Short Communication

Antimicrobial Resistance in *Salmonella* Strains Clinically Isolated in Hyogo, Japan (2009–2012)

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(Received June 12, 2013. Accepted August 13, 2013)

SUMMARY: The purpose of this study was to examine the in vitro susceptibilities to antimicrobial agents and genetic diversity of 195 clinical strains of *Salmonella* spp., which were isolated and examined for the extended-spectrum β -lactamase (ESBL) *bla*_{CTX-M} gene and the presence of *gyrA*, *gyrB*, *parC*, and *parE* genes mutations in Hyogo, Japan, from 2009 to 2012. Forty-three of the 195 strains were antimicrobial resistant. Two *Salmonella enterica* subsp. *enterica* strains, 1 serovar Schwarzengrund, and 1 serovar Enteritidis were identified as ESBL-producing strains possessing *bla*_{CTX-M-15} and *bla*_{CTX-M-2}, respectively. Among 8 nalidixic acid-resistant strains, 7 had mutations in *gyrA* alone or in *gyrA* and *parC*. In conclusion, we identified CTX-M ESBL-producing *Salmonella* clinical strains with multidrug resistance. Further studies are needed to monitor these serious drug-resistant *Salmonella* strains in Japan.

Salmonella is one of the principal pathogens implicated in human food-borne illnesses. Resistance to both fluoroquinolones (FQs) and β -lactam drugs has recently emerged among *Salmonella* spp. worldwide (1). *Salmonella* spp. that produce extended-spectrum β -lactamase (ESBL) or those that have mutations in FQ-resistance related genes have been isolated from hospitals (2). FQ resistance is conferred by point mutations in *gyrA* and *gyrB*, which encode DNA gyrase, and in *parC* and *parE*, which encode topoisomerase IV. The most frequent mutations in *gyrA*, which confer resistance to nalidixic acid (NA) and decreased susceptibility to FQs are located at the amino acids Ser 83 or Asp 87 (3). In the present study, we analyzed 195 *Salmonella enterica* subsp. *enterica* clinical strains obtained from 189 stool, 4 urine, and 2 blood specimens from the Hyogo Clinical Laboratory Corporation and Kobe University Hospital. The study was conducted from 2009 to 2012. *Salmonella*-positive strains were serogrouped with the use of O-antigen and H-antigen antiserum (Denka Seiken, Tokyo, Japan).

The antimicrobial susceptibility patterns of the strains were determined on Mueller–Hinton agar plates (BD: Becton, Dickinson and Company, Franklin Lakes, N.J., USA) using the Kirby–Bauer disk diffusion

method according to CLSI guidelines and interpretative criteria (2009) (4). Isolates were tested for sensitivities to antimicrobial disks (BD) containing tetracycline (TC), kanamycin (KM), gentamicin (GM), piperacillin (PIPC), amoxicillin (AMX), cefotaxime (CTX), ceftazidime (CAZ), ceftriaxone (CTRX), imipenem (IPM), meropenem (MERM), aztreonam (ATM), NA, norfloxacin (NOR), chloramphenicol (CP), and trimethoprim-sulfamethoxazole (ST). Antimicrobial minimum inhibitory concentrations (MICs) were determined by the standard agar plate dilution method using the antimicrobials of NA (Sigma-Aldrich Chemie GmbH, Munich, Germany) and ciprofloxacin (CIP) (Sigma-Aldrich). *Escherichia coli* strain ATCC 25922 was used as control. ESBL-producing strains were confirmed using antimicrobial disks of amoxicillin-clavulanic acid (BD), CTX, and CAZ by the double disc synergy test (DDST) (5).

A DNA template was obtained using the Illustra™ bacterial genomic Prep Mini Spin Kit (GE Healthcare Japan, Tokyo, Japan). Screening for *bla*_{CTX-M} was performed as previously described (6). Purification of the PCR products was performed by the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) and the sequencing was performed at Operon Biotechnologies, Inc. (Tokyo, Japan). *gyrA*, *gyrB*, *parC*, and *parE* were PCR-amplified, and DNA sequencing of all NA-resistant strains was performed using previously described primer sets (3). Purification and sequencing were performed as described above. Pulse field gel electrophoresis (PFGE) was performed to investigate subtypes of all the resistant strains and some of the sensitive

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Drug-Resistance of *Salmonella* Isolated in Hyogo

Table 1. Resistance pattern by serotype for *Salmonella* spp. in Japan

Isolate ¹⁾	Serotype	Disk susceptibility test	MIC ($\mu\text{g/mL}$)		ESBL detection	
		Resistance type ²⁾	NA	CIP ²⁾	DDST ³⁾	<i>bla</i> _{CTX-M} gene
S77	<i>S. Thompson</i>	TC, AMX, PIPC, CP	8	<0.063	—	—
S138	<i>S. Schwarzengrund</i>	TC, KM, AMX, PIPC, CTX, CTRX, ATM	4	<0.063	+	CTX-M-15
S197	<i>S. Saintpaul</i>	KM	8	<0.063	—	—
S94	<i>S. Infantis</i>	TC, KM	4	<0.063	—	—
S88	<i>S. Infantis</i>	NA	>128	0.25	—	—
S81	<i>S. Infantis</i>	TC, KM, AMX, ST	4	<0.063	—	—
S87	<i>S. Infantis</i>	TC, KM, ST	8	<0.063	—	—
S83	<i>S. Infantis</i>	TC, KM, ST	4	<0.063	—	—
S84	<i>S. Infantis</i>	GM, NA, ST	>128	0.25	—	—
S4	<i>S. Infantis</i>	TC	4	<0.063	—	—
S6	<i>S. Infantis</i>	TC, AMX	4	<0.063	—	—
S20	<i>S. Infantis</i>	TC, ST	2	<0.063	—	—
S27	<i>S. Infantis</i>	TC, AMX	4	<0.063	—	—
S29	<i>S. Infantis</i>	TC, KM	8	<0.063	—	—
S37	<i>S. Infantis</i>	TC	8	<0.063	—	—
S38	<i>S. Infantis</i>	TC, NA	>128	0.5	—	—
S41	<i>S. Infantis</i>	TC, NA	>128	0.5	—	—
S50	<i>S. Infantis</i>	NA	>128	0.25	—	—
S53	<i>S. Infantis</i>	TC, KM, CP, ST	8	<0.063	—	—
S54	<i>S. Infantis</i>	TC, KM	8	<0.063	—	—
S64	<i>S. Infantis</i>	TC, KM	4	<0.063	—	—
S68	<i>S. Infantis</i>	TC	8	<0.063	—	—
S69	<i>S. Infantis</i>	TC, KM	8	<0.063	—	—
S100	<i>S. Infantis</i>	TC, ST	8	<0.063	—	—
S107	<i>S. Infantis</i>	TC, KM	4	<0.063	—	—
S112	<i>S. Infantis</i>	TC, KM	<0.063	<0.063	—	—
S123	<i>S. Infantis</i>	TC, KM	4	<0.063	—	—
S158	<i>S. Infantis</i>	TC, AMX	8	<0.063	—	—
S159	<i>S. Infantis</i>	TC	8	<0.063	—	—
S165	<i>S. Infantis</i>	TC	8	<0.063	—	—
S176	<i>S. Infantis</i>	KM	8	<0.063	—	—
S194	<i>S. Infantis</i>	TC, KM	8	<0.063	—	—
S195	<i>S. Infantis</i>	TC, KM	8	<0.063	—	—
S200	<i>S. Infantis</i>	TC	8	<0.063	—	—
S3	O8:HNT ⁴⁾	TC	4	<0.063	—	—
S170	O8:HNT	TC	8	<0.063	—	—
S191	O8:HNT	TC	16	<0.063	—	—
S8	<i>S. Enteritidis</i>	AMX, PIPC	4	<0.063	—	—
S9	<i>S. Enteritidis</i>	AMX, PIPC	4	<0.063	—	—
S116	<i>S. Enteritidis</i>	NA	>128	0.25	—	—
S120	<i>S. Enteritidis</i>	NA	>128	0.25	—	—
S172	<i>S. Enteritidis</i>	CAZ	8	<0.063	—	—
S201	<i>S. Enteritidis</i>	TC, AMX, PIPC, CTX, CAZ, CTRX, ATM, NA	>128	0.25	+	CTX-M-2

¹⁾: Isolate names consists of an abbreviation for the study followed by the isolate number.

²⁾: TC, tetracycline; KM, kanamycin; GM, gentamicin; AMX, amoxicillin; PIPC, piperacillin; CTX, cefotaxime; CAZ, ceftazidime; CTRX, ceftriaxone; ATM, aztreonam; NA, nalidixic acid; ST, trimethoprim-sulfamethoxazole; CIP, ciprofloxacin; CP, chloramphenicol.

³⁾: Double disc synergy test.

⁴⁾: NT, not typing.

strains using the *BlnI* (TaKaRa, Shiga, Japan) in the GenePath™ System (Bio-Rad Laboratories, Inc., Hercules, Calif., USA). A dendrogram was generated using Gel compare II version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium) with the Dice coefficients described in a previous study (7). Resistance of *Salmonella* strains was observed against TC (n = 33), KM (n = 17), GM (n = 1), PIPC (n = 5), AMX (n = 9), CTX (n = 2), CAZ (n = 2), CTRX (n = 2), ATM (n = 2), and NA (n = 8) (Table 1). The ESBL-producing strains

from stool specimens were confirmed as CTX-M-15 (*Salmonella* Schwarzengrund) and CTX-M-2 (*S. Enteritidis*) types using DDST and *bla*_{CTX-M} gene sequencing (Table 1). In Japan, the CTX-M-14 type was reported in *S. Enteritidis* isolated from a stool culture (8). However, the reports regarding ESBL-producing *Salmonella* strains from human specimens in Japan, particularly with FQ resistance, is limited. The ESBL producers isolated in the present study may have acquired the transferable plasmid containing *bla*_{CTX-M}

Table 2. Mutation of quinolone resistant *Salmonella* strains

Isolate ¹⁾	Serotype	Mutation			
		<i>gyrA</i>	<i>gyrB</i>	<i>parC</i>	<i>parE</i>
S88	<i>S. Infantis</i>	—	—	—	—
S84	<i>S. Infantis</i>	Asp87-Asn	—	—	—
S38	<i>S. Infantis</i>	Ser83-Tyr	—	Thr57-Ser	—
S41	<i>S. Infantis</i>	Ser83-Tyr	—	Thr57-Ser	—
S50	<i>S. Infantis</i>	Ser83-Tyr	—	Thr57-Ser	—
S116	<i>S. Enteritidis</i>	Asp87-Gly	—	—	—
S120	<i>S. Enteritidis</i>	Asp87-Gly	—	—	—
S201	<i>S. Enteritidis</i>	Asp87-Tyr	—	—	—

¹⁾: Isolate name consists of an abbreviation for the study followed by the isolate number.

from another enteric bacterium in the patient's intestine. Furthermore, 195 *Salmonella* strains tested were divided on the basis of the approved CLSI breakpoints into 8 NA-resistant strains (MIC_{NA} of $\geq 32 \mu\text{g/mL}$) (Table 1). Among the resistant strains, the MICs of CIP ranged between 0.25 and 0.5 $\mu\text{g/mL}$ (Table 1). Seven of 8 NA-resistant strains had a mutation in *gyrA* at codon 83 or 87 (Table 2). Three of 8 NA-resistant strains had a mutation in *parC* at codon 57. ESBL-producing *S. Enteritidis* strains were NA-resistant and contained a mutation at codon Asp 87-Thr in *gyrA*. Such double mutations at codons 83 and 87 have been also found in clinical strains of *S. Typhimurium*, exhibiting a high level of resistance to FQs (9). In *S. Infantis* strains, the PFGE profiles of the antimicrobial-sensitive and -resistant

Fig. 1. Dendrograms show antimicrobial resistance type in *Salmonella* *Infantis* strains (A) and *S. Enteritidis* strains (B) and PFGE profiles. Genomic distance among the strains is shown by the scale at the top of the dendrogram by % similarity. Antibiotics were: TC, tetracycline; KM, kanamycin; GM, gentamicin; AMX, amoxicillin; PIPC, piperacillin; CTX, cefotaxime; CAZ, ceftazidime; CTRX, ceftriaxone; ATM, aztreonam; NA, nalidixic acid; CP, chloramphenicol; ST, trimethoprim-sulfamethoxazole.

strains were categorized into 6 clusters (1 to 6) by *BlnI* (Fig. 1A). Most of the antimicrobial-resistant strains belonged to cluster 4 (93.5%, 29/31 strains), suggesting a difference in subtypes between antimicrobial-sensitive and -resistant strains. In contrast, the PFGE patterns in *S. Enteritidis* strains were categorized into 2 clusters (A and B) (Fig. 1B), and there was no difference in subtypes between the antimicrobial-sensitive and -resistant strains. In Hokkaido, Japan, the number of *S. Typhimurium* isolates of bovine-origin that showed multidrug resistance in the same PFGE cluster was increased (10). Therefore, we need to carefully monitor the spread of this multidrug resistant *S. Enteritidis* with the CTX-M-2 type ESBL and *gyrA* and *parC* mutations.

We would like to emphasize the limitations of this study. First, the number of ESBL-producing *Salmonella* strains in the ESBL study was not sufficient enough to draw definitive conclusions. Second, the antibiotics tested, particularly quinolones, might not be adequate to provide a definitive resistance study. These limitations should be overcome in future studies.

In conclusion, we identified multidrug resistant *Salmonella* strains producing CTX-M ESBL and containing *gyrA* and *parC* mutations in human samples from Japan and classified *S. Infantis* to different clusters according to the antimicrobial resistance. However there was no difference in clustering among the antimicrobial-resistant and -sensitive *S. Enteritidis* strains. Further investigations of ESBL-producing and FQ-resistant *Salmonella* strains are needed to monitor the emergence of serious multidrug resistant *Salmonella* strains in Japan.

Acknowledgments This work was supported by JSPS KAKENHI Grant number 24590610.

Conflict of interest None to declare.

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