# Distribution of Extended-Spectrum Cephalosporin Resistant *Enterobacteriaceae* in Broiler Farms

（ブロイラー農場における広域セファロスポリン耐性菌の分布）

THE UNITED GRADUATE SCHOOL OF VETERINARY SCIENCES,

YAMAGUCHI UNIVERSITY

（山口大学大学院連合獣医学研究科）

MITSUHIRO KAMEYAMA

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# I. General Introduction

## 1. ß-lactamases

### 1) overview

For the past 70 years, antimicrobial drugs, such as antibiotics, have been successfully used to treat patients with bacterial and infectious diseases [Drlica *et al.* 2011]. Among the antibiotics, ß-lactam antibiotics, such as penicillins, cephalosporins, carbapenems and monobactams, represent 60% of all antimicrobial use by weight [Livermoer *et al.* 2006]. They are preferred because of their efficacy and safety and because their activity can be extended or restored by chemical manipulation.

Some bacteria show resistance to ß-lactam antibiotics. In gram-positive bacteria, the resistance arises by penicillin-binding protein (PBP) modification or substitution. Some gram-negative bacteria such as *Haemophilus* spp. and *Neisseria* spp. also acquire the PBP modification, however, the resistance among gram-negative bacteria depends on ß-lactamases and efflux [Zimmermann *et al.* 1977; Livermore *et al.* 1991].

Based on their amino acid sequences, ß-lactamases are classified into four classes, designated classes A to D, according to the scheme of Ambler *et al.* (1991). Extended-spectrum cephalosporins (ESC) such as cefotaxime and ceftazidime, which are inherently less susceptible to ß-lactamases, were introduced in the early 1980s to treat infections caused by gram-negative bacilli that were resistant to established ß-lactams and that produced class A, C and D ß-lactamases [Bonnet R. 2004]. Their repetitive and increased use induced the appearance of resistant strains, which overproduced class C AmpC ß-lactamases

[Hanson *et al.* 1999; Philippon *et al.* 2002], and/or which produced class A extended-spectrum ß-lactamases (ESBLs) [Naas *et al.* 1999: Bradford P. A. 2001].

**2) ESBL**

ESBLs were first described in the middle of 1980s. Most early enzymes were mutants of the TEM and SHV plasmid-mediated penicillinases with one or more amino acid substitutions [Kliebe *et al.* 1985; Sirot *et al.* 1987; Sougakoff *et al.* 1988]. These TEM and SHV-derivatives, which characterized much greater hydrolytic activity against ceftazidime than cefotaxime, became widespread in Europe, especially SHV-4 ESBL-producing *Klebsiella pneumoniae* and TEM-24 ESBL-producing *Enterobacter aerogenes* [Arlet *et al.* 1994; Yuan *et al.* 1998; Perilli *et al.* 2002].

In the late 1990s, other types of ESBLs, named CTX-M, were emerged and dramatically spread in the family *Enterobacteriaceae* over the world. CTX-M enzymes can be subclassified in five groups by amino acid sequence similarities [Bonnet *et al.* 2000]. (i) The CTX-M-1 group includes six plasmid-mediated enzymes (CTX-M-1, M-3, M-10, M-12, M-15 and FEC-1) [Matsumoto *et al.* 1988; Barthélémy *et al.* 1992; Gniadkowski *et al.* 1998; Karim *et al.* 2001; Kariuki *et al.* 2001; Oliver *et al.* 2001] and the unpublished enzymes (CTX-M-22, M-23 and M-28). (ii) The CTX-M-2 group includes eight plasmid-mediated CTX-M enzymes (CTX-M-2, M-4, M-4L, M-5, M-6, M-7, M-20 and Toho-1) [Ishii *et al.* 1995; Bauernfeind *et al.* 1996; Bradford *et al.* 1998; Gazouli *et al.* 1998a; Gazouli *et al.* 1998b; Tassios *et al.* 1999; Saladin *et al.* 2002]. (iii) The CTX-M-8 group includes one plasmid-mediated member [Bonnet *et al.*

2000]. (iv) The CTX-M-9 group includes nine plasmid-mediated enzymes (CTX-M-9, M-13, M-14, M-16, M-17, M-19, M-21, M-27 and Toho-2) [Ma *et al.* 1998; Labia *et al.* 1999; Sabaté *et al.* 2000; Bonnet *et al.* 2001; Pai *et al.* 2001; Poirel *et al.* 2001; Cao *et al.* 2002; Chanawong *et al.* 2002; Saladin *et al.* 2002; Bonnet *et al.* 2003] and one unpublished enzyme (CTX-M-24). (v) The CTX-M-25 group includes the CTX-M-25 and CTX-M-26 enzymes. It is thought that the CTX-M-1 and CTX-M-2 group enzymes evolved by the escape of chromosomal genes from *Kluyvera ascorbata* [Rodrigues *et al.* 2004], whereas the CTX-M-8 and CTX-M-9 group enzymes evolved via similar escapes from *K. georgiana* [Olson *et al.* 2005]. CTX-M ESBLs hydrolyze cefotaxime more rapidly than ceftazidime, reversing the pattern of many TEM types. However, some CTX-M types acquire a hydrolyzing activity against ceftazidime by point mutation, thus CTX-M-15 and M-32 differ from CTX-M-3 and M-1, respectively, solely by A240G substitution, but are 100-fold more active against ceftazidime [Poirel *et al.* 2002; Cartelle *et al.* 2004].

### 3) plasmid-mediated AmpC ß-lactamase

The first bacterial enzyme reported to destroy penicillin was the AmpC ß-lactamase of *Escherichia coli* in 1940 [Abraham *et al.* 1940]. AmpC ß-lactamases are found in almost *Enterobacteriaceae* family on chromosome. Some organisms can become resistant to all ß-lactam drugs, except for cefepime and carbapenems, due to overexpressing AmpC ß-lactamases [Girlich *et al.* 2000; Thomson *et al.* 2000]. Constitutive overexpression of AmpC ß-lactamases in Enterobacteriaceae occurs either by degradation of the *ampC* chromosomal gene

or by acquisition of a transferable *ampC* gene on a plasmid or other transferable element. The transferable *ampC* gene products are commonly called plasmid-mediated AmpC ß-lactamases [Bauernfeind *et al.* 1998; Thomson *et al.* 2000; Bush, K. 2001]. Plasmid-mediated AmpC genes have been known since 1989 [Philippon *et al.* 2002; Walther-Rasmussen *et al.* 2002].

The majority of plasmid-mediated *ampC* genes are found in nosocomial isolates of *E. coli* and *K. pneumonia*, also in strains of other genera of the family *Enterobacteriaceae*. Plasmid-mediated *ampC* genes are derived from the chromosomal *ampC* genes of several members of the family *Enterobacteriaceae*, including *Enterobacter cloacae*, *Citrobacter freundii*, *Morganella morganii*, and *Hafnia alvei* [Bauernfeind *et al.* 1998]. AmpC ß-lactamases are categorized into eight families, based on their amino acid sequences; CMY, MIR, MOX, LAT, FOX, DHA, ACT and ACC [Jacoby, G. A. 2009]. Among them, CMY families especially CMY-2 AmpC ß-lactamase have broadest geographic spread.

## 2. ESC-resistance in *Enterobacteriaceae*

### 1) humans

In Europe, until the late 1990, European surveys of ESBLs almost exclusively found TEM and SHV variants, often TEM-24, SHV-2 and SHV-5, and largely found these in *Klebsiella* spp. [Livermore *et al.* 2007]. Some clones have spread among hospitals, including *K. pneumoniae* serotype K25 with SHV-4, and *Enterobacter aerogenes* with TEM-24; both clones are widespread in France and Belgium [Arlet *et al.* 1994; De Gheldre *et al.* 2001]. CTX-M ESBLs were recorded rarely, although there were some pathogenic strains, *Salmonella* Typhimurium with CTX-M-4 and M-5 in some European countries in the mid-1990 [Bradford *et al.* 1998].

These patterns have now changed dramatically, with CTX-M enzymes replacing TEM and SHV mutants as the predominant ESBLs in worldwide includes Japan, with *E. coli* and *K. pneumoniae* as a major host, and with producers increasingly isolated from community patient. One type, CTX-M-2, spread hugely in Argentina in the early 1990s and is now also frequent in Israel [Quinteros *et al.* 2003; Chmelnitsky *et al.* 2005]. Other types are spreading elsewhere, predominantly CTX-M-9 and M-14 in East Asia and Iberia, and CTX-M-3 and M-15 in Europe and, anecdotally, in India and the Middle East [Munday *et al.* 2004; Hernandez *et al.* 2005]. Some clonal strains have been spreading over the world. The emergence of an international pandemic clonal group, CTX-M type ESBL-producing *E. coli* with sequence type 131 (ST131) belonging to the O25b serogroup and the B2 phylogenetic group, has contributed

greatly to the pandemic [Rogers *et al.* 2011]. These clones were also detected in Japanese hospitals [Matsumura *et al.* 2012].

Plasmid-mediated AmpC ß-lactamases have been found worldwide but are less common than ESBLs. CMY-2 enzyme has the broadest geographic spread and is an important cause of ß-lactam resistance in nontyphoidal *Salmonella* and *E. coli* strains in many countries [Miriagou *et al.* 2004; Egorova *et al.* 2008]. The United States reported significant problems with CMY-2-producing *S.* Newport in cattle and zoonotic infections [Devasia *et al.* 2005]. In Japan, it is thought that plasmid-mediated AmpC ß-lactamase producers are still rare in human cases [Matsumoto *et al.* 2012].

**2) food-producing animals**

In food-producing animals, ESCs and fluoroquinolones are the antibiotics of choice in the treatment of invasive salmonellosis and severe bacterial infections. In the United Kingdom, ESCs (e.g. cefelonium, cefquinome and ceftiofur) are approved exclusively for the treatment of animal diseases such as metritis and mastitis in cattle, respiratory diseases in ruminants, horses and swine, necrotic enteritis and colisepticaemia in poultry [Batchelor *et al.* 2005]. Also in Japan, 1st to 3rd generation cephalosporins are used for the treatment such as mastitis and phlegmone in cattle, and pleural pneumonia in swine. However, the use of cephalosporins is not approved for broilers.

In the late 1990s, the presence of CTX-M-2 ESBL-producing bacteria in food animals has been observed in Japan [Shiraki *et al.* 2004]. After that, many reports describe the spread of ESBLs producers from food-producing animals

worldwide. In Japan, CTX-M ESBLs have been detected frequently from in *E. coli* and *Salmonella* spp. in broiler and broiler chickens [Matsumoto *et al.* 2007; Ishiguro *et al.* 2010; Hiroi *et al.* 2011]. Also many papers describe the emergence and spread of plasmid mediated AmpC ß-lactamase, particularly CMY-2 [Batchelor *et al.* 2005]. It has been suggested that the increase in CMY-2 producers observed in the last decade in the United States is due to the use of ceftiofur, licensed in 1988 [Hornish *et al.* 2002]. The occurrence of CMY-2 producers has been noted in several countries, and diffusion of this ß-lactamase seems to be linked to efficient horizontal transmission of its encoding plasmids [Batchelor *et al.* 2005]. For the past few years, the reports of CMY-2 producers in broilers and retail chicken meat are increasing in Japan [Ahmed *et al.* 2009; Kojima *et al.* 2009; Asai *et al.* 2011].

## 3. Objective

The ESC-resistant bacteria have been emerged and spread in food-producing animals worldwide since the late 1990s. Broilers and retail chicken meat are considered as potential reservoirs of ESC-resistant *Salmonella* spp. and *E. coli*. It is still unclear why ESC-resistant bacteria exist in broilers although the cephalosporins are not used for broilers in Japan.

Chapter II; we found the ESC-resistant and susceptible *Salmonella* isolates from broilers in January 2009. To clarify the mechanisms of ESC-resistance in the isolates, we characterized the resistance gene, resistance related plasmid(s) and also discussed the genetic relationships of the isolates.

Chapter III; in the broiler settings, CTX-M ESBLs are prevalent in the ESC-resistant *Enterobacteriaceae* in Japan, whereas CMY-2 AmpC ß-lactamase is detected frequently in Europe as well as CTX-M ESBLs. There is a possibility that CMY-2 ß-lactamase is distributed also in Japanese broiler farms. To clarify the population of the ESC-resistant bacteria, we isolated the ESC-resistant *Enterobacteriaceae* in broiler chickens from four commercial farms using selective agars supplemented with cefotaxime (CTX) or ceftazidime (CAZ) from May-September 2011. To determine the resistance determinants in the isolates, the ß-lactamase types (plasmid-mediated AmpC ß-lactamase and ESBL) were identified using PCR and sequencing. Also to analyze the epidemiological relationship of the isolates at the farm level, pulsed-field gel electrophoresis analysis was performed using *Xba*I restriction enzyme.

# II. Emergence of ESC-resistant *Salmonella enterica* serovar Infantis in a broiler farm

# 1. Introduction

Amongst members of the *Enterobacteriaceae* family, *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* have been implicated as potential producers of extended-spectrum β-lactamases (ESBLs) which possess hydrolyzing activity against third-generation cephalosporins. In recent years, several reports have described other bacterial species within the *Enterobacteriaceae* family capable of producing ESBLs. Of these, *Salmonella* spp. producing ESBLs have been detected in salmonellosis cases both in humans [Romero *et al.* 2004; Izumiya *et al.* 2005; Jin *et al.* 2006] and domestic animals [Shahada *et al.* 2010].

*Salmonella enterica serovar* Infantis (*S.* Infantis) is one of the predominant serotypes isolated from broilers in Japan [Shahada *et al.* 2008]. In the late 1990s, *S.* Infantis isolates harboring ESBLs were recovered from hospitalized patients in South America [Moraes *et al.* 2000]. More recently, *S.* Infantis producing ESBLs were found not only in human patients but also in domestic animals and commercial meats [Hasman *et al.* 2005; Darshan *et al.* 2010]. Most of these produced the CTX-M-type ESBLs, indicating the probability that dissemination of third-generation cephalosporin-resistant *S.* Infantis associated with various types of β-lactamases is gradually expanding world-wide. In this article, we characterized *S.* Infantis isolates carrying CTX-M-14 ESBL derived from broiler chickens.

## 2. Materials and Methods

### 1) *S.* Infantis isolates

Ten *S.* Infantis isolates obtained from 30 broiler cecal samples collected at a poultry processing plant in western Japan in January 2010 were used in this study. The broiler chickens involved in this survey were sampled from three different flocks raised on the same commercial farm. The isolation of the bacteria was done as follows; approximately 1 g of cecal contents was aseptically mixed with 5 m*l* of sterilized distilled water. Then, 1 m*l* of suspension was enriched in 10 m*l* of tetrathionate broth (Merck KGaA, Darmstadt, Germany) and incubated at 42℃. After 24 hr of incubation, a loopful from each of enriched broth was streaked onto plates of selective deoxycholate hydrogen sulfide lactose (DHL) agar (Oxoid Ltd., Basingstoke, Hampshire, UK) and mannitol lysine crystal violet brilliant green (MLCB) agar (Eiken Chemical Co. Ltd., Tokyo, Japan), and incubated at 37℃ for 24 hr. Suspected colonies were selected from each plate and cloned on Mueller-Hinton agar (Oxoid Ltd.), and the following identification and serotyping of bacteria were performed as described elsewhere [Shahada *et al.* 2008]. These isolates were stocked in brain heart infusion broth containing 20% of glycerol at - 80℃ until use for the following tests.

### 2) Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by employing the Kirby-Bauer disk diffusion method on Mueller-Hinton (Oxoid Ltd.) agar plates using the following antimicrobial agents: ampicillin (AMP), cephalothin (CEF),

cefotaxime (CTX), ceftazidime (CAZ), streptomycin (STR), kanamycin (KAN), gentamicin (GEN), tetracycline (TET), chloramphenicol (CHL), nalidixic acid (NAL), ciprofloxacin (CIP), tosufloxacin (TFX) and trimethoprim-sulfamethoxazole (SXT). The ESBL confirmation test using CTX and CAZ disks with or without clavulanic acid disks was performed according to the new criteria established by the Clinical and Laboratory Standards Institute (CLSI) [CLSI. 2010].

### 3) Detection and identification of ESBL gene

The ESBL genes were detected by PCR [Yagi *et al.* 1999; Shibata *et al.* 2006], and obtained amplicons were directly sequenced using specific primers [Bou *et al.* 2002] with the BigDye Terminator v3.1 Ready Reaction Sequencing kit and the ABI 3500xl Automated DNA Sequencer (Applied Biosystems, Foster City, CA, U.S.A.). The sequencing data were compared with the published DNA sequences using the BLASTN database (www.ncbi.nlm.nih.gov).

### 4) Pulsed-field gel electrophoresis analysis

Pulsed-field gel electrophoresis (PFGE) using *Bln*I restriction enzyme was performed according to the standard PulseNet protocol [Ribot *et al.* 2006]. The banding patterns obtained were analyzed using the Molecular Analyst Fingerprinting Plus software (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.).

### 5) Characterization of the plasmid with ESBL gene

Isolation of plasmids was conducted using the alkaline lysis method as

previously described [Kado *et al.* 1981]. Conjugation experiments were conducted as described elsewhere [Shahada *et al.* 2010] using the isolates Y2 and Y6 as donors and the rifampicin-resistant *E. coli* DH5α strain as recipient. The minimum inhibitory concentrations for AMP, CTX, KAN, STR, TET and sulfamethoxazole (SUL) on *E. coli* transconjugants were determined by the agar dilution method. The PCR assay was performed to confirm the transmission of the $bla_{\text{CTX-M-14}}$, *aphA1*, *aadA1, tetA*, and *sul1* genes. The PCR-based replicon typing (PBRT) was carried out as previously described [Johnson *et al.* 2007].

## 3. Results

### 1) ESC-resistant *S.* Infantis isolates

The resistance patterns of *S.* Infantis isolates examined are shown in Table 1. All ten *S.* Infantis isolates were showed resistance to KAN, STR, TET and SXT, and of those, the four isolates (Y1, Y2, Y3 and Y4) exhibited resistance to three ß-lactam antibiotics; AMP, CEF and CTX. Preliminary ESBL confirmation test results indicated that the four CTX resistant isolates were potential ESBL producers. Ultimately, PCR and nucleotide sequence analysis revealed that the CTX resistant isolates carried the $bla_{\text{CTX-M-14}}$ gene.

### 2) Genetic relationship of the isolates

The fragment similarity above 94% was observed among the ten isolates, suggesting the likelihood of sharing the same origin (Fig.1 A).

### 3) Characterization of the plasmid with ESBL gene

Analysis of the plasmid profiles demonstrated that four isolates (Y1, Y2, Y3 and Y4) harbored two plasmids of approximately 140 kb and 95 kb. The other six isolates (Y5, Y6, Y7, Y8, Y9 and Y10) carried only one plasmid of approximately 140 kb (Fig. 1 B). For the results of the conjugation experiments, we observed three distinct types of *E. coli* transconjugants: T1 transconjugants contained both 140- and 95-kb plasmids whereas T2 carried 95-kb plasmid and T3 harbored 140-kb plasmid (Table 2). Out of the transconjugants, the T1 and T2 were obtained from Y2 donor and the T3 were from Y6 donor. These findings

indicate that both 95-kb and 140-kb plasmids associated with *S.* Infantis donor isolates were potentially self-transmissible. Resistance phenotypes and genes detected in *E. coli* transconjugants are summarized in Table 2. T2 transconjugants with a 95-kb plasmid showed resistance to AMP and CTX; and they also tested positive for the $bla_{\text{CTX-M-14}}$ gene, suggesting the location of $bla_{\text{CTX-M-14}}$ to be the 95-kb plasmid. Genetic analysis of the T3 transconjugants revealed that resistance traits *aphA1*, *aadA1, tetA*, and *sul1* were located on the 140-kb plasmid. The findings of PBRT demonstrated that $bla_{\text{CTX-M-14}}$ was associated with IncI1 plasmid. On the other hand, plasmids harboring *aphA1*, *aadA1, tetA*, and *sul1* were IncP type.

## 4. Discussion

Previous reports indicate that IncI1 plasmid harboring *bla*$_{\text{CTX-M-14}}$ was detected in *S.* Enteritidis [Hopkins *et al.* 2006] and *E. coli* bacteria [Navarro *et al.* 2007]. It's worth noting that several studies describing the occurrence of CTX-M-14 ESBL in *S.* Enteritidis isolated from human patients in Spain [Romero *et al.* 2004], Hong Kong [Jin *et al.* 2006] and Japan [Izumiya *et al.* 2005] have been reported since 2003. In Japan, serovar Enteritidis producing CTX-M-14 enzyme was isolated from chicken meat imported from China in 2004 [Matsumoto *et al.* 2007]. Moreover, a commentary was released in Japan explaining the occurrence of *S.* Infantis producing CTX-M-14 ESBL recovered from domestic poultry meat [Department of Bacteriology I, National Institute of Infectious Diseases, 2008].

Although the use of cephalosporins including CTX is not approved for broilers in Japan, we detected CTX-resistant *S.* Infantis isolates derived from the broiler farm. It is likely that the *bla*$_{\text{CTX-M-14}}$ gene was acquired by *S.* Infantis isolates through interspecies transmission of the potential IncI1 resistance plasmid. This hypothesis is supported by early reports which described the existence of CTX-M-14 ESBL producing *E. coli* from broilers and chicken meats in Japan [Ishiguro *et al.* 2010; Hiroi *et al.* 2011]. The spread of cephalosporin-resistant *Salmonella* spp. in poultry via the transmissible resistance plasmids raises serious veterinary and public health concerns. Thus, continuous monitoring of members of the *Enterobacteriaceae* family possessing IncI1 resistance plasmids is required in order to establish the magnitude of the

health hazard associated with these bacteria.

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

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

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

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

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

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

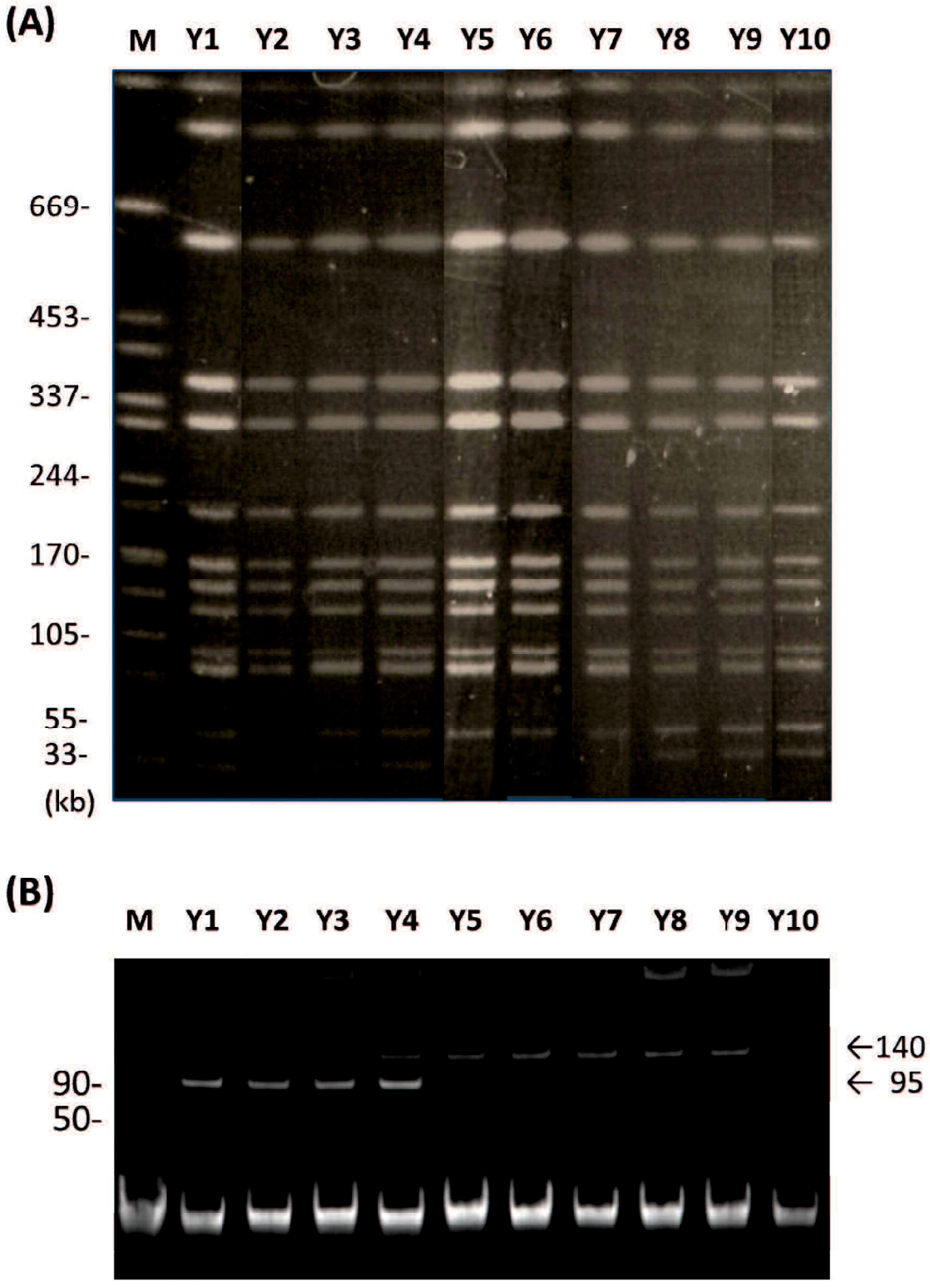

(A)
M Y1 Y2 Y3 Y4 Y5 Y6 Y7 Y8 Y9 Y10
669-
453-
337-
244-
170-
105-
55-
33-
(kb)
(B)
M Y1 Y2 Y3 Y4 Y5 Y6 Y7 Y8 Y9 Y10
90-
50-
←140
← 95

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

# III. Prevalence of ESC-resistant Escherichia coli in broiler farms

# 1. Introduction

In the late 1990s, extended-spectrum cephalosporin (ESC)-resistant *Enterobacteriaceae* emerged in food-producing animals worldwide. Spread of ESC-resistant bacteria amongst animals via their products has become a threat to public health, because of the risk of horizontal transfer of the antibiotic resistance genes to human pathogens [Izumiya *et al.* 2005]. Indeed, ESC-resistant pathogenic bacteria such as *Salmonella enterica* serovar Enteritidis and Shiga-toxin producing *Escherichia coli* have already been detected in patients with diarrhea [Ishii *et al.* 2005; Izumiya *et al.* 2005].

AmpC β-lactamases, which belong to the class C family of cephalosporinases, usually confer resistance to all β-lactams, except for cefepime and carbapenems [Jacoby G. A. 2009]. Overexpression of the AmpC enzyme in *E. coli* occurs either by deregulation of the chromosomal *ampC* gene, or through acquisition of a transferable plasmid containing the *ampC* gene. Recently, there have been reports of an increased prevalence of plasmidic AmpC β-lactamase in Gram-negative bacteria. In Europe, plasmid-mediated CMY-2 AmpC β-lactamases, as well as CTX-M type ESBL, have been detected in ESC-resistant *E. coli* from broiler chickens and their products [Smet *et al.* 2008; Bergenholtz *et al.* 2009]. There have been many reports from Japan describing the distribution of CTX-M ESBL in *E. coli* from broilers [Ishiguro *et al.* 2010; Hiroi *et al.* 2011; Hiroi *et al.* 2012], and in recent years, AmpC β-lactamase, especially CMY-2-type, from broilers and retail chicken meats has been detected [Ahmed *et al.* 2009; Kojima *et al.* 2009; Asai *et al.* 2011]. However, distribution of the AmpC

ß-lactamase in broilers is unknown. Moreover, the mechanisms that enable ESC-resistant bacteria to spread between broiler chickens remain unclear.

The aims of the present study were to i) investigate the prevalence of ESC-resistant bacteria in broiler chickens from four commercial farms, using selective agars supplemented with cefotaxime (CTX) or ceftazidime (CAZ), ii) determine the ß-lactamase types (plasmid-mediated AmpC ß-lactamase and ESBL) in ESC-resistant bacteria, and iii) analyze the epidemiological relationship of the isolates at the farm level.

## 2. Materials and Methods

### 1) Sampling

Forty-one rectal samples were collected from broiler chickens at a poultry processing plant located in western Japan from May–September 2011. The chickens originated from four commercial farms (10 or 11 samples per farm) that were located within a 10-km radius of the plant. Each of the four farms was under individual management; however, veterinarians and the workers that harvested the broilers did travel between the locations. All sampled animals were healthy and approximately 7 weeks of age.

### 2) Isolation and identification of *Enterobacteriaceae*

Approximately 1 g of cecal content was mixed with 3 m*l* of sterilized saline solution and homogenized by vortexing. The suspension was streaked onto each of two desoxycholate hydrogen sulfide lactose (DHL) agars (Eiken Chemical Co., Tokyo, Japan), supplemented with 4 $\mu$g/m*l* CTX (to isolate CTX-M ESBLs) or 16 $\mu$g/m*l* CAZ (to isolate non CTX-M ESBLs or AmpC ß-lactamases). The concentrations of CTX and CAZ were determined in reference to the resistant breakpoints of the antimicrobials described by the Clinical and Laboratory Standards Institute (CLSI) (CLSI. 2011). Following incubation for 18–20 hr at 37°C, two colonies of different morphologies (pink or colorless, rough or smooth, and large or small) were taken from each selective medium and purified on Mueller-Hinton (MH) agar plates (Oxoid Ltd., Basingstoke, UK), resulting in four isolates per sample. The biochemical phenotypes of the isolates were examined

using several analytical growth media, including triple-sugar iron slants (Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan), lysine indole motility medium (Eiken Chemical Co.) and sulfide indole motility medium (Kyokuto Pharmaceutical Industrial Co.), and were further identified using an EB-20 identification test (Nissui Pharmaceutical Co., Tokyo, Japan).

### 3) Antimicrobial susceptibility testing

Antimicrobial susceptibility of each of the isolates was examined using the Kirby-Bauer disk diffusion method (CLSI. 2008) on MH agar plates with the following sensi-disks (Becton Dickinson, Franklin Lakes, NJ, U.S.A): amoxicillin-clavulanic acid, CTX, CAZ, ceftriaxone, cefoxitin, cefepime, imipenem and meropenem. The phenotypic confirmatory test of ESBLs using CTX and CAZ with or without clavulanic acid disks (Becton Dickinson) was also carried out, as per the new criteria established by the CLSI (CLSI. 2011).

### 4) Detection of ß-lactamase genes

Template DNA was prepared by the boiling method using an InstaGene Matrix kit (Bio-Rad, Hercules, CA, U.S.A). ß-lactamase-encoding genes, including *bla*$_{\text{MOX}}$, *bla*$_{\text{CIT}}$, *bla*$_{\text{DHA}}$, *bla*$_{\text{ACC}}$, *bla*$_{\text{EBC}}$, *bla*$_{\text{FOX}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{CTX-M-1 group}}$, *bla*$_{\text{CTX-M-2 group}}$, *bla*$_{\text{CTX-M-8 group}}$, *bla*$_{\text{CTX-M-9 group}}$ and *bla*$_{\text{OXA-1}}$, were examined by PCR as described previously [Yagi *et al.* 2000; Pérez-Pérez *et al.* 2002; Colom *et al.* 2003; Pitout *et al.* 2004; Shibata *et al.* 2006]. The primers used are listed in Table 3. The isolates with a positive result in the *bla*$_{\text{CIT}}$ PCR were further analyzed by PCR to distinguish whether *bla*$_{\text{CMY-2}}$ or other genes were also present

[Pérez-Pérez *et al.* 2002]. The *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX\text{-}M\text{-}1\ group}$ and *bla*$_{CTX\text{-}M\text{-}2\ group}$ amplicons were sequenced using a BigDye Terminator v3.1 Ready Reaction Sequencing kit on an ABI 3500xl Automated DNA Sequencer (Applied Biosystems, Foster City, CA, U.S.A.). The sequencing data were compared with the published DNA sequences using the BLASTN database (www.ncbi.nlm.nih.gov).

**5) Pulsed-field gel electrophoresis analysis**

To distinguish the CMY-2- and/or CTX-M-type β-lactamase-producing bacteria, pulsed-field gel electrophoresis (PFGE) analysis using the *Xba*I restriction enzyme (Roche, Mannheim, Germany) was carried out according to the standard PulseNet protocol [Ribot *et al.* 2006). *S. enterica* serovar Braenderup strain H9812 was used as a standard size marker. Macro-restriction patterns were compared using Molecular Analyst Fingerprinting Plus software (Bio-Rad). A Dice coefficient and the unweighted-pair group method using arithmetic mean (UPGMA) algorithm were used with a 1.2% band tolerance and 1.2% optimization to generate a dendrogram. Isolates with $\geq$ 85% similarity were considered to be clonal for the purposes of this study.

## 3. Results

### 1) Isolation of ESC-resistant bacteria

ESC-resistant bacteria were isolated from all 41 samples using selective DHL agars containing either CTX or CAZ (Table 4). Of the 164 ESC-resistant isolates, 163 were *E. coli*, while the remaining strain, which was isolated from Farm 4, was identified as *E. cloacae*. The phenotypic confirmatory test of ESBLs showed that 34 (77.3%), 12 (30%), 14 (35%) and 13 (37.5%) of the ESC-resistant isolates originating from Farms 1, 2, 3 and 4, respectively, were putative ESBL producers (73 isolates in total, 44.5%).

### 2) Antimicrobial susceptibility testing of the isolates

Isolates were tested for their susceptibility to eight ß-lactam antimicrobial agents (Table 5). All isolates, except for two *E. coli* strains (99%), were resistant to CTX. More than 60% of the isolates also showed resistance to CAZ and ceftriaxone. Cefepime-resistant isolates were found in samples from Farms 1, 3 and 4, with prevalences ranging from 3–30%. No resistance to imipenem or meropenem was found.

### 3) Characterization of ß-lactamase genes

One AmpC ß-lactamase gene, $bla_{\text{CMY-2}}$ (107 isolates, 66%), and four ESBL genes, $bla_{\text{CTX-M-1}}$ (43 isolates, 26%), $bla_{\text{CTX-M-55}}$ (17 isolates, 10%), $bla_{\text{SHV-5}}$ (seven isolates, 4%) and $bla_{\text{CTX-M-2}}$ (five isolates, 3%), were detected in the 163 *E. coli* isolates. All of the identified $bla_{\text{TEM}}$ genes belonged to the non-ESBL group,

including *bla*$_{TEM-1}$ (22%) and *bla*$_{TEM-135}$ (3%) (Table 6). Among the *bla*$_{CMY-2}$–positive isolates (n=107), 85 contained the *bla*$_{CMY-2}$ gene alone, while 22 isolates carried *bla*$_{CMY-2}$ together with *bla*$_{CTX-M-1}$ (n=14), *bla*$_{CTX-M-55}$ (n=4), *bla*$_{SHV-5}$ (n=3) or *bla*$_{CTX-M-2}$ (n=1). The six isolates obtained from Farms 3 (n=3) and 4 (n=3) had *bla*$_{TEM-1}$ alone.

**4) Macro-restriction profiles of CMY-2 and CTX-M-type β-lactamase-producing isolates**

PFGE analysis with *Xba*I digestion revealed 41 (Y1–Y41) and 14 (X1–X14) clusters in the 107 CMY-2 and 65 CTX-M-carrying *E. coli* isolates, respectively (Fig. 2). Of the CTX-M clusters, CTX-M-1-carrying isolates were grouped into eight clusters (X-1, 2, 4, 5, 8, 12, 13 and 14), CTX-M-2-carrying isolates into two clusters (X-6 and 9) and CTX-M-55-carrying isolates into four clusters (X-3, 7, 10 and 11). Several clusters included isolates derived from more than one farm, including five CMY-2 clusters (Y-7, 9, 13, 23 and 37, Fig. 2 A), and three CTX-M clusters (X-2, 3 and 13, Fig. 2 B).

## 4. Discussion

In this study, ESC-resistant *E. coli* were isolated from all broiler samples (100%) from four different farms, indicating that ESC-resistant *E. coli* strains are prevalent on broiler chicken farms in Japan. The Japanese Veterinary Antimicrobial Resistance Monitoring (JVARM) Program, which was conducted from 1999–2002 across Japan, found that only 18/676 *E. coli* isolates (2.7%), derived from 12/354 broiler farms, were resistant to cefazolin (first generation cephalosporin) [Kojima et al. 2005]. In the decade following the JVARM program, ESC-resistant bacteria appear to have spread throughout broiler farms in Japan.

Recently, plasmid-mediated CMY-2 AmpC ß-lactamase-producing *E. coli* have been isolated from humans and domestic animals worldwide. In the United States, the enzyme was already prevalent in bacteria from humans and food-producing animals as early as 1998/1999 [Winokur *et al.* 2001]. In Belgium, Smet *et al.* reported that 49% of ceftiofur-resistant *E. coli* isolates derived from five broiler farms carried CMY-2 [Smet *et al.* 2008]. Our results showed a high prevalence (66%) of CMY-2 amongst *E. coli* from broilers in Japan, which surpasses the level in Belgium (49%) [Smet *et al.* 2008]. CMY-2-type AmpC ß-lactamase and CTX-M-type ESBL may play an important role in conferring the ESC resistance of *E. coli* in broilers.

Reports of CTX-M-type ß-lactamases have increased worldwide. In Japan, several Gram-negative bacterial species, including non-typhoidal salmonellae, *E. coli* and *Klebsiella pneumoniae*, carrying CTX-M ESBL (i.e. CTX-M-1, CTX-M-2, CTX-M-14 and CTX-M-15) were detected in samples from broiler chickens

[Shahada *et al.* 2010; Hiroi *et al.* 2011; Hiroi *et al.* 2012]. In the present study, CTX-M-1, CTX-M-2 and CTX-M-55 ESBLs were detected in *E. coli* isolates. CTX-M-55 ESBL was recently reported in *E. coli* and *K. pneumoniae* from patients in Thailand [Kiratisin *et al.* 2008] and Japan [Matsumura *et al.* 2012], and from turkey meat in Great Britain [Randall *et al.* 2011]. This is the first report of the CTX-M-55 ESBL in *E. coli* from broiler chickens in Japan. We previously isolated CTX-M-14 (CTX-M-9 group) ESBL-producing *S.* Infantis from broiler chickens originating from Farm 4 [Kameyama *et al.* 2012]. In the present study, no CTX-M-9 ESBLs were detected in *E. coli*, indicating that transmission of the $bla_{\text{CTX-M-14}}$ gene between *S.* Infantis and other bacteria has not occurred between the study periods.

According to results of PFGE analysis, the 107 CMY-2 β-lactamase-producing isolates and 65 CTX-M ESBL-producing isolates were categorized into 41 and 14 clusters, respectively. It was likely that higher diversity was seen in the CMY-2-producing isolates compared with the CTX-M ESBL-harboring isolates. The difference in the diversity may be caused by higher transferability of the plasmid(s) carrying the $bla_{\text{CMY-2}}$ gene than those carrying the $bla_{\text{CTX-M}}$ gene.

Previous studies have demonstrated cross-contamination of broiler chickens with *Campylobacter* and *Salmonella* species between farms [Slader *et al.* 2002]. In the current study, PFGE analysis revealed that some clonal *E. coli* strains that carried the CMY-2, CTX-M-1 or CTX-M-55 β-lactamase genes were found in samples from multiple farms. In Italy, genetically related SHV-12 ESBL-producing *E. coli* isolates were detected from geographically unrelated

farms [Bortolaia *et al.* 2010]. It appears that the ESBL types of the bacteria that contaminate broilers are specific to individual countries. Our result suggested that cross-contamination amongst the farms of some clonal strains that produce CMY-2 AmpC ß-lactamase or CTX-M ESBL had occurred.

In conclusion, we determined that ESC-resistant *E. coli* are prevalent in animals at broiler farms in Japan. Among the resistant bacteria, CMY-2-type AmpC ß-lactamase was one of the most important enzymes in conferring ESC resistance. Moreover, we confirmed that some clonal ESC-resistant strains were present at multiple farms. To verify whether a particular clonal plasmid is present amongst the ESC-resistant bacteria, further analysis of the characteristics of resistance plasmids is needed.

Table 3. Primers used for PCR and sequencing

| Target | Nucleotide sequence[a)] | Product size (bp) | $T_a$ (℃)[b)] | Reference |
|---|---|---|---|---|
| *bla* TEM | F- CCGTGTCGCCCTTATTCC, R- AGGCACCTATCTCAGCGA | 824 | 55 | [Yagi *et al*. 2000] |
| *bla* SHV | F- ATTTGTCGCTTCTTTACTCGC, R- TTTATGGCGTTACCTTTGACC | 1051 | 55 | [Yagi *et al*. 2000] |
| *bla* CTX-M-1 | F- GCTGTTGTTAGGAAGTGTGC, R- CCATTGCCCGAGGTGAAG | 516 | 55 | [Shibata *et al*. 2006] |
| *bla* CTX-M-2 | F- ACGCTACCCCTGCTATTT, R- CCTTTCCGCCTTCTGCTC | 779/780 | 55 | [Shibata *et al*. 2006] |
| *bla* CTX-M-825 | F- CGCTTTGCCATGTGCAGCACC, R- GCTCAGTACGATCGAGCC | 307 | 55 | [Pitout *et al*. 2004] |
| *bla* CTX-M-9 | F- GCAGATAATACGCAGGTG, R- CGGCGTGGTGGTGTCTCT | 393 | 55 | [Shibata *et al*. 2006] |
| *bla* OXA-1 | F- ATATCTCTACTGTTGCATCTC, R- AAACCCTTCAAACCATCC | 619 | 48 | [Colom *et al*. 2003] |
| *bla* MOX | F- GCTGCTCAAGGAGCACAGGAT, R- CACATTGACATAGGTGTGGTGC | 520 | 64 | [Pérez-Pérez *et al*. 2002] |
| *bla* CIT | F- TGGCCAGAACTGACAGGCAAA, R- TTTCTCCTGAACGTGGCTGGC | 462 | 64 | [Pérez-Pérez *et al*. 2002] |
| *bla* ACC | F- AACAGCCTCAGCAGCCGGTTA, R- TTCGCCGCAATCATCCCTAGC | 346 | 64 | [Pérez-Pérez *et al*. 2002] |
| *bla* CMY-2 | F- AACACACTGATTGCGTCTGAC, R- CTGGGCCTCATCGTCAGTTA | 1,226 | 60 | [Pérez-Pérez *et al*. 2002] |
| *bla* DHA | F- AACTTTCACAGGTGTGCTGGGT, R- CCGTACGCATACTGGCTTTGC | 405 | 64 | [Pérez-Pérez *et al*. 2002] |
| *bla* EBC | F- TCGGTAAAGCCGATGTTGCGG, R- CTTCCACTGCGGCTGCCAGTT | 302 | 64 | [Pérez-Pérez *et al*. 2002] |
| *bla* FOX | F- AACATGGGGTATCAGGGAGATG, R- CAAAGCGCGTAACCGGATTGG | 190 | 64 | [Pérez-Pérez *et al*. 2002] |

a) F, forward primer; R, reverse primer  b) annealing temperature

Table 4. Prevalence of ESC-resistant bacteria from four broiler farms

| Farm | No. of samples | No. of isolates collected on selective DHL agars containing CTX or CAZ[a)] | | | No. (%) of putative ESBL-producing isolates collected on selective DHL agars containing CTX or CAZ[b)] | | |
|---|---|---|---|---|---|---|---|
| | | 4 µg/mL CTX | 16 µg/mL CAZ | Total | 4 µg/mL CTX | 16 µg/mL CAZ | Total |
| 1 | 11 | 22 | 22 | 44 | 22 (100) | 12 (54.5) | 34 (77.3) |
| 2 | 10 | 20 | 20 | 40 | 8 (40) | 4 (20) | 12 (30) |
| 3 | 10 | 20 | 20 | 40 | 8 (40) | 6 (30) | 14 (35) |
| 4 | 10 | 20 | 20 | 40 | 8 (40) | 5 (25) | 13 (37.5) |
| Total | 41 | 82 | 82 | 164 | 46 (56.1) | 27 (32.9) | 73 (44.5) |

a) DHL, desoxicholate hydrogen sulfide lactose; CTX, cefotaxime; CAZ, ceftazidime

b) putative ESBL-producing isolates were determined by phenotypic confirmatory test

Table 5. Resistance of *E. coli* and *E. cloacae* isolates to β-lactam antimicrobials

| β-lactam antimicrobials | | % of resistant isolates | | | | | |
|---|---|---|---|---|---|---|---|
| | | Farm 1 | Farm 2 | Farm 3 | Farm 4 | | Total |
| | | *E. coli* | | | | *E. cloacae* | |
| | | (n=44) | (n=40) | (n=40) | (n=39) | (n=1) | (n=164) |
| Amoxicillin-clavulanic acid | (13)[a)] | 34.1 | 100 | 62.5 | 69.2 | 100 | 65.9 |
| Cefotaxime | (22) | 95.5 | 100 | 100 | 100 | 100 | 98.8 |
| Ceftazidime | (17) | 36.4 | 57.5 | 87.5 | 71.8 | 100 | 62.8 |
| Ceftriaxone | (19) | 90.9 | 80 | 77.5 | 84.6 | 100 | 83.5 |
| Cefoxitin | (14) | 47.7 | 65 | 75 | 69.2 | 100 | 64 |
| Cefepime | (14) | 29.5 | 0 | 27.5 | 2.6 | 0 | 15.2 |
| Imipenem | (19) | 0 | 0 | 0 | 0 | 0 | 0 |
| Meropenem | (19) | 0 | 0 | 0 | 0 | 0 | 0 |

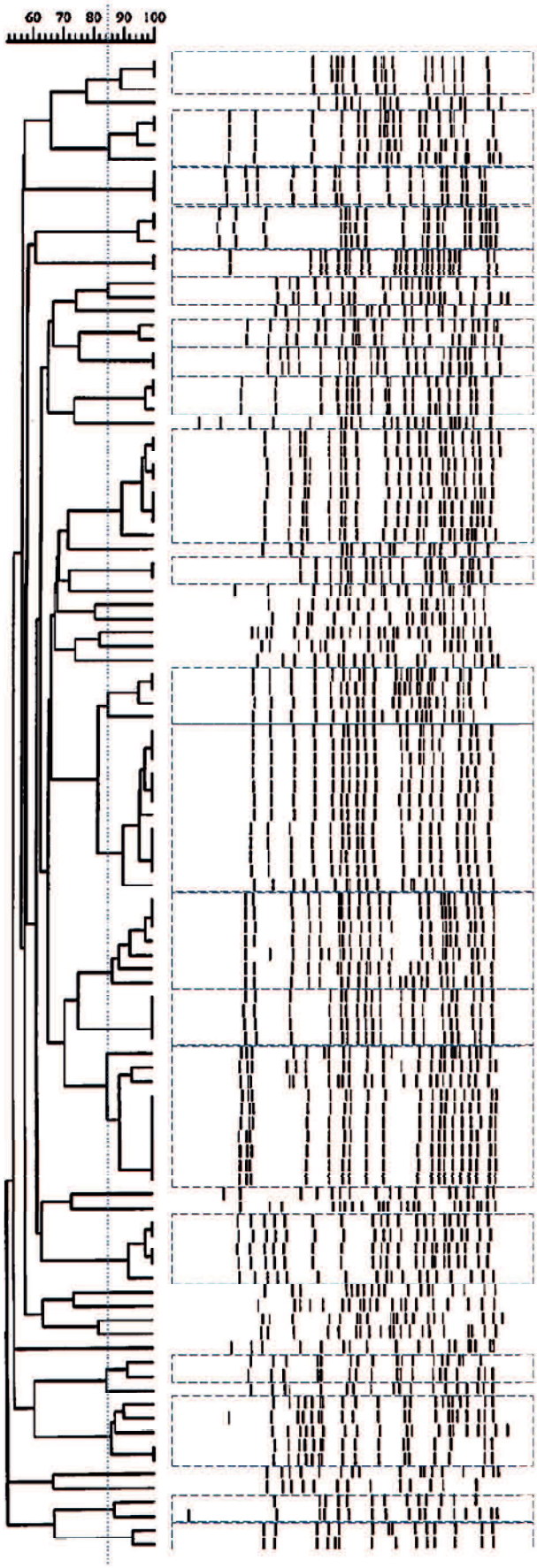
a) Resistance breakpoint (diameter, mm) defined by CLSI document M100-S21.

Table 6. CMY-2 β-lactamase and ESBL genes detected in 163 *E. coli* isolates from four broiler farms

| β-lactamase gene(s) | No. (%) of isolates | | | | |
|---|---|---|---|---|---|
| | Farm 1 (n=44) | Farm 2 (n=40) | Farm 3 (n=40) | Farm 4 (n=39) | Total (n=163) |
| CMY-2 | 9 (20.5) | 29 (72.5) | 24 (60) | 23 (59) | 85 (52.1) |
| CMY-2 + CTX-M-1 | 12 (27.3) | 0 | 0 | 2 (5.1) | 14 (8.6) |
| CMY-2 + CTX-M-2 | 1 (2.3) | 0 | 0 | 0 | 1 (0.6) |
| CMY-2 + CTX-M-55 | 0 | 0 | 4 (10) | 0 | 4 (2.5) |
| CMY-2 + SHV-5 | 1 (2.3) | 1 (2.5) | 0 | 1 (2.6) | 3 (1.8) |
| CTX-M-1 | 17 (38.6) | 7 (17.5) | 0 | 5 (12.8) | 29 (17.8) |
| CTX-M-2 | 4 (9.1) | 0 | 0 | 0 | 4 (2.5) |
| CTX-M-55 | 0 | 1 (2.5) | 9 (22.5) | 3 (7.7) | 13 (8) |
| SHV-5 | 0 | 2 (5) | 0 | 2 (5.1) | 4 (2.5) |
| ND[a)] | 0 | 0 | 3 (7.5) | 3 (7.7) | 6 (3.7) |

a) Not detected, the isolates carried only TEM-1 β-lactamase

A




| Cluster | No. of isolates from Farms 1 4 | | | | |
|---|---|---|---|---|---|
| | Total | 1 | 2 | 3 | 4 |
| Y-1 | 3 | · | · | 3 | · |
| Y-2 | 1 | · | · | · | 1 |
| Y-3 | 4 | · | 4 | · | · |
| Y-4 | 3 | · | · | 3 | · |
| Y-5 | 3 | · | · | · | 3 |
| Y-6 | 2 | 2 | · | · | · |
| Y-7 | 2 | 1 | · | · | 1 |
| Y-8 | 1 | · | · | 1 | · |
| Y-9 | 2 | · | 1 | · | 1 |
| Y-10 | 2 | · | · | · | 2 |
| Y-11 | 3 | · | · | 3 | · |
| Y-12 | 1 | · | · | · | 1 |
| Y-13 | 8 | · | 7 | · | 1 |
| Y-14 | 1 | · | · | 1 | · |
| Y-15 | 2 | · | · | · | 2 |
| Y-16 | 1 | · | · | 1 | · |
| Y-17 | 1 | · | · | 1 | · |
| Y-18 | 1 | · | · | 1 | · |
| Y-19 | 1 | · | 1 | · | · |
| Y-20 | 1 | 1 | · | · | · |
| Y-21 | 1 | · | 1 | · | · |
| Y-22 | 4 | · | · | · | 4 |
| Y-23 | 12 | · | 7 | · | 5 |
| Y-24 | 7 | 7 | · | · | · |
| Y-25 | 4 | · | · | 4 | · |
| Y-26 | 10 | 10 | · | · | · |
| Y-27 | 1 | 1 | · | · | · |
| Y-28 | 1 | · | · | · | 1 |
| Y-29 | 5 | · | · | 5 | · |
| Y-30 | 1 | 1 | · | · | · |
| Y-31 | 1 | · | 1 | · | · |
| Y-32 | 1 | · | · | · | 1 |
| Y-33 | 1 | · | · | · | 1 |
| Y-34 | 1 | · | · | 1 | · |
| Y-35 | 2 | · | · | 2 | · |
| Y-36 | 1 | · | 1 | · | · |
| Y-37 | 5 | · | 4 | 1 | · |
| Y-38 | 1 | · | 1 | · | · |
| Y-39 | 1 | · | · | 1 | · |
| Y-40 | 2 | · | · | · | 2 |
| Y-41 | 2 | · | 2 | · | · |

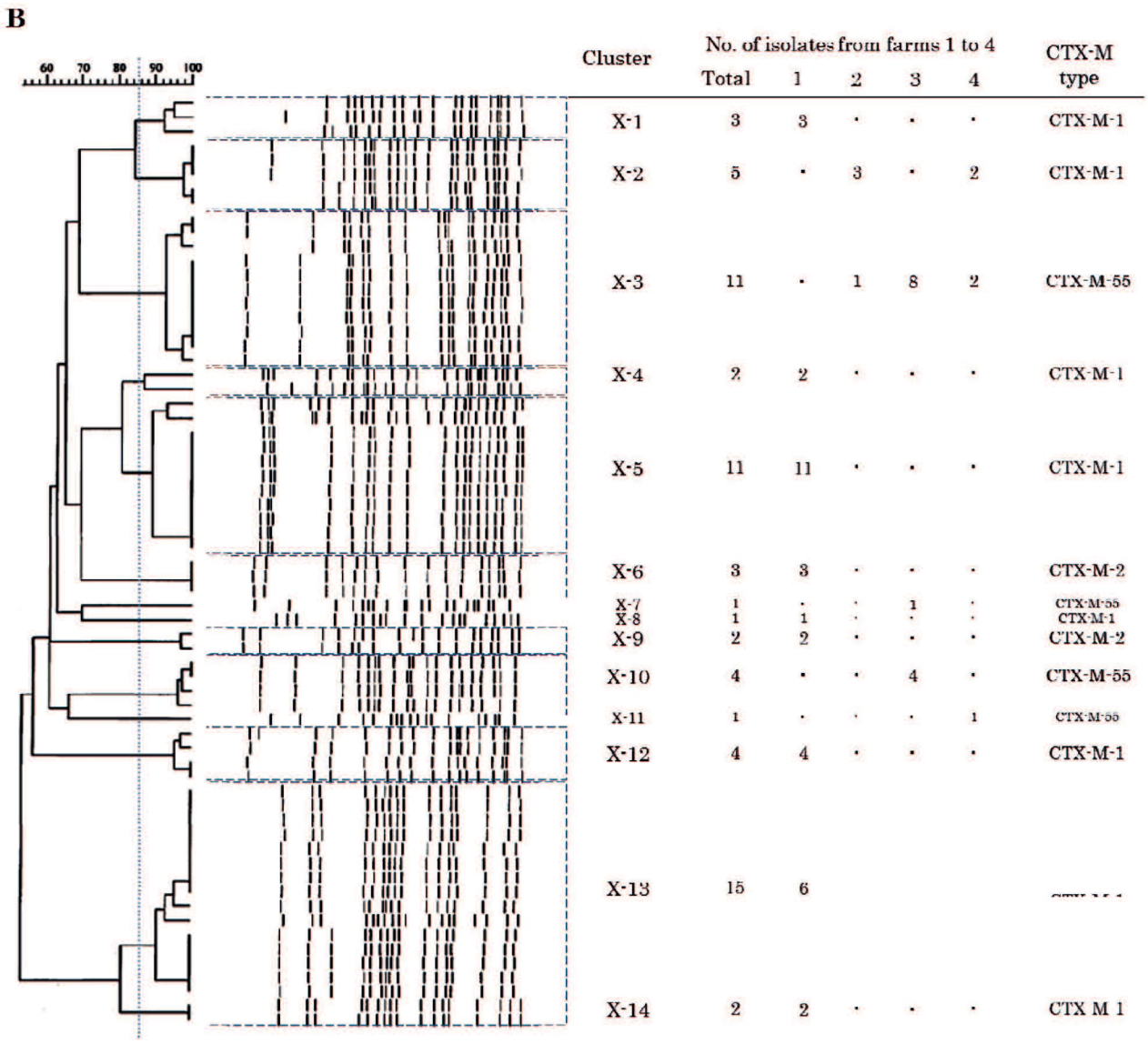



Fig. 2. Molecular macro-restriction profiles of 107 CMY-2 (A) and 65 CTX-M (B) producing *E. coli* isolates, as determined by PFGE analysis using *Xba*I digestion. The vertical, dotted line indicates the cutoff value (85%). The isolates with greater than 85% similarity are circled with a broken line. · indicates that no isolate was obtained. All isolates in a cluster produced the same CTX-M-type ESBL in Fig. 1B.

# IV. Conclusion

ESC-resistant *Enterobacteriaceae* are widely spread among food-producing animals and produce various types of ß-lactamase, includes ESBLs and AmpC ß-lactamase. Broiler has been recognized recently as a potential reservoir of ESC-resistant bacteria. To prevent the resistant bacterial infection for humans from the contaminated chicken meat, the identifying the resistance mechanisms and the surveillance of the bacteria on broiler farms are needed. In this study, we attempted to identify the mechanisms of ESC resistance in *S.* Infantis isolates from a broiler farm in the chapter II, and also attempted to investigate the distributions of the ESC-resistant *E. coli* derived from four broiler farms in the chapter III. In addition, we also demonstrated that the epidemiological relationships of the ESC-resistant *E. coli* isolates among four farms.

In 2009, we detected the CTX-M-14 ESBL-producing *S.* Infantis from broilers which derived from a farm. The resistance gene was on the IncI1 transferable plasmid. And non-ESBL isolates did not have the plasmid. Therefore, we suggested that the resistant isolates acquired the transferable plasmid from other *Enterobacteriaceae* on the farm. In 2011, we attempted to detect the CTX-M-14 ESBL-producing bacteria on four farms, but not detected. So, it is thought that transmission of the $bla_{\text{CTX-M-14}}$ gene between *S.* Infantis and other bacteria has not occurred between the study periods.

In the chapter III, ESC-resistant *E. coli* were recovered from all broiler samples derived from four farms. The Japanese Veterinary Antimicrobial Resistance Monitoring (JVARM) Program, which was conducted from 1999–2002 across Japan, found that only 2.7% of *E. coli* isolates were resistant to cefazolin

(first generation cephalosporin) [Kojima et al. 2005]. In the decade following the JVARM program, ESC-resistant bacteria appear to have spread remarkable throughout broiler farms in Japan.

CMY-2 AmpC ß-lactamase was predominant resistance determinant in the ESC-resistant *E. coli* in this study. In Japan, it is thought that plasmid-mediated AmpC ß-lactamase producers are still rare in human cases [Matsumoto *et al.* 2012], but the enzyme has been detected nowadays from patients in the United States and Europe. The United States has reported that significant problems with CMY-2-producing S. Newport in cattle and zoonotic infections [Devasia *et al.* 2005]. In the future, emergence of plasmid-mediated AmpC ß-lactamase includes CMY-2 may become a serious threat for humans and food-producing animals in Japan.

In this study, we clarified that transfer of plasmid carrying ESC-resistant determinants between bacteria and cross-contamination of the ESC-resistant bacteria occurred in the broiler farms we surveyed. It is possibly that the events occurred over Japanese farms, and caused the dissemination of the ESC-resistant bacteria in broiler farms.

It is important that some ESBL and AmpC ß-lactamase genes are located within mobile genetic elements, associated with other resistance genes, conferring resistance to antimicrobials that could be extensively used among humans and animals (e.g. trimethoprim, sulfamethoxazole, streptomycin), and which could play an important role in the co-selection of these ESBL and AmpC ß-lactamase genes [Carattoli, A. 2008]. Further analysis of the resistance genes on plasmid(s) with ESBL and AmpC ß-lactamase genes in the ESC-resistant *E. coli* is needed.

Among gram-negative bacteria, the emergence of resistance to ESC has been a major concern, and ESBL and AmpC ß-lactamase producers are expected to increase in the future in both animals and humans. The presence of ESBLs and *ampC* genes in the microbiota of food-producing animals may pose a human health hazard since these bacteria may represent a reservoir of resistance genes for pathogens causing diseases in humans. Indeed, ESBL-producing *S.* Enteritidis and Enterohemorrhagic *E. coli* serotype O26 were detected from patients with diarrhea in Japan. Therefore, more prudent use of antimicrobials in general may be necessary.

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