High prevalence of bla_{CTX-M} extended-spectrum β-lactamase genes in *Escherichia coli* isolates from pets and emergence of CTX-M-64 in China

Y. Sun[1], Z. Zeng[1], S. Chen[2], J. Ma[1]*, L. He[1], Y. Liu[1], Y. Deng[1], T. Lei[1], J. Zhao[1] and J.-H. Liu[1]

1) *College of Veterinary Medicine, National Reference Laboratory of Veterinary Drug Residues (SCAU), South China Agricultural University, Guangzhou and*

2) *Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong SAR, China*

Abstract

As a cause of community-acquired infections, extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* constitute an emerging public-health concern. Few data on the molecular epidemiology of ESBL-producing *E. coli* isolates from pets are available in China. Detection and characterization of ESBL genes (bla_{CTX-M}, bla_{SHV} and bla_{TEM}) was conducted among 240 *E. coli* isolates recovered from healthy and sick pets in South China from 2007 to 2008. The clonal relatedness of ESBL-producing *E. coli* isolates was assessed by pulsed field gel electrophoresis. ESBL-encoding genes were identified in 97 (40.4%) of the 240 isolates and 96 (40.0%) of them harbored CTX-M. The most common CTX-M types were CTX-M-14 (n = 45) and CTX-M-55 (n = 24). The recently reported CTX-M-64 was identified in three isolates. Isolates producing CTX-M-27, -15, -65, -24, -3 and -9 were also identified. Ten isolates carried two or three CTX-M types, with the combination of CTX-M-14 and CTX-M-55 being the most frequent (n = 6). IS*Ecp1* was identified in the upstream region of 93 out of the 107 bla_{CTX-M} genes (86.9%). The sequence of the spacer region (45 bp) between IS*Ecp1* and the start codon of all $bla_{CTX-M-55}$ genes (except four) was identical to that of $bla_{CTX-M-64}$. No major clonal relatedness was observed among these CTX-M producers. It is suggested that the horizontal transfer of bla_{CTX-M} genes, mediated by mobile elements, contributes to their dissemination among *E. coli* isolates from pets. Our finding of high prevalence of ESBL in *E. coli* of companion animal origin illustrates the importance of molecular surveillance in tracking CTX-M-producing *E. coli* strains in pets.

Keywords: Antimicrobial resistance, China, CTX-M, *Escherichia coli*, extended-spectrum β-lactamases, molecular epidemiology, pets

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Corresponding author: J.-H. Liu, College of Veterinary Medicine, National Reference Laboratory of Veterinary Drug Residues (SCAU), South China Agricultural University, Guangzhou 510642, China

E-mail: jhliu@scau.edu.cn

*Present address: Guangdong Key Laboratory of Marine Materia Medica, South China Sea Institute of Oceanology, the Chinese Academy of Sciences, Guangzhou, China.

Introduction

The Gram-negative bacterium *Escherichia coli* is a common intestinal microorganism of humans and animals, with the capability of acquiring and preserving transferable resistance genes found in other organisms and the environment. In addition, *E. coli* is also an important pathogen causing a variety of illnesses, including urinary and gastrointestinal infections and septicaemia. For the treatment of these infections, β-lactams including cephalosporins are frequently used in human and veterinary medicine [1]. The major mechanisms of resistance to oxyimino-cephalosporins in *E. coli* rely on the production of extended-spectrum β-lactamases (ESBLs) [2]. In the past few years, a rapid dissemination of *E. coli* isolates producing ESBLs has been reported in many parts of the world and CTX-M type enzymes have become the most dominant ESBLs [3,4]. Of particular concern is the emergence and dissemination of CTX-M family ESBLs among *E. coli* within the community [5]. The CTX-M family is composed of more than 80 heterogeneous ESBLs and can be divided into five different groups (CTX-M-1, M-2, M-8, M-9 and M-25) based on amino acid sequence similarities. Within each group, ESBLs share greater than 90% sequence identity [3,4]. Recently, a new type of CTX-M enzyme, CTX-M-64, that might be a chimera of CTX-M-15 and CTX-M-14 family ESBLs, was identified in Japan, suggesting a new mechanism of the evolution of CTX-M type ESBLs [6].

ESBLs are commonly detected in human clinical isolates. Recent reports of the detection of ESBLs in bacterial isolates

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from animals have raised concern regarding the co-transfer of ESBL genes between human and animal isolates [7]. Considering the shared environment of humans and pets and the close contact between them, companion animals could be potential sources of ESBL-producing *E. coli* isolates causing community-acquired infections. In the present study, the prevalence of ESBLs among *E. coli* isolates from companion animals and the phenotype and genotype of ESBLs were characterized to provide useful information about the epidemiology and evolution of ESBLs in bacteria.

Materials and Methods

Bacterial isolates

A total of 240 *E. coli* isolates were obtained from faecal or clinical samples of healthy (i.e. pets admitted to hospitals for vaccination or for sale) and diseased dogs and cats (for diagnostic investigation) in eight animal hospitals and one pet shop in Guangdong Province during November 2007 and June 2008. Bacterial strains were identified using classical biochemical methods and Microlog™, release 4.2 (Biolog, Hayward, CA, USA).

Antimicrobial susceptibility testing and ESBL confirmation

Antimicrobial susceptibility was determined using the agar dilution and disk diffusion methods and interpreted according to the CLSI recommendations [8,9]. *E. coli* ATCC25922 was used as quality control strain in antimicrobial susceptibility testing. Production of ESBLs in the isolates was determined with the phenotypic confirmatory test using both cefotaxime and ceftazidime, alone and in combination with clavulanic acid [9].

Detection of β-lactamase genes

PCR amplification of $bla_{CTX\text{-}M}$, bla_{TEM} and bla_{SHV} genes was carried out for all ESBL-producing isolates using Ex*Taq* DNA polymerase (Takara, Dalian, China) and primers as previously described [10]. Purified PCR products were directly sequenced from both ends or cloned in pMD18-T and then sequenced. The DNA sequences and deduced amino acid sequences were compared, using BLAST, with sequences available at GenBank and also compared with sequences at Lahey Clinic (available at http://www.lahey.org/studies/webt.html) to identify the subtypes of β-lactamase genes.

Analysis of the up- and downstream regions of $bla_{CTX\text{-}M}$ type genes

Analysis of the $bla_{CTX\text{-}M}$ upstream regions was performed using PCR with forward primers located in IS*Ecp1* (ISEcp1-F: 5′-CTA TCC GTA CAA GGG AGT GT-3′) or IS*CR1* (CR1-F: 5′-GTC AAT CGC CCA CTC AAA C-3′), together with reverse primers for group 1 (M1-R: 5′-CCA TTG CCC GAG GTG AAG-3′) and group 9 CTX-M genes (M9-R: 5′-CGG CGT GGT GGT GTC TCT-3′). The downstream regions of $bla_{CTX\text{-}M}$ genes was investigated by PCR amplification with the CTX-M forward and reverse primers specific for *orf477* (5′-CAG CGG AAG GAG AAC CAG-3′) or *IS903* (5′-TTT CCA CTC GCC TTC ACC-3′). PCR products were purified and subjected to bidirectional DNA sequencing. New CTX-M sequences were deposited into the GenBank database and assigned accession numbers GQ300937, GQ300938, GQ456156, GQ456157, GQ456158 and GQ456159.

Conjugation experiments

The transfer of $bla_{CTX\text{-}M}$ between bacteria was assessed by conjugation experiments using the filter method, as described elsewhere [11]. Briefly, the donor bacteria and the recipient, streptomycin-resistant *E. coli* C600, were grown in tryptic soy broth to logarithmic phase, mixed at a 1 : 4 ratio (v/v), collected on a filter and incubated at 37°C for 20 h. Transconjugants were selected on MacConkey agar containing cefotaxime (2 mg/L) and streptomycin (1000 mg/L). Selected transconjugants were further characterized in terms of their antimicrobial susceptibility, ESBL phenotype and the presence of $bla_{CTX\text{-}M}$ genes. The PCR identification of $bla_{CTX\text{-}M\text{-}64}$ in transconjugants was conducted with the forward primer for CTX-M-1 group genes and the reverse primer (M9-R) located in the central region of the CTX-M-9 group genes.

Epidemiological typing

The ESBL-producing *E. coli* isolates were characterized by pulsed field gel electrophoresis (PFGE) using the CHEF-MAPPER System (Bio-Rad Laboratories, Hercules, CA, USA) as described by Gautom [12]. Briefly, the chromosomal DNA of *E. coli* isolates was isolated and the plugs were subjected to digestion with *Xba*I for 16 h at 37°C. Electrophoresis was carried out at 6.0 V/cm for 22 h, with an angle of 120° at 14°C. The pulse time was increased from 0.5 to 60 s. A bacteriophage lambda DNA ladder consisting of 48.5-kb concatemers was used as a size marker. Gels were then stained in ethidium bromide (1.0 mg/L). The results were interpreted according to the criteria of Tenover *et al.* [13].

Results

A total of 240 *E. coli* were isolated from 185 dogs and 55 cats, with 226 (94.2%) animals from private owners (92 of them were healthy pets admitted to hospitals for vaccination and 134 were sick pets) and the remaining 14 healthy pets

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originating from one pet store. Approximately 93.0% of the samples were from faeces and the rest were from nasal mucus, wound swabs or other sources. Ninety-nine (41.3%) of the 240 *E. coli* isolates were confirmed to produce ESBLs using the double-disk synergy test. The sample sources of ESBL producers were: 88 from faeces, seven from nasal mucus, and four from swabs (wound, skin and pharynx). The ESBL producer rate was 54.5% (73/134) among isolates from sick animals, and significantly higher than among isolates from healthy animals (24.5%; 26/106) (χ^2 = 19.504, p <0.01).

Ninety-eight percent (97/99) of the isolates harbored one or more of the genes from the three families of TEM, SHV and CTX-M, whereas two isolates failed to show the presence of such genes, suggesting that these two isolates have (an)other ESBL-encoding gene(s) not detected with our primers, which need further investigation. CTX-M type genes were shown to be dominant in these isolates and 96 isolates carried one or more CTX-M genes. The most common CTX-M type was CTX-M-14 (*n* = 45), followed by CTX-M-55 (*n* = 24), CTX-M-27 (*n* = 8), CTX-M-24 (*n* = 8), CTX-M-15 (*n* = 6), CTX-M-65 (*n* = 6), CTX-M-3 (*n* = 5), CTX-M-64 (*n* = 3) and CTX-M-9 (*n* = 2). More than one type of CTX-M type gene was identified in ten isolates and their combinations were triple (CTX-M-55, -64, -14) in one isolate, and double [CTX-M-55 and -14 (*n* = 6), CTX-M-55 and -65 (*n* = 1), CTX-M-55 and -9 (*n* = 1) and CTX-M-64 and -14 (*n* = 1)] in nine isolates (Table 1). In addition to the CTX-M type genes, one *E. coli* isolate was positive for bla_{SHV-12} and one carried both bla_{SHV-12} and $bla_{CTX-M-9}$. A variant of $bla_{CTX-M-14}$, $bla_{CTX-M-14b}$, which differs from $bla_{CTX-M-14}$ at three nucleotide positions (A372G, G570A, G702A) was found in two *E. coli* isolates. Fifty-three isolates were positive for bla_{TEM}, and all were found to be bla_{TEM-1} by sequencing.

Analyses of antimicrobial susceptibility patterns of isolates carrying CTX-M-1 subgroup (CTX-M-3, -15, -55 and -64) and CTX-M-9 subgroup genes (CTX-M-14, 24, 27, 65), and isolates producing more than one ESBL are shown in Table 2. The isolates that carried CTX-M-1 subgroup genes showed significantly reduced susceptibilities to ceftazidime compared to the CTX-M-9 subgroup (χ^2 = 26.58573, p <0.01) but, interestingly, isolates carrying CTX-M-9 subgroup genes alone showed significantly reduced susceptibilities to amikacin (χ^2 = 5.0073, p 0.014).

Chromosomal DNAs of 93 isolates were available for PFGE typing. Banding patterns were not obtained from four ESBL-producing *E. coli* isolates because their DNAs were consistently autodigested. Based on CTX-M types, all clusters according to PFGE profiles of CTX-M-producing *E. coli* are shown in Table 1. The results reveal that a majority of isolates showed unique, unrelated PFGE profiles and were unlikely to be considered the cause of an epidemic. However, among the three CTX-M-64-producing *E. coli* isolates, two were clonally related.

Table 3 shows the genetic structure up- and downstream of the bla_{CTX-M} type genes detected among ESBL-positive isolates in the present study. The IS*Ecp1*-like and IS*CR1* elements were located upstream of 93 (86.9%) and one ($bla_{CTX-M-9}$) of the 107 bla_{CTX-M} genes, respectively. PCR amplification of IS*Ecp1*-like elements and IS*CR1* were negative for other bla_{CTX-M} genes. *orf477* and *IS903* were detected downstream of most $bla_{CTX-M-1}$ group and $bla_{CTX-M-9}$ group genes respectively.

Nucleotide sequence analysis of the upstream region of bla_{CTX-M} type genes revealed that the spacer region between the right inverted repeat (IRR) and bla_{CTX-M} were found to be 42–127 bp. All of the CTX-M-9 group genes (except CTX-M-9) were located 42 up downstream of IS*Ecp1*. A 127-bp intergenic region was found upstream of four $bla_{CTX-M-3}$ genes but, in the case of one $bla_{CTX-M-3}$ gene, a 117-bp intergenic region was found. The $bla_{CTX-M-15}$ genes were characterized by a 48-bp intergenic region, whereas all $bla_{CTX-M-55}$ (except four) and $bla_{CTX-M-64}$ genes were characterized by a 45-bp intergenic region.

The conjugation experiments were conducted for 96 CTX-M-producing isolates to determine the transferability of bla_{CTX-M}. The results obtained indicated that the cefotaxime resistance trait from 54 CTX-M-producing strains could be transferred to the recipient strain, and transconjugants were positive for bla_{CTX-M}, suggesting that the bla_{CTX-M} type genes could well be located on conjugative plasmids and transferred to other bacteria through conjugation. Two transconjugants carrying different bla_{CTX-M} genes, ($bla_{CTX-M-64}$ and $bla_{CTX-M-55}$, respectively) were obtained from the *E. coli* isolate 096 (Table 1). By contrast to the donor strains, the transconjugants of CTX-M-1 subgroup genes remained susceptible to ciprofloxacin, gentamicin, chloramphenicol and tetracycline, suggesting the corresponding resistance mechanisms are not transferable and located at a distance from the plasmid carrying the CTX-M-1 group genes. However, unlike the resistance in the CTX-M-1 subgroup transconjugants, non-β-lactam (i.e. amikacin and chloramphenicol) resistance was cotransferred with CTX-M-9 subgroup genes (Table 1), which is consistent with the antimicrobial susceptibility of their donors in that CTX-M-9 subgroup producers were more frequently resistant to these antimicrobials than CTX-M-1 group producers.

Discussion

ESBL-producing *E. coli*, particularly those producing CTX-M type ESBLs, are commonly associated with hospital- or

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TABLE 1. Characteristics of isolates with extended-spectrum β-lactamase genes

bla_{CTX-M} type[a]	PFGE type[b] (number of isolates)	Isolate[c]	Origin[d] (number of isolates)	Isolation date	Hospital (number of isolates)	Resistance phenotype[e]
CTX-M-3 (*n* = 5)	3NR	210, 225, 916	**Cat**, **Dog**, Cat	2008.2-2008.4	Y, F, F	CTX GEN (CHL) TET (CIP)
	3NT	153, 110	**Cat**, **Dog**	2008.1	Y	CTX GEN AMK TET CIP
CTX-M-15 (*n* = 6)	15A (2)	108, 081	Dog	2007.11-2008.5	Z	CTX CAZ GEN (AMI CHL FLR) TET CIP
	15NR (4)	127	Dog	2008.1	F	CTX CAZ GEN AMK CHL TET CIP
		124, f57, 525	**Dog**, Dog, **Cat**	2007.11-2008.7	Z, Z, T	CTX CAZ GEN (AMK) CHL (FLR) TET CIP
CTX-M-55 (*n* = 15)	55A (2)	02R, 424	Dog	2008. 2-2008.4	F	CTX CAZ GEN AMK CHL FLR TET CIP
	55NR (12)	122, 129, 160, 212, 312, 066, 107, 203	Dog (6), **Dog**, **Cat**	2007.11-2008.5	Z (3), F (2), Y (2)	CTX CAZ (GEN CHL FLR) TET (CIP)
		226, 061, 104, 138	Dog (3), **Dog**	2007.11-2008. 5	F, Z, Z, F	CTX CAZ GEN (AMK CHL) TET (CIP)
	55NT	410	Dog	2008.4	Z	CTX CAZ TET CIP
CTX-M-64	64A	hf2	**Dog**	2008.6	T	CTX CAZ TET
CTX-M-64, -55, -14	64B1	096	**Cat**	2008.5	Y	CTX CAZ FOX GEN AMK TET CIP
CTX-M-64, -14	64B2	218	Dog	2008.2	F	CTX CAZ FOX TET CIP
CTX-M-55,-14 (*n* = 6)	55B (2)	120, 124	Dog	2008.1	F	CTX CAZ GEN AMK CHL TET CIP
	55NR (4)	304, 408, 313, 132	Dog	2008.3-2008.4	Z (3), F	CTX CAZ (FOX GEN AMK) CHL TET CIP
CTX-M-65, -55	65A	143	Dog	2008.1	F	CTX CAZ GEN AMK CHL TET
CTX-M-9, -55	9A	069	Dog	2008.5	Z	CTX CAZ FOX GEN AMK TET CIP
CTX-M-9, SHV-12	9B	111	Dog	2008.1	Y	CTX CAZ FOX GEN AMK CHL TET CIP
CTX-M-24a (*n* = 7)	24A (2)	308, 201	Dog	2008. 2, 3	F, Z	CTX (CAZ FOX) GEN AMK CHL TET CIP
	24B1 (2)	117, 404	**Cat**, Dog	2008.5, 4	Shop, Z	CTX (CHL TET) (CIP)
	24B2	118	**Cat**	2008.5	Shop	CTX
	24C	114	Dog	2008.1	F	CTX GEN AMK CHL TET CIP
	24D	146	**Dog**	2008.1	F	CTX CAZ FOX GEN AMK TET CIP
CTX-M-24e	24D[f]	113	**Cat**	2008.5	Shop	CTX CHL TET
CTX-M-27 (*n* = 8)	27NR (8)	109	Dog	2008. 1.18	Y	CTX CAZ FOX GEN AMK CHL FLR CIP
		SS6, 518	**Dog**, Dog	2008.1-2008.5	F, Z	CTX CAZ GEN AMK (CHL) TET CIP
		105, 252	Dog, **Dog**	2008. 1-2008.4	Y, Z	CTX GEN AMK (CHL) TET (CIP)
		303, 830, 202	Dog	2007.12-2008.3	Z	CTX (CAZ) GEN AMK (CHL FLR TET) CIP
CTX-M-65 (*n* = 5)	65A	084	Cat	2008.5.8	Z	CTX GEN AMK CHL TET CIP
	65B1	181	Dog	2008.5.18	Z	CTX GEN CHL FLR CIP
	65B2	429	Cat	2008.4.29	C	CTX GEN AMK CHL TET CIP
	65NR	233, 409	Dog	2008.2-2008.3	F, Z	CTX GEN AMK CHL (FLR) TET CIP
CTX-M-14 (*n* = 35)	14A1	083	Dog	2008.5,	Z	CTX GEN AMK CHL TET CIP
	14A2 (3)	f29, 301, 134	Cat, Dog, Dog	2008.1-2008.3	Y, Z, F	CTX (GEN AMK CHL TET CIP)
	14B (2)	309, 228	Dog	2008. 3, 2	Z, F	CTX GEN AMK (CHL FLR TET) CIP
	14C1 (2)	1201, 426	Dog, Dog	2007.11, 2008.4	Z	CTX GEN AMK (CHL FLR TET) CIP
	14C2	155	Dog	2008.1	A	CTX GEN TET CIP
	14NR (25)	1111, 112	Dog	2007.11-2008.1	Z, Y	CTX GEN AMK CHL FLR TET CIP
		13D, 116, 157	Dog, Dog, **Dog**	2008.1	F, F, A	CTX GEN AMK (CHL FLR TET) CIP
		128	Dog	2007.11	Z	CTX GEN CHL FLR TET CIP
		0141, 429,	Dog	2008.1-2008.4	F	CTX (FOX) GEN CHL (FLR) TET CIP
		145, 525	**Cat**, **Dog**	2008.1-2008.5	F, Z	CTX (FOX) GEN (AMK) CHL FLR TET CIP
		SS3, 158, 1120	**Dog**, **Dog**, Dog	2007.11-2008.1	Y, A, Z	CTX (GEN AMK CHL FLR TET) CIP
		1129, 1201, 0115, 0154, 101, 102, 8304, 204, 305R, 311, 403, 5083, 184	Dog (8), **Dog** (5)	2007.11-2008.5	Z (8), F (2), A (2), Y (2)	CTX (CAZ FOX GEN AMK CHL FLR TET CIP)
CTX-M-14b (*n* = 2)	14D	106, 08D	Dog	2008.1	Y	CTX GEN AMK CHL FLR TET CIP
SHV-12	NA	Tfs	Dog	2008.5	T	GEN CIP

CTX, cefotaxime; CAZ, ceftazidime; FOX, cefoxitin; GEN, gentamicin; AMK, amikacin; CHL, chloramphenicol; FLR, florfenicol; TET, tetracycline; CIP, ciprofloxacin.
[a]The extended-spectrum β-lactamase genes (ESBL) genes that were transferred to a recipient by conjugation are underlined.
[b]Patterns that differed by fewer than six bands were considered to represent subtypes within the main group (e.g., 14A1, 14A2). Patterns that differed from pattern 14A by six or more bands were considered to represent different strains. NT, isolates did not generate DNA bands by the pulsed field gel electrophoresis (PFGE) method used in this study. NA, not applicable. 14NR, isolates with patterns that differ from 14A, 14B, 14C and each other by six or more bands. NA, not applicable.
[c]Isolates from which the ESBL gene was transferred to a recipient by conjugation are underlined.
[d]Healthy animals are in bold.
[e]Isolates were not susceptible according to National Committee on Clinical Laboratory Standards/CLSI guidelines. The criterium for florfenicol resistance is MIC ≥16 mg/L.
[f]Resistance phenotypes transferred to recipient by conjugation are underlined. A variable presence or transferability of the resistance phenotype is indicated by parentheses.

community-related infection in humans [2,4]. Recently, ESBLs have also been reported in *Enterobacteriaceae* isolated from companion animals in some countries [14–16]. In China, only ceftiofur-resistant *Enterobacteriaceae* isolated from companion animals have been reported [17]. In the present study, we provide the first report of the surveillance of ESBL-gene dissemination in *E. coli* isolates from healthy and sick companion animals in China.

Approximately 41.3% of the isolates screened were ESBL producers, which is very similar to that found in human clinical isolates from Guangzhou (39.5%) but somewhat lower than in isolates from China as a whole (55%) [18]. However, this percentage is much higher than that in isolates from food animals in China [10,19] and companion animals in other countries [15,16]. Only 3.3% and 11.0% of *E. coli* isolates from food animals were ESBL-positive in 2003–2005

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TABLE 2. Antimicrobial susceptibilities of isolates producing extended-spectrum β-lactamase genes

Antimicrobial	Susceptibility (%)			
	CTX-M-1[a] group (*n* = 27)	CTX-M-9 group (*n* = 58)	More than one ESBL gene (*n* = 11)	Total (*n* = 99)
Cefotaxime	0	0	0	1.0
Ceftazidime	**22.2**	**82.8**	**0**	57.6
Cefoxitin	100.0	87.9	33.3	84.8
Amoxicillin–clavulanic acid	61.5	67.2	33.3	61.6
Imipenem	100.0	100.0	100.0	100.0
Gentamicin	14.8	17.2	8.3	15.2
Amikacin	**66.7**	**38.3**	**25.0**	47.5
Ciprofloxacin	18.5	19.0	16.7	20.2
Chloramphenicol	40.7	29.3	16.7	32.3
Florfenicol	66.7	60.3	66.7	73.7
Tetracycline	0	6.9	0	6.1

[a]As $bla_{CTX-M-64}$ was amplified with CTX-M-1 group-specific primers targeting the 5′ and 3′ ends of bla_{CTX-M}, it was included in CTX-M-1 group in the present study.
The data with significant difference between different groups were bold.

TABLE 3. Analysis of the genetic structure upstream and downstream of the bla_{CTX-M} type genes

Type of bla_{CTX-M}	Number of isolates		Size of the intergenic spacer region between IS*Ecp1* and bla_{CTX-M} (bp)	Number of isolates	
	IS*CR1*	IS*Ecp1*		*orf477*	*IS903*
CTX-M-3 (*n* = 5)	0	4	127	5	NA
		1	114		
CTX-M-15 (*n* = 6)	0	5	48	6	NA
CTX-M-55 (*n* = 24)	0	18	45	22	NA
		4	48		
CTX-M-64 (*n* = 3)	0	3	45	3	NA
CTX-M-14 (*n* = 45)	0	37	42	NA	28
CTX-M-24 (*n* = 8)	0	8	42	NA	6
CTX-M-27 (*n* = 8)	0	7	42	NA	7
CTX-M-65 (*n* = 6)	0	6	42	NA	5
CTX-M-9 (*n* = 2)	1	0	NA	NA	0

NA, not applicable.

and 2007, respectively [10,19]. This may be a result of the use of cephalosporins as common treatment for companion animals; half of the diseased animals in the present study have been treated with cephalosporins within 12 months prior to sampling (data not shown). This is also consistent with the fact that approximately 54.5% of the isolates from sick animals carried ESBL genes, which is significantly higher than the corresponding 24.5% in healthy companion animals.

The CTX-M family was shown to have become dominant (98%) in ESBL-producing companion animal isolates in the present study, consistent with other reports concerning human clinical isolates (76.8%) in China in which the CTX-M-9 group accounts for the most prevalent gene types (64.4%) [20,21]. Nine CTX-M types of specific ESBLs were detected in *E. coli* isolates in the present study. The most predominant ESBL was CTX-M-14 (45.0%), which is similar to reports of human isolates in China [20,21]. CTX-M-55 of was also found to be prevalent (24.2%) in the present study.

CTX-M-55 is a derivative of CTX-M-15 with enhanced activity against ceftazidime and was first identified in *E. coli* and *Klebsiella pneumoniae* isolates in Thailand in 2007 [22]. CTX-M-55 has recently also been detected in other countries [23–25]. Most interestingly, subsequent to the first discovery of a novel CTX-M-64 in a *Shigella sonnei* isolate from Japan [6], we detected CTX-M-64 in three *E. coli* isolates from pets. Further research is needed to understand the mechanism(s) of the development of this novel type of ESBL.

IS*Ecp1* is frequently found in the upstream region of bla_{CTX-M} type genes and plays an important role in bla_{CTX-M} expression and gene transfer [11,26,27]. In agreement with previous studies [11,28], IS*Ecp1* was detected 42 bp upstream of CTX-M-9 group (CTX-M-14, -24, -27 and -65) genes and this is the first time that IS*Ecp1* was identified upstream of $bla_{CTX-M-65}$.

For the CTX-M-1group of *bla* genes, consistent with other studies, a 48-bp sequence, previously called the W sequence, was detected between $bla_{CTX-M-15}$ and IS*Ecp1* [11,28], whereas a 45-bp spacer sequence has been identified in 18 of 22 $bla_{CTX-M-55}$ copies, as well as in other *bla* genes such as $bla_{CTX-M-64}$ [6], $bla_{CTX-M-79}$ (GenBank accession no. FJ169498) and $bla_{CTX-M-82}$ (GenBank accession number DQ256091). Interestingly, both CTX-M-79 and CTX-M-82 were originally and recently reported from China [21,29]. In the remaining four isolates, a 48-bp W sequence was detected upstream of $bla_{CTX-M-55}$, which was the same as that described in isolates from Thailand and the UK [22,23,28]. PFGE analysis showed very diverse patterns in these bla_{CTX-M}-positive *E. coli* isolates and suggested that the high prevalence of bla_{CTX-M} in *E. coli* is a result of horizontal transfer of ESBL genes among bacteria instead of the pandemic spread of single clones.

The present study revealed that more than 60% of the isolates carrying CTX-M-9 subgroup genes showed reduced susceptibilities to amikacin, a transferable trait (data not shown).

A recent study reported the co-localization of $bla_{CTX-M-14}$ and *rmtB* on a broad-host-range IncA/C plasmid [30]. The association of different resistance markers on the same plasmid can also promote the spread of ESBLs by co-selection. Approximately 30% of the diseased animals in the present study had been treated with amikacin (data not shown); thus, amikacin might have positively selected for the ESBL producers.

In conclusion, a surprisingly high prevalence of ESBL producers was detected in *E. coli* isolates from companion

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animals in China, with CTX-M type enzymes being most predominant. It is necessary to examine the factors that contributed to their selection and dissemination. This is the first report of the emergence of CTX-M-64 in China and in animals. The clinical significance of the finding that CTX-M-55 and CTX-M-14 were simultaneously detected in six isolates is yet to be ascertained. The present study extends our knowledge of the prevalence and evolution of ESBLs in both China and throughout the world. Attention should be drawn to this serious situation of ESBL-producing *E. coli* as well as other bacteria in companion animals.

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Transparency Declaration

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References

1. Collignon P, Aarestrup FM. Extended-spectrum beta-lactamases, food, and cephalosporin use in food animals. *Clin Infect Dis* 2007; 44: 1391–1392.
2. Perez F, Endimiani A, Hujer KM *et al.* The continuing challenge of ESBLs. *Curr Opin Pharmacol* 2007; 7: 459–469.
3. Bonnet R. Growing group of extended-spectrum ß-lactamases: the CTX-M enzymes. *Antimicrob Agents Chemother* 2004; 48: 1–14.
4. Cantón R, Coque TM. The CTX-M beta-lactamase pandemic. *Curr Opin Pharmacol* 2006; 9: 466–475.
5. Pitout JD, Laupland KB. Extended-spectrum beta-lactamase-producing *Enterobacteriaceae*: an emerging public-health concern. *Lancet Infect Dis* 2008; 8: 159–166.
6. Nagano Y, Nagano N, Wachino J *et al.* Novel chimeric beta-lactamase CTX-M-64, a hybrid of CTX-M-15-like and CTX-M-14 beta-lactamases, found in a *Shigella sonnei* strain resistant to various oxyimino-cephalosporins, including ceftazidime. *Antimicrob Agents Chemother* 2009; 53: 69–74.
7. Carattoli A. Animal reservoirs for extended spectrum beta-lactamase producers. *Clin Microbiol Infect* 2008; 14 (suppl 1): 117–123.
8. Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial disk susceptibility tests for bacteria isolated from animals; Approved standard*, 3rd edn. Document M31-A3. Wayne, PA: CLSI, 2008.
9. Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing: eighteenth informational supplement M100-S18*. Wayne, PA: CLSI, 2008.
10. Liu JH, Wei SY, Ma JY *et al.* Detection and characterization of CTX-M and CMY-2 ß-lactamases among *Escherichia coli* isolates from farm animals in Guangdong Province of China. *Int J Antimicrob Agents* 2007; 29: 576–581.
11. Eckert C, Gautier V, Arlet G. DNA sequence analysis of the genetic environment of various bla_{CTX-M} genes. *J Antimicrob Chemother* 2006; 57: 14–23.
12. Gautom RK. Rapid pulsed-field gel electrophoresis protocol for typing of *Escherichia coli* O157:H7 and other gram-negative organisms in 1 day. *J Clin Microbiol* 1997; 35: 2977–2980.
13. Tenover FC, Arbeit RD, Goering RV *et al.* Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995; 33: 2233–2239.
14. Carattoli A, Lovari S, Franco A *et al.* Extended-spectrum ß-lactamases in *Escherichia coli* isolated from dogs and cats in Rome, Italy, from 2001 to 2003. *Antimicrob Agents Chemother* 2005; 49: 833–835.
15. Costa D, Poeta P, Brinas L *et al.* Detection of CTX-M-1 and TEM-52 beta-lactamases in *Escherichia coli* strains from healthy pets in Portugal. *J Antimicrob Chemother* 2004; 54: 960–961.
16. Lim SK, Lee HS, Nam HM *et al.* CTX-M-type beta-lactamase in *Escherichia coli* isolated from sick animals in Korea. *Microb Drug Resist* 2009; 15: 139–142.
17. Ma J, Zeng Z, Chen Z *et al.* High prevalence of plasmid-mediated quinolone resistance determinants *qnr*, *aac(6′)-Ib-cr*, and *qepA* among ceftiofur-resistant Enterobacteriaceae isolates from companion and food-producing animals. *Antimicrob Agents Chemother* 2009; 53: 519–524.
18. Wang F, Zhu D, Hu F *et al.* CHINET 2007 surveillance of bacterial resistance in China. *Chin J Infect Chemother* 2008; 18: 325–333.
19. Tian GB, Wang HN, Zou LK *et al.* Detection of CTX-M-15, CTX-M-22, and SHV-2 extended-spectrum beta-lactamases (ESBLs) in *Escherichia coli* fecal-sample isolates from pig farms in China. *Foodborne Pathog Dis* 2009; 6: 297–304.
20. Yu YS, Ji SJ, Chen YG *et al.* Resistance of strains producing extended-spectrum β-lactamases and genotype distribution in China. *J Infect* 2007; 54: 53–57.
21. Liu W, Chen L, Li H *et al.* Novel CTX-M {beta}-lactamase genotype distribution and spread into multiple species of *Enterobacteriaceae* in Changsha, Southern China. *J Antimicrob Chemother* 2009; 63: 895–900.
22. Kiratisin P, Apisarnthanarak A, Saifon P *et al.* The emergence of a novel ceftazidime-resistant CTX-M extended-spectrum beta-lactamase, CTX-M-55, in both community-onset and hospital-acquired infections in Thailand. *Diagn Microbio Infect Dis* 2007; 58: 349–355.
23. Hopkins KL, Threlfall EJ, Karisik E *et al.* Identification of novel plasmid-mediated extended-spectrum beta-lactamase CTX-M-57 in *Salmonella enterica* serovar Typhimurium. *Int J Antimicrob Agents* 2008; 31: 85–86.
24. Song W, Lee H, Lee K *et al.* CTX-M-14 and CTX-M-15 enzymes are the dominant type of extended-spectrum beta-lactamase in clinical isolates of *Escherichia coli* from Korea. *J Med Microbiol* 2009; 58: 261–266.
25. Shi WF, Zhou J, Qin JP. Transconjugation and genotyping of the plasmid-mediated AmpC beta-lactamase and extended-spectrum beta-lactamase genes in *Klebsiella pneumoniae*. *Chin Med J* 2009; 122: 1092–1096.

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26. Poirel L, Decousser JW, Nordmann P. Insertion sequence IS*Ecp1B* is involved in expression and mobilization of a bla_{CTX-M} betalactamase gene. *Antimicrob Agents Chemother* 2003; 47: 2938–2945.
27. Vinue′ L, Sa′enz Y, Martı′nez S *et al.* Prevalence and diversity of extended spectrum β-lactamases in faecal *Escherichia coli* isolates from healthy humans in Spain. *Clin Microbiol Infect* 2009; 15: 1–4.
28. Kiratisin P, Apisarnthanarak A, Laesripa C *et al.* Molecular characterization and epidemiology of extended-spectrum-beta-lactamase-producing Escherichia coli and *Klebsiella pneumoniae* isolates causing health care-associated infection in Thailand, where the CTX-M family is endemic. *Antimicrob Agents Chemother* 2008; 52: 2818–2824.
29. Tian SF, Chen BY, Chu YZ *et al.* Prevalence of rectal carriage of extended-spectrum beta-lactamase-producing *Escherichia coli* among elderly people in community settings in China. *Can J Microbiol* 2008; 54: 781–785.
30. Kang HY, Kim J, Seol SY *et al.* Characterization of conjugative plasmids carrying antibiotic resistance genes encoding 16S rRNA methylase, extended-spectrum beta-lactamase, and/or plasmid-mediated AmpC beta-lactamase. *J Microbiol* 2009; 47: 68–75.

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