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Prevalence of ESBL/AmpC genes and specific clones among the third-generation cephalosporin-resistant *Enterobacteriaceae* from canine and feline clinical specimens in Japan

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ABSTRACT

In recent years, besides the widespread occurrence of extended-spectrum β-lactamase (ESBL)- and/or plasmid-mediated AmpC (pAmpC)-producing *Enterobacteriaceae* in both healthcare and community settings of humans, the third-generation cephalosporin (3GC)-resistant microbes have also been reported from companion animals worldwide. Here, we characterized ESBL- and/or pAmpC-producing *Enterobacteriaceae* clinical isolates from companion animals. Among the 487 clinical isolates mainly from urine of dogs and cats between May and September 2016, 104 non-repetitive isolates were resistant to the 3GC, and they consisted of 81 of 381 (21.3%) *Escherichia coli*, 21 of 50 (42.0%) *Klebsiella pneumoniae*, and 2 of 56 (3.6%) *Proteus mirabilis* isolates. In the 81 *E. coli*, the predominant *bla* genes were $bla_{CTX-M-27}$ and bla_{CMY-2} (n = 15 each), followed by $bla_{CTX-M-15}$ (n = 14), $bla_{CTX-M-14}$ (n = 10), and $bla_{CTX-M-55}$ (n = 5). In 21 *K. pneumoniae*, 10 *bla* gene types including $bla_{CTX-M-15}$ (n = 4), $bla_{CTX-M-2}$ (n = 4), and $bla_{CTX-M-14}$ (n = 3) were found. The $bla_{CTX-M-2}$ was identified in 2 *P. mirabilis*. Twenty-four of the 42 *E. coli* belonging to phylogroup B2 were O25b-ST131 clone, mostly associated with uropathogenic *E. coli* pathotype, and 22 isolates of this clone were identified as specific *H*30R subclone. High prevalence of the $bla_{CTX-M-27}$-harboring isolates were noted among the *H*30R/non-Rx lineage (13/19, 68.4%) ($p < 0.05$). The genetic environment of $bla_{CTX-M-27}$ of most isolates of this lineage was identical to that of human isolates, but unique flanking genetic structures were also identified. Newly emerging virulent lineage B2-non-O25b-ST1193 was also confirmed in 5 isolates. The *fosA3* and/or *armA* genes were detected in *E. coli* and *K. pneumoniae* isolates. These data suggest that companion animals serve as a potential reservoir of antimicrobial resistant *E. coli* and *K. pneumoniae*. This also has considerable veterinary importance, since urinary tract infections are an important disease causing therapeutic challenges worldwide.

1. Introduction

Over the last two decades, extended-spectrum-β-lactamase (ESBL)- and plasmid-mediated AmpC (pAmpC)-producing *Enterobacteriaceae* exhibiting resistance to the third-generation cephalosporins (3GC) have been increasingly isolated in humans. In recent years, besides the widespread occurrence of ESBL- and/or pAmpC-producing *Enterobacteriaceae* in both healthcare and community settings of humans, the 3GC-resistant microbes have also been reported from food animals, food products, companion animals, and environmental sources worldwide (Bevan et al., 2017).

Among the pAmpC β-lactamase genes, particularly bla_{CMY-2} and bla_{DHA-1} are the most common in *Escherichia coli* and *Klebsiella pneumoniae*, respectively, of human and companion animal isolates (Li et al., 2008; Wohlwend et al., 2015; Harada et al., 2016). In particular, frequency of bla_{CMY-2} in *E. coli* isolates from companion animal has been reported to be significantly higher than in those from human (Bortolaia et al., 2014).

Nowadays, CTX-M-15 and CTX-M-14 are the most common types of ESBLs among human isolates, though CTX-M-14 has mainly been reported in Japan and other Asian countries (Zhao and Hu, 2013). A pandemic-specific O25b-ST131 clone with multi-resistance and a high

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virulence potential is largely associated with the global increase of these ESBL producers. The $bla_{CTX-M-15}$ gene is most closely related with this clone, however, $bla_{CTX-M-14}$ was also reported in Japan, Canada, and Spain (Suzuki et al., 2009; Peirano et al., 2010; Dahbi et al., 2013). The O25b-ST131 clone harboring $bla_{CTX-M-15}$ or $bla_{CTX-M-14}$ has also been confirmed among companion animals in many countries (Ewers et al., 2010; Harada et al., 2012; Kawamura et al., 2017). Transmission of the *E. coli* ST131 clone among family members and companion animals has been documented, increasing the risk in human of causing severe infections difficult to be treated (Johnson et al., 2009). The $bla_{CTX-M-27}$, a single-nucleotide variant of $bla_{CTX-M-14}$ has been increasingly identified among the isolates from human and companion animals in Asian countries including Japan, European countries, and the US (Harada et al., 2012; Matsumura et al., 2015; Bevan et al., 2017; Kawamura et al., 2017).

In Japan, strict regulation of antimicrobial usage in food animals has been executed by the Government. However, little attention has so far been paid to the use of antimicrobials in companion animals, which was left to the prescription of individual veterinarian. This study was aimed to investigate the presence of ESBL and/or pAmpC genes among the 3GC-resistant *Enterobacteriaceae* derived from companion animals for better understanding the dissemination dynamics of not only the antimicrobial resistance genes, but also their host bacterial clones. The findings obtained would be useful to assess the possible circulation of those resistance genes and bacterial clones across human and companion animals.

2. Materials and methods

2.1. Bacterial isolates

During May–September 2016, bacterial isolates were recovered from specimen of companion animals in clinical settings and hospitals throughout Japan. Most of the samples were urine, but also some from other clinical infections were investigated. They were identified using NegCombo BPC1J panel and Microscan WalkAway plus system according to the manufacturer's instructions (Beckman Coulter, Inc, Tokyo, Japan). For some *E. coli* isolates, the identification was confirmed by MALDI-TOF MS assay using ≥2.000 score cut-offs for species-level identification as recommended by the manufacturer (Bruker Daltonics, Bremen, Germany). A total of 381 *E. coli* isolates including 296 and 85 of canine and feline origin, respectively, 50 *K. pneumoniae* isolates including 42 and 8 of canine and feline origin, respectively, and 56 *Proteus mirabilis* isolates including 53 and 3 of canine and feline origin, respectively, were subjected to the study.

2.2. Antimicrobial susceptibility testing

MICs for the isolates were determined by the CLSI broth microdilution method according to CLSI document M07-A10 (Clinical and Laboratory Standards Institute (CLSI, 2015) using the same panel and system described for bacterial identification, and the results were interpreted using CLSI M100-S27 standard (Clinical and Laboratory Standards Institute (CLSI, 2017) for human pathogens except for the interpretation of MICs of colistin (≤2 and > 2 mg/L for colistin susceptible and resistant, respectively) followed by the EUCAST standard (European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2017).

For the 3GC-resistant isolates, ESBL and/or AmpC production was confirmed by MADISCS ID™ ESBL and AmpC Detection Discs (Kanto Chemical Co., Inc., Tokyo, Japan).

2.3. PCR detection and sequencing of ESBL and pAmpC genes

ESBL genes bla_{TEM} and bla_{SHV} types, and $bla_{CTX-M-1}$, $bla_{CTX-M-2}$, $bla_{CTX-M-9}$, and $bla_{CTX-M-8}$ groups were sought by PCR as described previously (Nagano et al., 2009). Screening of pAmpC gene groups, ACC, FOX, MOX, DHA, CIT, and EBC, was performed by multiplex PCR (Perez-Perez and Hanson, 2002).

The nucleotide sequence of full-length *bla* gene was determined by direct sequencing of PCR products obtained using the primer sets listed in Supplementary Table S1. Briefly, PCR amplicons derived from each *bla* gene were purified with a Wizard SV Gel and PCR Clean Up system (Promega, Madison, WI, USA), and sequenced directly on both strands with a BigDye Terminator v1.1 Cycle Sequencing Kit and an ABI 3730*xl* genetic analyzer (Applied Biosystems, Foster City, CA, USA). The nucleotide and deduced amino acid sequences were analyzed with the BLAST programs (http://www.ncbi.nlm.nih.gov/blast). The ClustalW program (http://www.ebi.ac.uk/clustalw) was used for the analysis of the alignment of the deduced amino acid sequences of β-lactamases.

The genetic environments surrounding bla_{CTX-M} genes were analyzed by PCR mapping and DNA sequencing using primers described in Table S2 with reference to commonly detectable structures (Zhao and Hu, 2013).

2.4. Detection of plasmidic 16S rRNA methyltransferase and fosfomycin resistance genes

For aminoglycoside-resistant isolates, plasmid-mediated 16S rRNA methyltransferase genes, *armA*, *rmtB*, and *rmtC*, were detected as described previously (Doi and Arakawa, 2007). For fosfomycin-resistant isolates, plasmid-mediated fosfomycin resistance genes, *fosA3* and *fosC2*, were detected as described previously (Wachino et al., 2010).

2.5. Classification of ExPEC and UPEC pathotypes

For all the 3GC-resistant *E. coli* isolates belonging to phylogroup B2, virulence genes responsible for extraintestinal infections were analyzed by multiplex PCR (Johnson et al., 2015). Pathotypes of *E. coli* were presumed to be extraintestinal pathogenic *E coli* (ExPEC) if positive for ≥2 of 5 markers, including *papAH* and/or *papC*, *sfa/focDE*, *afa/draBC*, *kpsM* II and *iutA*, and to be uropathogenic *E coli* (UPEC) if positive for ≥3 of 4 markers, including *chuA*, *fyuA*, *vat* and *yfcV* (Johnson et al., 2015).

2.6. Molecular typing of E. coli and K. pneumoniae isolates

Phylogenetic grouping of *E. coli* isolates harboring ESBL and/or pAmpC genes was done by multiplex PCR as described previously (Clermont et al., 2013).

Sequence types (STs) of *K. pneumoniae* isolates harboring ESBL and/or pAmpC genes were determined by the multi-locus sequencing typing (MLST) method according to the *K. pneumoniae* MLST website (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html). For *E. coli* isolates harboring ESBL and/or pAmpC genes and belonging to the phylogroup B2, STs were determined according to the *E. coli* MLST website (http://mlst.warwick.ac.uk/mlst/mlst/dbs/Ecoli). Additionally, the *fimH*30 (*H*30) subclone of ST131 was detected by PCR, and ciprofloxacin-resistant *H*30 isolates were identified as *H*30R (Colpan et al., 2013). The *H*30Rx lineage within *H*30R subclone was identified by sequencing of PCR product for the detection of SNP-264 as described previously (Price et al., 2013). Distributions of bla_{CTX-M} genes among the *H*30R and non-*H*30R subclones of B2-O25b-ST131clone were analyzed using the Chi-square test with Yates correction.

2.7. E. coli O antigen typing

ESBL and/or pAmpC gene-positive *E. coli* isolates belonging to serotype O25 were identified using *E. coli* antisera (Denka Seiken, Tokyo, Japan) according to the manufacturer's instructions.

Additionally, for *E. coli* isolates belonging to ST131, the O25b molecular subtype was identified by PCR as described previously

(Clermont et al., 2008).

3. Results

3.1. Prevalence of ESBL- and/or pAmpC-producing Enterobacteriaceae

Over the five-month period, a total of 487 *Enterobacteriaceae* isolates were collected, all of them were susceptible to carbapenems except for some *P. mirabilis* isolates which exhibited reduced susceptibility to carbapenems, as noted in CLSI M100-S27 (Clinical and Laboratory Standards Institute (CLSI, 2017). A total of 104 non-repetitive isolates resistant to the 3GC were found from canine and feline clinical samples. Of these animals 61 were hospitalized, 20 were not hospitalized and from 23 the status was unknown. Most isolates were recovered from urine specimen (59/104) (Table S3). Of 381 *E. coli* isolates identified 81 (59 and 22 of canine and feline origin, respectively) were 3GC-resistant (21.3%). Among 50 *K. pneumoniae* isolates, 21 (16 and 5 of canine and feline origin, respectively) were 3GC-resistant (42.0%). For *P. mirabilis* isolates, 2 (canine origin) of 56 (3.6%) were resistant to the 3GC. Phenotypic test revealed that 78 isolates including 59 *E. coli*, 17 *K. pneumoniae*, and 2 *P. mirabilis* were confirmed to be ESBL producers, and 21 isolates including 19 *E. coli* and 2 *K. pneumoniae* were pAmpC producers. Co-production of both ESBL and pAmpC was detected in 3 *E. coli* and 2 *K. pneumoniae* isolates (Table S3).

3.2. Antimicrobial susceptibilities of ESBL- and/or pAmpC-producers

As shown in Supplementary Table S4, the ESBL-producing organisms were resistant to cefpodoxime, ceftazidime, cefotaxime, cefepime, and aztreonam with MIC_{90} values of > 4 mg/L, > 8 mg/L, > 2 mg/L, > 16 mg/L, and > 8 mg/L, respectively. They also exhibited co-resistance to gentamicin and levofloxacin. The *E. coli* pAmpC producers had the same resistance profiles against those antimicrobials except for cefepime. One isolate of *K. pneumoniae* co-producing ESBL and pAmpC showed resistance to colistin.

3.3. Molecular characterization of the ESBL and pAmpC genes

Among the 78 ESBL-producing isolates, the most common types of ESBL genes identified were $bla_{CTX\text{-}M\text{-}15}$ (n = 18), $bla_{CTX\text{-}M\text{-}27}$ (n = 15), and $bla_{CTX\text{-}M\text{-}14}$ (n = 13), which were followed by $bla_{CTX\text{-}M\text{-}2}$ (n = 9) and $bla_{CTX\text{-}M\text{-}55}$ (n = 7). A high frequency of $bla_{CMY\text{-}2}$ (n = 15), followed by $bla_{DHA\text{-}1}$ (n = 6) was found in the 21 pAmpC producers. The $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}27}$, and $bla_{CTX\text{-}M\text{-}55}$ were also found among co-producers of different ESBL types or those of ESBL and pAmpC (Table S3).

In the 81 *E. coli* isolates resistant to the 3GC, the predominant *bla* gene was $bla_{CTX\text{-}M\text{-}27}$ and $bla_{CMY\text{-}2}$ (n = 15 each), and followed by $bla_{CTX\text{-}M\text{-}15}$ (n = 14), $bla_{CTX\text{-}M\text{-}14}$ (n = 10), and $bla_{CTX\text{-}M\text{-}55}$ (n = 5). Co-harboring $bla_{CTX\text{-}M\text{-}55}$ and $bla_{CMY\text{-}2}$, $bla_{CTX\text{-}M\text{-}3}$ and $bla_{CTX\text{-}M\text{-}27}$, and $bla_{CTX\text{-}M\text{-}55}$ and $bla_{CTX\text{-}M\text{-}2}$ were found in 3, 1, and 1, respectively (Tables 1 and 2). Ten *bla* gene types including $bla_{CTX\text{-}M\text{-}15}$ (n = 4), $bla_{CTX\text{-}M\text{-}2}$ (n = 4), and $bla_{CTX\text{-}M\text{-}14}$ (n = 3) were identified in 21 *K. pneumoniae* isolates. Co-harboring $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CMY\text{-}2}$, and $bla_{DHA\text{-}1}$ and $bla_{SHV\text{-}12}$ were detected in one isolate each (Table 3). The $bla_{CTX\text{-}M\text{-}2}$ was identified in 2 *P. mirabilis* isolates.

3.4. Genetic environment of $bla_{CTX\text{-}M}$

Among 15 isolates harboring the $bla_{CTX\text{-}M\text{-}27}$, 12 isolates of *E. coli* B2-O25b-ST131-*H*30R/non-Rx shared flanking genetic structures, IS*26*-ΔIS*Ecp1*-$bla_{CTX\text{-}M\text{-}27}$-ΔIS*903D*-IS*26*, with variation in the length of ΔIS*903D* (338 bp, 340 bp or 391 bp) (Table 4). The $bla_{CTX\text{-}M\text{-}14}$ was associated with the common elements IS*Ecp1* and ΔIS*903* or IS*903* in all of 13 isolates among diverse clones including *E. coli* ST131, but with new amino acid substitution K156N in *tnpA* of IS*903* in 3 *K. pneumoniae* ST655 isolates. The $bla_{CTX\text{-}M\text{-}15}$ had classical flanking genetic structures in 17 including 2 *E. coli* B2-O25b-ST131-*H*30R/Rx, that is, it was bracketed upstream and downstream by IS*Ecp1* and *orf477*, respectively, and upstream by IS*26*-truncated IS*Ecp1* in 2 isolates including one *E. coli* B2-O25b-ST131-*H*30R/Rx. In one *K. pneumoniae* ST709 isolate, the $bla_{CTX\text{-}M\text{-}15}$ gene was preceded uniquely by IS*Ecp1*-IS*1*-IS*Ecp1*. The flanking genetic structures of $bla_{CTX\text{-}M\text{-}55}$ in 11 isolates was basically the same as that of $bla_{CTX\text{-}M\text{-}15}$. Namely, IS*Ecp1*-$bla_{CTX\text{-}M\text{-}55}$-*orf477* was discovered in 8 isolates with variation in the length of IS*Ecp1*-$bla_{CTX\text{-}M\text{-}55}$ spacer regions, 45 bp or 48 bp. In the remaining 3 isolates, IS*26*-truncated IS*Ecp1* was identified upstream of the $bla_{CTX\text{-}M\text{-}55}$, and IS*26*-flanked *fosA3* was further located downstream of *orf477* in one *E. coli* isolate.

3.5. Molecular lineage analysis of E. coli isolates

The 81 *E. coli* isolates harboring *bla* genes that showed resistance to the 3GC mainly assigned phylogroup B2 (42 isolates, 51.9%), followed by phylogroups F (16 isolates, 19.8%), D (9 isolates, 11.1%), and B1 (8 isolates, 10.0%) as shown in Tables 1 and 2. Phylogroup B2 isolates were comprised of 24 O25b isolates, where presumed UPEC and ExPEC were 22 (91.7%) and 15 isolates (62.5%), respectively, and 18 non-O25b isolates, where presumed UPEC and ExPEC were 14 (77.8%) and 12 isolates (66.7%), respectively (Table 1). All of those 24 O25b isolates belonged to the pandemic ESBL-producing *E. coli* B2-O25b-ST131 clone, though two of them did not agglutinate with the O25 antiserum. Furthermore, 19 isolates of the 24 B2-O25b-ST131 clone mostly associated with UPEC pathotype were identified as specific B2-O25b-ST131-*H*30R/non-Rx lineage, among the 19 high prevalence of the $bla_{CTX\text{-}M\text{-}27}$-harboring isolates was found (13/19, 68.4%) ($p < 0.05$). Of 5 $bla_{CTX\text{-}M\text{-}15}$-harboring isolates belonging to B2-O25b-ST131-*H*30R subclone, only 3 were assigned to *H*30R/Rx lineage. The STs and *bla* gene types were diverse among 18 B2-non-O25b isolates, where ST1193 (5/18, 27.8%) and ST131 (4/18, 22.2%) were identified (Table 1). The other 9 B2-non-O25b isolates were assigned to ST38 (2/18), ST12 (2/18), ST372 (2/18), ST95 (1/18), ST127 (1/18), and ST1262 (1/18). The *bla* genes were diversely detected among non-O25 isolates belonging to the phylogroups F, which was predominantly detected, D, B1, clade I or II, A, and C (Table 2). Plasmid-mediated fosfomycin-resistance determinant *fosA3* gene was detected in an isolate co-harboring $bla_{CTX\text{-}M\text{-}55}$ plus $bla_{CMY\text{-}2}$ genes, and a $bla_{CTX\text{-}M\text{-}27}$-harboring isolate, both of which belonged to the phylogroup F.

3.6. ST distribution of K. pneumoniae isolates

The STs of the 21 *bla*-harboring *K. pneumoniae* isolates showing resistance to the 3GC were classified into 9 types, in which the 7 isolates (33.3%) were identified to belong to international clone ST15. A $bla_{DHA\text{-}1}$-harboring ST15 isolate had the plasmid-mediated 16S rRNA methylase gene *armA*, and a $bla_{DHA\text{-}1}$- and $bla_{SHV\text{-}12}$-harboring ST37 isolate had *armA* and *fosA3* (Table 3).

4. Discussion

In Japan, the antimicrobial resistance (AMR) action plan lists companion animals into the subjects in which researches and surveys to countermeasure AMR need to be conducted. Despite sharing their habitation with human, study findings on antimicrobial resistant microbes in companion animals have been limited. Thus, we were prompted to conduct the present study to investigate the prevalence status of ESBL and pAmpC genes, and ESBL- and pAmpC-producing lineages among 104 3GC-resistant *Enterobacteriaceae* clinical isolates recovered from canine and feline patients which are particularly in close contact with humans.

The Japan Nosocomial Infections Surveillance (JANIS) data shows a notable increase in the frequency of cefotaxime resistance in *E. coli* and *K. pneumoniae* human clinical isolates from 12.6% and 3.3% in 2014 to

Table 1
Serotypes, sequence types, and pathotypes among the third-generation cephalosporin-resistant *Escherichia coli* phylogroup B2 harboring *bla* genes.

Phylogroup	Serotype	*n*	Sequence type	*n*	Subclone/ lineage	*n*	*bla* genes	*n*	Pathotypes[a]	
									UPEC	ExPEC
B2	O25b	24	ST131	24	*H*30R/ non-Rx	19	CTX-M-27	12[b,*]	12	10
							CTX-M-3 + CTX-M-27	1[*]	1	0
							CTX-M-15	2	2	1
							CTX-M-14	1	0	1
							CTX-M-3	3	2	0
					*H*30R/Rx	3	CTX-M-15	3[b]	3	3
					non-*H*30	2	CTX-M-14	1	1	0
							CMY-2	1	1	0
	non-O25b	18	ST131	4	non-*H*30	4	CTX-M-15	1	0	1
							CTX-M-14	2	0	0
							CTX-M-2	1	1	0
			ST1193	5	NA		CTX-M-27	2	2	2
							CTX-M-15	1	1	1
							CMY-2	1	1	1
							CTX-M-55	1	1	1
			ST38	2	NA		CTX-M-15	1	1	1
							CMY-2	1	1	0
			ST12	2	NA		CMY-2	1	1	1
							DHA-1	1	1	1
			ST372	2	NA		CMY-2	2	2	1
			ST95	1	NA		CTX-M-55	1	1	0
			ST127	1	NA		CTX-M-15	1	0	1
			ST1262	1	NA		CTX-M-14	1	1	1

ExPEC, extraintestinal pathogenic *E coli*; UPEC, uropathogenic *E coli*; NA, not analyzed.
[a] Pathotypes of ExPEC (if positive for ≥2 of 5 markers, including *papAH* and/or *papC, sfa/focDE, afa/draBC, kpsM* II and *iutA*) and UPEC (if positive for ≥3 of 4 markers, including *chuA, fyuA, vat* and *yfcV*) were determined (Johnson et al., 2015).
[b] Including one which did not cause bacterial agglutination with *E. coli* O25 antiserum, but was positive for the O25b molecular subtype.
* Significant association was observed between $bla_{\text{CTX-M-27}}$ and a specific lineage, B2-O25b-ST131-*H*30R/non-Rx ($p < 0.05$).

24.5% and 8.0% in 2015, respectively. For the clinical isolates from companion animals in 2016 in this study, the resistance rate of *E. coli* isolates to cefotaxime was found to be 21.3%, which was closely to the results reported in the JANIS as well as in the previous studies (Kawamura et al., 2017). However, in *K. pneumoniae*, the resistance rate to cefotaxime of 42.0% from canine and feline clinical isolates was far higher than those from JANIS data on human clinical isolates. A similar rate of cefotaxime resistance (34.0%) in *K. pneumoniae* isolates from companion animals has been reported in Japan, whereas resistance rate to 3GC has been 21.4% even in Italy (Donati et al., 2014; Harada et al., 2016). Thus, companion animals as a potential reservoir of antimicrobial resistant *K. pneumoniae* should be taken into account in Japan.

CTX-M-15-producing *K. pneumoniae* international clone ST15 is currently the most widespread clone among human and companion animals, involved in nosocomial infections in both (Haenni et al., 2012). Our results showed diversity in STs among *K. pneumoniae* isolates harboring ESBL and/or pAmpC genes, though ST15 were detected in relatively lower frequency among the isolates. The finding is inconsistent with previous study, where the predominance of CTX-M-15-producing *K. pneumoniae* ST15 clone has been noted in companion animals (Harada et al., 2016). The $bla_{\text{CTX-M-27}}$, a major ESBL gene in *E. coli* in our present study was not identified among the 21 *K. pneumoniae* isolates.

In *E. coli* isolates, their ESBL gene types were diverse, whereas $bla_{\text{CMY-2}}$ gene constituted a majority of the pAmpC genes, followed by $bla_{\text{DHA-1}}$. Frequency of main single ESBL or pAmpC genes in the 3GC-resistant *E. coli* was 15 (18.8%) for $bla_{\text{CTX-M-27}}$ and $bla_{\text{CMY-2}}$ each, 14 (17.3%) for $bla_{\text{CTX-M-15}}$, 10 (12.3%) for $bla_{\text{CTX-M-14}}$, and 5 (6.2%) for $bla_{\text{CTX-M-55}}$. Similar ESBL gene types but not their frequency have been reported in previous studies on companion animals and human (Harada et al., 2012; Matsumura et al., 2015; Kawamura et al., 2017). High frequency of $bla_{\text{CMY-2}}$ has also been found among the 3GC-resistant *E. coli* from companion animals in Japan (Okubo et al., 2014).

Though aminoglycosides and fosfomycin are important therapeutic options for human infections caused by 3GC-resistant Gram-negative

Table 2
Other phylogroups of the third-generation cephalosporin-resistant *Escherichia coli* harboring *bla* genes.

Phylo-group	Serotype	*n*	*bla* genes													
			CMY-2	CTX-M-15	CTX-M-14	CTX-M-1	CTX-M-55	DHA-1	CTX-M-55 + CMY-2	CTX-M-2	CTX-M-3	CTX-M-24	CTX-M-27	CTX-M-65	CTX-M-55 + CTX-M-2	SHV-12
F	non-O25	16	3	3	1	2	1	2	1[a]		1[a]	1	1			
D		9	3	1	4		1									
B1		8	2					1	2	1					1	1
Clade I or II		3[b]	1	1		1										
A		2					1							1		
C		1								1						
Total		39	9	5	5	3	3	3	3	2	1	1	1	1	1	1

[a] Harboring plasmid-mediated fosfomycin-resistance determinant *fosA3*.
[b] Identified as *E. coli* by MALDI-TOF MS assay.

Table 3
Sequence types of the third-generation cephalosporin-resistant *Klebsiella pneumoniae* harboring *bla* genes.

MLST sequence type	*n*	*bla* gene									
		CTX-M-2	CTX-M-15	CTX-M-14	CTX-M-3	CTX-M-55	DHA-1	CTX-M-15 + CTX-M-2	CTX-M-15 + CMY-2	DHA-1 + SHV-12	SHV-12
ST15	7	1				2	2[a]		1		1[b]
ST655	3			3							
ST11	2	2									
ST147	2				2						
ST307	2		2								
ST709	2	2						1			
ST4	1		1								
ST37	1									1[c]	
ST45	1		1								
Total	21	4	4	3	2	2	2	1	1	1	1

[a] Including one harboring plasmid-mediated 16S rRNA methylase gene *armA*.
[b] Belonging to a single-locus variant of ST15.
[c] Harboring plasmid-mediated 16S rRNA methylase gene *armA* and fosfomycin-resistance determinant *fosA3*.

bacteria, the status of use of those antimicrobials is not clear in companion animals. Moreover, reports on the detection of plasmid-mediated 16S rRNA methyltransferase genes conferring high-level resistance to various aminoglycosides and plasmid-mediated fosfomycin resistance genes were few in isolates from companion animals (Hou et al., 2012; Hidalgo et al., 2013). Our analysis revealed *fosA3* in 2 *E. coli* isolates, and *armA* in a bla_{DHA-1}-harboring *K. pneumoniae* ST15 isolate for the first time in Japan. In addition, both *fosA3* and *armA* were detected in a bla_{DHA-1}- and bla_{SHV-12}-harboring *K. pneumoniae* ST37 isolate, which also showed resistance to colistin and tigecycline by disrupting *mgrB* and *ramR* with IS*10R* insertions (Taniguchi et al., 2017). The detection frequency of those resistance genes was low, but their transmission between companion animals and humans could become a significant future public health concern.

E. coli B2-non-O25b-ST1193, a newly emerging virulent and resistant lineage in China was noted in 5 isolates belonging to both UPEC and ExPEC (Wu et al., 2017). This lineage has phenotypic characteristics of fluoroquinolone resistance and lactose non-fermenting, and has been prevalent in human clinical isolates associated with urinary tract infection in Korea (Chang et al., 2014). Our study confirmed the

Table 4
Flanking genetic structures of bla_{CTX-M} genes.

bla_{CTX-M}	Flanking genetic structures	Origin (no. of animals)		Bacterial host (no. of isolates)
		Dogs	Cats	
$bla_{CTX-M-27}$	IS*26*-ΔIS*Ecp1*(208 bp)-$bla_{CTX-M-27}$ -ΔIS*903D* (391 bp)-IS*26*	9	1	*E. coli H*30R/non-Rx (10)
	IS*26*-ΔIS*Ecp1*(208 bp)-$bla_{CTX-M-27}$ -ΔIS*903D* (338 bp)-IS*26*	1		*E. coli H*30R/non-Rx (1)
	IS*26*-ΔIS*Ecp1*(208 bp)-$bla_{CTX-M-27}$ -ΔIS*903D* (340bp)-IS*26*		1	*E. coli H*30R/non-Rx (1)
	IS*26*-ΔIS*Ecp1*(388 bp)-$bla_{CTX-M-27}$-IS*903D*	2	1	*E. coli* B2-ST1193 (2)/*E. coli* F (1)
$bla_{CTX-M-14}$	IS*Ecp1*-$bla_{CTX-M-14}$-IS*903*	1	2	*E. coli H*30R/non-Rx (1)/*E. col* non-O25b-ST131 (1)/*E. coli* B2-ST1262 (1)
	IS*Ecp1*-$bla_{CTX-M-14}$-IS*903*[a]	3		*K. pneumoniae* ST655 (3)
	IS*Ecp1*-$bla_{CTX-M-14}$-ΔIS*903*	6	1	*E. coli* O25b-ST131-non-*H*30 (2)/*E. coli* D (4)/F (1)
$bla_{CTX-M-15}$	IS*Ecp1*-(48 bp)- $bla_{CTX-M-15}$-*orf477*	13	3	*E.coli H*30R/Rx (2)/*E. coli H*30R-nonRx (2)/*E. coli* non-O25b-ST131 (1)/*E. coli* B2-ST127 (1)/*E. coli* B2-ST1193 (1)/*E. coli* B2-ST38 (1)/*E. coli* F (2)/D (1) *K. pneumoniae* ST4 (1)/ST15 (1)/ST45 (1)/ST307 (2)
	IS*Ecp1*-(48 bp)- $bla_{CTX-M-15}$-*orf477*[b]	1		*E. coli* F (1)
	IS*Ecp1*- IS*1*-IS*Ecp1*-(127 bp)-$bla_{CTX-M-15}$-*orf477*		1	*K. pneumoniae* ST709 (1)
	IS*26*-ΔIS*Ecp1*(497 bp)-(48 bp)- $bla_{CTX-M-15}$-*orf477*		2	*E. coli H*30R/Rx (1)/*E. coli* Clade I or II (1)
$bla_{CTX-M-55}$	IS*Ecp1*-(45 bp)-$bla_{CTX-M-55}$-*orf477*	2	3	*E.coli* B2-ST95 (1)/*E. coli* B1 (2) *K. pneumoniae* ST15 (2)
	IS*Ecp1*-(48 bp)-$bla_{CTX-M-55}$-*orf477*	2	1	*E. coli* B2-ST1193 (1)/*E. coli* A(1)/F(1)
	IS*26*-ΔIS*Ecp1*(497 bp)-(48 bp)-$bla_{CTX-M-55}$-*orf477*	1		*E. coli* B1 (1)
	IS*26*-ΔIS*Ecp1*(242 bp)-(127bp)-$bla_{CTX-M-55}$-*orf477* -Δbla_{TEM}-*IS26-fosA3*	1		*E. coli* F (1)
	IS*26*-ΔIS*Ecp1*(242 bp)-(127 bp)-$bla_{CTX-M-55}$-*orf477*		1	*E. coli* D (1)
$bla_{CTX-M-2}$	IS*CR1*- $bla_{CTX-M-2}$-Δ*orf3*		1	*E. coli* B1 (1)
	IS*CR1*- $bla_{CTX-M-2}$	6	4	*E. coli* non-O25b-ST131 (1)/*E. coli* B1 (1)/C (1) *K. pneumoniae* ST11 (2)/ST15 (1)/ST709 (2) *P. mirabilis* (2)

*H*30R/non-Rx, B2-O25b-ST131-*H*30R: *H*30R/Rx, B2-O25b-ST131-*H*30Rx; non-O25b-ST131, B2-non-O25b-ST131; O25b-ST131-non-*H*30, B2- O25b-ST131-non-*H*30; A, B1, B2, C, D, F, and Clade I or II, phylogroups A, B1, B2, C, D, F, and Clade I or II.
[a] Containing K156N in *tnpA* of IS*903*.
[b] Containing E271K in *tnpA* of IS*Ecp1*.

emergence of *E. coli* B2-non-O25b-ST1193 among companion animals.

Highly similar antimicrobial resistance genes and genotypes of *E. coli* including pandemic UPEC lineage ST131 or those of *K. pneumoniae* have been reported among companion animals and human (Harada et al., 2012, 2016). This study revealed the dominant prevalence of *E. coli* harboring $bla_{CTX\text{-}M\text{-}27}$ among pandemic lineage, B2-O25b-ST131-*H*30R. This lineage also included $bla_{CTX\text{-}M\text{-}15}$-, $bla_{CTX\text{-}M\text{-}3}$-, and $bla_{CTX\text{-}M\text{-}14}$-encoding *E. coli.* Global widespread of ESBL-producing *E. coli* is associated with global clonal expansion of B2-O25b-ST131-*H*30R isolates harboring $bla_{CTX\text{-}M\text{-}15}$ (Mathers et al., 2015). Instead, *E. coli* harboring $bla_{CTX\text{-}M\text{-}14}$ combined with this lineage has been predominant in Japan (Matsumura et al., 2015). The $bla_{CTX\text{-}M\text{-}27}$-carrying *E. coli* B2-O25b-ST131-*H*30R/non-Rx, which emerged in the early 2000s has become increasingly prevalent domestically in human, where Matsumura et al. have observed that the $bla_{CTX\text{-}M\text{-}27}$ is confined to O25b-ST131-*H*30R/non-Rx lineage (Matsumura et al., 2015). However, the $bla_{CTX\text{-}M\text{-}27}$ was also detected among B2-non-O25-ST1193 (2 isolates) and F-non-O25 (1 isolate) though it was located within a rare flanking genetic structure, IS*26*-ΔIS*Ecp1* (388 bp)-$bla_{CTX\text{-}M\text{-}27}$-IS*903D* (Matsumura et al., 2016). In contrast, the surrounding genetic background of $bla_{CTX\text{-}M\text{-}27}$ genes, IS*26*-ΔIS*Ecp1*-$bla_{CTX\text{-}M\text{-}27}$ -ΔIS*903D* (391 bp)-IS*26* harbored by most *E. coli* B2-O25b-ST131-*H*30R/non-Rx in this study were identical to those harbored by human clinical isolates (Matsumura et al., 2015), suggesting circulation of $bla_{CTX\text{-}M\text{-}27}$-encoding *E. coli* B2-O25b-ST131-*H*30R/non-Rx lineage between human and companion animals in Japan. Analysis of the genetic environment of $bla_{CTX\text{-}M}$ genes also allowed us to detect variation in the length of truncated IS*903D*, 338 bp or 340 bp in two isolates of this pandemic *E. coli* lineage, which was unique to canine and feline isolates. So, such unique flanking genetic structures may possibly be derived from external sources such as pet food or environmental pool of antimicrobial resistance genes and/or clones (Baede et al., 2017; Gomi et al., 2017). Because of the close contact not only with human but also with those external sources, companion animals may play an important role in prevalence dynamics of antimicrobial resistance genes including *bla* genes and/or high-risk clonal lineage that could cause human infections.

In conclusion, our results showed high prevalence of the $bla_{CTX\text{-}M\text{-}27}$-harboring isolates among the pandemic *E. coli* B2-O25b-ST131-H30R/non-Rx lineage. The genetic environment of $bla_{CTX\text{-}M\text{-}27}$ harbored by most of isolates of this lineage was identical to those harbored by human isolates, but flanking genetic structures unique to companion animals were also identified. Newly emerging virulent and resistant lineage B2-non-O25b-ST1193 was also confirmed in 5 isolates. The *fosA3* and/or *armA* genes were detected from *E. coli* and *K. pneumoniae* isolates. These data suggest that companion animals serve as a potential reservoir of antimicrobial resistant *E. coli* and *K. pneumoniae*. This also has considerable veterinary importance, since urinary tract infections are an important disease causing therapeutic challenges worldwide.

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Conflict of interest statement

None.

Ethical approval

Not required.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.vetmic.2018.02.020.

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Table S1. Primers for sequencing of ESBL and pAmpC genes

Gene		Primers[a] (5' to 3')	Annealing temperature	Amplicon size (bp)
bla$_{CTX-M-1}$	Fw	CTTCCAGAATAAGGAATC	55℃	908 bp
	Rev	CCGTTTCCGCTATTACAA		
bla$_{CTX-M-2}$	Fw	TTAATGATGACTCAGAGCATTC		902 bp
	Rev	GATACCTCGCTCCATTTATTG		
bla$_{CTX-M-9}$	Fw	CTGATGTAACACGGATTGAC		913 bp
	Rev	TTACAGCCCTTCGGCGATGA		
bla$_{TEM}$	Fw	ATAAAATTCTTGAAGACGAAA		1080 bp
	Rev	GACAGTTACCAATGCTTAATCA		
bla$_{SHV}$	Fw	ATTTGTCG CTTCTTTACTCGC		1051 bp
	Rev	TTTATGGCGTTACCTTTGACC		
bla$_{CMY-2}$	Fw	ATGATGAAAAAATCGTTATGCT		562 bp
	Rev	TTATTGCAGCTTTTCAAGAATGCG		
bla$_{DHA-1}$	Fw	CACGGAAGGTTAATTCTGATG		1167 bp
	Rev	GGAAAAAAATTATTCCAGTGC		

[a]Fw, forward primer; Rev; reverse primer.

Table S2. Primers used for analyzing flanking genetic structures of bla_{CTX-M} genes

PCR target	Primer name	Sequence (5' to 3')
IS*Ecp1*	IS*Ecp1*-F	GCAGGTCTTTTTCTGCTCCTTG
	tnpA IS*Ecp1*-F	TCTCGCGTACTGAAACCAGA
IS*26*	IS*26*-F	AACGCGGAGTGAATGTCGAT
	tnpA IS*26*-F	CTGCTTACCAGGCGCATTTC
	IS*26*-R	GGAAGGGTTACGCCAGTACC
IS*CR1*	IS*CR1*-F	CCGGTTACACCAAAGCCTCT
IS*903*	IS*903*-R	CGCAACCTGACCATCGTA
	ΔIS*903*-R	CATCATCCAGCCAGAAAGTT
fosA3	*fosA3*-R	TCAATCAAAAAAGACCATC
Orf477	Orf477-R	AAACCTGTACGGTGCTGGAG
	Orf477 (inner)-R	CCGTACAGGTTTCCCCCAAT
TEM	TEM-R	CAGTGCTGCAATGATACCGC
Orf3	Orf3-R	ATCGAACTCGAGCGTTGACA
$bla_{CTX-M-1}$	CTX-M-1 (inner)-F	GCTGTTGTTAGGAAGTGTGC
	CTX-M-1 (inner)-R	CCATTGCCCGAGGTGAAG
$bla_{CTX-M-2}$	CTX-M-2 (inner)-F	TTTGCGATGTGCAGTACCAGTAA
	CTX-M-2 (inner)-R	AAATAGCAGGGGTAGCGTCG
$bla_{CTX-M-9}$	CTX-M-9 (inner)-F	ACCTATTTTACCCAGCCGCA
	CTX-M-9 (inner)-R	CACTCGTCTGCGCATAAAGC

Table S3. Characteristics of ESBL- and/or pAmpC-producing *E. coli* and *K. pneumoniae* isolates from companion animals

Phenotype	*bla* genes	Bacterial species (no. of isolates)	Origin (no. of animals)[a]	Source of isolate (no. of specimens)
ESBL (n=78)	CTX-M-15	*E. coli* (14) *K. pneumoniae* (4)	Dogs (14), Cats (4)	Urine (9), pus (4), ear discharge (3), nasal discharge (1), vaginal discharge (1)
	CTX-M-27	*E. coli* (15)	Dogs (12), Cats (3)	Urine (9), nasal discharge (3), cornea (1), ear discharge (1), bile (1)
	CTX-M-14	*E. coli* (10) *K. pneumoniae* (3)	Dogs (10), Cats (3)	Urine (8), ear discharge (2), pus (1), nasal discharge (1), prostate discharge (1)
	CTX-M-2	*K. pneumoniae* (4) *E. coli* (3) *P. mirabilis* (2)	Dogs (5), Cats (4)	Urine (8), ascites (1)
	CTX-M-55	*E. coli* (5) *K. pneumoniae* (2)	Dogs (3), Cats (4)	Urine (5), pus (2)
	CTX-M-3	*E. coli* (4) *K. pneumoniae* (2)	Dogs (4), Cats (2)	Urine (2), ear discharge (2), pus (2)
	CTX-M-1	*E. coli* (3)	Dogs (2), Cat (1)	Pus (3)
	SHV-12	*E. coli* (1) *K. pneumoniae* (1)	Dogs (2)	Urine (1), prostate discharge (1)
	CTX-M-15+CTX-M-2	*K. pneumoniae* (1)	Cat (1)	Urine (1)
	CTX-M-3+CTX-M-27	*E. coli* (1)	Dog (1)	Buccal swab (1)
	CTX-M-55+CTX-M-2	*E. coli* (1)	Dog (1)	Urine (1)
	CTX-M-65	*E. coli* (1)	Dog (1)	Pus (1)
	CTX-M-24	*E. coli* (1)	Dog (1)	Urine (1)
pAmpC (n=21)	CMY-2	*E. coli* (15)	Dogs (13), Cats (2)	Urine (9), pus (3), ear discharge (1), nasal discharge (1), milk (1)
	DHA-1	*E. coli* (4) *K. pneumoniae* (2[b])	Dogs (5), Cat (1)	Urine (2), pus (2), nasal discharge (1), ascites (1)
ESBL+pAmpC (n=5)	CTX-M-55+CMY-2	*E. coli* (3[c])	Dogs (2), Cat (1)	Urine (1), ear discharge (1), nasal discharge (1)
	CTX-M-15+CMY-2	*K. pneumoniae* (1)	Cat (1)	Urine (1)
	SHV-12+DHA-1	*K. pneumoniae* (1[d])	Dog (1)	Urine (1)

[a]comprising 61 inpatients, 20 outpatients, and 23 unknown patients
[b]including one harboring plasmid-mediated 16S rRNA methylase gene *armA*
[c]including one harboring plasmid-mediated fosfomycin-resistance determinant *fosA3*
[d]harboring plasmid-mediated 16S rRNA methylase gene *armA* and fosfomycin-resistance determinant *fosA3*

Table S4. MIC ranges and MIC_{90} values of antimicrobial agents for the third-generation cephalosporin-resistant *Escherichia coli* and *Klebsiella pneumoniae* isolated from companion animals

Antimicrobials	ESBL producers				pAmpC producers			ESBL+pAmpC producers	
	E. coli (n=59)		*K. pneumoniae* (n=17)		*E. coli* (n=19)		*K. pneumoniae* (n=2)	*E. coli* (n=3)	*K. pneumoniae* (n=2)
	MIC range (mg/L)	MIC_{90} (mg/L)	MIC range (mg/L)	MIC_{90} (mg/L)	MIC range (mg/L)	MIC_{90} (mg/L)	MIC range (mg/L)	MIC range (mg/L)	MIC range (mg/L)
piperacillin	>64	>64	>64	>64	≤16->64	>64	>64	>64	>64
piperacillin/tazobactam	≤16->64	≤16	≤16->64	≤16	≤16->64	≤16	≤16	≤16	64
cefpodoxime	>4	>4	>4	>4	>4	>4	>4	>4	>4
ceftazidime	≤4->8	>8	≤4->8	>8	≤4->8	>8	>8	>8	>8
cefotaxime	>2	>2	>2	>2	2->2	>2	>2	>2	>2
cefepime	≤8->16	>16	≤8->16	>16	≤8	≤8	≤8	>16	≤8->16
cefpirome	≤8->16	>16	16->16	>16	≤8	≤8	≤8	>16	16->16
cefmetazole	≤16	≤16	≤16	≤16	≤16->32	>32	>32	≤16->32	32->32
aztreonam	≤4->8	>8	≤4->8	>8	≤4->8	>8	8	>8	>8
imipenem	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1
meropenem	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1
gentamicin	≤4->8	>8	≤4->8	>8	≤4->8	>8	≤4->8	≤4	≤4->8
amikacin	≤16	≤16	≤16	≤16	≤16	≤16	≤16->32	≤16	≤16->32
levofloxacin	≤2->4	>4	≤2->4	>4	≤2->4	>4	>4	>4	>4
fosfomycin	≤4->16	≤4	≤4->16	>16	≤4->16	≤4	16	≤4->16	>16
colistin	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2->2