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NOTE

Bacteriology

The occurrence of CTX-M-25-producing Enterobacteriaceae in day-old broiler chicks in Japan

Montira YOSSAPOL[1,2], Michiyo SUGIYAMA[1] and Tetsuo ASAI[1,3]*

[1]Department of Applied Veterinary Science, the United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[2]Office of Academic Affairs, Faculty of Veterinary Sciences, Mahasarakham University, Talad sub-district, Mueang district, Maha Sarakham 44000, Thailand
[3]Education and Research Center for Food Animal Health (GeFAH), Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

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ABSTRACT. Day-old chicks from 3 hatcheries were placed on bedding paper and brought to a commercial broiler farm between January and July 2016. Sixty-six samples of the paper, which were stained with meconium droppings of the chicks, were collected and examined for isolation of cephalosporin-resistant Enterobacteriaceae. Cefotaxime (CTX)-resistant *Klebsiella pneumoniae* (1 isolate) and *Enterobacter cloacae* (4 isolates) were isolated from 5 (7.58%) of the 66 samples. Conjugation experiments revealed that the $bla_{CTX\text{-}M\text{-}25}$ gene conferring CTX resistance was transferred from the *K. pneumoniae* isolate and 2 of the 4 *E. cloacae* isolates to *Escherichia coli* DH5α via IncA/C plasmids carrying the gene. Our results suggested that the $bla_{CTX\text{-}M\text{-}25}$ gene originating from chicks may be spread among commercial broiler farms.

KEY WORDS: antimicrobial resistance, chick, Enterobacteriaceae, ESBL

Antimicrobial resistance is a major issue in modern medicine, since it limits the treatment options for bacterial infections. Food-producing animals are reservoirs of antimicrobial-resistant (AMR) bacteria [9, 11, 13, 25]. Accordingly, it is essential to control and monitor these bacteria in animals in order to reduce the transfer of AMR bacteria and resistance determinants to humans via the consumption of animal products. In Japan, in the last decade, many studies have reported a high prevalence of AmpC β-lactamase- and extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* in broilers [11, 13] and in retail chicken meat [1, 14]. Several studies in Europe have reported contamination with ESBL-producing Enterobacteriaceae at various stages of the broiler production system, e.g., at the breeder [9, 20, 25], hatchery [17, 20], and farm levels [9, 25]. Although CMY-2 is the major broad-spectrum β-lactamase found in the broiler industry in Japan, various ESBLs of the CTX-M- and SHV-types are also prevalent [11, 13]. In addition, since the initial discovery of CTX-M-2 in broilers, diverse ESBL-producing CTX-M subtypes have been observed in broilers in Japan [10]. However, the origin and prevalence of AMR bacteria in broiler farms are unclear. Therefore, in this study, potential ESBL gene transmission vehicles in commercial farms were identified by examining the prevalence and characteristics of cephalosporin-resistant Enterobacteriaceae in day-old chicks.

Day-old chicks from 3 hatcheries located in the different cities in Japan were placed on bedding paper and brought to a commercial broiler farm between January and July 2016. Sixty-six samples of the paper (34, 8 and 24 samples from hatcheries A, B and C, respectively), which were stained with meconium droppings of the chicks, were collected on arrival at the farm and examined for isolation of cephalosporin-resistant Enterobacteriaceae. After cutting out two 25-cm^2 pieces of the bedding paper, (the paper) samples were further cut into smaller pieces and equal amount of the samples were put into two separate test tubes. Five milliliters of 0.85% NaCl were added to the first test tube and mixed, and 50 μl of the mixture was spread onto deoxycholate hydrogen sulfide lactose (DHL) agar (Eiken Chemical Co., Ltd., Ootawara, Japan) containing 50 μg/m*l* cephalexin (CEX) (C-DHL). In the second test tube, 5 m*l* of Müller–Hinton broth (Becton, Dickinson and Co., Franklin Lakes, NJ, U.S.A.) containing 50 μg/m*l* CEX were added and incubated overnight at 37°C. After the incubation period, a loop from the culture was streaked onto C-DHL. Bacterial colonies on C-DHL plates were picked, and species-level identification was based on biochemical parameters using the API 20E (bioMérieux, Marcy-l'Étoile, France).

Minimum inhibitory concentrations (MICs) were determined using a commercially available broth microdilution test (Eiken Chemical Co., Ltd.) for the following 12 antimicrobial agents: ampicillin (ABPC), cefazolin (CEZ), cefotaxime (CTX),

*Correspondence to: Asai, T.: tasai@gifu-u.ac.jp

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Table 1. Characteristics of CEX-resistant strains isolated from the meconium of day-old chicks

Organism	Strain	Broiler hatchery	β-lactamase type	MIC of antimicrobials (Resistance breakpoint)[a)]									
				CTX (≥4)	MEPM (≥4)	GM (≥16)	KM (≥64)	TC (≥16)	NA (≥32)	CPFX (≥4)	CL (>2)	CP (≥32)	ST (≥76/4)
K. pneumoniae	CC37	A	CTX-M-25, SHV-11	64	≤0.25	2	≥128	≥64	4	≤0.03	1	2	19/1
E. cloacae	CC23	A	CTX-M-25, TEM-1	≥64	≤0.25	4	≥128	4	16	≤0.03	1	8	19/1
	CC5	B	CTX-M-25	≥64	≤0.25	32	64	4	2	≤0.03	2	8	38/2
	CC6	B	CTX-M-25	≥64	≤0.25	32	64	4	2	≤0.03	1	8	38/2
	CC32	C	CTX-M-25	≥64	≤0.25	≤0.5	≥128	2	4	≤0.03	≥16	8	9.5/0.5

CTX, cefotaxime; MEPM, meropenem; GM, gentamicin; KM, kanamycin; TC, tetracycline; NA, nalidixic acid; CPFX, ciprofloxacin; CL, colistin; CP, chloramphenicol; ST, sulfamethoxazole and trimethoprim. a) Resistance breakpoint of CL (mg/*l*) was defined by EUCAST [23], while that of other antimicrobials (μg/m*l*) was defined by CLSI [7].

meropenem (MEPM), gentamicin (GM), kanamycin (KM), tetracycline (TC), nalidixic acid (NA), ciprofloxacin (CPFX), colistin (CL), chloramphenicol (CP), and sulfamethoxazole-trimethoprim (ST). The resistance breakpoint for CL was defined as per the guidelines of the European Committee on Antimicrobial Susceptibility Testing [23]. Determination of resistance breakpoints for the other antimicrobials and confirmation of the ESBL phenotype by the disk diffusion test using CTX/clavulanate (30/10 μg/disk), and CTX (30 μg/disk) (Nissui Pharmaceutical, Tokyo, Japan) were performed according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [7].

Genes encoding TEM and SHV β-lactamases were detected by polymerase chain reaction (PCR) using specific primers [15]. CTX-M β-lactamase genes were detected using multiplex PCR [8], and subtypes of CTX-M-8/25 group β-lactamases were determined by sequencing analysis using the primer pair CTXM825F (5′-CGCTTTGCCATGTGCAGCACC-3′) and CTXM20 (5′-ATAACCGTCGGTGACAATT-3′), described in a previous report [2]. Both strands of the amplified DNA fragments were sequenced at the Life Science Research Center of Gifu University, and the resulting amino acid sequences were analyzed using BLAST (National Center for Biotechnology Information, Bethesda, MD, U.S.A.).

Genotypes of the ESBL-producing isolates were identified by pulsed-field gel electrophoresis (PFGE) according to the PulseNet standardized protocol [21]. Briefly, after digestion with the *XbaI* restriction enzyme (Takara Bio, Inc., Shiga, Japan), electrophoresis was performed at 6 V/cm for 18.5 hr, with a pulse-time ranging from 2.2 to 54.2 sec, using a CHEF-DR III system (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Results were interpreted by generating a UPGMA dendrogram with 0.5% optimization and 0.5% band filtering tolerance using BioNumerics version 7.6.1 (Applied Maths NV, Sint-Martens-Latem, Belgium).

A conjugation experiment was performed using the broth-mating method with rifampicin (RIF)- and NA-resistant *Escherichia coli* DH5α as recipients, as described previously [24]. Briefly, 4-hr-old Luria–Bertani broth (Becton, Dickinson and Co.) cultures of the donor (0.5 m*l*) and the recipient (4.5 m*l*) were mixed in an L-shaped tube and incubated in a water bath at 37°C for 1–2 hr under mild shaking. Dilutions of the donor and recipient mixture were spread on transconjugant-selective MacConkey agar (Eiken Chemical Co., Ltd.) plates containing RIF (50 μg/m*l*) and CEX (50 μg/m*l*) and recipient-selective MacConkey agar plates containing RIF (50 μg/m*l*). Tranconjugants were subjected to a PCR analysis using primers for CTX-M-8/25, SHV, and TEM and were examined for antimicrobial susceptibility. PCR-based plasmid replicon typing was performed using primer sets described previously [4].

CTX-resistant *Klebsiella pneumoniae* and *Enterobacter cloacae* isolates (MIC≥4 μg/m*l*) were found in 1 (1.52%) and 4 (6.06%) of 66 bedding paper samples of day-old broilers, respectively. In a Dutch study, CMY-2-producing *Escherichia coli* were isolated from 1.88% (8/425) of individual fresh meconium samples taken from the bedding paper of day-old broilers [9]. In the present study, because meconium dropping from more than one chick were present on the bedding paper samples, the isolation rates cannot be compared.

CTX-resistant *K. pneumoniae* was found only in hatchery A (1/34, 2.94%), whereas CTX-resistant *E. cloacae* was found in 1 of 34 (2.94%), 2 of 8 (25.00%), and 1 of 24 (4.17%) samples obtained from hatcheries A, B and C, respectively. Among CTX-resistant isolates from hatchery A, a *K. pneumoniae* strain harbored both $bla_{\text{CTX-M-25}}$ and $bla_{\text{SHV-11}}$ and exhibited resistance to KM and TC, and an *E. cloacae* strain harbored $bla_{\text{CTX-M-25}}$ and $bla_{\text{TEM-1}}$ and showed resistance to KM. Other CTX-resistant *E. cloacae* strains harbored $bla_{\text{CTX-M-25}}$ from hatcheries B and C and exhibited resistant to KM with GM and KM with CL, respectively (Table 1). In addition, PFGE profiles of CTX-resistant *E. cloacae* strains from the chicks in the three hatcheries were different from each other (Fig. 1), suggesting that the prevailing CTX-resistant *E. cloacae* strains from distinct hatcheries were genetically unrelated.

In the present study, CTX-M-25-producing *K. pneumoniae* and *E. cloacae* were detected in day-old chicks. CTX-M-25, one of the enzymes belonging to the CTX-M-25 group, was first found in *E. coli* isolated from a hospitalized patient in Canada in 2000 [18], and CTX-M-25 ESBL producers have been detected in *E. cloacae, K. pneumoniae* [19] and *Proteus mirabilis* [16] isolated from patients in several countries. In Japan, CTX-M-25 ESBL producers are not commonly detected in humans; the enzymes assigned in the CTX-M-9 group are most frequently found in *E. coli* isolated from human patients [5, 22]. In Japan, since CTX-M-25-producing *E. coli* was isolated from diseased poultry in 2005 and 2006 [2], $bla_{\text{CTX-M-25}}$ has been detected in *Salmonella* Infantis and *E. coli* from healthy broilers in 2007 and 2008 [6] and in 2010 and 2012 [12], respectively. These results suggested that

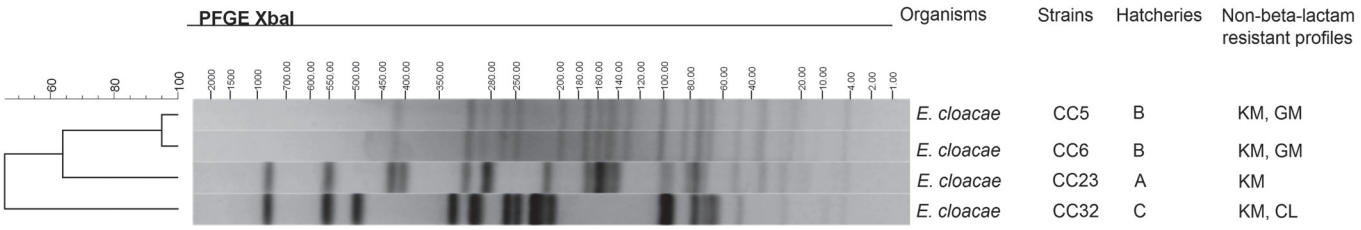

Fig. 1. Dendrogram of *XbaI*-digested pulsed-field gel electrophoresis (PFGE) profiles of CTX-M-25-harboring isolates of *Enterobacter cloacae* obtained from day-old chicks from three hatcheries. Kb, kilobases; KM, kanamycin; TC, tetracycline; GM, gentamycin; CL, colistin.

Table 2. Characteristics of donor strains and their transconjugants

Organism	Donors			Transconjugants			
	Strain	β-lactamase type	Resistance patterns[a)]	Strain	β-lactamase type	Resistance patterns[a)]	Plasmid replicon type
K. pneumoniae	CC37	CTX-M-25, SHV-11	CTX, KM, TC	TC37	CTX-M-25	CTX, KM	IncA/C
E. cloacae	CC23	CTX-M-25, TEM-1	CTX, KM	TC23	CTX-M-25	CTX, KM	IncA/C
	CC32	CTX-M-25	CTX, KM, CL	TC32	CTX-M-25	CTX, KM	IncA/C

CTX, cefotaxime; KM, kanamycin; TC, tetracycline; CL, colistin. a) Resistance breakpoint of CL (mg/*l*) was defined by EUCAST [23], while that of other antimicrobials (μg/m*l*) was defined by CLSI [7].

$bla_{\text{CTX-M-25}}$ is persistently distributed among various bacterial species in poultry in Japan, although CTX-M-2 is the most dominant enzyme in ESBL-producing *E. coli* from poultry [11, 12].

As the spread of $bla_{\text{CTX-Ms}}$ in Enterobacteriaceae occurs via plasmid dissemination [3], conjugation tests were performed. Transconjugants were obtained from the *K. pneumoniae* strain and two of the four *E. cloacae* strains and they were also resistant to KM as their donor strains. PCR-based plasmid replicon typing of transconjugants showed the IncA/C plasmid (Table 2). Thus, $bla_{\text{CTX-M-25}}$ genes could be transferred via the IncA/C plasmid among Enterobacteriaceae.

Transconjugants obtained in the present study showed resistance to CTX along with KM. Co-resistance often contributes to the prevalence of AMR bacteria [20]. Hiki *et al.* reported an increase in KM and streptomycin resistance in CTX-resistant *E. coli* isolated from broilers following the discontinuation of in ovo injections of ceftiofur coupled with the vaccine for Marek's disease and fowl pox at a hatchery [12]. The mechanism by which CTX-resistant Enterobacteriaceae increased in the broiler industry is unknown.

In conclusion, our results suggested that the $bla_{\text{CTX-M-25}}$ gene originating from chicks may be spread among commercial broiler farms through IncA/C plasmid harboring this gene.

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