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Molecular Characterization of *Escherichia coli* Strains Isolated from Retail Meat That Harbor bla_{CTX-M} and *fosA3* Genes

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A total of 55 cefotaxime-resistant *Escherichia coli* isolates were obtained from retail meat products purchased in Shenzhen, China, during the period November 2012 to May 2013. Thirty-seven of these 55 isolates were found to harbor a bla_{CTX-M} gene, with the $bla_{CTX-M-1}$ group being the most common type. bla_{CMY-2} was detected in 16 isolates, alone or in combination with other extended-spectrum β-lactamase (ESBL) determinants. Importantly, the *fosA3* gene, which encodes fosfomycin resistance, was detected in 12 isolates, with several being found to reside in the conjugative plasmid that harbored the bla_{CTX-M} gene. The insertion sequence IS*26* was observed upstream of some of the $bla_{CTX-M-55}$ and *fosA3* genes. Conjugation experiments showed that bla_{CTX-M} genes from 15 isolates were transferrable, with Inc I1 and Inc FII being the most prevalent replicons. High clonal diversity was observed among the bla_{CTX-M} producers, suggesting that horizontal transfer of the bla_{CTX-M} genes among *E. coli* strains in retail meats is a common event and that such strains may constitute an important reservoir of bla_{CTX-M} genes, which may be readily disseminated to other potential human pathogens.

Escherichia coli causes a wide range of opportunistic infections and may be regarded as a key vector in the dissemination of resistance elements among Gram-negative human pathogens. Among the range of antibiotic-resistant Gram-negative bacterial pathogens known to date, organisms producing CTX-M-type extended-spectrum β-lactamases (ESBLs) pose a particularly serious public health threat worldwide (1). In the past few years, rapid dissemination of *E. coli* strains that produce ESBLs has been reported in various parts of the world, with the CTX-M-type enzymes being the most common ESBLs detected among clinical isolates (2, 3). The CTX-M family can be divided into five major groups (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25), with less than 90% identity between different groups and more than 95% identity among members of a group (4).

Fosfomycin is a naturally occurring antibacterial agent with a broad spectrum of antimicrobial activity against both Gram-positive and Gram-negative bacteria, except *Acinetobacter baumannii* (5). The agent has been used to treat urinary tract infections, but development of bacterial resistance during therapy frequently occurred, rendering it unsuitable for sustained therapy of severe infections (6, 7). Recently, renewed attention has been paid to fosfomycin for the treatment of both urinary tract and systemic infections due to rapid dissemination of multidrug-resistant Gram-negative bacteria, especially strains of *Enterobacteriaceae* species that are resistant to traditionally used agents (8). Compared to other agents, fosfomycin seems to have retained antimicrobial activity against a substantial percentage of clinical isolates, in particular *E. coli*. Recently, a fosfomycin resistance gene, *fosA3*, has been reported in *E. coli* and *Klebsiella pneumoniae* isolates (9–13). The gene is normally plasmid mediated and flanked by the IS*26* transposase genes and is often detected in bla_{CTX-M}-producing and multidrug-resistant *E. coli* strains recovered from animals, as well as patients, in China, Japan, and Korea (9–13). It has been suggested that the increasing prevalence of the *fosA3* gene is due to dissemination of the Inc I, Inc N, and Inc FII plasmids among *E. coli* isolates rather than to clonal expansion of specific strains (11, 14).

The origins of ESBL and *fosA3* genes in clinical strains, as well as the factors facilitating the dissemination of such elements, remain ill defined. Recent reports of recovery of ESBL-producing bacterial isolates in animals have raised concerns about the possibility that such isolates are becoming causative agents of human clinical infections (15). In particular, we postulate that meat products constitute a potential source of bla_{CTX-M}- and *fosA3*-bearing organisms that mediate drug-resistant infections. In the present study, we tested this hypothesis by examining the prevalence of *E. coli* strains that harbor such elements in retail meat, followed by characterization of their phenotypic and genotypic features.

MATERIALS AND METHODS

Bacterial isolation. *E. coli* isolates were collected from fresh pork and chicken samples purchased from wet markets (markets that sell fresh and unprocessed meat products) in Shenzhen, Guangdong Province, China, during the period November 2012 to May 2013. *E. coli* was isolated on MacConkey agar plates and identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) using a Bruker MicroFlex LT mass spectrometer (Bruker Daltonics). *E. coli* iso-

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lates were further confirmed with an API20E test strip (bioMérieux, Inc.). Only one *E. coli* isolate from each sample was used for further analysis.

Antimicrobial susceptibility testing. Antimicrobial susceptibility was determined using the agar dilution method and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) recommendations (16). *E. coli* strain ATCC 25922 was used as a quality control strain in antimicrobial susceptibility testing. Isolates that exhibited resistance to at least three different classes of agents were classified as multidrug resistant.

Detection of β-lactamase genes. β-Lactamase genes in the *E. coli* isolates were determined by PCR as previously described (17). The PCR amplicons were then sequenced and subjected to BLAST analysis against the NR database available at GenBank to investigate the degree of genetic similarity between the detected sequence and known ESBL genes.

Conjugation experiments. The horizontal-transfer efficiencies of the *bla*$_{CTX-M}$ genes were assessed by performing conjugation using the filter-mating method as previously described (18). Transconjugants were selected on MacConkey agar containing cefotaxime (2 μg/ml) and sodium azide (100 μg/ml) and tested for antimicrobial susceptibility and the presence of *bla*$_{CTX-M}$ genes.

Epidemiological typing. The genetic relatedness between the test isolates and the corresponding transconjugants was determined by pulsed-field gel electrophoresis (PFGE) using the CHEF-MAP-PER System (Bio-Rad Laboratories, Hercules, CA, USA) as described previously (19). The results were interpreted according to the criteria of Tenover et al. (20). Plasmids harbored by the transconjugants were analyzed by S1 nuclease-PFGE and Southern blot hybridization, using probes that target specific resistance genes.

Plasmid replicon typing. Plasmids extracted from transconjugants were characterized by plasmid-based replicon typing. Eighteen pairs of primers were designed to perform 5 multiplex and 3 simplex PCRs targeting the FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA replicons, as previously described (21).

Multilocus sequence typing and phylogenetic grouping. *E. coli* strains were subjected to multilocus sequence typing (MLST) and phylogenetic grouping. Internal fragments of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) were sequenced (22), followed by assignment of sequence types (STs) in accordance with the *E. coli* multilocus sequence typing scheme (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). Phylogenetic grouping was performed for all the isolates as described previously (23).

Analysis of the genetic environment of *bla*$_{CTX-M}$ genes. The genetic environment of *bla*$_{CTX-M}$ genes was probed by PCR, using specific primer pairs (IS*Ecp1*, IS*CR1*, IS*26*, ORF477, IS*903*, and those targeting group 1 and group 9 *bla*$_{CTX-M}$ genes, as well as the *fosA3* gene). The PCR products were subjected to bidirectional DNA sequencing and BLAST analysis to identify the insertion sequence concerned.

RESULTS

A total of 70 pork and 50 chicken samples were purchased during the isolation period. Eighty *E. coli* isolates were obtained—45 *E. coli* isolates from pork (64% isolation rate) and 35 *E. coli* isolates from chicken (70% isolation rate). Among these 80 *E. coli* isolates, 55 were resistant to cefotaxime and were further characterized in this study. These cefotaxime-resistant *E. coli* isolates also showed resistance to other antibiotics, such as tetracycline (78%), nalidixic acid (71%), sulfamethoxazole-trimethoprim (65%), chloramphenicol (58%), ciprofloxacin (56%), kanamycin (47%), and fosfomycin (22%). However, all the isolates were susceptible to meropenem, and only two strains (4%) were resistant to amikacin. Thirty-seven out of the 55 isolates harbored the *bla*$_{CTX-M}$ gene, 16 harbored a *bla*$_{CMY-2}$-like gene, and 4 possessed a *bla*$_{ACT-2}$-like gene (see Table S1 in the supplemental material). Three isolates harbored only *bla*$_{TEM-1}$, suggesting that cephalosporin resistance mechanisms other than those we screened for may be present. Further investigation will be needed to discover the exact cefotaxime resistance mechanisms in these isolates. The combined presence of different β-lactamase genes was commonly observed in the isolates, as shown in Table S1 in the supplemental material. The *fosA3* gene was also detected in 12 of the 55 *E. coli* isolates tested, representing 22% and 33% of the cefotaxime-resistant and *bla*$_{CTX-M}$-positive *E. coli* isolates, respectively. It should be noted that *fosA3* was detected only in *bla*$_{CTX-M}$-positive isolates and not in strains containing the *bla*$_{ACT-2}$-or *bla*$_{CMY-2}$-like gene.

Among the 37 *bla*$_{CTX-M}$-positive *E. coli* isolates tested, 15 were found to be able to successfully transfer their cefotaxime resistance phenotypes to the *E. coli* recipient strain J53 via conjugation. PFGE analysis of these 37 isolates revealed high genetic diversity of the isolates, with 29 arbitrary pulsotypes at a 90% threshold and 3 untypeable isolates (see Fig. S1 in the supplemental material). Among the 15 *E. coli* isolates with conjugative plasmids, 14 arbitrary pulsotypes at a 90% threshold were detected (Fig. 1). These data suggested that the *bla*$_{CTX-M}$ genes recovered in the test isolates were acquired as a result of horizontal transfer from existing resistant organisms, rather than through clonal expansion of specific resistant strains. The results of phylogenetic and MLST analyses also revealed a great diversity of MLST types. The most prevalent sequence type was ST156, followed by ST155 and ST88. Most of these strains belonged to the clonal complexes ST156cc, ST155cc, ST23cc, ST101cc, and ST469cc and to phylogenetic group B1, followed by A and D (Fig. 1).

Sequencing of the full-length β-lactamase genes revealed that *bla*$_{CTX-M-55}$ was the most prevalent (8/15) in these 15 *E. coli* isolates, followed by *bla*$_{CTX-M-15}$ (4/15), *bla*$_{CTX-M-14}$ (2/15), and *bla*$_{CTX-M-123}$ (1/15). For transconjugants, the most commonly detected *bla*$_{CTX-M}$ gene was *bla*$_{CTX-M-55}$, followed by *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-14}$, and *bla*$_{CTX-M-123}$. It should be noted that all except one gene in the *bla*$_{CTX-M-1}$ group were transferrable to the recipient, whereas conjugation was successful in only two out of four strains of the *bla*$_{CTX-M-9}$ group, suggesting that the *bla*$_{CTX-M-1}$-type genes were located more commonly on conjugative plasmids (Table 1). A notable case was isolate 63, the parental strain of which produced both *bla*$_{CTX-M-14}$ and *bla*$_{CTX-M-15}$, but no β-lactamase gene could be detected in the recipient strain by PCR, suggesting the existence of a novel β-lactamase gene in the conjugative plasmid.

S1-PFGE analysis showed that the parental strains carried multiple plasmids with sizes ranging from 78 to 244 kb (Table 1). In comparison, conjugative plasmids of four major sizes, (78, 104, 130, and 138 kb) were recovered (Table 1; see Fig. S2 in the supplemental material). Plasmid typing indicated that all five 78-kb conjugative plasmids were Inc I1 plasmids that harbored β-lactamase genes of the *bla*$_{CTX-M-1}$ group, including *bla*$_{CTX-M-55}$ and *bla*$_{CTX-M-15}$; four 138-kb plasmids, which were untypeable, were found to harbor all *bla*$_{CTX-M-1}$ group genes, including *bla*$_{CTX-M-15}$ and *bla*$_{CTX-M-55}$, with two of them also harboring the *fosA3* gene; three 130-kb conjugative plasmids were found to belong to the Inc I1, FII, and FIV types and to harbor *bla*$_{CTX-M-1}$ group β-lactamase genes, including *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-55}$, and *bla*$_{CTX-M123}$; two 104-kb plasmids were of the FII type, which harbored the *bla*$_{CTX-M-55}$ element. One strain, TC75, was found to carry two conjugative plasmids with sizes of 244 kb and 50 kb, with the 244-kb plasmid comprising a *bla*$_{CTX-M-9}$ group β-lactamase gene, *bla*$_{CTX-M-14}$, together with a *fosA* gene (Table 1; see Fig. S2 in the supplemental material). The Inc FIV plasmid has rarely

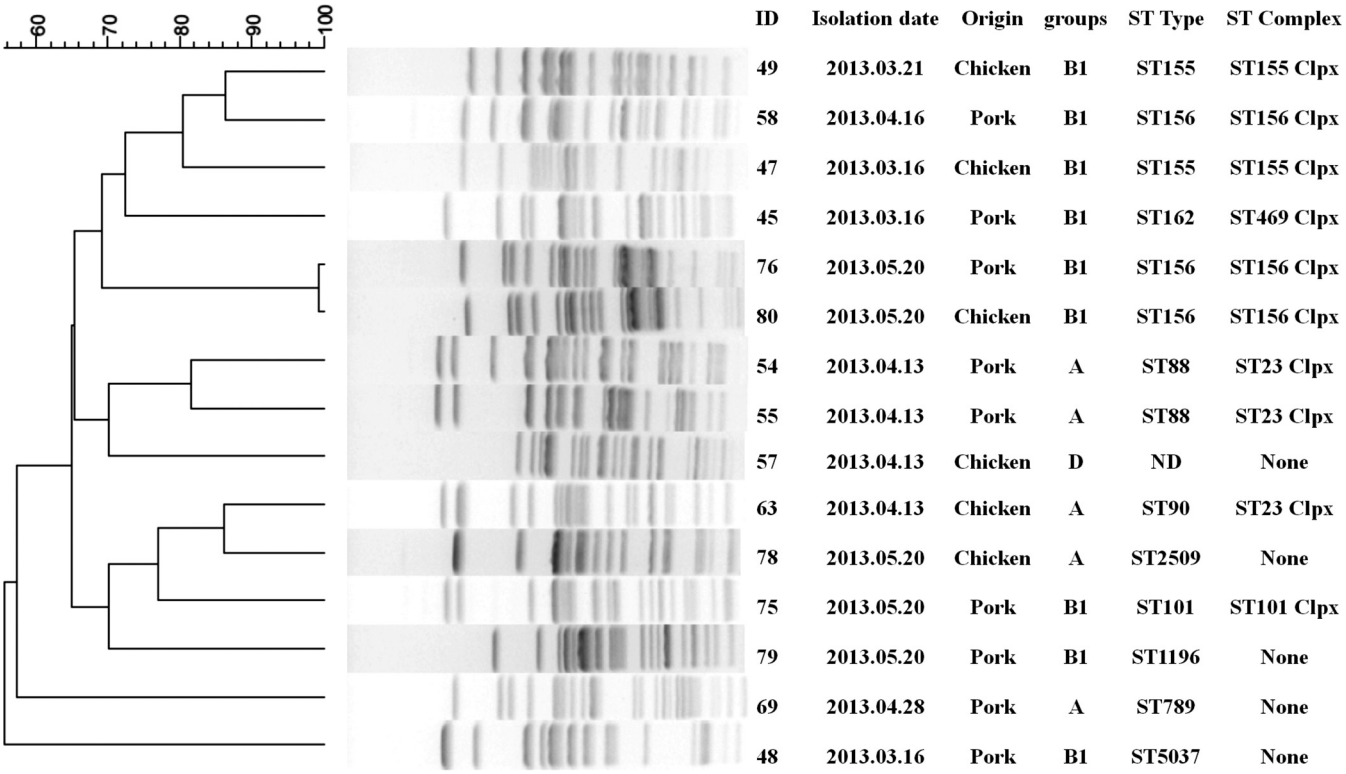

FIG 1 Origins, molecular characteristics, and genetic relatedness of 15 ESBL-producing *E. coli* strains that harbored conjugative plasmids.

been described and has not been detected with any bla_{CTX-M} ESBL gene before.

Southern hybridization was performed and confirmed that bla_{CTX-M} and *fosA3* genes were located in plasmids of various sizes. bla_{CTX-M}-bearing plasmids of the same sizes as those in the parental strains could also be detected in transconjugants, with the exception of two strains, namely, 48 and 58. In these two cases, the $bla_{CTX-M-55}$ gene was found to be located in plasmids with sizes of ~78 kb and ~104 kb, respectively, in the parental strains but in plasmids with a size of ~138 kb in the transconjugants TC48 and TC58 (Table 1; see Fig. S3 in the supplemental material). Southern hybridization also confirmed that both bla_{CTX-M} and *fosA* genes recovered from five strains (47, 57, 75, 76, and 80) were all located on the same plasmids, which could all be transferred to the transconjugants (see Fig. S4 in the supplemental material).

In an attempt to probe the genetic environment of the resistance genes, an IS*26* element was detected upstream of five $bla_{CTX-M-55}$ genes, with ORF477 and a 47-bp spacer detected downstream of the $bla_{CTX-M-55}$ genes. However, IS*Ecp1*, IS*CR1*, and IS*903* were not detected in the rest of the 10 transconjugants. IS*26* was detected upstream of the *fosA3* gene in two transconjugants, C76 and C80, but not in the other three transconjugants in which IS*26* was detectable (see Fig. S5 in the supplemental material).

DISCUSSION

Food animals colonized with ESBL-producing *E. coli* bacteria have been considered potential sources of resistant strains that commonly cause infections in the community (24). ESBL producers were rarely detected in animal isolates in China before 2005 (25–27) but have emerged at a dramatically increasing rate in recent years (27–29), presumably due to overuse of third-generation cephalosporins in raising food animals (30). A recent report that *E. coli* isolated from Dutch patients shared identical ESBL genes and plasmids with strains recovered from retail chicken meat and poultry suggests that transmission of ESBL-producing organisms from poultry to humans is a common event (31). In a similar study, Shiraki et al. (32) suggested that genetic determinants encoding CTX-M enzymes may have originated from animals and were transmitted to humans via the food chain. Therefore, tracking the transmission routes of food-borne *E. coli* strains in community settings is urgently required to prevent widespread dissemination of ESBL-producing organisms in China.

Resistance conferred by ESBLs is often associated with resistance to other classes of antibiotics, such as trimethoprim-sulfamethoxazole, aminoglycosides, and fluoroquinolones (33). In China, plasmid-mediated fosfomycin resistance has been frequently detected among CTX-M-producing isolates in animals (10, 34). It was unexpected, since the use of fosfomycin in food-producing animals is not approved in China. Nevertheless, we also observed a high rate of fosfomycin resistance among the bla_{CTX-M}-producing isolates. Our finding is also consistent with those of previous studies, in which bla_{CTX-M} genes were found to be located predominantly in Inc FII- or Inc I1-type plasmids in *E. coli* isolates recovered from food animals (35). bla_{CTX-M} genes were often found in plasmids of different sizes (from 78 kb to 138 kb), implying that a diversity of bla_{CTX-M} genetic environments exist. The IS*Ecp1* element is known to play an important role in bla_{CTX-M} expression and gene transfer and is frequently located upstream of bla_{CTX-M} genes (18, 36, 37). Surprisingly, such an element was not

TABLE 1 Antibiotic susceptibility and β-lactamase gene profiles of plasmid-mediated ESBL-producing *E. coli* strains and corresponding transconjugants

Strain or transconjugant[a]	Susceptibility [MIC (μg/ml)][b]										β-Lactamase gene(s) detected in plasmid(s)	Estimated plasmid size(s) (kb)[c]	Plasmid type[d]
	AMK	CTX	CIP	KAN	STR	CRO	TET	CHL	NAL	SXT			
45	2	>16	0.03	2	4	>16	2	4	4	16	TEM-1, CTX-M-55	244, 138, **78**	
TC45	1	>16	0.015	4	16	>16	2	4	4	0.5	CTX-M-55	**78**	Inc I1
47	>128	>16	>16	>128	>128	>16	32	>64	>64	>16	TEM-1, CTX-M55, *fosA3*	**130**, 104, 78	
TC47	0.5	>16	0.03	1	1	>16	0.5	1	4	2	CTX-M-55	**130**	Inc FIV
48	2	>16	0.5	>128	64	>16	16	32	8	>16	CTX-M-55	**78**	
TC48	1	>16	0.06	128	32	>16	16	64	16	1	CTX-M-55	**138**	ND
49	1	>16	0.03	>128	64	>16	16	32	2	>16	CTX-M-123, *fosA3*	216,**130**	
TC49	0.5	>16	0.03	>128	1	>16	8	1	4	2	CTX-M-123, *fosA3*	**130**	Inc I1
54	1	>16	1	16	128	>16	16	32	8	>16	CTX-M15, CMY-2, CTXM14	167,138,**78**	
TC54	0.5	>16	0.03	1	1	>16	1	1	4	≤0.25	CTX-M-15	**78**	Inc I1
55	1	>16	>16	>128	128	>16	32	32	>64	>16	TEM-1, CTX-M55	**200, 78**	
TC55	0.5	>16	0.015	1	2	>16	1	2	4	≤0.25	CTX-M-55	**78**	Inc I1
57	1	>16	>16	>128	32	>16	16	>64	>64	4	CTX-M-55, *fosA3*	138, **130**, 104, 78	
TC57	1	>16	0.06	128	32	>16	16	64	16	1	CTX-M-55, *fosA3*	**130**	Inc FII
58	2	>16	>16	>128	>128	>16	>32	>64	>64	>16	TEM-1, CTX-15	**130**	
TC58	1	>16	0.06	128	32	>16	16	64	16	1	CTX-M-15	**138**	ND
63	4	>16	>16	>128	64	>16	32	>64	>64	1	CTX-M-15, CTX-M-14	138, **104**	
TC63	1	4	0.015	1	2	16	1	2	4	0.25	/	**104**	Inc I1
69	4	16	2	2	16	8	16	2	>64	0.5	TEM-1, OXA-1, CTX-M-55	104, **78**	
TC69	1	>16	0.015	4	16	>16	2	4	4	0.5	CTX-M-55	**78**	Inc I1
75	4	8	2	>128	128	>16	>32	>64	64	>32	TEM-1, CTX-M-14, *fosA3*	**244**, 104	
TC75	0.25	4	0.03	>128	32	4	16	32	4	>16	TEM-1, CTX-M-14, *fosA3*	**244**	ND
76	4	>16	>16	16	128	>16	>32	64	>64	>32	TEM-1, CTX-M-15, *fosA3*	**138**	
TC76	0.5	>16	0.06	128	64	>16	16	64	16	2	CTX-M-15, *fosA3*	**138**	ND
78	4	>16	>16	>128	>128	>16	32	>64	>64	>32	TEM-1, CTX-M-55	**100**	
TC78	0.5	>16	0.015	>128	64	>16	16	32	4	≤0.25	TEM-1, CTX-M-55	**104**	Inc FII
79	4	>16	>16	64	8	>16	>32	>64	>64	>32	CTX-M-14	**104**, 78	
TC79	2	8	0.03	32	2	16	1	4	4	≤0.25	CTX-M14	**104**	Inc FII
80	4	>16	8	>128	>128	>16	>32	>64	>64	>32	TEM-1, CTX-M-55, *fosA3*	142, **138**, 104	
TC80	0.5	>16	0.06	128	32	>16	8	64	32	2	CTX-M-55, *fosA3*	**138**	ND

[a] TC, transconjugant.
[b] AMK, amikacin; CTX, cefotaxime; CIP, ciprofloxacin; KAN, kanamycin; STR, streptomycin; CRO, ceftriaxone; TET, tetracycline; CHL, chloramphenicol; NAL, nalidixic acid; SXT, sulfamethoxazole-trimethoprim.
[c] Number in bold denotes plasmid transferable to recipient strain.
[d] ND, not determined.

detected upstream of the *bla*$_{CTX-M}$ gene in these conjugative plasmids. No *bla*$_{CTX-M}$ gene was found to be associated with IS*CR1* in the conjugative plasmids, which is consistent with the previous observation in that IS*CR1* was much less commonly associated with *bla*$_{CTX-M}$ genes. In previous studies, ORF477 and IS*903* were frequently detected downstream of the *bla*$_{CTX-M-1}$ and *bla*$_{CTX-M-9}$ group genes, respectively. ORF477 was also frequently detected in this study, whereas IS*903* was not, presumably because only two transconjugants that belonged to the *bla*$_{CTX-M-9}$ group were analyzed. Surprisingly, the *bla*$_{CTX-M}$ gene in a strain that contained the *fosA3* element, with the genetic structure IS*26*–*bla*$_{CTX-M-55}$–ORF477, located in a plasmid of 138 kb, was different from those reported in all previous studies in that the *bla*$_{CTX-M}$ gene in the same genetic environment was often located in plasmids 60 kb to 100 kb in size. In addition, *bla*$_{CTX-M-1}$ group genes were found to be more transferable than the *bla*$_{CTX-M-9}$ group genes in this study, presumably due to the difference in the genetic environments.

In this study, most of the transferable isolates belonged to the phylogenetic group B1, followed by A, and none belonged to phylogenetic group B2, ST131, which is the most prevalent type worldwide at present. Since virulent organisms causing infections are mainly known to belong to group B2, and to a lesser extent to group D, whereas most commensal strains belong to groups B1 and A (38), our data indicate that most of the transferable isolates tested in this study belonged to commensal strains. This finding is therefore consistent with previous reports in that commensal *E. coli* strains in food animals were likely to be a reservoir of ESBL genes (24) and to play a role in dissemination of such resistance

elements. ST156 was the most prevalent ST detected and the only type isolated from humans (39, 40). ST131 (26%) and ST156 (11.1%) were the most prevalent STs identified in carbapenemase-producing *E. coli* strains in 83 hospitals in Spain (41). Various other reports also showed that ST156 was detected in water and animals (42–44). The strong linkage of $bla_{\text{CTX-M-15}}$ ST156 isolates from humans, animals, and the environment warrants further attention to and research on this type of *E. coli* bacteria.

The $bla_{\text{CTX-M}}$ producers observed in this study pose a serious challenge for treatment of human infections, since the test strains were resistant not only to cephalosporins, but also to other classes of antibiotics. Continued investigations and surveillance for better understanding of the transmission dynamics and evolutionary characteristics of such strains and the resistance elements that they harbor are necessary.

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