International Journal of Food Microbiology 274 (2018) 45–51

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

# Decrease in the prevalence of extended-spectrum cephalosporin-resistant *Salmonella* following cessation of ceftiofur use by the Japanese poultry industry

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ARTICLE INFO

*Keywords:*
AmpC β-lactamase
Chicken meat
Food safety
Plasmid
Extended-spectrum β-lactamase
*Salmonella*

ABSTRACT

Extended-spectrum cephalosporin (ESC)-resistant *Salmonella* in chicken meat is a significant food safety concern. We previously reported that the prevalence of ESC-resistant *Salmonella* in chicken meat, giblets, and processed chicken (chicken meat products) increased in Japan between 2005 and 2010, with 27.9% (17/61) of *Salmonella* isolated from chicken meat products in 2010 showing resistance to ESC. The aims of the present study were to clarify trends in the prevalence of ESC-resistant *Salmonella* in chicken meat products in Japan between 2011 and 2015, and to determine the genetic profiles of *bla*-harboring plasmids, including replicon types, using next-generation sequencing. Our results showed that the prevalence of ESC-resistant *Salmonella*, mainly consisting of AmpC β-lactamase CMY-2-producing isolates, in chicken meat products had increased to 45.5% (10/22) by 2011. However, following the voluntary cessation of ceftiofur use by the Japanese poultry industry in 2012, the prevalence of ESC-resistant *Salmonella* steadily decreased each year, to 29.2% (7/24), 18.2% (4/22), 10.5% (2/19), and 10.5% (2/19) in 2012, 2013, 2014, and 2015, respectively. Furthermore, no AmpC β-lactamase CMY-2-producing isolates were identified in 2014 and 2015. However, the prevalence of *Salmonella enterica* subspecies *enterica* serovar Manhattan isolates harboring a $bla_{TEM-52}$-carrying IncX1 plasmid remained steady even after the cessation of ceftiofur use. Therefore, continuous monitoring of ESC resistance amongst *Salmonella* isolates from chicken meat products is required for food safety.

## 1. Introduction

The emergence of extended-spectrum cephalosporin (ESC)-resistant *Salmonella* strains is of global concern. *Salmonella* is a zoonotic food-borne pathogen, and although infections are usually self-limiting, antibiotic therapy is required in severe cases. ESCs are usually administrated in severe cases involving children and patients infected with fluoroquinolone-resistant *Salmonella* isolates (Hohmann, 2001; Izumiya et al., 2005). Ceftiofur (CEF), an ESC, was extensively used off-label in Japan until March 2012 as a disinfectant for embryonated eggs and newborn chicks (Hiki et al., 2015). The isolation ratio of CEF-resistant *Escherichia coli* isolates derived from healthy chickens increased

*Abbreviations:* CAZ, ceftazidime; CEF, ceftiofur; CFIX, cefixime; CFPM, cefepime; CFX, cefoxitin; CLSI, Clinical and Laboratory Standards Institute; CMZ, cefmetazole; CPD, cefpodoxime; CTT, cefotetan; CTX, cefotaxime; CVA, clavulanic acid; DDBJ, DNA Data Bank of Japan; ESBL, extended-spectrum β-lactamase; ESC, extended-spectrum cephalosporin; IPM, imipenem; LMOX, moxalactam; LPP, large plasmid profile; MEPM, meropenem; PAPM, panipenem; PFGE, pulsed-field gel electrophoresis

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https://doi.org/10.1016/j.ijfoodmicro.2018.03.011
Received 21 October 2017; Received in revised form 14 March 2018; Accepted 16 March 2018
Available online 17 March 2018
0168-1605/ 

each year until 2011, but decreased after 2012 following the voluntary cessation of CEF use by the Japanese poultry industry (Hiki et al., 2015). In Canada, the incidence of CEF-resistant *E. coli* and *Salmonella* isolation from chicken meat also decreased after the withdrawal of CEF from poultry farms in 2005 (Dutil et al., 2010). We previously reported that the prevalence of ESC-resistant *Salmonella* in chicken meat in Japan significantly increased between 2004 and 2010, with 28% of chicken meat-derived *Salmonella* isolates showing resistance to ESC in 2010 (Noda et al., 2015). However, little information is available regarding trends in the prevalence of ESC-resistant *Salmonella* in chicken meat in Japan after 2010, and further studies are needed to examine this issue. Therefore, in the current study, we investigated whether the prevalence of ESC resistance amongst *Salmonella* isolates decreased following cessation of CEF use in Japan, as was observed for *E. coli*.

Transferable plasmids containing genes encoding AmpC β-lactamases and extended-spectrum β-lactamases (ESBL) have contributed to the dissemination of ESC resistance amongst *Salmonella* strains of human and other origins in many countries (Cloeckaert et al., 2007; Hasman et al., 2005; Noda et al., 2015; Randall et al., 2011; Shahada et al., 2013; Sirichote et al., 2010; Weill et al., 2004; Yu et al., 2011; Zhao et al., 2003). We previously found that several *bla* genes, including $bla_{\text{CTX-M-2}}$, $bla_{\text{SHV-12}}$, and $bla_{\text{TEM-52}}$, were present in *Salmonella* isolated from chicken meat in Japan between 2004 and 2010. During the same period, we also observed an increase in the number of *Salmonella* isolates harboring an ~280-kb $bla_{\text{CMY-2}}$-carrying plasmid (Noda et al., 2015). IncFIB plasmids carrying $bla_{\text{CTX-M-65}}$ were discovered in *Salmonella enterica* subspecies *enterica* serovar Infantis isolates in the United States between 2012 and 2015 (Tate et al., 2017). In addition, several plasmids belonging to different replicon types, including an IncFIB plasmid carrying $bla_{\text{CTX-M-27}}$, an IncN plasmid carrying $bla_{\text{CTX-M-65}}$, and a non-typable plasmid carrying $bla_{\text{CTX-M-27}}$, were found in *Salmonella* isolates from food-producing animals in China in 2014 (Zhang et al., 2016). Because *bla* genes are usually disseminated amongst bacteria *via* these transferable plasmids, plasmid replicon typing is important when investigating *bla*-harboring plasmids in *Salmonella* isolates worldwide. Therefore, the aims of this study were to examine trends in ESC resistance amongst *Salmonella* isolated from chicken meat products in Japan after 2011, and to determine the genetic profiles and replicon types of plasmids harboring *bla* genes using a next-generation sequencing approach. The findings of this study may aid in determining whether banning antibiotic use on poultry farms can help control the spread and/or development of antibiotic resistance.

## 2. Materials and methods

### 2.1. Sampling of chicken meat products

A total of 181 chicken meat product samples were collected between 2011 and 2015, with 38, 41, 32, 35, and 35 samples collected in 2011, 2012, 2013, 2014, and 2015, respectively. The 181 samples included chicken meat ($n = 163$), *sashimi* (raw chicken meat, $n = 3$), and *tataki* (slices of raw chicken with a briefly seared surface, $n = 15$). The 181 samples were randomly collected from 126 retailers in Fukuoka Prefecture, Japan, including supermarkets and butcher shops, by Fukuoka prefectural food hygiene inspectors. Of the 181 samples, 165 were derived from chickens that had been bred in Japan, while the remaining samples were from chickens reared outside of Japan (eight samples were imported from Brazil, and eight samples were of unknown origin).

### 2.2. Isolation of Salmonella from chicken samples

Isolations from samples collected from January 2011 to December 2015 were performed as described below. For samples collected between May and July during the 5-year period, 25 g of sample were homogenized with 225 mL of buffered peptone water (Oxoid Ltd., Basingstoke, Hampshire, UK) for 1 min in a stomacher, and then incubated at 35 °C for 18 h. Following incubation, 0.1 mL and 1 mL aliquots of the buffered peptone water suspensions were inoculated into 10 mL of Rappaport-Vassiliadis enrichment broth (Oxoid Ltd.) and 10 mL of tetrathionate broth (Oxoid Ltd.), respectively, and incubated at 42 °C for 18 h. Aliquots from each culture were then inoculated onto xylose lysine tergitol-4 agar (BD Diagnostic Systems, Sparks, MD, USA) and *Salmonella* Detection and Identification agar (bioMérieux, Lyon, France) and incubated at 35 °C for 18–48 h.

Isolations from samples collected between September and December during the 5-year period were carried out in accordance with a Japanese standard method, NIHSJ-01-ST4 (National Institute of Health Sciences, 2016). CHROMagar *Salmonella* (CHROMagar, Paris, France) and deoxycholate hydrogen sulfide lactose agar (Eiken Chemical Co., Tokyo, Japan) were used in place of xylose lysine tergitol-4 agar and *Salmonella* Detection and Identification agar, respectively. The plates were cultured at 37 °C for 18–48 h, and then colonies (1–4 colonies per sample) putatively identified as *Salmonella* were subjected to further biochemical identification assays, as described in our previous reports (Murakami et al., 2001; Murakami et al., 2013; Noda et al., 2015). The isolates confirmed as *Salmonella* were serotyped using somatic and flagella antisera as per the manufacturer's instructions (Denka Seiken Co., Tokyo, Japan).

### 2.3. Antibiotic susceptibility testing, detection of antimicrobial resistance genes, and DNA sequence analysis

Antimicrobial susceptibility testing was performed using the disk diffusion method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2012; CLSI, 2013). The following 12 antimicrobials were used: cefpodoxime (CPD, 10 μg), cefotaxime (CTX, 30 μg), ceftazidime (CAZ, 30 μg), cefixime (CFIX, 5 μg), cefepime (CFPM, 30 μg), cefoxitin (CFX, 30 μg), cefmetazole (CMZ, 30 μg), cefotetan (CTT, 30 μg), moxalactam (LMOX, 30 μg), imipenem (IPM, 10 μg), meropenem (MEPM, 10 μg), and panipenem (PAPM, 10 μg). Antimicrobial disks were obtained from Becton, Dickinson and Co. (Franklin Lakes, New Jersey, US). Phenotypic confirmatory tests were carried out to further examine the resistant isolates using CTX (30 μg)-clavulanic acid (CVA, Becton, Dickinson and Co.), CAZ (30 μg)-CVA (Becton, Dickinson and Co.), and CPD (30 μg)-CVA (Nissui Pharmaceutical Co., Tokyo, Japan) disks, as previously described (Noda et al., 2015). A boronic acid test was also performed as described previously (Coudron, 2005), although CPD and CFX disks were used in place of CTT. *E. coli* ATCC 25922 was used as a quality control strain in all assays in accordance with CLSI guidelines (CLSI, 2012; CLSI, 2013). *Klebsiella pneumoniae* ATCC 700603 and *E. coli* ATCC 35218 were used as additional reference strains.

A polymerase chain reaction assay was performed to detect β-lactamase genes ($bla_{\text{CMY}}$, $bla_{\text{CTX-M}}$, $bla_{\text{TEM}}$, and $bla_{\text{SHV}}$) in the resistant isolates as previously described (Noda et al., 2015). Isolates harboring β-lactamase genes were further examined for susceptibility to CEF (30 μg, Eiken Chemical Co.) as described above. The CEF disks were kindly provided by Zoetis Japan Inc. (Tokyo, Japan). The resistance genes were then sequenced using previously reported DNA sequencing primers (Noda et al., 2015), and the resulting sequences were analyzed using the BLAST program available from the DNA Data Bank of Japan (DDBJ) (2017). The corresponding amino acid sequences were then analyzed using the β-Lactamase Classification and Amino Acid Sequences for TEM, SHV, and OXA Extended-Spectrum and Inhibitor Resistant Enzymes website (Jacoby, 2017).

### 2.4. Comparison with data collected between 2004 and 2010

We compared data from the present study, conducted from 2011 to 2015, with findings from our previous study, conducted from 2004 to 2010 (Noda et al., 2015). The previous study used the same analysis