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: blaSHV-12, blaCTX-M-2, blaCTX-M-14, blaCTX-M-15 and blaCTX-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 blaCTX-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.


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, cephemycins, 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...
onto Chromocult Coliform Agar ES (Merck KGaA, Darmstadt, Germany) supplemented with 1 μg/ml 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 (bioMérieux, 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/ml for cefpodoxime: CPDX, or MIC≥2 μg/ml 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 blaTEM, blaqVI, blacCTX, blacM1, blacCTXM1, blacCTXM2, blacCTXM3, blacCMY1, blacCMY2, blacPER1, and blacPER2 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 INVoF (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 ml 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/ml) and cefotaxime (1 μg/ml). 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, 5.7, 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 EcoRI, SphI and ClaI restriction enzymes (Takara Bio Inc., Shiga, Japan) and subjected to electrophoresis on a 1% agarose gel.

PFGE with XbaI (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 μl of bacterial suspension and 100 μl 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 ml of lysis buffer (0.5 M EDTA pH 8.0, 1% N-lauroyl sarcosine and 1 mg/ml 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 XbaI 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 1 (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer’s instructions. Primers for the preparation of probes from the blaCTXM2 and blaCTXM14 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-
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: \(\text{bla}_{\text{CTX-M-1}}\) in 24 isolates, \(\text{bla}_{\text{CTX-M-14}}\) in 4 isolates, \(\text{bla}_{\text{SHV-12}}\) in 9 isolates, \(\text{bla}_{\text{CTX-M-15}}\) in 3 isolates, \(\text{bla}_{\text{CTX-M-44}}\) in 3 isolates, and \(\text{bla}_{\text{TEM-1}}\) in 8 isolates (Table 2). The \(\text{bla}_{\text{SHV-12}}\) gene was found in combination with the \(\text{bla}_{\text{CTX-M-2}}\) gene in 4 isolates, and the \(\text{bla}_{\text{CTX-M-14}}\) gene in 1 isolate. In addition, the \(\text{bla}_{\text{TEM-1}}\) gene was found alone in 2 isolates, in combination with the \(\text{bla}_{\text{CTX-M-15}}\) gene in 3 isolates, and the \(\text{bla}_{\text{CTX-M-2}}\) gene in 3 isolates. None of the acquired β-lactamase genes examined in this study were detectable in 1 isolate. In the K. pneumoniae strain, the \(\text{bla}_{\text{CTX-M-2}}\) gene and \(\text{bla}_{\text{SHV-12}}\) gene were detected.

β-Lactam resistance transfer assay: Southern blot analysis, plasmid DNA analysis and PFGE: Southern blot hybridization with primers encoding CTX-M- or SHV-type ESBLs. Undigested plasmid profiles and Southern blot hybridization of the \(\text{K. pneumoniae}\) 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 \(\text{bla}_{\text{CTX-M-2}}\) and \(\text{bla}_{\text{CTX-M-14}}\) genes was performed. Hybridization was positive in 9 plasmids that originated from 5 isolates harboring the β-lactamase genes harbored in E. coli isolates from broilers

<table>
<thead>
<tr>
<th>β-lactamase genes</th>
<th>Farm No. of isolates</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTX-M-2</td>
<td>B: 5, G: 5, H: 5, J: 2</td>
<td>17</td>
</tr>
<tr>
<td>CTX-M-2 + SHV-12</td>
<td>B: 4</td>
<td>4</td>
</tr>
<tr>
<td>SHV-12</td>
<td>C: 3, E: 1</td>
<td>4</td>
</tr>
<tr>
<td>CTX-M-14</td>
<td>E: 2, I: 1</td>
<td>3</td>
</tr>
<tr>
<td>CTX-M-15 + TEM-1</td>
<td>C: 3</td>
<td>3</td>
</tr>
<tr>
<td>CTX-M-44</td>
<td>I: 3</td>
<td>3</td>
</tr>
<tr>
<td>CTX-M-2 + TEM-1</td>
<td>J: 3</td>
<td>3</td>
</tr>
<tr>
<td>CTX-M-14 + SHV-12</td>
<td>E: 1</td>
<td>1</td>
</tr>
<tr>
<td>TEM-1</td>
<td>H: 2</td>
<td>2</td>
</tr>
</tbody>
</table>

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, \(\text{bla}_{\text{CTX-M-2}}, \text{bla}_{\text{CTX-M-14}}, \text{bla}_{\text{SHV-12}}, \text{bla}_{\text{CTX-M-15}}\) and \(\text{bla}_{\text{CTX-M-44}}\) detected in broilers in this study have already been observed as clinical iso-
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-nega-
tive bacteria is increasing in Japan [29], this is the first report of E. coli isolates from broilers harboring \( \text{bla}_{\text{SHV-12}}, \text{bla}_{\text{CTX-M-15}} \) and \( \text{bla}_{\text{CTX-M-44}} \) genes in Japan. We detected isolates that were harboring the same \( \text{bla}_{\text{CTX-M-14}} \) 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 \( \text{bla}_{\text{CTX-M-2}} \) or \( \text{bla}_{\text{CTX-M-14}} \) 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

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![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 INV F'). 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 EcoRI, CiaI and SphI. 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.]
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 \textit{E. coli} and \textit{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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