NOTE *Public Health*

Emergence of *Salmonella enterica* Serovar Infantis Harboring IncI1 Plasmid with $bla_{\text{CTX-M-14}}$ in a Broiler Farm in Japan

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ABSTRACT. Cefotaxime (CTX)-resistant and -susceptible *Salmonella enterica* serovar Infantis isolates obtained from broilers raised on a farm in January 2010 in Japan were characterized to establish their resistance determinants. The CTX-resistant isolates produced CTX-M-14 extended-spectrum β-lactamase and harbored 2 distinct plasmid of approximately 140- and 95-kb, whereas the CTX-susceptible isolates harbored one 140-kb plasmid. The 95-kb plasmids were replicon typed as IncI1 carrying the $bla_{\text{CTX-M-14}}$ gene, while the 140-kb plasmids were IncP and harbored the *aphA1*, *aadA1, tetA*, and *sul1* genes. Genetic fingerprinting by pulsed-field gel electrophoresis revealed similar macrorestriction profiles amongst CTX-resistant and susceptible isolates, suggesting a clonal relationship. The presence of CTX-resistant *S*. Infantis on a broiler farm has occurred through the acquisition of IncI1 resistance plasmid.
KEY WORDS: β-lactamase, broiler, CTX-M-14, extended-spectrum plasmid replicon typing, *Salmonella* Infantis.

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Amongst members of the *Enterobacteriaceae* family, *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* have been implicated as potential producers of extended-spectrum *β*-lactamases (ESBLs) which possess hydrolyzing activity against third-generation cephalosporins. In recent years, several reports have described other bacterial species within the *Enterobacteriaceae* family capable of producing ESBLs. Of these, *Salmonella* spp. producing ESBLs have been detected in salmonellosis cases both in humans [9, 10, 17] and domestic animals [18].

Salmonella enterica serovar Infantis (*S.* Infantis) is one of the predominant serotypes isolated from broilers in Japan [19]. In the late 1990s, *S*. Infantis isolates harboring ESBLs were recovered from hospitalized patients in South America [14]. More recently, *S*. Infantis producing ESBLs were found not only in human patients but also in domestic animals and commercial meats [3, 5]. Most of these produced the CTX-M-type ESBLs, indicating the probability that dissemination of 3rd-generation cephalosporin-resistant *S*. Infantis associated with various types of β-lactamases is gradually expanding world-wide. In this article, we characterized *S*. Infantis isolates carrying CTX-M-14 ESBL derived from broiler chickens.

Ten *S*. Infantis isolates obtained from 30 broiler cecal samples collected at a poultry processing plant in western Japan in January 2010 were used in this study. The broiler chickens involved in this survey were sampled from 3 different flocks raised on the same commercial farm. The isolation of the bacteria was done as follows; approximately 1 g of cecal contents was aseptically mixed with 5 m*l* of sterilized distilled water. Then, 1 m*l* of suspension was enriched in 10 m*l* of tetrathionate broth (Merck KGaA, Darmstadt, Germany) and incubated at 42°C. After 24 hr of incubation, a loopful from each of enriched broth was streaked onto plates of selective deoxycholate hydrogen sulfide lactose (DHL) agar (Oxoid Ltd., Basingstoke, Hampshire, UK) and mannitol lysine crystal violet brilliant green (MLCB) agar (Eiken Chemical Co., Ltd., Tokyo, Japan), and incubated at 37°C for 24 hr. Suspected colonies were selected from each plate and cloned on Mueller-Hinton agar (Oxoid Ltd.), and the following identification and serotyping of bacteria were performed as described elsewhere [19]. These isolates were stocked in brain heart infusion broth containing 20% of glycerol at −80°C until use the following tests. Antimicrobial susceptibility testing was performed by employing the Kirby-Bauer disk diffusion method on Mueller-Hinton (Oxoid Ltd.) agar plates using the following antimicrobial agents: ampicillin (AMP), cephalothin (CEF), cefotaxime (CTX), ceftazidime (CAZ), streptomycin (STR), kanamycin (KAN), gentamicin (GEN), tetracycline (TET), chloramphenicol (CHL), nalidixic acid (NAL), ciprofloxacin (CIP), tosufloxacin (TFX) and trimethoprim-sulfamethoxazole (SXT). The double-disk synergy test using CTX and CAZ disks with or without clavulanic acid disks was performed according to the new criteria established by the Clinical and Laboratory Standards Institute (CLSI) [2]. The ESBL genes were detected by polymerase chain reaction (PCR)

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Table 1. Resistance profiles and ESBL type of *S.* Infantis isolates

Isolates	Source			Resistance phenotypes[a)]	ESBL type
	Date	Flock	Age of broiler (d)		
Y1, Y2, Y3	20-Jan-2010	a	51	STR, KAN, TET, SXT - AMP, CEF, CTX	CTX-M-14
Y5, Y6, Y7	20-Jan-2010	a	51	STR, KAN, TET, SXT	-
Y4	25-Jan-2010	b	37	STR, KAN, TET, SXT - AMP, CEF, CTX	CTX-M-14
Y8, Y9, Y10	25-Jan-2010	c	49	STR, KAN, TET, SXT	-

a) STR, streptomycin; KAN, kanamycin; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole; AMP,ampicillin; CEF, cephalothin; CTX, cefotaxime.

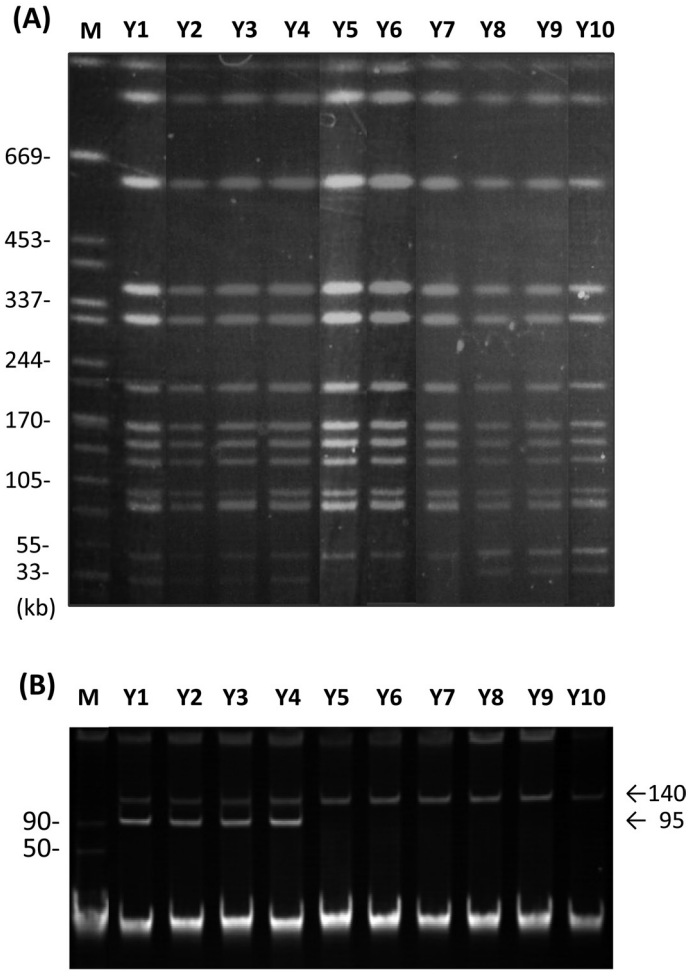

Fig. 1. (A) PFGE analysis of *S.* Infantis genomic DNA digested by *Bln*I enzyme. Lane M, *Salmonella enterica* serovar Braenderup H9812 used as a size marker; Lane 1, isolate Y1; Lane 2, Y2; Lane 3, Y3; Lane 4, Y4; Lane 5, Y5; Lane 6, Y6; Lane 7, Y7; Lane 8, Y8; Lane 9, Y9; Lane 10, Y10. Numbers on the left indicate the size of the bands in lane M. (B) Plasmid profiles of *S.* Infantis isolates. Lane M, standard *Salmonella enterica* serovar Cholerasuis (50 kb) and serovar Typhimurium DT 104 (90 kb); Lane 1, isolate Y1; Lane 2, Y2; Lane 3, Y3; Lane 4, Y4; Lane 5, Y5; Lane 6, Y6; Lane 7, Y7; Lane 8, Y8; Lane 9, Y9; Lane 10, Y10. Numbers on the left indicate the sizes in lane M and arrow on the right indicate detected band sizes (approximately).

Table 2. MIC of antimicrobials and their resistance genes harbored plasmids of the donor isolates and transconjugants

Strain		Plasmid (s)		MIC (μg/ml) [a]						Resistance gene (s)
		Size (kb)	Replicon type	AMP	CTX	KAN	STR	TET	SUL	
Donor	Y2	95, 140	I1, P	>512	256	>512	64	128	>512	$bla_{CTX\text{-}M\text{-}14}$, *aphA1*, *aadA1*, *tetA*, *sul1*
Transconjugant	T1	95, 140	I1, P	>512	16	>512	32	64	256	$bla_{CTX\text{-}M\text{-}14}$, *aphA1*, *aadA1*, *tetA*, *sul1*
	T2	95	I1	>512	32	2	4	1	0.5	$bla_{CTX\text{-}M\text{-}14}$
Donor	Y6	140	P	4	<0.25	>512	64	128	>512	*aphA1*, *aadA1*, *tetA*, *sul1*
Transconjugant	T3	140	P	4	<0.25	>512	32	32	256	*aphA1*, *aadA1*, *tetA*, *sul1*

a) AMP, ampicillin; CTX, cefotaxime; KAN, kanamycin; STR, streptomycin; TET, tetracycline; SUL, sulfamethoxazole.

[20, 21]; and obtained amplicons were directly sequenced using specific primers [1] with the BigDye Terminator v3.1 Ready Reaction Sequencing kit and the ABI 3500 × l Automated DNA Sequencer (Applied Biosystems, Foster City, CA, U.S.A.). The sequencing data were compared with the published DNA sequences using the BLASTN database (www.ncbi.nlm.nih.gov). The resistance patterns of *S*. Infantis isolates examined are shown in Table 1. All 10 *S*. Infantis isolates were shown resistance to KAN, STR, TET and SXT, and of those, the 4 isolates (Y1, Y2, Y3 and Y4) exhibited resistance to three β-lactam antibiotics; AMP, CEF and CTX. Preliminary phenotypic double-disk synergy test results indicated that the 4 CTX resistant isolates were potential ESBL producers. Ultimately, PCR and nucleotide sequence analysis revealed that the CTX resistant isolates carried the $bla_{CTX\text{-}M\text{-}14}$ gene.

Pulsed-field gel electrophoresis (PFGE) using *Bln*I restriction enzyme was performed according to the standard PulseNet protocol [16]. The banding patterns obtained were analyzed using the Molecular Analyst Fingerprinting Plus software (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.). The fragment similarity above 94% was observed among the isolates, suggesting the likelihood of sharing the same origin (Fig. 1A). Isolation of plasmids was conducted using the alkaline lysis method as previously described [12]. Analysis of the plasmid profiles demonstrated that 4 isolates (Y1, Y2, Y3 and Y4) harbored two plasmids of approximately 140 kb and 95 kb. The other 6 isolates (Y5, Y6, Y7, Y8, Y9 and Y10) carried only one plasmid of approximately 140 kb (Fig. 1B).

Conjugation experiments were conducted as described elsewhere [18] using the isolates Y2 and Y6 as donors and the rifampicin-resistant *E. coli* DH5α strain as recipient. We observed 3 distinct types of *E. coli* transconjugants: T1 transconjugants contained both 140- and 95-kb plasmids, whereas T2 carried 95-kb plasmid and T3 harbored 140-kb plasmid (Table 2). Out of the transconjugants, the T1 and T2 were obtained from Y2 donor, and the T3 were from Y6 donor. These findings indicate that both 95-kb and 140-kb plasmids associated with *S*. Infantis donor isolates were potentially self-transmissible. The minimum inhibitory concentrations for AMP, CTX, KAN, STR, TET and sulfamethoxazole (SUL) on *E. coli* transconjugants were determined by the agar dilution method. The PCR assay was performed to confirm the transmission of the $bla_{CTX\text{-}M\text{-}14}$, *aphA1*, *aadA1, tetA*, and *sul1* genes. Resistance phenotypes and genes detected in *E. coli* transconjugants are summarized in Table 2. T2 transconjugants with a 95-kb plasmid showed resistance to AMP and CTX; and they also tested positive for the $bla_{CTX\text{-}M\text{-}14}$ gene, suggesting the location of the $bla_{CTX\text{-}M\text{-}14}$ to be the 95-kb plasmid. Genetic analysis of the T3 transconjugants revealed that resistance traits *aphA1*, *aadA1, tetA*, and *sul1* were located on the 140-kb plasmid.

The PCR-based replicon typing (PBRT) was carried out as previously described [11]. The findings of PBRT demonstrated that $bla_{CTX\text{-}M\text{-}14}$ was associated with IncI1 plasmid. On the other hand, plasmids harboring *aphA1*, *aadA1, tetA*, and *sul1* were IncP type. Previous reports indicated that IncI1 plasmids harboring $bla_{CTX\text{-}M\text{-}14}$ were detected in *Salmonella enterica* serovar Enteritidis (*S*. Enteritidis) [7] and *E. coli* bacteria [15]. It's worth noting that several studies describing the occurrence of CTX-M-14 ESBL in *S*. Enteritidis isolated from human patients in Spain [17], Hong Kong [10] and Japan [9] have been reported since 2003. In Japan, serovar Enteritidis producing CTX-M-14 enzyme was isolated from chicken meat imported from China in 2004 [13]. Moreover, a commentary was released in Japan explaining the occurrence of *S*. Infantis producing CTX-M-14 ESBL recovered from domestic poultry meat [4].

Although the use of cephalosporins including CTX is not approved for broilers in Japan, we detected CTX-resistant *S*. Infantis isolates derived from the broiler farm. It is likely that the $bla_{CTX\text{-}M\text{-}14}$ gene was acquired by *S*. Infantis isolates through interspecies transmission of the potential IncI1 resistance plasmid. This hypothesis is supported by early reports which described the existence of CTX-M-14 ESBL producing *E. coli* from broilers and chicken meats in Japan [6, 8]. The spread of cephalosporin-resistant *Salmonella* spp. in poultry via the transmissible resistance plasmids raises serious veterinary and public health concerns. Thus, continuous monitoring of members of the *Enterobacteriaceae* family possessing IncI1 resistance plasmids is required in order to establish the magnitude of the health hazard associated with these bacteria.

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