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Molecular characteristics of extended-spectrum β-lactamase/AmpC-producing *Salmonella enterica* serovar Virchow isolated from food-producing animals during 2010–2017 in South Korea

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ABSTRACT

Global dissemination of non-typhoidal *Salmonella* producing extended-spectrum β-lactamase (ESBL) is a public-health concern. Recently, the prevalence of *Salmonella* spp. resistant to third-generation cephalosporins has been increasing in food-producing animals in Korea. In this study, we investigated resistance mechanisms and molecular characteristics of *S.* Virchow isolates resistant to extended-spectrum cephalosporins (ESCs). We obtained 265 *S.* Virchow isolates from fecal and carcasses samples of cattle (n = 2), pigs (n = 7), and chickens (n = 256) during 2010–2017, and observed high ESC-resistance (63.8%, 169/265); most of the resistant isolates (96.4%) were obtained from chickens. ESC-resistant *S.* Virchow isolates (n = 169) showed significantly higher resistance rates to other antimicrobials (especially aminoglycosides and tetracycline, p-value < 0.0001), as well as prevalence of multidrug resistance, than did ESC-susceptible *S.* Virchow isolates (n = 96). All ESC-resistant *S.* Virchow produced CTX-M-15-type ESBL (n = 147) and/or CMY-2-type AmpC β-lactamase (n = 23). ESC-resistant *S.* Virchow represented seven pulsotypes, predominantly composed of type II (58.6%) and III (26.0%), detected in 69 farms in 10 provinces, and 33 farms in 7 provinces, respectively. Genes encoding ESC-resistance were horizontally transferred by conjugation to recipient *E. coli* J53; this was demonstrated in 28.8% (42/146) of $bla_{CTX\text{-}M\text{-}15}$-positive isolates and in 50.0% (11/22) of $bla_{CMY\text{-}2}$-positive isolates. All conjugative plasmids carrying $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CMY\text{-}2}$ genes belonged to ST2-IncHI2 and ST12/CC12-IncI1, respectively. Genetic features of transferred *bla* genes were involved with IS*Ecp1* in both $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CMY\text{-}2}$; IS*Ecp1* plays a critical role in the efficient capture, expression, and mobilization of *bla* genes. In addition to $bla_{CTX\text{-}M\text{-}15}$ genes, resistance markers to aminoglycosides and/or tetracycline were co-transferred to recipient *E. coli* J53.

Our results show a high prevalence of ESBL-producing *S.* Virchow in chickens and chicken carcasses. Specific $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CMY\text{-}2}$-carrying *S.* Virchow clones and plasmids were predominant in food-producing animals nationwide. Restriction of antimicrobial use and proper biosecurity practices at the farm level should be urgently implemented in the poultry industry.

1. Introduction

Extended-spectrum β-lactamases (ESBLs), which mediate resistance to third-generation cephalosporins and monobactams, are widely disseminated in *Enterobacteriaceae* in both humans and animals (Seiffert et al., 2013). Most ESBLs belong to class A Ambler classification and are mutants of classic TEM and SHV β-lactamase enzymes. However, during the past two decades, CTX-M-type ESBLs and AmpC β-lactamases have been increasingly found across the world (Chong et al., 2018; Philippon et al., 2002; Rossolini et al., 2008).

Non-typhoidal *Salmonella* (NTS) are common pathogens in humans and animals, even in developed countries (Threlfall, 2002). Extended-spectrum cephalosporins (ESCs) are the drugs of choice for such *Salmonella* infections. Since ESBL production in *Salmonella* spp. was first identified in 1988 (Hammami et al., 1991), ESBL-producing NTS has been increasingly reported worldwide (Carattoli, 2008; McDermott

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Abbreviations

CLSI	Clinical and Laboratory Standards Institute
DANMAP	Danish Integrated Antimicrobial Resistance Monitoring and Research Programme
ESCs	Extended-spectrum cephalosporins
ESBL	Extended-spectrum β-lactamase
MS	Mass spectrometry
MALDI-TOF	Matrix-assisted laser desorption ionization time-of-flight
MICs	Minimum inhibitory concentrations
MSRV	Modified Semisolid Rappaport Vassiliadis medium
MDR	Multi-drug resistance
NGS	Next-generation sequencing
NTS	Non-typhoidal *Salmonella*
pDLST	Plasmid double-locus sequence typing
pMLST	Plasmid multi-locus sequence typing
PFGE	Pulsed-field gel electrophoresis

et al., 2018), including in South Korea (Choi et al., 2015; Jeon et al., 2019; Tamang et al., 2011b). In recent years, resistance in *Salmonella* spp. caused by plasmid-mediated ESBLs, such as cefotaximases or AmpC β-lactamases, and the likelihood of transmission of these organisms between animals and humans, have been of great concern (Seiffert et al., 2013).

In Korea, NTS is the second most common cause of food poisoning in humans. *S. enterica* serovar Enteritidis and *S.* Typhimurium are the two most prevalent serotypes causing salmonellosis in humans and livestock (Yang et al., 2002). Identical plasmid replicon types of CTX-M-producing *S.* Enteritidis isolated from human patients and chickens were reported previously by our group (Tamang et al., 2011b). Recently, ESC-resistant *S.* Virchow has shown a rapid increase in prevalence in patients with acute diarrhea during 2010–2014 in South Korea (Kim et al., 2016). Thus, our objective in this study was to investigate the antimicrobial resistance and molecular characteristics of ESC-resistant *S.* Virchow isolated from food-producing animals in South Korea.

2. Materials and methods

2.1. Salmonella isolation and serotyping

A total of 265 *S.* Virchow isolates were collected from all the provinces of South Korea from 2010 to 2017. (Table 1). Seven isolates were collected from the Animal and Plant Quarantine Agency, and 258 from nine laboratories/centers participating in the Korean Veterinary Antimicrobial Resistance Monitoring System. These *S.* Virchow organisms were isolated from the feces and carcasses of healthy cattle (n = 2), pigs (n = 7), and chickens (n = 249), and from the feces of diseased chickens (n = 7) throughout South Korea. No more than five fecal and carcass samples were collected from a single farm, and one *Salmonella* strain was isolated from each sample. > 10,000 samples were collected from approximately 5000 farms annually, and > 200 of *Salmonella* spp. were isolated every year (http://www.qia.go.kr/anp/rchStatus/listwebQiaCom.do?type=86_1ndyjsy&clear=1). The isolation of *Salmonella* was conducted as described previously using buffered peptone water (Becton Dickinson, Sparks, MD), Modified Semisolid Rappaport Vassiliadis medium (MSRV; Becton Dickinson, Sparks, MD), and Rambach agar (Merk, Darmstadt, Germany) (Lim et al., 2011). Species identification of *S. enterica* isolates was performed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) using a Vitek MS system (bioMerieux, Marcy-l'Etoile, France). *Salmonella* serogroup C1 was determined by PCR using specific primers (Ranieri et al., 2013). *S.* Virchow were confirmed by agglutination identifying somatic antigen O and flagellar antigens H (phase 1 and 2) using *Salmonella* O Group C1 antiserum and *Salmonella* H antiserum (Becton Dickinson, Sparks, MD), respectively, according to the White-Kauffmann-Le Minor typing scheme (Grimont and Weill, 2007). *S.* Virchow isolates used in this study are listed in Table 1.

2.2. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) for 12 antimicrobial agents were determined using broth microdilution with a commercially available Sensititre® panel KRVP4F or KRVP5F (TREK Diagnostic Systems, West Sussex, UK) according to the manufacturer's instructions. The following antimicrobials were tested: ampicillin (2–64 μg/ml), amoxicillin/clavulanic acid (2/1–32/16 μg/ml), cefoxitin (1–32 μg/ml), ceftiofur (0.5–8 μg/ml), chloramphenicol (2–64 μg/ml), ciprofloxacin (0.12–16 μg/ml), colistin (2–16 μg/ml), gentamicin (1–64 μg/ml), nalidixic acid (2–128 μg/ml), streptomycin (16–128 μg/ml), tetracycline (2–128 μg/ml), and trimethoprim/sulfamethoxazole (0.12/2.38–4/76 μg/ml). The reference strain, *E. coli* ATCC 25922, was used as quality control for determining MICs. MICs were interpreted according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2017). When CLSI breakpoints were not available, MICs were interpreted according to the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) 2014 (DANMAP, 2014). Multi-drug resistance (MDR) was defined as resistance to three or more antimicrobial subclasses.

Following antimicrobial-susceptibility assessments, double-disc synergy assays were performed to detect ESBL production by ceftiofur-resistant bacteria. For this, cefotaxime-cefotaxime/clavulanic acid and ceftazidime-ceftazidime/clavulanic acid discs were used according to CLSI guidelines (CLSI, 2017). ESBL-producing Salmonella isolates were then used in consequent analyses.

2.3. Detection of resistance genes

The presence of the $bla_{\text{CTX-M}}$ gene was detected by PCR amplification using group-specific primers for CTX-M-1 and CTX-M-9 families as described previously (Batchelor et al., 2005; Branger et al., 2005). Then, previously-described primers were used to amplify and sequence the complete $bla_{\text{CTX-M}}$ genes (Tamang et al., 2011a). Screening for genes encoding six AmpC families was performed using multiplex PCR (Pérez-Pérez and Hanson, 2002). To amplify the entire bla_{AmpC} gene, PCR-positive isolates were amplified using specific primers as described previously (Pérez-Pérez and Hanson, 2002). PCR amplification of entire bla_{TEM} and bla_{SHV} genes was performed as described previously (Rayamajhi et al., 2008). Sequence analysis was performed using BLAST programs in the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST).

2.4. Conjugation experiment

Conjugation was performed using filter-mating as described previously (Tamang et al., 2014) with sodium azide-resistant *E. coli* J53 as recipient strain. Briefly, donor and recipient strains were cultured overnight, inoculated with fresh tryptone soy (TS) broth (Becton Dickinson, Sparks, MD), and cultured for 4 h at 37 °C. The freshly cultured bacteria were mated with a 1:4 donor/recipient ratio and trapped on a membrane filter. The bacteria on the filters were incubated on TS agar plates overnight and then suspended in phosphate buffered saline. To select putative transconjugants, appropriate dilutions of the mixture were transferred to MacConkey agar plates supplemented with sodium azide (150 μg/ml) and cefotaxime (2 μg/ml). All selected transconjugants were examined for the presence of β-lactamase genes as described above. The antimicrobial-susceptibility

Table 1
Salmonella enteria serovar Virchow isolates obtained from samples of healthy and diseased animals during 2010–2017 in South Korea.

Year	Healthy food-producing animals and their carcasses								Diseased animals			Total		
	Cattle			Pigs			Chickens		Chickens					
	No. of slaughterhouses	No. of farms	No. of *S.* Virchow isolates (%)	No. of slaughterhouses	No. of farms	No. of *S.* Virchow isolates (%)	No. of slaughterhouses	No. of farms	No. of *S.* Virchow isolates (%)	No. of slaughterhouses	No. of farms	No. of *S.* Virchow isolates (%)	No. of slaughterhouses	No. of farms	No. of *S.* Virchow isolates (%)
2010	–	–	- (0)	–	–	- (0)	2	2	2 (0.8)	–	–	- (0)	2	2	2 (0.8)
2011	–	–	- (0)	1	1	1 (14.3)	2	6	7 (2.8)	–	1	1 (14.3)	3	8	9 (3.4)
2012	–	–	- (0)	–	–	- (0)	5	28	45 (18.1)	–	–	- (0)	5	28	45 (17)
2013	–	–	- (0)	2	2	2 (28.6)	3	13	22 (8.8)	–	–	- (0)	5	15	24 (9.1)
2014	2	2	2 (100)	2	3	3 (42.9)	11	55	90 (36.1)	–	2	2 (28.6)	15	62	97 (36.6)
2015	–	–	- (0)	1	1	1 (14.3)	10	34	39 (15.7)	–	2	2 (28.6)	11	37	42 (15.8)
2016	–	–	- (0)	–	–	- (0)	2	20	21 (8.4)	–	–	- (0)	2	20	21 (7.9)
2017	–	–	- (0)	–	–	- (0)	4	22	23 (9.2)	–	2	2 (28.6)	4	24	25 (9.4)
Total	2	2	2 (100)	5	7	7 (100)	20	175	249 (100)	–	7	7 (100)	26	190	265 (100)

patterns of transconjugants were determined using disc diffusion according to the CLSI guidelines (CLSI, 2017).

2.5. Molecular characterization of ceftiofur-resistant Salmonella enterica serovar Virchow

Plasmid DNA was extracted using the QuickGene® plasmid isolation system (FUJIFILM Corporation, Tokyo, Japan) according to the manufacturer's protocol. Replicon typing of the isolated plasmid was performed using PCR-based replicon typing with 18 pairs of primers as described previously (Carattoli et al., 2005). The genetic environment of the $bla_{\text{CTX-M-15/CMY-2}}$ gene was investigated using PCR and Sanger sequencing. A combination of IS*26* (Eckert et al., 2006) or IS*Ecp1* (Saladin et al., 2002) forward primers, and a CTX-M reverse consensus (MA2) primer (Saladin et al., 2002) or CMY-2 reverse primer (Pérez-Pérez and Hanson, 2002), were used to investigate regions upstream of the *bla* genes. A MA1 primer (Saladin et al., 2002) or CMY-2 forward primer (Pérez-Pérez and Hanson, 2002), and reverse primers of IS*903*, *tnpA* IS*903*, *orf477*, *or mucA* (Eckert et al., 2006), were used to characterize regions downstream of the *bla* genes. Plasmid multi-locus sequence typing (pMLST) and plasmid double-locus sequence typing (pDLST) were performed using specific primers as described previously for IncI1 and IncHI2 plasmids, respectively (García-Fernández et al., 2008; García-Fernández and Carattoli, 2010). Allele and profile analysis for pMLST and pDLST was conducted via http://pubmlst.org/plasmid/. Pulsed-field gel electrophoresis (PFGE) was performed using the *Xba*I enzyme (Takara Bio Inc., Shiga, Japan) as described previously (Gautom, 1997). PFGE banding profiles were analyzed using Bionumerics software, and relatedness was calculated using unweighted pair-group method with arithmetic averages algorithm based on the Dice similarity index.

2.6. Statistical analysis

Statistical analyses were performed using Rex (Version 3.0.3, RexSoft Inc., Seoul, South Korea). Comparisons between groups were performed using chi-square test. A value of $p < 0.01$ was considered statistically significant.

3. Results

3.1. Prevalence of ceftiofur-resistant Salmonella enterica serovar Virchow

A total of 265 *S*. Virchow isolates were isolated from fecal samples and carcasses of food-producing animals during 2010–2017 (Table 1). Until 2011, < 10 *S*. Virchow strains were isolated per year; however, this number gradually increased to 97 in 2014, and then decreased to 25 in 2017. *S*. Virchow isolates showed a dramatically higher presence in chicken samples ($n = 256$) than in cattle ($n = 2$) and pigs ($n = 7$). Among the 265 *S*. Virchow isolates, 169 (63.8%) showed resistance to ceftiofur, and most of the resistant isolates (163/169, 96.4%) were obtained from chickens (Table 2). Although the number of isolates obtained from cattle and pigs was scant (two and seven, respectively), the prevalence of ceftiofur-resistant isolates in all animals was high [100% (2/2) of cattle, 57.1% (4/7) of pigs, and 63.7% (163/256) of chickens]. Overall resistance to ceftiofur began to increase in 2012, reached its highest level of 88.1% in 2015, and then decreased gradually. In cattle and pigs, ceftiofur-resistant *S*. Virchow isolates were observed only in 2014 and 2015 among the seven years examined in this study (Table 2).

3.2. Antimicrobial resistance of Salmonella enterica serovar Virchow

Antimicrobial resistance of 265 *S*. Virchow isolates to 12 antimicrobial agents is shown in Table 3. All isolates except two were resistant to one or more antimicrobials, and a MDR phenotype was

Table 2
Prevalence of ceftiofur-resistant *Salmonella enterica* serovar Virchow isolated from food-producing animals during 2010–2017 in South Korea.

Year	Prevalence of ceftiofur-resistant *S.* Virchow (No. of resistant isolates/No. of tested isolates)										
	Cattle			Pigs			Chickens				Total
	Feces	Carcasses	Subtotal	Feces	Carcasses	Subtotal	Feces	Carcasses	Diseased chickens	Subtotal	
2010	0% (0/0)	0% (0/0)	0% (0/0)	0% (0/0)	0% (0/0)	0% (0/0)	0% (0/2)	0% (0/0)	0% (0/0)	0% (0/2)	0% (0/2)
2011	0% (0/0)	0% (0/0)	0% (0/0)	0% (0/1)	0% (0/0)	0% (0/1)	0% (0/2)	40% (2/5)	0% (0/1)	25% (2/8)	22.2% (2/9)
2012	0% (0/0)	0% (0/0)	0% (0/0)	0% (0/0)	0% (0/0)	0% (0/0)	76.9% (10/13)	43.8% (14/32)	0% (0/0)	53.3% (24/45)	53.3% (24/45)
2013	0% (0/0)	0% (0/0)	0% (0/0)	0% (0/2)	0% (0/0)	0% (0/2)	80% (12/15)	85.7% (6/7)	0% (0/0)	81.8% (18/22)	75.0% (18/24)
2014	100% (1/1)	100% (1/1)	100% (2/2)	100% (2/2)	100% (1/1)	100% (3/3)	66.7% (26/39)	86.3% (44/51)	100% (2/2)	78.3% (72/92)	79.4% (77/97)
2015	0% (0/0)	0% (0/0)	0% (0/0)	100% (1/1)	0% (0/0)	100% (1/1)	85.7% (12/14)	88.0% (22/25)	100% (2/2)	87.8% (36/41)	88.1% (37/42)
2016	0% (0/0)	0% (0/0)	0% (0/0)	0% (0/0)	0% (0/0)	0% (0/0)	44.4% (4/9)	16.7% (2/12)	0% (0/0)	28.6% (6/21)	28.6% (6/21)
2017	0% (0/0)	0% (0/0)	0% (0/0)	0% (0/0)	0% (0/0)	0% (0/0)	50% (1/2)	9.5% (2/21)	100% (2/2)	20% (5/25)	20.0% (5/25)
Total	100% (1/1)	100% (1/1)	100% (2/2)	50% (3/6)	100% (1/1)	57.1% (4/7)	67.7% (65/96)	60.1% (92/153)	85.7% (6/7)	63.7% (163/256)	63.8% (169/265)

Table 3
Antimicrobial resistance of *Salmonella enterica* serovar Virchow isolated from food-producing animals during 2010–2017 in South Korea.[a]

Antimicrobial agents	Range tested (μg/ml)	Break-points (μg/ml)	Comparison between resistance rates of susceptible and resistant isolates to ceftiofur							Total *S.* Virchow tested (n = 265)		
			Ceftiofur-susceptible *S.* Virchow (n = 96)			Ceftiofur-resistant *S.* Virchow (n = 169)			p-value[c]			
			MIC_{50}[b]	MIC_{90}[b]	% Resist. (n)	MIC_{50}[b]	MIC_{90}[b]	% Resist. (n)		MIC_{50}[b]	MIC_{90}[b]	% Resist. (n)
Aminoglycosides												
Gentamicin	1–64	≥16	≤1	32	11.5 (11)	2	64	49.1 (83)	< 0.0001	≤1	64	35.5 (94)
Streptomycin	16–128	≥32	≤16	> 128	18.8 (18)	> 128	> 128	96.4 (163)	< 0.0001	> 128	> 128	68.3 (181)
Aminopenicillin												
Ampicillin	2–64	≥32	≤2	≤2	8.3 (8)	> 64	> 64	100 (169)	ND	> 64	> 64	66.8 (177)
β-lactam/β-lactamase inhibitor												
Amoxicillin/clavulanic acid	2/1–32/16	≥32/16	≤2	≤2	0 (0)	4	64	14.8 (25)	< 0.0001	4	8	9.4 (25)
Cephamycin												
Cefoxitin	1–32	≥32	2	4	0 (0)	2	> 32	14.8 (25)	ND	2	16	9.4 (25)
Cephalosporin III												
Ceftiofur	0.5–8	≥8	≤0.5	1	0 (0)	> 8	> 8	100 (169)	ND	> 8	> 8	63.8 (169)
Fluoroquinolone												
Ciprofloxacin	0.12–16	≥1	0.25	0.5	0 (0)	0.25	0.5	2.4 (4)	0.1288	0.25	0.5	1.5 (4)
Quinolone												
Nalidixic acid	2–128	≥32	> 128	> 128	91.7 (88)	> 128	> 128	100 (169)	0.0001	> 128	> 128	97.0 (257)
Polymyxins												
Colistin	2–16	≥4	≤2	≤2	0 (0)	≤2	≤2	0 (0)	ND	≤2	≤2	0 (0)
Folate pathway inhibitors												
Trimethoprim/sulfamethoxazole	0.12/2.38–4/76	≥4/76	≤0.125	0.25	1 (1)	0.25	0.25	1.2 (2)	0.9165	0.25	0.25	1.1 (3)
Phenicols												
Chloramphenicol	2–64	≥32	8	8	2.1 (2)	8	8	0.6 (1)	0.2699	8	8	1.1 (3)
Tetracyclines												
Tetracycline	2–128	≥16	≤2	≤2	8.3 (8)	64	128	87.0 (147)	< 0.0001	64	128	58.5 (155)
MDR isolates					8.3 (8)			100 (169)	< 0.0001			66.8 (177)

[a] Abbreviations: MIC, minimum inhibitory concentration.
[b] MIC_{50} and MIC_{90} are the concentrations at which 50% and 90% of the isolates were inhibited.
[c] The p-values were calculated by chi-square test using Rex (Version 3.0.3, RexSoft Inc., Seoul, South Korea).

observed in 66.8% (177/265) of the isolates (Table S1). We next compared the resistance rates of ceftifur-resistant group (n = 169) and ceftiofur-susceptible group (n = 96). Overall resistance to non-β-lactam antimicrobials was higher in ceftiofur-resistant group than in ceftiofur-susceptible group (Table 3). Although resistance to chloramphenicol was higher in ceftiofur-susceptible group than in ceftiofur-resistant group, resistance rates to gentamicin (11.5% vs. 49.1%, p-value < 0.0001), streptomycin (18.8% vs. 96.4%, p-value < 0.0001), amoxicillin/clavulanic acid (0% vs. 14.8%, p-value < 0.0001), nalidixic acid (91.7% vs. 100%, p-value = 0.0001), and tetracycline (8.3% vs. 87.0%, p-value < 0.0001) were significantly higher in the ceftiofur-resistant group than in the ceftiofur-susceptible group. However, resistance to colistin, ciprofloxacin, and trimethoprim/sulfamethoxazole was rarely, or not at all, observed in either group. Furthermore, the MDR phenotype was observed in only 8.3% of ceftiofur-susceptible group, but all of the isolates were resistant to more than five antimicrobial subclasses in ceftiofur-resistant group (Table S1).

3.3. Molecular characteristics of ceftiofur-resistant Salmonella enterica serovar Virchow

Molecular characteristics of 169 ceftiofur-resistant *S*. Virchow isolates are shown in Table 4. The results from PCR and sequencing indicate that all of ceftiofur-resistant *S*. Virchow isolates produced β-lactamases, namely CTX-M-15 (n = 146) and CMY-2 (n = 22), and one isolate produced both CTX-M-15 and CMY-2 β-lactamases. None of the isolates produced TEM or SHV β-lactamases.

We identified seven types of patterns for ceftiofur-resistant *S*. Virchow based on PFGE (pulsotype I – VII) (Table 4, Fig. S1). The predominant pattern was pulsotype II in both $bla_{\text{CTX-M-15}}$-positive isolates (89/146, 61.0%) and $bla_{\text{CMY-2}}$-positive isolates (9/22, 40.9%). Pulsotype II and III were consistently detected for the examined time periods (2012–2016 and 2011–2017, respectively), and persisted in 69 farms (one cattle farm, four pig farms, and 64 chicken farms) in 10 provinces, and 33 farms (all from chicken farms) in 7 provinces, respectively. Moreover, pulsotype II was detected in all the animal species examined in this study.

Transfer of $bla_{\text{CTX-M-15}}$ and $bla_{\text{CMY-2}}$ genes was demonstrated in 42 out of 146 $bla_{\text{CTX-M-15}}$-positive *S*. Virchow isolates, and in 11 out of 22 $bla_{\text{CMY-2}}$-positive *S*. Virchow isolates to recipient *E. coli* J53 by conjugation (Table 4). We then performed molecular characterization of 53 transconjugants, and the results are shown in Table 5. Thirty-four transferable $bla_{\text{CTX-M-15}}$-positive plasmids belonged to replicon IncHI2/ST2, but the remaining eight plasmids had no *smr0199* loci.

The structure of the genetic environment of $bla_{\text{CTX-M-15}}$ was observed as three distinct types among the transconjugants carrying the $bla_{\text{CTX-M-15}}$ gene. IS*Ecp1*-$bla_{\text{CTX-M-15}}$-*orf477* elements were the most prevalent (38/42, 90.5%) among the elements examined in this study. IS*Ecp1*-$bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-15}}$-*orf477* elements were detected in two transconjugants, respectively. All transconjugants of CTX-M-15 showed resistance to aminoglycosides and/or tetracycline. The $bla_{\text{CMY-2}}$-carrying plasmids of 11 transconjugants belonged to IncI1/ST12 (clonal complex-12). All $bla_{\text{CMY-2}}$ gene expression was driven by the IS*Ecp1* insertion sequence, but *orf477* and IS*903* elements were not found downstream of $bla_{\text{CMY-2}}$ genes. Resistance to non-β-lactam agents was not transferred in 11 transconjugants of CMY-2 producing isolates (Table 5).

4. Discussion

Our findings showed a high prevalence of ESBL-producing *S*. Virchow in chickens and chicken carcasses; we found the identical pulsotypes (pulsotype II and III) and plasmid replicon type (IncHI2-type) among these isolates in Korea. *S*. Virchow was previously a rare serotype in Korean livestock. However, the presence of this serotype increased dramatically during 2012–2015, peaked in 2014, and was

Table 4
Distribution of ceftiofur-resistant *Salmonella enterica* serovar Virchow based on pulsed-field gel electrophoresis and their extended-spectrum β-lactam-resistance genes.

Pulsotype	Source	No. of isolates	No. of farms	No. of slaughterhouses	No. of provinces	Isolation periods	No. of isolates carrying ceftiofur-resistance gene			Self-transfer	
							$bla_{\text{CTX-M-15}}$	$bla_{\text{CMY-2}}$	$bla_{\text{CTX-M-15}}$ + $bla_{\text{CMY-2}}$	$bla_{\text{CTX-M-15}}$ positive	$bla_{\text{CMY-2}}$ positive
I	Cattle	1	1	1	1	2014 (n = 1)	1	–	–	–	–
	Chickens	13	13	4	5	2014 (n = 5), 2015 (n = 7), 2017 (n = 1)	13	–	–	–	–
II	Cattle	1	1	1	1	2014 (n = 1)	1	–	–	–	–
	Pigs	4	4	3	3	2014 (n = 3), 2015 (n = 1)	4	–	–	–	–
	Chickens	94	64	12	10	2012 (n = 7), 2013 (n = 13), 2014 (n = 53), 2015 (n = 19), 2016 (n = 2)	84	9	1	19	4
III	Chickens	44	33	6	7	2011 (n = 2), 2012 (n = 17), 2013 (n = 3), 2014 (n = 5), 2015 (n = 9), 2016 (n = 4), 2017 (n = 4)	38	6	–	22	1
IV	Chickens	8	3	2	2	2013 (n = 1), 2014 (n = 7)	1	7	–	–	6
V	Chickens	2	2	2	2	2013 (n = 1), 2014 (n = 1)	2	–	–	–	–
VI	Chickens	1	1	1	1	2014 (n = 1)	1	–	–	1	–
VII	Chickens	1	1	1	1	2015 (n = 1)	1	–	–	–	–
Total		169	114	19	10		146	22	1	42	11

Table 5
Characteristics of *E. coli* J53 transconjugants carrying $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CMY\text{-}2}$ genes described in this study.[a]

Transconjugants	Donor strains	Pulsotype	Resistance gene	Replicon type	pMLST	*bla* gene environment		Non-β-lactam resistance transferred
						Upstream	Downstream	
11SV115-1J	11V02S03002	III	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	GEN STR TET
11SV116-1J	11V02S03003	III	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	GEN STR TET
12SV260-1J	12V05S03013	III	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	-	STR TET
12SV261-1J	12V05S03014	III	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	-	STR TET
12SV262-1J	12V05S03015	III	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	STR TET
12SV263-1J	12V05S03016	III	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	STR TET
12SV215-1J	12V05S03018	III	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	STR TET
12SV167-1J	12V06A03001	III	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	STR TET
12SV105-1J	12V06A03007	II	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	-	orf477	GEN STR TET
12SV114-1J	12V06A03017	III	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	-	orf477	GEN STR TET
12SV267-1J	12V06S03008	III	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	GEN STR TET
12SV108-1J	12V06S03014	III	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	STR
13SV89-1J	13V02A03008	II	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	GEN STR TET
13SV90-1J	13V06A03002	II	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	GEN STR TET
13SV91-1J	13V06A03003	II	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	GEN STR TET
13SV92-1J	13V06A03010	II	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	TET
13SV99-1J	13V06S03002	III	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	GEN STR TET
13SV101-1J	13V06S03005	II	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	GEN STR TET
13SV102-1J	13V06S03006	III	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	GEN STR TET
14SV3-1J	14V02A03016	II	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	GEN STR TET
14SV4-1J	14V02A03017	II	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	STR TET
14SV5-1J	14V02A03018	II	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	GEN STR TET
14SV6-1J	14V02A03019	II	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	GEN STR TET
14SV7-1J	14V02A03020	VI	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	GEN STR TET
14SV55-1J	14V05S03001	II	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	GEN TET
14SV57-1J	14V05S03003	II	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	GEN STR TET
14SV58-1J	14V05S03004	II	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	GEN STR TET
14SV59-1J	14V05S03005	II	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	GEN STR TET
15SV118-1J	15V02A03001	III	$bla_{CTX\text{-}M\text{-}15}$	HI2	Untypable[b]	ISEcp1	orf477	STR TET
15SV122-1J	15V02A03005	III	$bla_{CTX\text{-}M\text{-}15}$	HI2	Untypable[b]	ISEcp1	orf477	STR TET
15SV126-1J	15V04S03009	II	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	TET
15SV130-1J	15V04S03043	II	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	GEN STR TET
15SV142-1J	15V06R03033	III	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	STR TET
15SV144-1J	15V09A03007	II	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	GEN STR TET
15SV145-1J	15V09A03008	II	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	STR TET
15SV146-1J	15V09A03009	II	$bla_{CTX\text{-}M\text{-}15}$	HI2	ST2	ISEcp1	orf477	STR TET
15SV147-1J	15V09R03001	III	$bla_{CTX\text{-}M\text{-}15}$	HI2	Untypable[b]	ISEcp1	orf477	STR TET
16SV277-1J	16V04A03012	III	$bla_{CTX\text{-}M\text{-}15}$	HI2	Untypable[b]	ISEcp1	orf477	STR TET
16SV278-1J	16V06A03006	III	$bla_{CTX\text{-}M\text{-}15}$	HI2	Untypable[b]	ISEcp1	orf477	STR TET
16SV279-1J	16V06A03007	III	$bla_{CTX\text{-}M\text{-}15}$	HI2	Untypable[b]	ISEcp1	orf477	STR TET
17SV300-1J	17V06R03007	III	$bla_{CTX\text{-}M\text{-}15}$	HI2	Untypable[b]	ISEcp1	orf477	STR TET
17SV301-1J	17V06R03008	III	$bla_{CTX\text{-}M\text{-}15}$	HI2	Untypable[b]	ISEcp1	orf477	STR TET
13SV94-1J	13V06A03014	III	$bla_{CMY\text{-}2}$	I1	ST12, CC-12	ISEcp1	–	–
14SV11-1J	14V02A03028	II	$bla_{CMY\text{-}2}$	I1	ST12, CC-12	ISEcp1	–	–
14SV12-1J	14V02A03029	II	$bla_{CMY\text{-}2}$	I1	ST12, CC-12	ISEcp1	–	–
14SV13-1J	14V02A03030	IV	$bla_{CMY\text{-}2}$	I1	ST12, CC-12	ISEcp1	–	–
14SV14-1J	14V02A03031	IV	$bla_{CMY\text{-}2}$	I1	ST12, CC-12	ISEcp1	–	–
14SV16-1J	14V02A03033	IV	$bla_{CMY\text{-}2}$	I1	ST12, CC-12	ISEcp1	–	–
14SV17-1J	14V02A03034	IV	$bla_{CMY\text{-}2}$	I1	ST12, CC-12	ISEcp1	–	–
14SV18-1J	14V02A03036	IV	$bla_{CMY\text{-}2}$	I1	ST12, CC-12	ISEcp1	–	–
14SV19-1J	14V02A03037	IV	$bla_{CMY\text{-}2}$	I1	ST12, CC-12	ISEcp1	–	–
14SV20-1J	14V02A03038	II	$bla_{CMY\text{-}2}$	I1	ST12, CC-12	ISEcp1	–	–
14SV21-1J	14V02A03039	II	$bla_{CMY\text{-}2}$	I1	ST12, CC-12	ISEcp1	–	–

[a] Abbreviations: GEN, gentamicin; pMLST, plasmid multi-locus sequence type; STR, streptomycin; and TET, tetracycline.
[b] Untypable, no *smr0199* loci of the IncHI2-type plasmid.

recently one of the main serovars isolated from chickens in Korea (APQA, 2017; Jeon et al., 2019). This increased presence has also been shown in a previous study on *S*. Virchow isolates from human patients in South Korean (Kim et al., 2016). *S*. Virchow is mainly found in broiler chickens and in poultry-producing environments, such as hatcheries and farms in Korea (Ha et al., 2018), Thailand (Jerngklinchan et al., 1994), and the European Union (Snow et al., 2008; Threlfall, 2002). However, it is unclear why and how *S*. Virchow distribution increased during 2012–2015. It may have occurred via contamination of broiler hatcheries or farms caused by lack of hygiene or improper sanitation practices at these facilities.

As the presence of *S*. Virchow increased in livestock, ceftiofur resistance in *S*. Virchow has also increased; 96.4% of ceftiofur-resistant *S*. Virchow strains were isolated from chicken feces and chicken carcasses. High ESC resistance in *Salmonella* spp. isolated from retail chickens in the United States (37.9%) and Italy (27.3%) was shown previously (McDermott et al., 2018). This may have been due to increased use of these antimicrobials. In Korea, the amount of ceftiour used to treat food animals is greater than that used in other countries, and the amount used in Korea has been doubled compared to that used before 2009 (APQA, 2017). In addition, the prevalence of ceftiofur-resistant *S*. Virchow isolates from human patients in Korea increased dramatically from 21.4% in 2011 to 82.3% in 2014 (Kim et al., 2016).

In this study, we found that ESC-resistant *S*. Virchow isolates

showed significantly higher resistance to other antimicrobials (especially aminoglycosides and tetracycline) than did ESC-susceptible *S.* Virchow isolates. Similarly, ESBL-producing *E. coli* and *K. pneumoniae* isolates are reported to have higher MICs to gentamicin and trimethoprim/sulfamethoxazole than those of ESC-susceptible isolates (Deshpande et al., 2000). Another report has shown that resistance to quinolones and tetracyclines is high in ESBL-producing *Enterobacteriaceae* isolated from chickens (Vitas et al., 2018). Additionally, all ESC-resistant *S.* Virchow isolates showed multiple resistance patterns, while 8.3% of ESC-susceptible *S.* Virchow were MDR isolates. Our results agree with data reported by other studies, showing that all ESBL-producing *Salmonella* spp. isolated from chickens showed MDR phenotypes (Choi et al., 2015; Jeon et al., 2019).

In our present study, only one type of bla_{CTX-M} gene, namely $bla_{CTX-M-15}$, was detected in 87.0% (147/169) of ceftiofur-resistant *S.* Virchow isolates. The CTX-M-15 enzyme is the most predominant type in both animals and humans infected with *Enterobacteriace,* including *Salmonella*, worldwide (Seiffert et al., 2013), including South Korea (Lee et al., 2016). Since CTX-M-9-producing *S.* Virchow was first reported in Spain (Simarro et al., 2000), various bla_{CTX-M} genes have been detected in *S.* Virchow; these include $bla_{CTX-M-3}$ in Turkey (Bahar et al., 2006), $bla_{CTX-M-10}$ in Spain and Russia (Cartelle et al., 2006; Egorova et al., 2007), and $bla_{CTX-M-2}$ in Belgium, France, and the Netherlands (Bertrand et al., 2006; Coque et al., 2008). However, in Korea, 93.3% and 100% of ESC-resistant *S.* Virchow isolates obtained from human patients and chickens, respectively, produce CTX-M-15-type ESBL (Choi et al., 2015; Kim et al., 2016).

In this study, 23 isolates, including one *S.* Virchow isolate producing both CTX-M-15 and CMY-2 β-lactamase, produced AmpC β-lactamase, namely CMY-2. CMY-2 β-lactamase is the most prevalent and most widely disseminated AmpC enzyme (Philippon et al., 2002). Recently, epidemic *Salmonella* spp. producing CMY-2 β-lactamases were reported in Brazil (*S.* Heidelberg), the European Union (*S.* Heidelberg and *S.* Minnesota), and South Korea (*S.* Virchow) (Campos et al., 2018; Kim et al., 2016; Tiba-Casas et al., 2019).

Interestingly, the 169 ceftiofur-resistant *S.* Virchow isolates represented seven pulsotypes, predominantly consisting of type II (58.6%) and III (26.0%). These pulsotypes were distributed nationwide at approximately 100 farms in 10 provinces in Korea. These results suggest that a specific $bla_{CTX-M-15}$ *S.* Virchow clone may have been clonally disseminated among Korean livestock. Furthermore, these pulsotypes were detected over all the time periods examined in our present study. Clonal spread of multidrug resistant *Salmonella* spp. has been previously reported in both animals and humans worldwide (Butaye et al., 2006; Kim et al., 2016; Riaño et al., 2009). However, the discriminatory power of PFGE for typing *Salmonella* spp. is limited compared to that of other molecular typing methods, such as multilocus variable-number of tandem-repeats analysis or whole genome sequencing. Thus, further investigation was needed to identify the predominant specific clones by combining PFGE with other methods or by digestion with a second restriction enzyme, such as *Bln*1 (Zhao et al., 2006).

Of the 169 CTX-M-15 and/or CMY-2 β-lactamase-producing *S.* Virchow, 42 CTX-M-15-producing isolates and 11 CMY-2-producing isolates transmitted their plasmids containing *bla* genes to J53 *E. coli* recipients by conjugation. The $bla_{CTX-M-15}$ gene is located predominantly on plasmids belonging to the IncF group (Carattoli, 2009), but in our present study, all conjugative plasmids carrying the $bla_{CTX-M-15}$ genes belonged to IncHI2-type group. Of the 42 conjugative IncHI2-type plasmids, 34 belonged to ST2. The plasmid type and *bla* gene environments of the $bla_{CTX-M-15}$-carrying plasmids were identical to those reported previously for plasmids in *S.* Virchow and *S.* Enteritidis isolated from human patients and poultry meat in South Korea (Kim et al., 2016). Eight plasmids were not typable because there were no *smr0199* loci. Although next-generation sequencing (NGS) was not performed for these plasmids, the NGS report on IncHI2 plasmids without *smr0199* loci in China (Yi et al., 2017) may be useful for clarifying similar cases.

In this study, the IS*Ecp1*-$bla_{CTX-M-15}$-*orf477* transposable units predominated (38/42). We found that IS*Ecp1* was located upstream of the $bla_{CTX-M-15}$ gene, as were other bla_{CTX-M} types of the CTX-M-1, CTX-M-2, CTX-M-9, and CTX-M-25 groups (Rossolini et al., 2008). IS*Ecp1* is involved in the mobilization and expression of the bla_{CTX-M} gene (Eckert et al., 2006; Poirel et al., 2003).

We found that resistance to aminoglycosides and/or tetracycline was co-transferred with the $bla_{CTX-M-15}$ gene. Co-transfer of aminoglycoside- and tetracycline-resistance with the *bla* genes was reported in a previous study on *S. Indiana* in China (Bai et al., 2016). Furthermore, Bouzidi et al. (2011) suggested that horizontal spread of $bla_{CTX-M-15}$ and *armA* (16S rRNA methylase gene causing high-level resistance to aminoglycosides) co-occurred in *S.* Enteritidis in Algeria. These results indicate that $bla_{CTX-M-15}$-carrying plasmids were related to high resistance to aminoglycosides and tetracycline in ceftiofur-resistant *S.* Virchow examined in our present study. Furthermore, widespread use of these antimicrobials may contribute to co-selection of organisms resistant to extended-spectrum cephalosporins.

The bla_{CMY-2} gene is associated with IncA/C and IncI1 plasmids (Carattoli, 2009; Seiffert et al., 2013). IncI1 plasmids are widespread in animal strains of *Enterobacteriaceae* and may be associated with dissemination of the bla_{CMY-2} gene (Carattoli, 2009). The bla_{CMY-2}-carrying ST12 (clonal complex 12)-IncI1 plasmids were also recently detected in *S.* Paratyphi B strains in Belgium (Doublet et al., 2014) and in *S.* Minnesota in Brazil (Moura et al., 2018). In Korea, IncI1-Iγ plasmids carrying the bla_{CMY-2} gene have been detected in *E. coli*, which showed a genetic environment of *bla* genes and co-resistance pattern that was similar to those observed by our team (Tamang et al., 2011a). In this study, the bla_{CMY-2}-positive *S.* Virchow isolates were mainly detected in one slaughterhouse in 2014 (15/21), and most isolates were transferred to the *E. coli* recipient strain (data not shown). Thus, our results suggest that the bla_{CMY-2} gene may be predominantly contained in specific types of IncI1 plasmids, and may be disseminated horizontally in South Korea.

In conclusion, the prevalence of CTX-M enzymes among the *S.* Virchow organisms isolated from food-producing animals, especially chickens, in South Korea has increased since 2010 and is reaching worrisomely high levels. This rapidly growing presence of CTX-M-15 and widespread co-resistance in CTX-M-producing isolates, as well as increasing incidence of identical conjugative plasmids in humans and animals, indicate that intervention strategies should be urgently applied to food-producing animals in Korea. This is especially important in the chicken production industry, where restriction of antimicrobial use and improved biosecurity practices need to be implemented at the farm level.

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Declaration of competing interest

Not applicable.

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