animal environment (Collignon, et al., 2013), as noticed in the present study. In 2012, the Food and Drug Administration (FDA) in United States approved a novel legislation restricting the use of cephalosporins in food-producing animals, including chickens, aiming to reduce the prophylactic use of antimicrobial drugs in animal production and also the dissemination of resistant bacteria (US Food and Drug Administration, 2012).

CTX-M-producing enterobacteria are widespread among human populations but an increasing number of reports describes the presence in the animal environment and in food of animal origin (Lazarus, et al., 2015). Our findings show that the poultry is a reservoir of resistance genes $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-8}}$ and $bla_{\text{CTX-M-15}}$ in enterobacteria

isolated from the intestinal tract. As shown in Table 1, a wide genetic diversity was found among all ESBL isolates, classified among different PFGE-types.

CTX-M-2 is the dominant ESBL group among human clinical *Enterobacteriaceae* isolates in South America (Minarini, et al., 2009). The gene $bla_{\text{CTX-M-2}}$ was found in 37.5% ESBL isolates, from three different *Enterobacteriaceae* species. All *E. coli*, *E. fergusonii* and *K. pneumoniae* carrying $bla_{\text{CTX-M-2}}$, shared in common a class 1 integron and IS*CR1* associated with the resistance gene, suggesting that both these MGE may have been disseminated in the farms, transferring the resistance gene to different plasmids. Moreover, the same MGE carrying the resistance gene were found in a polyclonal bacterial population, *E. coli* from different PFGE-types and 2 different species. IS*CR1* was previously attributed to the mobilization of resistance genes (e.g. $bla_{\text{CTX-M-2}}$) into plasmids, consequently increasing the dissemination of antimicrobial resistance mechanisms (Toleman, et al., 2006). Similar to the present work, class 1 integron-IS*CR1* complex was found associated to $bla_{\text{CTX-M-2}}$ in Argentina (Arduino, et al., 2003). The association of $bla_{\text{CTX-M}}$ genes with class 1 integron and IS*CR1* may contribute to increase the mobility of the resistance gene and recombination with other plasmid Inc groups in different bacterial hosts, and also increases the risk of insertions into bacterial chromosome, as previously shown (Ferreira, et al., 2014). Therefore, given the non-clonal nature of the disseminated resistant isolates in the studied farms, plasmids and other MGE may be involved. Thus not only clonal dissemination by selection of a single resistant isolate should be considered for the antimicrobial resistance dissemination (Canton, et al., 2006) in the animal environment.

The CTX-M-8 enzyme was first described in the year 2000 from clinical isolates, in Brazil (Bonnet, et al., 2000). However, it has also been identified in Europe and USA since then, but the prevalence is still low in other territories (Eller, et al.,

2013). Our findings show that *bla*$_{\text{CTX-M-8}}$ was found in higher prevalence (56%) among the ESBL isolates from animal source. This resistance gene was found in 9 *E. coli* isolates from farms 1 and 2, however these were classified into 6 different PFGE-types (A, D, E, H, L and one was not determined). The in-depth plasmid analysis showed that *bla*$_{\text{CTX-M-8}}$ was harbored in IncI1 plasmids with similar sizes (50kb) and the insertion element IS*10* was associated with the resistance gene in all isolates except in MA32. Despite similarities, the pMLST revealed four different STs, including three novel STs (130, 131 and 132) among these plasmids. The variable genotypes and phenotypes of the resistant *E. coli* isolates found in the present study demonstrates the rich genetic diversity involved in the dissemination of antimicrobial resistance genes. This diversity may be a consequence of the polyclonal *E. coli* populations present in the intestinal microbiota.

CTX-M-15 is one of the most common ESBL detected worldwide, in human clinical infections by different pathogens. In Brazil, this enzyme was described for the first time in 2010, found in clinical isolates from hospitalized patients (Cergole-Novella, et al., 2010). In 2008, a study evaluated the presence of resistant bacteria in raw chicken meat from the UK and other countries, including Brazil, but *E. coli* carrying *bla*$_{\text{CTX-M-15}}$ were not found. Only in 2015, CTX-M-15 producing *E. coli* was isolated for the first time in Brazil from frozen chicken carcasses (Botelho, et al., 2015). However, in the present study we characterized *bla*$_{\text{CTX-M-15}}$ in one *E. coli* isolate from live healthy poultry in Brazil for the first time, which might consequently be transferred to carcasses and *in natura* meat. Our results corroborate with the current literature, and show the risk of dissemination of resistance genes and bacteria present in livestock through the food chain. In fact, this gene has also been recently described in food-producing animals in other countries (Kang, et al., 2013).

Eleven different Inc groups of plasmids were found among the isolates, including IncI1 and IncK. The IncI1 plasmid family is frequently associated with resistance genes (Carattoli, 2013). The pMLST database up-to-date has more than 320 STs, among these, 191 belong to IncI1 profile. In this study, the $bla_{\text{CTX-M-8}}$ was present in IncI1 plasmids, within 4 different STs, from which three (ST 130, ST 131 and ST 132) were identified for the first time. IncI1 plasmid family was also described in UK, carrying $bla_{\text{CTX-M-8}}$ in isolates from raw chicken imported from South America (Dhanji, et al., 2010). These plasmids (IncI1) were highly disseminated during the last decade and previous study hypothesize the presence in animal reservoirs (Leverstein-van Hall, et al., 2011), which was confirmed by the present study. IncK plasmids were associated to the dissemination of CTX-M in the UK, in animals, humans and environment (Cottell, et al., 2011). In the United States these plasmids were identified in multidrug-resistant *E. coli* isolated from chickens (Lindsey, et al., 2011).

Few reports show *E. fergusonii* or *K. pneumoniae* carrying resistance genes in farm animals. Resistant and enterotoxigenic *E. fergusonii* were isolated from chickens in South Korea and India (Oh, et al., 2012). In Japan, CTX-M-2-producing *K. pneumoniae* was identified in broiler chickens (Hiroi, et al., 2012). Although *E. fergusonii* may be present in the intestinal tract as commensal microbiota, different reports suggest a high pathogenic potential, describing many virulence factors, associated to multidrug resistance in these bacteria. Considering the capacity to cause disease in both humans and animals *E. fergusonii* may emerge as a novel concern for public health (Forgetta, et al., 2012). In the present work we found MDR *E. fergusonii* carrying $bla_{\text{CTX-M-2}}$ in a large plasmid (260kb), contributing to better understand resistance to antimicrobial agents in this species, which still has few data in the current literature (Gaastra, et al., 2014). Despite not being typeable by PBRT, this large plasmid

harbored the class I integron and IS*CR1* associated with the resistance gene, increasing the risks of mobility and recombination with other plasmids.

The presence of CTX-M-producing enterobacteria in food producing animals and raw food products was suggested as potential source of dissemination of resistance genes worldwide (Dhanji, et al., 2010; Eller, et al., 2013). Furthermore, similarities between ESBL-producing *E. coli* isolated from chickens and humans have been described, particularly the MGEs that carries the resistance genes (Kluytmans, et al., 2013). ESBLs from groups CTX-M-2 and CTX-M-8 were previously identified in *E. coli* isolated from raw chicken meat imported from South America to the UK (Dhanji, et al., 2010) and more recently, in enterobacteria isolated from retail chicken meat collected in marketplace from Brazil (Casella, et al., 2015).

In conclusion we showed the presence of ESBL encoding-genes in commensal enterobacteria isolated from live chickens from Brazil and found different plasmids carrying ESBL genes ($bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-8}}$ and $bla_{\text{CTX-M-15}}$). The epidemiological evaluation showed that the plasmids size carrying resistance genes were diverse, ranging from 35 to 260kb. Despite the large size of the plasmids, some of these showed to be conjugative and were present in different populations of *E. coli* and also *E. fergusonii* and *K. pneumoniae*. Furthermore, IS*CR1* or IS*10* were detected in association with resistance genes. The IncI plasmids carrying $bla_{\text{CTX-M-8}}$ were highly similar and had the same size (50kb), however the pMLST analysis revealed that 4 different plasmids acquired the same resistance gene. These evidences support the increasing public health concern considering the dissemination of resistance genes through the food chain and animal environment.

Conflict of interest statement

None to declare.

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Table 1. Microbiological and molecular characteristics of ESBL-producing isolates from poultry farms in Brazil

Isolates						**beta-lactamase gene**		**Plasmid**		
Species	number	Resistances	Farms	Phylogenetic group*	PFGE	*bla*	Genetic enviroment	Replicon type	Size (kb)	pMLST
E.coli	MA1	AMC, CTX, CAZ, FEP, ATM, NAL, CIP, TET	1	B1	Y	CTX- M-15		I1,FIB,A/C,F,NT	50	NA
E.coli	MA41	AMC, CTX, CAZ, FEP, FOX, ATM, NAL, CIP, LEV, TET, CHL, GEN	1	D	M	CTX-M-2	Int class 1+ IS*CR1*	K,FIB,F	35	NA
E.coli	MA86	AMC, CTX, FEP, ATM, NAL, CIP, LEV,TET, GEN	1	A	O	CTX-M-2	Int class 1+ IS*CR1*	FIB,FIC,F,NT	200	NA
E.coli	MA93	AMC, CTX, FEP, ATM, NAL, CIP, LEV, TET, CHL, SXT	1	A	P	CTX-M-2	Int class 1+ IS*CR1*	I1,HI1,FIB,F,NT	240	NA
E. fergusonii	MA40a	AMC,CTX, FEP, ATM, NAL, CIP, LEV, TET, GEN, SXT	1		K	CTX-M-2	Int class 1+ IS*CR1*	I1,FIB,F,NT	250	NA
E. fergusonii	MA100b	AMC,CTX, FEP, ATM, NAL, CIP, TET, GEN, SXT	1		K	CTX-M-2	Int class 1+ IS*CR1*	I1,FIB,F,NT	250	NA
K.pneumoniae	LO 166b	AMC,CTX, FEP, ATM, TET, CHL, GEN, SXT	2		T	CTX-M-2	Int class 1+ IS*CR1*	NT	260	NA
E.coli	MA10b	CTX, FEP, ATM, NAL, CIP, LEV	1	B1		CTX-M-8	IS*10*	I1,FIB,Y,F	50	ST 113
E.coli	MA19a	AMC, CTX, CAZ, FEP, NAL, CIP, LEV, TET,	1	A	D	CTX-M-8	IS*10*	I1,FIB,F	50	ST 132
E.coli	MA32	AMC, CTX, FEP, NAL, CIP, LEV, TET	1	A	H	CTX-M-8		I1,F	50	ST 132
E.coli	MA58	AMC,CTX, NAL, CIP, LEV,GEN,SXT	1	A	L	CTX-M-8	IS*10*	I1	50	ST 130
E.coli	LO116b	AMC,CTX, FEP, NAL, CIP, LEV,TET	2	D	A	CTX-M-8	IS*10*	I1,FIB,Y,F	50	ST 131
E.coli	LO 137b	AMC, CTX, FEP, ATM, NAL, CIP, LEV, TET	2	D	A	CTX-M-8	IS*10*	I1,FIB,Y,F	50	ST 131
E.coli	LO168	AMC,CTX, NAL, CIP, LEV,TET	2	D	A	CTX-M-8	IS*10*	I1,FIB,Y,F	50	ST 131
E.coli	LO 182	AMC,CTX, FEP, ATM, NAL, CIP, LEV, TET	2	A	E	CTX-M-8	IS*10*	I1,F	50	ND
E.coli	LO 194	AMC,CTX, FEP, ATM, NAL, CIP, LEV, TET	2	A	E	CTX-M-8	IS*10*	I1,F	50	ND

AMC: amoxicillin-clavulanic acid, CTX: cefotaxime, CAZ: ceftazidime, FEP: cefepime, FOX: cefoxitin, ATM: aztreonam, NAL : nalidixic acid, CIP ciprofloxacin, LEV: levofloxacin, TET: tetracycline, CHL: chloramphenicol, SXT: trimethoprim-sulfamethoxazole, GEN: gentamicin.

NT: ESBL gene-carrying plasmids non-typeable by PBRT; NA: pMLST analysis not available for the replicon carrying the resistance gene;

ND: not determined using the primers from the current pMLST database; *Phylogenetic groups were assigned for *E. coli* isolates.

The replicon type and the size of the plasmids that carried bla_{CTX-M} genes are underlined.

The replicon carrying the resistance gene in *E. coli* isolates number MA1, MA86 and MA93 was not detectable by hybridization.