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CMY-2 β -Lactamase-Producing *Salmonella enterica* Serovar Infantis Isolated from Poultry in Japan

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Infection with salmonellae causes a wide range of symptoms from self-limiting acute enteritis to systemic sepsis. Extended-spectrum cephalosporins (ESCs) may be used in treatment of a severe case. *Salmonella* spp. strains resistant to ESCs have recently been identified in several countries, and the presence of these strains is considered to be highly problematic in treating patients (1-3).

There have been only two reports of ESC-resistant *Salmonella* spp. in Japan. One was regarding CMY-2 β -lactamase-producing *S. Newport* isolates, and the other a CTX-M-14 β -lactamase-producing *S. Enteritidis* isolate (4,5).

We have been conducting surveillance regarding the antimicrobial susceptibility of *Salmonella* spp. isolates from meat specimens of domestic poultry purchased from retail stores in Osaka Prefecture, a western part of Japan. Of the 83 specimens collected from April 2004 to March 2005, 29 (34.9%) were found to be positive for the organisms. The most common serotype was *S. Infantis*, which was isolated from 21 specimens. Two of the serotypes were resistant to ESCs. Here we report the results of our detailed bacteriological and genetical analysis of the two isolates.

Antimicrobial susceptibility testing was performed using

a disk diffusion method, as previously described according to the standards outlined by the Clinical and Laboratory Standards Institute (formerly, the National Committee for Clinical Laboratory Standards) (6). Disks were purchased from Becton Dickinson Microbiology Systems, Cockeysville, Md., USA. MICs were determined with an E-test (AB Biodisk, Solna, Sweden). All the 21 *S. Infantis* isolates showed resistance to two or more of the antimicrobials tested. Among them, seven were resistant to kanamycin (KAN), streptomycin (STR), sulfamethoxazole-trimethoprim (STX) and tetracycline (TET); six were resistant to KAN, STR and TET; three were resistant to STR and TET; one was resistant to ampicillin (AMP), KAN, STR, STX and TET (strain No. 16A-166); one was resistant to AMP, chloramphenicol (CHL), STR and TET (strain No. 16A-157); one was resistant to AMP, KAN, STR and TET; one was resistant to KAN, nalidixic acid (NAL), STR, STX and TET; and one was resistant to NAL, STR, STX and TET (strain No. 16A-158). Since the strains No. 16A-157 and No. 16A-166 showed intermediate susceptibility to cefotaxime (CTX) in the disk diffusion method, cephalothin (CEF) and ceftiofuran (FOX) were also applied in the MIC tests. Both strains were resistant to CTX at MIC 16 μ g/mL and the resistance was not inhibited by clavulanic acid, while MICs for CEF and FOX were higher than 256 μ g/mL for both strains (Table 1).

PCR detection of the *bla*_{CMY} gene and sequence analysis was performed as follows. The *bla*_{CMY} gene was detected by

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Table 1. Characteristics of *S. Infantis* 16A-166, 16A-157, 16A-158 and 16A-157/158 transconjugant

Strain	MIC ($\mu\text{g/mL}$) ¹⁾											ESBL test		PCR for <i>bla</i> _{CMY} genes
	AMP	CHL	STR	TET	KAN	NAL	CIP	CTX	CEF	FOX	CTX	CTL		
	<i>S. Infantis</i> 16A-166	256	1	32	128	>256	4	0.016	16	>256	256	16	>1	
<i>S. Infantis</i> 16A-157	256	128	>256	256	2	4	0.008	16	>256	>256	16	>1	+	
<i>S. Infantis</i> 16A-158 ²⁾	1	2	64	256	2	>256	0.125	0.064	4	4	NT ³⁾	NT	-	
<i>S. Infantis</i> 16A-157/158 transconjugant	256	128	>256	256	2	>256	0.125	16	>256	>256	16	>1	+	

¹⁾: Determined by E test.

²⁾: *S. Infantis* 16A-158: recipient of transconjugation.

³⁾: NT, not tested.

AMP, ampicillin; CHL, chloramphenicol; STR, streptomycin; TET, tetracycline; KAN, kanamycin; NAL, nalidixic acid; CIP, ciprofloxacin; CTX, cefotaxime; CEF, cephalothin; FOX, ceftiofloxacin; CTL, cefotaxime plus clavulanic acid.

PCR using primers described in Zhao et al. (7). Nucleotide sequencing was performed as follows. A 1.3-kb DNA fragment containing the open reading frame of *bla* was amplified by PCR using a primer pair of ampC-5' (5'-tctgctgctaaatgaaccg-3') and ampC-3' (5'-ttttgttaagtgtagatgac-3'). The amplified fragment was purified using Microspin Columns (Amersham Biosciences Corp., Piscataway, N. J., USA), and sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 3100-Avant (Applied Biosystems, Foster City, Calif., USA). The resulting sequences were searched for homologues in the EMBL database with the WU-BLAST program. In both strains of No. 16A-157 and No. 16A-166, a 1-kb fragment was amplified by a PCR method for detection of the *bla*_{CMY} gene (Table 1). In addition, the nucleotide sequences of the DNA fragments amplified by primers of ampC-5' and ampC-3' were 100% identical to that previously reported for the *bla*_{CMY-2} gene in *Klebsiella pneumoniae* (8).

Plasmids were extracted according to the Kado and Liu method (9), and were separated in a 0.5% agarose gel in TBE buffer. Plasmid transconjugation tests were performed using *S. Infantis* strains No. 16A-157 and No. 16A-166 as donors, and *S. Infantis* strain No. 16A-158 as a recipient. The resulting transconjugants were selected on trypticase soy agar plates (Nissui, Tokyo, Japan) containing 20 $\mu\text{g/ml}$ NAL and 5 $\mu\text{g/ml}$ CTX, and were used for antimicrobial susceptibility testing, plasmid detection, and *bla*_{CMY} gene detection. The strain No. 16A-157 possessed a plasmid over 90 kb in length, while no plasmid was observed in the strain No. 16A-166 (Fig. 1).

In the transconjugation test, the conjugation between donor 16A-157 and recipient 16A-158 transferred antimicrobial resistance at a frequency of 10^{-4} , while conjugation using donor 16A-166 did not result in any transfer of resistance. In the 16A-157/158 transconjugant, a plasmid over 90 kb in length was detected as in the donor strain (Fig. 1). In antimicrobial susceptibility testing, MICs of AMP, CHL, CTX, CEF, FOX, and STR in the transconjugant were quite similar to those in the donor strain. In addition, the *bla*_{CMY} gene was detected in the transconjugants (Table 1).

S. Infantis is one of the serovars that are detected in high frequency every year in Japan. The frequency of *S. Infantis* isolation from domestic poultry has been increasing since the middle 1970s. Now the organism has become a representative etiologic agent causing food poisoning and seriously affects food hygiene (10). In the year 2005, the serovar was ranked second following *S. Enteritidis* (full data are available at <http://idsc.nih.go.jp/iasr/virus/graph/salm2003.gif>). In August 2004, Sakai City in Osaka Prefecture experienced

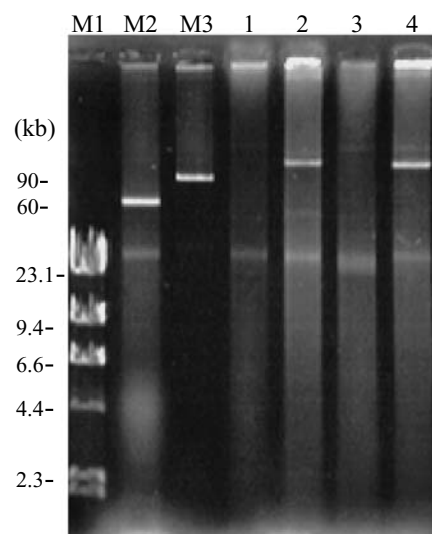


Fig. 1. Plasmid profiles of *S. Infantis* 16A-166, 16A-157, 16A-158 and 16A-157/158 transconjugant. Lane 1, *S. Infantis* 16A-166; Lane 2, *S. Infantis* 16A-157; Lane 3, *S. Infantis* 16A-158; Lane 4, *S. Infantis* 16A-157/158 transconjugant; Lane M1, a size marker of lambda DNA digested with *Hind*III; Lane M2, size marker *S. Enteritidis* L-156 with the size of 60 kb; Lane M3, size marker *E. coli* K12 CSH-2 (NR1) with the size of 90 kb.

a large-scale outbreak of food poisoning that involved 366 patients.

In this study, we identified two isolates of *S. Infantis* resistant to ESCs, which is the first finding of these resistant strains in Japan, and they were found to possess the *bla*_{CMY-2} gene. It has previously been reported that most *S. Infantis* isolates from poultry show multi-drug resistance in Japan (10), and, accordingly, all 21 isolates of *S. Infantis* in the present study were multi-drug resistant. In addition, the two ESC-resistant isolates were also resistant to AMP, STR, TET, KAN, and STX (No. 16A-166), and AMP, CHL, STR and TET (No. 16A-157). It is well known that resistance genes including *bla*_{CMY-2} are usually carried on plasmids (1). The ESC-resistance in strain No. 16A-157 appeared to be derived from a conjugative plasmid. Furthermore, the plasmid of 16A-157 seemed to also have resistance determinants to at least AMP and CHL. However, no plasmids were detected in another resistant strain, No. 16A-166, the resistance of which was not transferred in a transconjugation test. Further study is needed to elucidate how drug resistance had been acquired in this strain.

There has thus far been only one report (2) describing *S.*

Infantis from a pediatric patient in Honduras that produced CMY-2 β -lactamase. To our knowledge, the present study is the first to report the isolation of such an organism from poultry.

In Japan, six cephalosporins are approved for parenteral use only to cattle and pigs for food-producing animals. However, ESC-resistant *E. coli* strains were isolated from broilers in a nationwide survey (11). The reasons why these *E. coli* and *S. Infantis* strains have become resistant to ESCs are not clear at present. Nevertheless, considering the fact that poultry is frequently contaminated by *S. Infantis*, the isolation of ESC-resistant strains raises a serious public health concern, in that they could spread through foods based on poultry.

The cephalosporins used in this study were mainly for investigation of *Salmonella* isolated by humans. In a survey of the spread of ESC-resistant *Salmonella* it would be necessary to have greater variety in the antimicrobials utilized, not only for humans but also for food-producing animals.

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