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Prevalence of Extended-Spectrum β-Lactamase-Producing *Escherichia coli* and *Klebsiella pneumoniae* in Food-Producing Animals

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ABSTRACT. To evaluate the diversity of extended-spectrum β-lactamases (ESBL) genes among food-producing animals, 48 isolates of ESBL-producing *Escherichia coli* isolates were obtained from rectal samples of broilers, layers, beef cattle and pigs, at the slaughterhouse level. ESBL-carrying *E. coli* were isolated from 60.0% of individual broiler rectal samples, 5.9% of layers, 12.5% of beef cattle and 3% of pigs. One ESBL-producing *Klebsiella pneumoniae* was isolated from a broiler. The ESBL-positive *E. coli* isolates from broilers harbored various ESBL genes: $bla_{\text{SHV-12}}$, $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-44}}$. The plasmid DNAs were analyzed by restriction patterns. Homogeneous band patterns were yielded in those of *K. pneumoniae* and *E. coli* isolates harboring the $bla_{\text{CTX-M-2}}$ gene from different farms. No genetic relation between the 2 CTX-M-14 ESBL-producing strains was found by pulsed-field gel electrophoresis, although 2 plasmids in these strains, obtained from different broiler farms, were similar to each other. This study provides evidence that the proliferation of CTX-M-producing *E. coli* is due to the growth of indigenous CTX-M-producing strains and the possible emergence of strains that acquired CTX-M genes by horizontal transfer in different broiler farms. CTX-M-producing coliforms in broilers should be controlled due to the critical importance of cephalosporins and the zoonotic potential of ESBL-producing bacteria.
KEY WORDS: antimicrobial resistance, broiler, ESBL, *Escherichia coli*, *Klebsiella pneumoniae.*

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Recently, reports concerning *Escherichia coli* carrying broad-spectrum β-lactamases isolated from food-producing animals and humans have been published worldwide [22]. Extended-spectrum-β-lactamases (ESBLs) are a new group of enzymes that confer resistance to extended-spectrum cephalosporins, while remaining generally susceptible to carbapenems, cephamycins, and β-lactamase inhibitors such as clavulanic acid [5]. The vast majority of ESBLs were derivatives of TEM-1 or SHV-1; the common plasmid-mediated β-lactamase of organisms such as *E. coli* and the common chromosome-mediated β-lactamase of *Klebsiella pneumoniae*, respectively [34].

Reports of CTX-M groups with ESBL-resistant phenotypes are becoming more common [4]. In Japan, CTX-M-type ESBL-producing *Enterobacteriaceae* is important for nosocomial infections. ESBL-producing *K. pneumoniae*, *E. coli* and *Proteus mirabilis* have been implicated in numerous outbreaks of nosocomial infections over the last 2 decades [13, 23, 37], and recently an increase in the number of reports of CTX-M groups with ESBL-resistant phenotypes has occurred. Clinical isolates of ESBL- and Shiga toxin-producing *Escherichia coli* O26, *Shigella sonnei*, and *Salmonella* serotype Enteritidis have been reported [15, 17, 19, 21]. In food-producing animals, Shiraki *et al*. first reported the isolation of CTX-M-2-producing *E. coli* from cattle in Japan [32]. The Japanese Veterinary Antimicrobial Resistance Monitoring Program (JVARM) reported ESBL-producing *E. coli* isolated from poultry in Japan [20]. In addition, a study from our laboratory suggested a potential increase in the ESBL-producing *E. coli* isolated from broilers [14].

The concern is that, in farm environments, commensal and environmental bacteria may be reservoirs for the transfer of antimicrobial resistance genes to pathogenic bacteria [3, 22, 33]. However, there are few reports of ESBL-producing commensal enteric bacteria in food-producing animals in Japan. In this study, we characterized the prevalence and genetic similarities of ESBL-producing *E. coli* and *K. pneumoniae* strains in food-producing animals.

MATERIALS AND METHODS

Bacterial strains: Fresh rectal samples were collected in slaughterhouses in Shizuoka Prefecture, Japan. Samples from broilers (n=30) were derived from 10 farms between May and August 2007. Samples from layers (n=17), beef cattle (n=16) and pigs (n=33) were derived from 13 farms between July and October 2007. Rectal samples were plated

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onto Chromocult Coliform Agar ES (Merck KGaA, Darmstadt, Germany) supplemented with 1 μg/m*l* cefotaxime (CTX) and incubated for 24 hr at 35°C. The β-D-glucuronidase- and β-D-galactosidase-positive colonies were typical *E. coli* morphology which shows dark blue to violet color on this chromogenic culture medium.

Up to 3 colonies with typical *E. coli* morphology from each sample were selected and purified. In addition, the isolates were confirmed biochemically by using such as triple sugar iron agar (Difco Laboratories, Detroit, MI, U.S.A.) slants and lysine indole motility medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan), the results for lactose reaction (+), saccharose reaction (+), gas (+), lysine decarboxylation (+) and indole (+). The identification of the *Klebsiella*-like colonies were then confirmed using API ID 32E (bioMérioux, Marcy-l'Etoile, France) and conventional biochemical methods.

Antimicrobial susceptibility testing: Isolates growing at or above the screening concentrations may indicate ESBL production (i.e., MIC≥8 μg/m*l* for cefpodoxime: CPDX, or MIC≥2 μg/m*l* for ceftazidime, aztreonam, cefotaxime or ceftriaxone) [8]. The putative ESBL-producers and their derivative transconjugants were examined by the CLSI-recommended disk confirmatory tests by a standard disk diffusion method [7]. BD Sensi-Disc (Becton Dickinson, NJ, U.S.A.), ESBLs-CTX/CVA'Eiken', ESBLs-CAZ/CVA'Eiken' and ESBLs-CPX/CVA'Eiken' (Eiken Chemical Co., Ltd. Tokyo, Japan) were used for the disk diffusion test. Strains of *K. pneumoniae* (ATCC 700603) and *E. coli* (ATCC 25922) were used as quality controls for the ESBL tests as positive and negative controls, respectively.

Characterization of β-lactamase genes: Several β-lactamase genes, including bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-9}}$, $bla_{\text{CMY-1}}$, $bla_{\text{CMY-2}}$, $bla_{\text{PSE-1}}$, and bla_{FOX}, were detected by PCR as described previously [10, 20] in ESBL-producing isolates and their transconjugants obtained from broilers. The amplified PCR products were sequenced with an Applied Biosystems 3730xl DNA Analyzer. Nucleotide sequences were examined using the BLAST program [1].

Conjugation, plasmid analysis and pulsed-field gel electrophoresis (PFGE): To determine the transferability of genes encoding antimicrobial resistance, transconjugation experiments were performed. Forty-two ESBL-positive isolates from broilers were used as donors, and a rifampicin-resistant mutant of *E. coli* INVαF' (Invitrogen Corp., Carlsbad, CA, U.S.A.) generated in our laboratory was used as the recipient in the transconjugation experiments. The organisms were inoculated into 4 m*l* of Luria-Bertani (LB) broth and incubated for 18 hr at 37°C while shaking. Two organisms were then mixed together 1:1 and incubated for 18–24 hr at 37°C. After incubation, transconjugants were selected on BTB lactose agar (Merck, Darmstadt, Germany) containing rifampicin (50 μg/m*l*) and cefotaxime (1 μg/m*l*). The donor strains, lactose-positive and the recipient strain, lactose-negative formed yellow and colorless colonies on BTB lactose agar, respectively. The transconjugant strains also formed colorless colonies. The colorless colonies were selected from the agar plates and inoculated into BTB lactose agar again for colony purification of transconjugant strains. The plasmid electrophoresis and PFGE were then further analyzed.

Plasmid DNA of the *E. coli* transconjugants was obtained as described by Sasakawa [28]. Transconjugants harboring a single plasmid that originated from isolates were successfully selected. *E. coli* V517 (35.8, 4.8, 3.7, 3.4, 2.6, 2.0 and 1.8 MDa), *E. coli* R1 (62 MDa) and *E. coli* Rts1 (120 MDa) were used as the standards for plasmid size analysis. Plasmid DNA was then digested by *Eco*RI, *Sph*I and *Cla*I restriction enzymes (Takara Bio Inc., Shiga, Japan) and subjected to electrophoresis on a 1% agarose gel.

PFGE with *Xba*I (Takara Bio Inc.) was carried out according to the Centers for Disease Control and Prevention's PulseNet protocol with some modifications, as described previously [16, 18, 35]. Briefly, 100 μ*l* of bacterial suspension and 100 μ*l* of melted 1% (w/v) SeaKem Gold agarose (Cambrex, NJ, U.S.A.) were mixed. The mixture was poured into the wells of a sample plug caster (Bio-Rad Laboratories, Hercules, CA, U.S.A.) and kept at 4°C to solidify. The plug was incubated with 1 m*l* of lysis buffer (0.5 M EDTA pH 8.0, 1% *N*-lauroyl sarcosine and 1 mg/m*l* proteinase K) at 50°C for 18 to 20 hr and washed with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 4 mM Pefabloc SC (Roche Diagnostics, Basel, Switzerland). Then, the plug was cut and treated with the *Xba* I enzyme (30 units per sample) at 37°C for 2 hr or more. Electrophoresis was performed in a 1% SeaKem Gold agarose gel with 0.5 × TBE (50 mM Tris, 45 mM boric acid, 0.5 mM EDTA) using the CHEF-DR III system (Bio-Rad Laboratories) at pulse time of 2.2–54.2 sec and 6 V/cm for 20 hr. After electrophoresis, the gel was stained with an ethidium bromide solution and photographed under a transilluminator. PFGE patterns were compared with the Fingerprinting II software version 3.0 (Bio-Rad Laboratories) using the Dice coefficient, according to manufacturer's instructions, and a dendrogram was constructed using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm. Strains sharing different 3 or fewer bands in PFGE pattern were considered related, whereas those with more than 3 bands were considered unrelated [36].

Southern blot analysis: Digoxigenin (DIG)-labeled probes were generated using the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. Primers for the preparation of probes from the $bla_{\text{CTX-M-2}}$ and $bla_{\text{CTX-M-14}}$ genes were described previously [20]. DNA Molecular Weight Marker III, Digoxigenin-labeled (Roche Diagnostics GmbH) was used for molecular weight marker.

RESULTS

Origin of isolates and antimicrobial susceptibility: From 96 rectal samples, 109 *E. coli* were isolated by using Chromocult Coliform Agar ES (Merck KGaA) with CTX. The num-

Table 1. Source of the samples and no. of samples of isolated *E. coli* and ESBL producers from different origins

Origin of samples	No. of samples of isolated *E. coli* (%)[a)]	No. of samples of isolated ESBL producers (%)	No. of farms from where *E. coli* samples were isolated (%)[a)]	No. of farms from where ESBL producers were isolated (%)
Broilers	30/30 (100)	18/30 (60.0)	10/10 (100)	7/10 (70)
Layers	3/17 (17.6)	1/17 (5.9)	3/7 (42.9)	1/7 (14.3)
Beef cattle	5/16 (31.3)	2/16 (12.5)	4/5 (80.0)	2/5 (40)
Pigs	3/33 (9.1)	1/33 (3.0)	3/12 (25)	1/12 (8.3)

a) *E. coli* isolated by Chromocult Coliform Agar ES with 1 mg/L CTX.

bers of *E. coli* isolates were 5 from layers, 7 from beef cattle, 7 from pigs and 90 from broilers. *E. coli*-positive samples were 3 of 17 layers (17.6%), 5 of 16 beef cattle (31.3%), 3 of 33 pigs (9.1%) and 30 of 30 broilers (100%) (Table 1). From broilers only, all 30 samples were *E. coli*-positive. All *E. coli* isolates were resistant to CPDX. A strain of *K. pneumoniae* was isolated from the sample of a broiler.

A total of 48 ESBL-producing *E. coli* strains were detected. ESBL-producing isolates were derived from samples; 41 strains from 30 broilers, 1 strain from 17 layers, 4 strains from 16 beef cattle, and 2 strains from 33 pigs. The *K. pneumoniae* strain isolated from a broiler was an ESBL-producer.

Characterization of β-lactamase genes: The PCR products from the ESBL-producing *E. coli* strains were directly sequenced, analyzed, and identified as: $bla_{\text{CTX-M-2}}$ in 24 isolates, $bla_{\text{CTX-M-14}}$ in 4 isolates, $bla_{\text{SHV-12}}$ in 9 isolates, $bla_{\text{CTX-M-15}}$ in 3 isolates, $bla_{\text{CTX-M-44}}$ in 3 isolates, and $bla_{\text{TEM-1}}$ in 8 isolates (Table 2). The $bla_{\text{SHV-12}}$ gene was found in combination with the $bla_{\text{CTX-M-2}}$ gene in 4 isolates, and the $bla_{\text{CTX-M-14}}$ gene in 1 isolate. In addition, the $bla_{\text{TEM-1}}$ gene was found alone in 2 isolates, in combination with the $bla_{\text{CTX-M-15}}$ gene in 3 isolates, and the $bla_{\text{CTX-M-2}}$ gene in 3 isolates. None of the acquired β-lactamase genes examined in this study was detectable in 1 isolate. In the *K. pneumoniae* strain, the $bla_{\text{CTX-M-2}}$ gene and $bla_{\text{SHV-108}}$ gene were detected.

β-Lactam resistance transfer assays, Southern blot analysis, plasmid DNA analysis and PFGE: Seventeen of forty-one *E. coli* isolates and one *K. pneumoniae* isolate were able to transfer the ESBL phenotype to a recipient. The presence of the respective β-lactamase genes was confirmed in all transconjugants by PCR analysis with primers encoding CTX-M- or SHV-type ESBLs. Undigested plasmid profiles and Southern blot hybridization from the *K. pneumoniae* isolate no. 13-1k; *E. coli* isolates nos. 7–1, 7–4, 7–7 and 7–8; and transconjugants of isolates nos. 13–1k, 7–1, 7–4, 7–7 and 7–8 are shown in Fig. 1. Plasmid DNA, which was obtained from the transconjugants, harbored at least 1 large-sized plasmid band comparable to those previously described [20] (Fig. 1). Eighteen transconjugants harboring 1 large-sized plasmid encoding CTX-M- or SHV-type ESBLs were selected for obtaining plasmid restriction patterns.

Southern blot hybridization analysis with a 876-bp probe prepared by PCR amplification of the $bla_{\text{CTX-M-2}}$ and $bla_{\text{CTX-M-14}}$ genes was performed. Hybridization was positive in 9 plasmids that originated from 5 isolates harboring the $bla_{\text{CTX-M-2}}$ gene and 4 isolates harboring the $bla_{\text{CTX-M-14}}$ gene. The $bla_{\text{CTX-M-2}}$ and $bla_{\text{CTX-M-14}}$ genes signals were detected from the plasmid that originated from the transconjugants of 9 isolates. From these results, plasmids encoding CTX-M-2 or CTX-M-14 were conjugatively transferred from *E. coli* and *K. pneumoniae* isolates to recipient strains.

Table 2. β-lactamase genes harbored in *E. coli* isolates from broilers

β-lactamase genes	Farm: No. of isolates	Total
CTX-M-2	B: 5, G: 5, H: 5, J: 2	17
CTX-M-2 + SHV-12	B: 4	4
SHV-12	C: 3, E: 1	4
CTX-M-14	E: 2, I: 1	3
CTX-M-15 + TEM-1	C: 3	3
CTX-M-44	I: 3	3
CTX-M-2 + TEM-1	J: 3	3
CTX-M-14 + SHV-12	E: 1	1
TEM-1	H: 2	2

Restriction profiles of plasmids from CTX-M-2-producing transconjugants showed that 5 strains, *K. pneumoniae* isolate no. 13–1k, *E. coli* isolate nos. 7–1, 7–4, 7–7 and 7–8 isolated from broiler, harbored a highly similar plasmid (Fig. 2). Although *K. pneumoniae* strain was isolated from different farm than *E. coli* strains, plasmid in *K. pneumoniae* strain may have been derived from a common origin of *E. coli* strains.

Restriction profiles of plasmids from CTX-M-14-producing transconjugants showed that 4 strains, *E. coli* isolate nos. 7–9, 10–1, 10–2 and 10–3 isolated from broiler, harbored a closely similar plasmid (Fig. 3). In addition, PFGE patterns of chromosomal DNA restriction fragments from isolate nos. 7–9, 10–1, and 10–2 were different from each other. Although *E. coli* isolate no. 7–9 was isolated from different farm than *E. coli* isolate nos. 10–1, 10–2 and 10–3, these plasmids of the *E. coli* isolates may have a common origin.

DISCUSSION

In our survey, the prevalence of ESBL-producing bacteria in broilers (60.0%) was higher than in other food-producing animals. We focused on ESBL-producing bacteria in broilers to improve public health, because of the high incidence of *E. coli* contamination on the raw chicken meats [14]. Several β-lactamase genes, namely, $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{SHV-12}}$, $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-44}}$, detected in broilers in this study have already been observed as clinical iso-

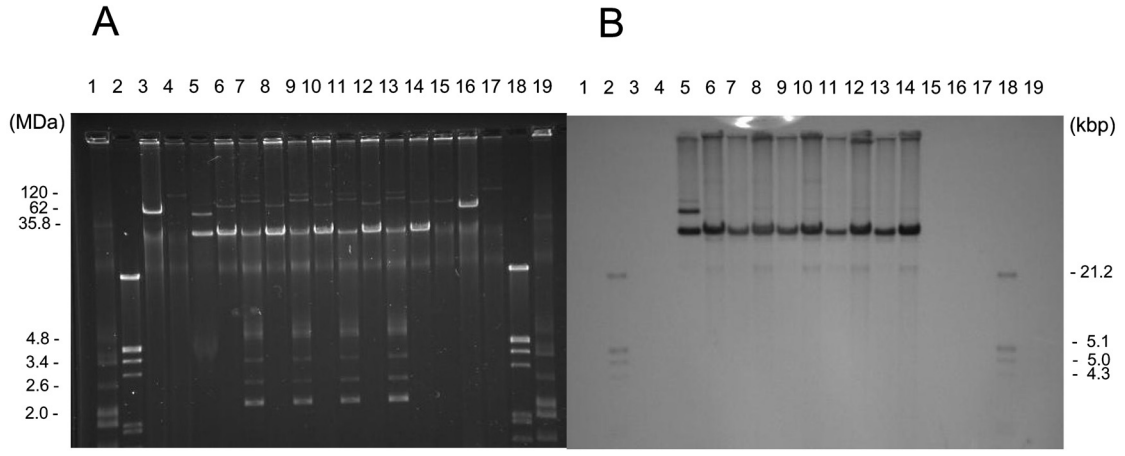

Fig. 1. Plasmid profiles (A) and Southern blot hybridization (B) of CTX-M-2 encoding plasmids obtained from *K. pneumoniae* and *E. coli* isolates from broilers and transconjugants. (A) Undigested plasmid profiles from isolate strain nos. 13–1k (lane 5), 7–1 (lane 7), 7–4 (lane 9), 7–7 (lane 11) and 7–8 (lane 13) and transconjugants of isolate strain nos. 13–1k (lane 6), 7–1 (lane 8), 7–4 (lane 10), 7–7 (lane 12) and 7–8 (lane 14), respectively. Lanes 1–4 and 16–19 are molecular weight markers. Lanes 1 and 19, *E. coli* V517; lanes 2 and 18, DNA Molecular Weight Marker III, Digoxigenin-labeled (Roche Diagnostics GmbH); lanes 3 and 16, *E. coli* R1; lanes 4 and 17, *E. coli* Rts1. (B) Southern blot hybridization of undigested CTX-M-2 encoding plasmids from isolate strain nos. 13–1k (lane 5), 7–1 (lane 7), 7–4 (lane 9), 7–7 (lane 11) and 7–8 (lane 13) and transconjugants of isolate strain nos. 13–1k (lane 6), 7–1 (lane 8), 7–4 (lane 10), 7–7 (lane 12) and 7–8 (lane 14), respectively. Lane 15, recipient strain (*E. coli* INVαF'). Lanes 1–4 and 16–19 are molecular weight markers. Lanes 1 and 19, *E. coli* V517; lanes 2 and 18, DNA Molecular Weight Marker III, Digoxigenin-labeled (Roche Diagnostics GmbH); lanes 3 and 16, *E. coli* R1; lanes 4 and 17, *E. coli* Rts1.

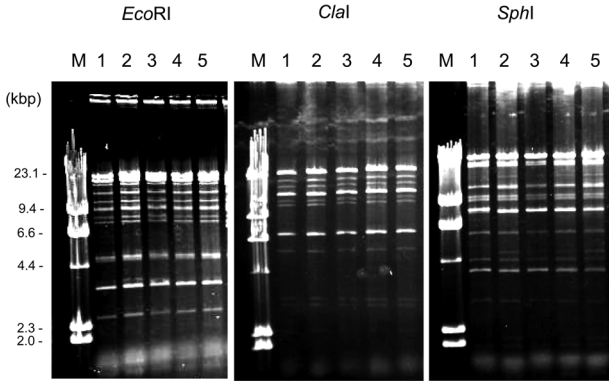

Fig. 2. Restriction profiles of plasmids from CTX-M-2-producing transconjugants of isolates from broilers digested with *Eco*RI, *Cla*I and *Sph*I. Lane 1, *E. coli* transconjugant of *K. pneumoniae* isolate strain no. 13–1k; lanes 2 to 5, *E. coli* transconjugants of *E. coli* isolate strain nos. 7–1, 7–4, 7–7 and 7–8, respectively. M: lambda DNA digested with Hind III marker.

lates in Japan [12, 24, 30, 31, 38, 40], and ESBL-producing *E. coli* strains have also been previously isolated from broilers in Japan [20]. Kojima *et al*. reported the distribution of ESBL-producing *E. coli* in food-producing animals at a low prevalence level in 2005. While the detection methods in our study were different from those used by Kojima *et al*. [20], a higher prevalence of ESBL-producing *E. coli* in broilers and different ESBL genes is observed in our study. Although expression of ESBL enzymes among gram-negative bacteria is increasing in Japan [29], this is the first report of *E. coli* isolates from broilers harboring $bla_{\text{SHV-12}}$, $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-44}}$ genes in Japan. We detected isolates that were harboring the same $bla_{\text{CTX-M-14}}$ gene but had PFGE patterns that differed from each other. Plasmid restriction profiles of the CTX-M-2-producing transconjugants of *K. pneumoniae* and *E. coli* isolates from different farms were identical. They suggest that transferable plasmids harboring the $bla_{\text{CTX-M-2}}$ or $bla_{\text{CTX-M-14}}$ gene are distributed amongst multiple bacteria species, located at the same farm and at different broiler farms.

The use of cephalosporins generally selects for cephalosporins-resistant bacteria. In this study, all *E. coli* isolated by using Chromocult Coliform Agar ES with CTX were resistant to CPDX. This is interesting, since ceftiofur and other cephalosporins with an extended spectrum are used in pigs, cattle, and pet animals, but are not allowed for use in broilers in Japan. Although the cause of the high prevalence of ESBL-producing or CPDX-resistant bacteria in broilers was not clarified in this study, the high prevalence of ESBL-producing bacteria in broilers and CPDX-resistance of the *E. coli* isolates obtained from the broilers indicate that broilers may be a potential reservoir of *E. coli* strains harboring CTX-M genes and cephalosporin-resistant *E. coli* in Japan.

Since *E. coli* and *K. pneumoniae* are commensal bacteria, they are not generally targeted for antimicrobial–resistance investigation. Like vancomycin-resistant *Enterococcus* and methicillin-resistant *Staphylococcus aureus*, ESBL-producing bacteria usually emerged after nosocomial infections. Therefore, the prevalence of ESBL-producing commensal

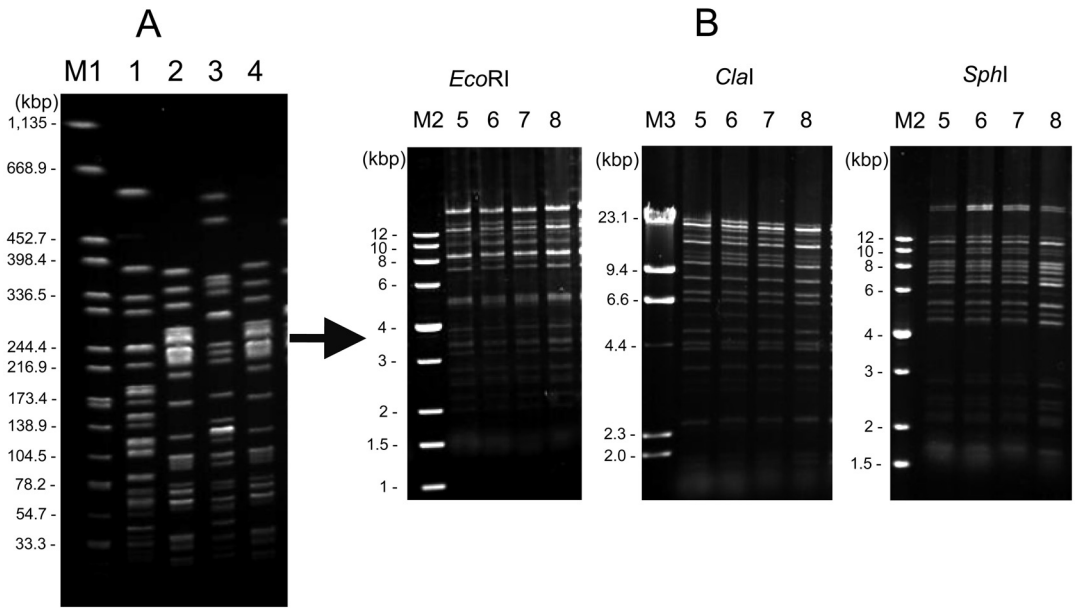

Fig. 3. (A) PFGE patterns of chromosomal DNA restriction fragments from CTX-M-14-producing *E. coli* isolates from broilers and (B) restriction profiles of plasmids from CTX-M-14-producing transconjugants of *E. coli* isolates from broilers digested with *Eco*RI, *Cla*I and *Sph*I. (A) PFGE patterns of chromosomal DNA restriction fragments from *E. coli* isolates from broiler strain nos. 7–9 (lane 1), 10–1 (lane 2), 10–2 (lane 3) and 10–3 (lane 4), respectively. M1: *Salmonella* serotype Braenderup reference standard (H9812) restricted with *Xba*I. (B) Restriction profiles of plasmids from CTX-M-14-producing transconjugants of *E. coli* isolates from broilers digested with *Eco*RI, *Cla*I and *Sph*I. Lane 5 to 8, *E. coli* transconjugants of isolate strain no. 7–9, 10–1, 10–2 and 10–3, respectively. M2: Perfect DNA Markers 0.5–12 kb (Merck); M3: lambda DNA digested with Hind III marker.

bacteria is unknown. It has been suggested that both clonal spread of epidemic strains and transfer of transposable genetic elements might contribute to the proliferation of ESBLs [2, 9, 11, 39]. Delayed treatment of infections caused by ESBL-producing organisms is associated with an increased rate of mortality [26, 27]. Currently, concerns regarding human infection of ESBL-producing bacteria from food-producing animals [6] have emerged, increasing when a high prevalence of ESBL genes was recently reported for chicken meat (79.8%) [25]. In our survey, we clarified the high prevalence and characteristics of ESBL genes of *E. coli* and *K. pneumoniae* in broilers in the Chubu region of Japan. Mobile drug-resistance genes are capable of crossing bacterial species and are likely to accelerate dissemination of drug-resistance between animals and humans through chicken meat. It is important to monitor the spread of expanded-spectrum cephalosporin-resistant bacteria, and further studies of genetic basis, including animals, humans, and the environments, are necessary for the control of drug-resistant bacteria.

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