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Original Article

Detection of *Escherichia coli* Producing CTX-M-1-Group Extended-Spectrum β-Lactamases from Pigs in Aichi Prefecture, Japan, between 2015 and 2016

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SUMMARY: We investigated the prevalence and characteristics of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* isolates from Japanese pigs. A total of 345 pig fecal specimens were collected from 30 farms in the Aichi prefecture of Japan between June 2015 and April 2016, and 22 unique ESBL-producing *E. coli* were isolated from 16 samples spanning 8 farms. The ESBL types included CTX-M-15 (54.5%), CTX-M-55 (27.2%), CTX-M-3 (0.9%), and CTX-M-14 (0.9%). The predominant plasmid replicon type was IncN, and the isolates carried $bla_{\text{CTX-M-55}}$. Nine sequence type (ST)s, including ST117, ST1706, ST38, and ST10, were detected in the ESBL-producers, but no B2-O25-ST131 was found. ESBL producers were highly resistant to cefotaxime, ceftiofur, and tetracycline, but were susceptible to imipenem, amikacin, and fosfomycin (FOM), although 2 ST354 isolates showed resistance to ciprofloxacin. All 11 chloramphenicol-resistant isolates, including ST117 (n = 6) and ST38 (n = 3) isolates, harbored *floR*, and the 2 FOM-resistant ST38 isolates harbored *fosA3*. Our results suggest that pigs do not act as direct reservoirs in the transmission of ESBL genes to *E. coli* in humans. However, ST117 *E. coli* carrying IncN-type plasmids mediating $bla_{\text{CTX-M-55}}$ were isolated from several different farms, suggesting the potential for future spread in Japan. Therefore, plasmid sequence analyses and continuous surveillance are necessary from an epidemiological point of view and are required to better protect against ESBL-producer transmission.

INTRODUCTION

Extended-spectrum β-lactamases (ESBLs), which include the TEM-, SHV-, and CTX-M-types, can hydrolyze the β-lactam ring of the broad-spectrum β-lactams, such as the oxyimino-cephalosporins, and the AmpC-type β-lactamases. Recently, ESBL-producing *Escherichia coli* isolates have been increasingly detected in clinical settings, healthy humans, food-producing animals, companion animals, and the environment (1,2). The third-generation cephalosporins are frequently used in both clinical settings and the veterinary field; therefore, the widespread distribution of ESBL producers has become a major public health concern. Notably, the CTX-M-type ESBL producers have acquired co-resistance to additional classes of antimicrobial agents, such as the fluoroquinolones and aminoglycosides (2), necessitating special attention for the treatment of infectious diseases caused by these organisms.

The spread of the CTX-M-type ESBL gene is associated with the transmission of CTX-M-type ESBL-producing bacteria from various sources. Recently, the frequency of ESBL-producing *E. coli* isolates detected from food-producing animals including chickens, pigs, and cattle has increased, leading to the consideration of such animals as possible reservoirs of plasmid-mediated antimicrobial resistance genes for the dissemination of ESBL producers (3,4). Indeed, the transmission of bacteria between livestock and humans has been documented in the Netherlands (5,6). Because the food chain could be a major route of bacterial transmission to humans, we speculated that food-producing animals might also act as reservoirs for these drug-resistant microorganisms in Japan. Some investigations have found that ESBL-producing *E. coli* have been increasingly isolated from chicken meat samples (7,8). However, epidemiological data describing ESBL-producing *E. coli* isolated from pigs remain quite limited in Japan. To address this issue, we investigated the prevalence of ESBL-producing *E. coli* isolates among pig farms and conducted a molecular characterization of these isolates.

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MATERIALS AND METHODS

Bacterial strains: A total of 345 pigs from 30 farms were subjected to sampling of their intestinal content (fecal samples) just after slaughter in a slaughterhouse in the Aichi prefecture of Japan between June 2015 and April 2016 (Fig. 1). Only the name of each pig farm was

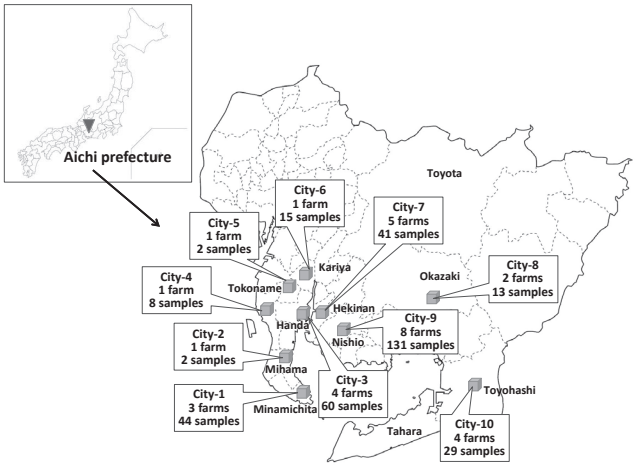


Fig. 1. Geographical locations of the 30 pig farms included in the present study in Aichi prefecture, Japan.

written on the sampling tube, and no detailed information about breeding conditions in the pig farms was provided. Within 24h of sample collection, fecal samples (~1 g) were inoculated onto MacConkey agar plates (Eiken Chemical Co., Ltd, Tokyo, Japan) supplemented with 1 mg/L of cefotaxime (CTX, Wako Pure Chemical Industries, Osaka, Japan) as described previously (9), and the plates were incubated at 35°C for 24 h. The bacterial species forming colonies on the CTX-MacConkey agar plates were determined using VITEK MS (SYSMEX bioMérieux Co., Ltd., Tokyo, Japan).

The genetic uniqueness of each isolate was evaluated by PCR-based open reading frame typing (POT) (Cica Geneus *E. coli* POT KIT; Kanto Chemical Co., Inc, Tokyo, Japan) and pulsed-field gel electrophoresis (PFGE). PFGE of each ESBL-producing isolate was performed as described previously (9). Strain H9812 of the *Salmonella enterica* serotype Braenderup was used as the control strain. A dendrogram showing the genetic relatedness among the isolates was prepared using the Fingerprinting II software (Bio-Rad Laboratories, Tokyo, Japan), and isolates obtained from each fecal sample were determined to have the same background when they possessed pulsotypes with ≥ 85% similarity.

Detection of ESBL producers and plasmid replicon typing: Isolates identified as *E. coli* were further evaluated for ESBL production with a phenotypic confirmatory test using CTX and ceftazidime (CAZ, Wako) alone and in combination with clavulanate according to the Clinical Laboratory Standards Institute (CLSI) guidelines (10). In addition, disks containing aminophenylboronic acid (Fluorochem Ltd., Hadfield, Derbyshire, UK) were used to detect isolates producing the AmpC β-lactamase as described previously (11).

The presence of the CTX-M β-lactamase genes was detected by PCR amplification (9). After classification into the CTX-M-1, -2, -8, and -9 groups, the PCR amplicons of genes from the CTX-M-1- and CTX-M-9 group ESBLs were subjected to nucleotide sequencing as described previously (12,13). Sequence analyses and comparisons to known sequences were performed with the BLAST program from the National Center for Biotechnology Information (USA) website (http://www.ncbi.nlm.nih/gov/BLAST).

Transmission of the *bla*$_{\text{CTX-M}}$-bearing plasmids was performed by conjugation using the broth mating method with the *E. coli* J53 strain (azide resistant) as a recipient. Transconjugants were selected on Luria-Bertani (LB) (Becton, Dickinson and Company, Sparks, MD, USA) agar containing 1 mg/L of CTX and 150 mg/L of azide (Kanto Chemical Co., Inc.), and the pulsotype of the resulting transconjugant was compared to that of the recipient strain. The plasmid replicon type of each transconjugant was determined by PCR-based replicon typing using 18 primer pairs (14).

Multilocus sequence typing (MLST), determination of phylogenetic groups, and O-serotyping: MLST was performed through the analysis of 7 housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). Phylogenetic groups of the ESBL-producing *E. coli* isolates were determined by multiplex PCR as described previously (15). Serotype O25 was identified using *E. coli* antisera (Denka Seiken, Tokyo, Japan), according to the manufacturer's instructions. Genetic O25b serotyping was also confirmed by PCR (16).

Antimicrobial susceptibility testing and detection of plasmid-mediated resistance genes: The antimicrobial susceptibility of each isolate was determined using the agar dilution method in accordance with CLSI guidelines (10), and the minimum inhibitory concentrations were determined according to CLSI criteria (17). The antimicrobial agents used were as follows: CTX, CTF, imipenem (IPM), gentamicin (GEN), kanamycin (KAN), amikacin (AMK), nalidixic acid (NA), fosfomycin (FOM), chloramphenicol (CP), and tetracycline (TC) (Wako Pure Chemical Industries); CAZ, florfenicol (FFC), and ciprofloxacin (CIP) (LKT Laboratories, St. Paul, MN, USA); apramycin (APM) (Duchefa Biochemie, Haarlem, the Netherlands); and nitrofurantoin (NFT) (MP Biomedicals, Solon, OH, USA). *E. coli* ATCC25922 was used as the control strain. The plasmid-mediated quinolone resistance genes, *qnrA*, *qnrB*, *qnrS*, *qepA*, and *aac(6′)-Ib-cr*, as well as the FOM resistance genes, *fosA3* and *fosC2*, and a FFC resistance gene, *floR*, were detected by PCR as previously described (18–21).

Statistical analysis: Data were analyzed using the statistical analysis software "R version 3.3.1." Fisher's exact test was used to analyze the distribution of sequence types, plasmid replicon types, and phylogenetic groups among different *bla*$_{\text{CTX-M}}$-harboring isolates or among different sequence types. It is likely that a high false discovery rate would occur when a multiple comparison is performed; therefore, we corrected the multiple comparison *p*-values using the Benjamini-Hochberg procedure (22). The adjusted *p*-values obtained were then considered under the condition that *p*-values < 0.05 indicated statistical significance.

RESULTS

Prevalence of ESBL-producing *E. coli*: Among the 345 fecal samples tested, 16 samples (4.6%) collected from 8 farms formed colonies on the CTX-MacConkey agar plates. Among these 16 samples, 10 produced many colonies on the CTX-MacConkey agar plates, and 5 distinct colonies were selected from each plate. For the

remaining 6 samples, fewer than 5 colonies grew on the plates; therefore, the colony selection was performed as follows: 4 colonies were selected from each of 2 plates, 3 colonies from each of 2 plates, and 2 colonies from each of 2 plates. The colonies obtained were subjected to bacterial species identification and phenotypic confirmatory tests for ESBL production. All 68 isolates tested were identified as ESBL-producing *E. coli*. Next, the genetic uniqueness of each isolate from each fecal sample was evaluated by POT. When POT produced the same banding patterns in multiple isolates recovered from a single fecal sample, a single representative isolate was selected. Finally, 22 non-repetitive isolates identified as ESBL producers were obtained from the 16 fecal samples from 8 farms in the present study (Fig. 2). All but 2 isolates (No. 71-1 and 79-1) had PFGE profiles with clear bands. Six isolates belonging to the ST 117 were isolated from 5 different farms (Farm IDs: K, S, AF, AL, and S), but evidence strongly indicated they share the same genetic background (Fig. 3). In addition, 4 ST1706 isolates and 2 ST4684 isolates with the identical phylogenetic profile of B1 were collected from the same farm (Farm ID: A) on different days (Fig. 3).

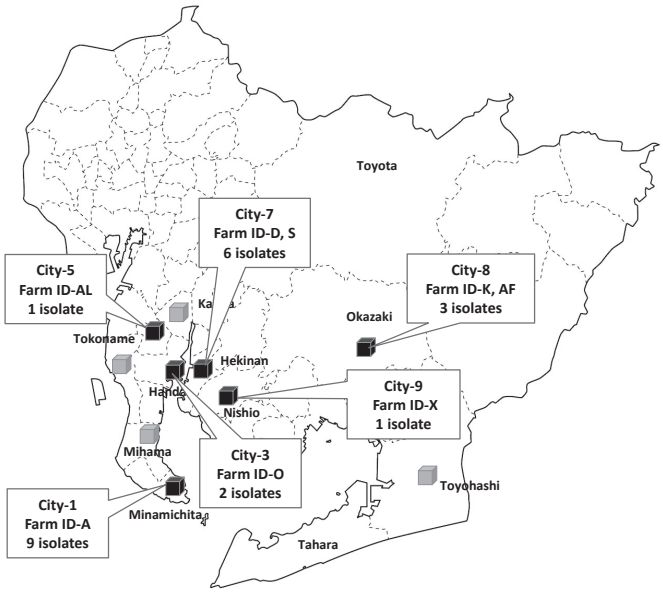
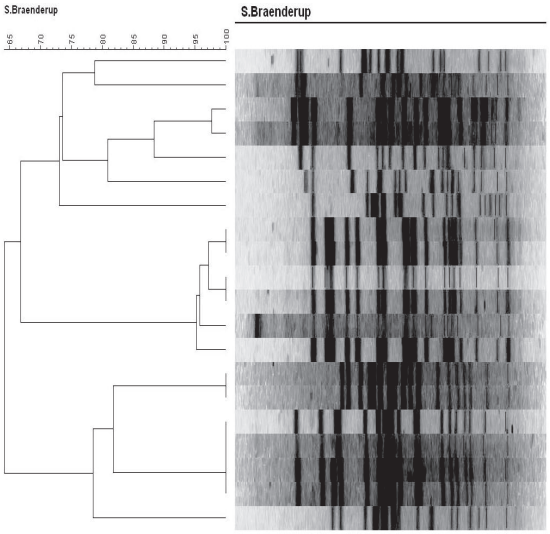


Fig. 2. Distribution of CTX-M-type ESBL-producing *E. coli* isolates. Black symbols indicate the locations of the pig farms where the CTX-M-type ESBL-producing *E. coli* isolates were obtained.

Characteristics of ESBL producers: Twenty isolates obtained from 14 fecal samples harbored the gene for CTX-M group 1, and 2 isolates obtained from 2 fecal samples harbored the gene for CTX-M group 9. There were 4 variants of $bla_{\text{CTX-M}}$, including $bla_{\text{CTX-M-15}}$ (12/22, 54.5%), $bla_{\text{CTX-M-55}}$ (6/22, 27.3%), $bla_{\text{CTX-M-3}}$ (2/22, 0.9%), and $bla_{\text{CTX-M-14}}$ (2/22, 0.9%) (Table 1). Neither the TEM- and SHV-type ESBL genes nor the AmpC β-lactamase gene was found in the present study. Conjugation experiments of plasmids that mediate CTX resistance were successful for 10 (45.5%) of the 22 donor isolates. The plasmid replicon types of the obtained transconjugants fell into the IncN (6/22, 27.3%), IncF group (3/22, 13.6%), and I1-Iγ (1/22, 4.5%), and all 6 IncN plasmids harbored $bla_{\text{CTX-M-55}}$ (Table 1).

MLST analysis revealed 9 STs, ST117 (n = 6, 27.3%); ST1706 (n = 4, 18.2%); ST38 (n = 3, 13.6 %); ST10, ST354, and ST4684 (n = 2 each, 9.1%); ST155 and ST167, which belong to the ST10 complex (n = 1 each, 4.5%); and ST744 (n = 1, 4.5%) (Fig. 3). With respect to the relationships between STs and $bla_{\text{CTX-M}}$ genes, all 6 isolates harboring $bla_{\text{CTX-M-55}}$ belonged to ST117, but the isolates harboring $bla_{\text{CTX-M-15}}$ belonged to different STs, ST10, ST38, ST155, ST1706, and ST4684 (Table 1). Thus, ST117 isolates harbored the IncN plasmid mediating the $bla_{\text{CTX-M-55}}$ variant at a higher frequency than the isolates of other STs, such as ST10, ST38, and ST1706; however, the observed differences were not statistically significant. The 22 isolates were divided into 4 phylogenetic groups, group A (n = 4, 18.2%), group



Isolate No.	City No.	Farm ID	Collected day	MLST	Phylotype	*E. coli*-POT typing		
117-1	1	A	2015/10/13	155	B1	8	1	20
62-4	1	A	2015/8/7	10	A	26	17	16
15-1	3	O	2015/6/10	38	D	16	49	26
41-1	3	O	2015/7/10	38	D	16	49	26
308-1	7	D	2016/2/8	38	D	16	17	18
308-3	7	D	2016/2/8	10	A	26	17	2
128-1	9	X	2015/10/20	167	A	24	81	152
137-1	8	K	2015/11/4	117	F	24	69	58
346-1	7	S	2016/2/9	117	F	24	69	58
105-1	8	AF	2015/10/7	117	F	24	69	58
153-1	5	AL	2015/11/11	117	F	24	69	58
11-2	8	K	2015/6/10	117	F	24	69	58
312-1	7	S	2016/3/8	117	F	24	69	58
25-1	1	A	2015/7/7	4684	B1	8	5	48
73-2	1	A	2015/9/7	4684	B1	8	5	48
117-2	1	A	2015/10/13	1706	B1	10	1	16
25-2	1	A	2015/7/7	1706	B1	10	1	16
62-1	1	A	2015/8/7	1706	B1	10	1	16
73-1	1	A	2015/9/7	1706	B1	10	1	16
117-4	1	A	2015/10/13	744	A	24	17	8

Fig. 3. Dendrogram of PFGE profiles of ESBL producers from pig fecal samples. Among 22 isolates, 20 isolates had PFGE profiles with clear bands, while in 2 isolates (No. 71-1 and 79-1), the band showed a smeared shape. Corresponding isolate number, city number, farm ID, collection date, MLST, phylotype, and POT number information is listed on the right.

B1 (n = 7, 31.8%), group D (n = 3, 13.6%), and group F (n = 8, 36.4%) (Table 1). Although all 6 isolates belonging to ST117 were classified in group F, there was no statistically significant difference in the distribution of ST117 between different phylogenetic groups, as shown by multiple comparisons with the other STs. However, among the 22 ESBL-producing *E. coli* isolates, no isolates from phylogenetic group B2, serotype O25:H4, and sequence type 131 (ST131), which has spread globally as a pandemic clone among human clinical isolates (23), were detected.

Antimicrobial susceptibility and carriage of plasmid-mediated resistance genes: All 22 CTX-M-producing *E. coli* isolates from the fecal samples of pigs were susceptible to IPM, AMK, FOM, NFT, and CIP, except for 3 isolates belonging to ST354 or ST744 that showed CIP resistance. However, these isolates showed high resistance rates to CTX (100%), CTF (100%), and TC (73%), and the resistance rates to CAZ, GEN, CP, FFC, and NA were all greater than 40% (Table 2). Among the 22 isolates, 5 isolates were resistant to 2 or 3 kinds of cephalosporins and TC, and 11 isolates showed co-resistance to more than 8 antimicrobial agents, including GM, TC, CP, CIP, and FFC. With respect to the relationships between the STs and resistance genes, the CP-resistant isolates from ST117 (n = 6) and ST38 (n = 3) harbored *floR*. Among the 9 *floR* harboring isolates, 2 ST38 isolates showed resistance to FOM and harbored *fosA3*. Two isolates harboring *qnrS* were susceptible to CIP, and there were no isolates that harbored *qnrA*, *qnrB*, *qepA*, *aac(6′)-Ib-cr*, or *fosC2*.

DISCUSSION

Recently, the contamination of foods with ESBL-producing *E. coli* has become a worldwide concern. In the present study, the isolation rate of *E. coli* producing any of the CTX-M-type ESBLs from pigs was 4.6%. This rate is lower than the rates observed in China (12.6%) (24), India (6%) (25), and Switzerland (15.3%) (4), but higher than the rate observed in Finland (0%) (26). In addition, this is approximately equal to the isolation rate

Table 1. Relationship among STs, ESBL genes, and replicon types of ESBL-encoding plasmids in 22 *Escherichia coli* isolates

ST	Phylogenetic group of *E. coli* (total number of isolates)	Number of *E. coli* isolates							
		Type of ESBL				Conjugation experiments			
		CTX-M-1group			CTX-M-9 group	Replicon type of plasmid mediating bla_{CTX-M} gene			Number of no transformant or transconjugant obtained
		CTX-M-3	CTX-M-15	CTX-M-55	CTX-M-14	IncF group	IncI1-Iγ	N	
10	A (2)		2						2
38	D (3)	2	1			2			1 (mediating $bla_{CTX-M-15}$)
117	F (6)			6				6	
155	B1 (1)		1						1
167	A (1)		1				1		
354	F (2)				2	1			1
744	A (1)		1						1
1706	B1 (4)		4						4
4684	B1 (2)		2						2
Total	(22)	2	12	6	2	3	1	6	12

Table 2. Antibiotic susceptibility profiles of 22 ESBL-producing *Escherichia coli* isolates

Antimicrobial agent	Range (μg/mL)	Susceptible (%)	Intermediate (%)	Resistant (%)
cefotaxime	16−>256	0 (0)	0 (0)	22 (100)
ceftazidime	2−32	3 (13.6)	8 (36.4)	11 (50.0)
ceftiofur	16−>256	0 (0)	0 (0)	22 (100)
imipenem	≦0.25	22 (100)	0 (0)	0 (0)
gentamicin	1−128	12 (54.5)	0 (0)	10 (45.5)
amikacin	0.5−4	22 (100)	0 (0)	0 (0)
apramycin	4−8	22 (100)	0 (0)	0 (0)
kanamycin	4−>256	19 (86.4)	0 (0)	3 (13.6)
tetracycline	0.5−256	6 (27.3)	0 (0)	16 (72.7)
fosfomycin	0.5−>256	20 (90.9)	0 (0)	2 (9.1)
nalidixic acid	1−>256	13 (59.1)	0 (0)	9 (40.9)
ciprofloxacin	≦0.25−32	19 (86.4)	0 (0)	3 (13.6)
chloramphenicol	4−>256	11 (50.0)	0 (0)	11 (50.0)
florfenicol	4−>256	11 (50.0)	0 (0)	11 (50.0)
nitrofurantoin	4−16	22 (100)	0 (0)	0 (0)

Among the 22 isolates, 5 isolates were resistant to 2 or 3 kinds of cephalosporins and TC, and 11 isolates showed co-resistance to more than 8 antimicrobial agents, including GM, TC, CP, CIP, and FFC.

(4.8%) from diseased pigs collected in the German national monitoring program (2008–2014) (1). Päivärinta et al. noted that reduced antimicrobial agent usage, individualized medication recommendations, and reduced import of pigs among Finnish livestock served to reduce the isolation rate of ESBL producers in Finland (26). Zheng et al. observed that the detection rate of ESBL producers among *E. coli* isolates collected from 2007 to 2009 (12.6%) was much higher than that among isolates collected from 2003 to 2005 (2.4%) in China (24) and suggested that the increasing incidence of ESBL producers isolated from food animals is caused by the increasing use of the third-generation cephalosporins in recent years. Certainly, isolation rates of ESBL producers from pigs may be related to the use of antimicrobial agents as feed additives or therapeutics in each country. In Japan, the isolation rates from pigs were similar to the resistance rates for cefazoline and CTX found in isolates from poultry, especially in broilers (27), but were much lower than the isolation rates reported previously in chicken meat samples (8). It may be that breeding conditions in pig farms, such as the use of antimicrobial agents as feed additives and individual therapeutics, are superior to those used in chicken farms. However, the oxyimino-cephalosporins, such as CTF and cefquinome, have been approved for the treatment of bacterial diseases in cattle and pigs in Japan. Previous investigations were limited by the fact that the number of participant pig farms and samples collected from each farm were insufficient. Therefore, further continuous and nationwide monitoring/surveillance using sensitive screening methods and ensuring the participation of and collection from enough pig farms and fecal samples, respectively, would be useful to investigate the actual prevalence of ESBL producers in pigs.

In the present study, the subtypes of CTX-M-type ESBLs from *E. coli* from pigs included CTX-M-3, CTX-M-15, and CTX-M-55, which belong to CTX-M group 1. Our results differ from the results of previous studies in which CTX-M-1 was predominant among livestock in Switzerland and Denmark (4,28) but are similar to the results from China (24). Zheng et al. has reported that the spread of *E. coli* isolates producing the CTX-M-55-type ESBL, which can hydrolyze CAZ, may be influenced by antimicrobial usage in livestock (24). In addition, Lv et al. (2013) noted that the predominant plasmid replicon types among *E. coli* isolates harboring $bla_{\text{CTX-M-55}}$ isolated from pigs were IncFII and IncI1, suggesting that these plasmids play an important role in the spread of $bla_{\text{CTX-M-55}}$ (29). In the present study, the plasmids harboring $bla_{\text{CTX-M-55}}$ belonged to the IncN, which is inconsistent with the results from China (29). All the *E. coli* isolates producing CTX-M-55 were susceptible to CIP and identified as ST117, but they were isolated from several different farms, suggesting that this bacterial strain spreads clonally among livestock in this area. In Japan, the isolation of *E. coli* isolates producing $bla_{\text{CTX-M-55}}$ from livestock has been limited, and the isolation rates among healthy humans and in clinical settings have remained low to date (30,31). Moreover, IncF-group plasmids harboring $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-27}}$ have been predominant among both healthy humans and clinical isolates (30,31). These findings suggest that pigs do not act as the main reservoir for ESBL producers found in humans. However, García-Fernández et al. noted that IncN plasmids are typically derived from a broad host range and are associated with resistance to many antimicrobial agents, including carbapenems, fluoroquinolones, and aminoglycosides (32). The occurrence of IncN plasmids carrying $bla_{\text{CTX-M-55}}$ in food-producing animals such as pigs is therefore a future concern in Japan. The increasing occurrence of IncN plasmids carrying $bla_{\text{CTX-M-55}}$ in healthy humans and in clinical settings, as well as in food-producing animals, is worthy of careful monitoring hereafter.

The worldwide spread of the B2-O25b:H4-ST131 CTX-M-15-producing *E. coli* clone has become a serious public health concern because this clone generally shows co-resistance to fluoroquinolones and aminoglycosides (33). Our investigation revealed some isolates belonging to the ST10 and ST38 complexes, but did not detect any isolates belonging to the pandemic clone ST131. Many studies have reported the presence of ST10 and ST38 in both humans and animals, suggesting that *E. coli* isolates belonging to these STs are transmissible between animals and humans and could adapt to the intestinal tracts of either species (5,34). In contrast, ST117 has been commonly identified among animals such as cattle and is relatively rare in pigs (35,36). Our findings that ST117 was the predominant ST in ESBL producers isolated from pigs and showed multiple resistance profiles are unique. In the present study, all 9 CP-resistant isolates, including ST117 and ST38, harbored the *floR* gene, supporting previous reports that frequent use of CP in livestock results in the selection of isolates harboring *floR* (37). In addition, the ST38 isolates harbored the *fosA3* gene, which has often been identified in healthy humans and clinical settings (21). Therefore, it is necessary to organize a nationwide monitoring system to track the carriage of drug-resistant microorganisms, especially the ESBL producers, in Japanese food-producing animals as well as in healthy humans and clinical settings.

One limitation of this study is that the pig fecal samples were collected from a limited region in the Aichi prefecture. Therefore, as a next step, a nationwide survey is necessary to more accurately evaluate the prevalence of *E. coli* producing ESBLs. Additionally, although we conducted a genetic characterization of ESBL producers, resistance genes may be driven by plasmids and mobile genetic elements such as insertion sequences, transposons, and integrons. In additional studies, the structures of plasmids in pig and human isolates should be compared using whole-plasmid genome sequencing to clarify details of their transmission among *E. coli* isolates from various genetic backgrounds.

In conclusion, the prevalence of ESBL-producing *E. coli* among Japanese pigs may be low because of the decreased use as feed additives and the limited therapeutic application of antimicrobial agents, suggesting that pigs do not act as the main reservoir for the transmission and spread of ESBL genes. However, multidrug-resistant *E. coli* ST117 isolates carrying IncN-type plasmids harboring $bla_{\text{CTX-M-55}}$ were isolated from the pigs of several different farms, indicating that they may become a future concern in Japan. Moreover, the spread of drug resistance genes mediated by mobile genetic elements could accelerate the dissemination of ESBL producers in the veterinary field. Therefore, whole-plasmid sequence

analysis, along with careful monitoring via continuous surveillance, is necessary to enable rapid efforts to control the transmission routes of the ESBL producers of swine origin.

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Conflict of interest None to declare.

REFERENCES

1. Michael GB, Kaspar H, Siqueira AK, et al. Extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* isolates collected from diseased food-producing animals in the GERM-Vet monitoring program 2008–2014. Vet Microbiol. 2017;200:142-50.
2. Woerther PL, Burdet C, Chachaty E, et al. Trends in human fecal carriage of extended-spectrum β-lactamases in the community: toward the globalization of CTX-M. Clin Microbiol Rev. 2013;26:744-58.
3. Horton RA, Randall LP, Snary EL, et al. Fecal carriage and shedding density of CTX-M extended-spectrum β-lactamase-producing *Escherichia coli* in cattle, chickens, and pigs: implications for environmental contamination and food production. Appl Environ Microbiol. 2011;77:3715-9.
4. Geser N, Stephan R, Hächler H. Occurrence and characteristics of extended-spectrum β-lactamase (ESBL) producing *Enterobacteriaceae* in food producing animals, minced meat and raw milk. BMC Vet Res. 2012;8:21.
5. Leverstein-van Hall MA, Dierikx CM, Cohen Stuart J, et al. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. Clin Microbiol Infect. 2011;17:873-80.
6. Overdevest I, Willemsen I, Rijnsburger M, et al. Extended-spectrum β-lactamase genes of *Escherichia coli* in chicken meat and humans, The Netherlands. Emerg Infect Dis. 2011;17:1216-22.
7. Hiroi M, Yamazaki F, Harada T, et al. Prevalence of extended-spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in food-producing animals. J Vet Med Sci. 2012;74:189-95.
8. Kawamura K, Goto K, Nakane K, et al. Molecular epidemiology of extended-spectrum β-lactamases and *Escherichia coli* isolated from retail foods including chicken meat in Japan. Foodborne Pathog Dis. 2014;11:104-10.
9. Nakane K, Kawamura K, Goto K, et al. Long-term colonization by $bla_{\text{CTX-M}}$-harboring *Escherichia coli* in healthy Japanese people engaged in food handling. Appl Environ Microbiol. 2016;82:1818-27.
10. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; 25th informational supplement. Document M100-S25. Wayne, PA: CLSI; 2015.
11. Yagi T, Wachino J, Kurokawa H, et al. Practical methods using boronic acid compounds or identification of class C β-lactamase-producing *Klebsiella pneumoniae* and *Escherichia coli*. J Clin Microbiol. 2005;43:2551-8.
12. Suzuki S, Shibata N, Yamane K, et al. Change in the prevalence of extended-spectrum-β-lactamase-producing *Escherichia coli* in Japan by clonal spread. J Antimicrob Chemother. 2009;63:72-9.
13. Kawamura K, Sugawara T, Matsuo N, et al. Spread of CTX-type extended-spectrum β-lactamase-producing *Escherichia coli* isolates of epidemic clone B2-O25-ST131 among dogs and cats in Japan. Microb Drug Resist. 2017;23:1059-66.
14. Carattoli A, Bertini A, Villa L, et al. Identification of plasmids by PCR-based replicon typing. J Microbiol Methods. 2005;63:219-28.
15. Clermont O, Christenson JK, Denamur E, et al. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. Environ Microbiol Rep. 2013;5:58-65.
16. Clermont O, Lavollay M, Vimont S, et al. The CTX-M-15-producing *Escherichia coli* diffusing clone belongs to a highly virulent B2 phylogenetic subgroup. J Antimicrob Chemother. 2008;61:1024-8.
17. Clinical and Laboratory Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; 10th approved standard. Document M07-A10.Wayne, PA: CLSI; 2015.
18. Blickwede M, Schwarz S. Molecular analysis of florfenicol-resistant *Escherichia coli* isolates from pigs. J Antimicrob Chemother. 2004;53:58-64.
19. Park CH, Robicsek A, Jacoby GA, et al. Prevalence in the United States of *aac(6′)-Ib-cr* encoding a ciprofloxacin- modifying enzyme. Antimicrob Agents Chemother. 2006;50:3953-5.
20. Cattoir V, Poirel L, Rotimi V, et al. Multiplex PCR for detection of plasmid-mediated quinolone resistance *qnr* genes in ESBL-producing enterobacterial isolates. J Antimicrob Chemother. 2007;60:394-7.
21. Wachino J, Yamane K, Suzuki S, et al. Prevalence of fosfomycin resistance among CTX-M-producing *Escherichia coli* clinical isolates in Japan and identification of novel plasmid-mediated fosfomycin-modifying enzymes. Antimicrob Agents Chemother. 2010;54:3061-4.
22. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Series B Stat Methodol. 1995;57:289-300.
23. Peirano G, Pitout JD. Molecular epidemiology of *Escherichia coli* producing CTX-M β-lactamases: the worldwide emergence of clone ST131 O25:H4. Int J Antimicrob Agents. 2010:35; 316-21.
24. Zheng H, Zeng Z, Chen S, et al. Prevalence and characterization of CTX-M β-lactamases amongst *Escherichia coli* isolates from healthy food animals in China. Inter J Antimicrob Agents. 2012;39:305-10.
25. Samanta I, Joardar SN, Mahanti A, et al. Approaches to characterize extended spectrum beta-lactamase/beta-lactamase producing *Escherichia coli* in healthy organized vis-a-vis backyard farmed pigs in India. Infect Genet Evol. 2015;36:224-30.
26. Päivärinta M, Pohjola L, Fredriksson-Ahomaa M, et al. Low occurrence of extended-spectrum β-lactamase-producing *Escherichia coli* in finnish food-producing animals. Zoonoses and Public Health. 2016;63:624-31.
27. Tamura Y. Present situations and control measures of drug resistant bacteria originated from food-producing animals in Japan. Modern Media. 2015;61:161-8. Japanese.
28. Hammerum AM, Larsen J, Andersen VD, et al. Characterization of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* obtained from Danish pigs, pig farmers and their families from farms with high or no consumption of third- or fourth-generation cephalosporins. J Antimicrob Chemother. 2014;69:2650-7.
29. Lv L, Partridge SR, He L, et al. Genetic characterization of IncI2 plasmids carrying $bla_{\text{CTX-M-55}}$ spreading in both pets and food animals in China. Antimicrob Agents Chemother. 2013;57:2824-7.
30. Matsumura Y, Yamamoto M, Nagao M, et al, Emergence and spread of B2-ST131-O25b, B2-ST131-O16 and D-ST405 clonal groups among extended-spectrum β-lactamase-producing *Escherichia coli* in Japan. J Antimicrob Chemother. 2012;67:2612-20.
31. Matsumura Y, Yamamoto M, Nagao M, et al, Association of fluoroquinolone resistance, virulence genes, and IncF plasmids with extended-spectrum-β-lactamase-producing *Escherichia coli* sequence type 131 (ST131) and ST405 clonal groups. Antimicrobial Agents Chemother. 2013;57:4736-42.
32. García-Fernández A, Villa L, Moodley A, et al. Multilocus sequence typing of IncN plasmids. J Antimicrob Chemother. 2011;66:1987-91.
33. Pitout JD, Laupland KB. Extended-spectrum β-lactamase-producing *Enterobacteriaceae*: an emerging public-health concern. Lancet Infect Dis. 2008;8:159-66.
34. Ewers C, Bethe A, Semmler T, et al. Extended-spectrum β-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. Clin Microbiol Infect. 2012;18:646-55.
35. Wu G, Ehricht R, Mafura M, et al. *Escherichia coli* isolates from extraintestinal organs of livestock animals harbor diverse virulence genes and belong to multiple genetic lineages. Vet Microbiol. 2012;160:197-206.
36. Fischer J, Hille K, Ruddat I, et al. Simultaneous occurrence of MRSA and ESBL-producing *Enterobacteriaceae* on pig farms and in nasal and stool samples from farmers. Vet Microbiol. 2017;200:107-13.
37. Harada K, Asai T, Kojima A, et al. Role of coresistance in the development of resistance to chloramphenicol in *Escherichia coli* isolated from sick cattle and pigs. Am J Vet Res. 2006;67:230-5.