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**Short Communication**

# Third-Generation Cephalosporin-Resistant Non-Typhoidal *Salmonella* Isolated from Human Feces in Japan

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**SUMMARY:** β-lactamase genes were detected and characterized from 10 non-typhoidal *Salmonella* (NTS) clinical isolates resistant to third-generation cephalosporins collected between 2012 and 2014 in Japan. Five strains showed cefotaxime minimum inhibitory concentration (MIC) ≥ 64 μg/ml and positive clavulanic acid inhibition results. The $bla_{\text{CTX-M-2}}$ was detected in 3 strains (serotypes Stanley and Muenchen), whereas $bla_{\text{TEM-52}}$ (serotype Manhattan) and $bla_{\text{SHV-12}}$ (serotype Infantis) were each found in 1 strain. $bla_{\text{CMY-2}}$ was detected in the remaining 5 strains (serotypes Infantis, Rissen, Newport, and Saintpaul) with cefotaxime MICs of 4–32 μg/ml and positive cloxacillin- and 3-aminophenylboronic acid-based inhibition tests. IS*Ecp1* was located upstream of the $bla_{\text{CMY-2}}$ in 4 strains and of the $bla_{\text{CTX-M-2}}$ in 1 strain. Incompatibility (Inc)A/C, IncP, and IncI1 plasmids were present in the strains harboring $bla_{\text{CMY-2,}}$ which were detected predominantly in this study. Acquisition of resistance to third-generation cephalosporins by invasive NTS may limit therapeutic options for severe systemic infections and causing serious public health problems. Though such resistant clinical isolates are still rare in *Salmonella* species in Japan, our findings reveal the presence of cephem-resistant NTS in food handlers, thus emphasizing the necessity of more systematic nationwide investigations.

Non-typhoidal *Salmonella* (NTS) are globally recognized as the major cause of foodborne diseases, commonly involving self-limited gastroenteritis. Although most young adult cases usually improve without antimicrobial prescriptions, cases in infants, elderly adults, and immunocompromised patients can cause more serious, life-threatening situations including sepsis and/or meningitis that often require antimicrobial therapy. Therefore, resistance to third-generation cephalosporins in invasive infections with NTS may limit therapeutic options, especially for those with severe systemic infections and cause serious public health problems.

Acquisition of third-generation cephalosporin resistance in NTS clinical isolates has been gradually increasing due to acquisition of resistance genes encoding extended-spectrum β-lactamases (ESBLs) and/or plasmid-mediated AmpC (pAmpC) β-lactamases. During the past two decades, SHV-type ESBLs (including SHV-2, SHV-5, and SHV-12), TEM-type ESBLs (including TEM-3, TEM-25, and TEM-52), CTX-M-type ESBLs (including CTX-M-2, CTX-M-14, CTX-M-3, CTX-M-15, and CTX-M-55), PER-type ESBLs, and pAmpC β-lactamases (including CMY-2) have been detected in NTS isolates of human origin in various countries and regions. In Japan, ESBL- or pAmpC β-lactamase-producing clinical isolates are still rare among *Salmonella* spp. compared to among other members of the family *Enterobacteriaceae* such as *Escherichia coli* and *Klebsiella pneumoniae*. Only a few studies have identified these enzyme types in the NTS clinical isolates: one strain each of CTX-M-14-, CTX-M-2-, CTX-M-15-, and CMY-2-producers from fecal samples and a CTX-M-55-producer from liver abscess fluid (1–4).

This study characterized the third-generation cephalosporin-resistant human NTS isolates collected from separate regions in Japan to gain insight into the prevalence of ESBL, and pAmpC β-lactamase. Interestingly, CMY-2 producers were the most prevalent followed by CTX-M-2 producers in *Salmonella* spp.

Ten human isolates of NTS with resistance to third-generation cephalosporins (4 for O-antigen serogroup 7 [O7], 4 for O8, and 2 for O4) collected between 2012 and 2014 were subjected to our analyses. *Salmonella* serotypes were determined based on the combination of O and H antigens using *Salmonella* antiserum "Seiken" (Denka-Seiken, Tokyo, Japan). Five isolates were detected from fecal specimens of healthy adults

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Table 1. Origins and MICs of β-lactams for strains of non-typhoidal *Salmonella* used in this study

| Strain no. | Geographic origin | Isolation, yr | Sero-group | Sero-type | MIC (μg/ml) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | PIP | CTX | CTX /CLA | CAZ | CAZ /CLA | CPD | CRO | ATM | FEP | FOX | CMZ | MEM |
| 1 | Healthy carrier/Shizuoka | 2012 | O7 | Infantis | ≤ 8 | 4 | 4/4 | 16 | 8/4 | > 64 | 16 | 2 | ≤ 1 | 32 | 4 | ≤ 0.5 |
| 2 | Healthy carrier/Akita | 2012 | O7 | Rissen | > 64 | 32 | 16/4 | 64 | > 32/4 | 64 | 16 | 16 | ≤ 1 | > 32 | 16 | ≤ 0.5 |
| 3 | Healthy carrier/Nara | 2012 | O4 | Stanley | > 64 | > 128 | ≤ 0.125/4 | 16 | 0.25/4 | > 64 | > 64 | > 64 | > 32 | ≤ 2 | ≤ 0.5 | ≤ 0.5 |
| 4 | Healthy carrier/Shiga | 2012 | O8 | Manhattan | > 64 | > 128 | ≤ 0.125/4 | 32 | ≤ 0.125/4 | > 64 | > 64 | 4 | > 32 | ≤ 2 | ≤ 0.5 | ≤ 0.5 |
| 5 | Healthy carrier/Osaka | 2012 | O7 | Infantis | > 64 | 64 | ≤ 0.125/4 | 128 | 0.25/4 | > 64 | 64 | > 64 | 8 | 4 | 1 | ≤ 0.5 |
| 6 | Clin iso./hospital A, Chiba | 2013 | O8 | Newport | > 64 | 32 | 16/4 | 64 | > 32/4 | > 64 | 32 | 16 | ≤ 1 | > 32 | 8 | ≤ 0.5 |
| 7 | Clin iso./hospital B, Chiba | 2013 | O7 | Infantis | ≤ 8 | 4 | 2/4 | 8 | 8/4 | 64 | 8 | 2 | ≤ 1 | 16 | 4 | ≤ 0.5 |
| 8 | Clin iso./hospital B, Chiba | 2013 | O8 | Muenchen | > 64 | > 128 | 0.5/4 | 32 | 1/4 | > 64 | > 64 | > 64 | > 32 | 8 | 4 | ≤ 0.5 |
| 9 | Clin iso./hospital C, Chiba | 2013 | O4 | Saintpaul | > 64 | 32 | 16/4 | 64 | > 32/4 | > 64 | 32 | 16 | 2 | > 32 | 16 | ≤ 0.5 |
| 10 | Clin iso./hospital D, Chiba | 2014 | O8 | Muenchen | > 64 | > 128 | 0.25/4 | 16 | 1/4 | > 64 | > 64 | > 64 | > 32 | 4 | 1 | ≤ 0.5 |

PIP, piperacillin; CTX, cefotaxime; CLA, clavulanic acid (at a fixed concentration of 4 μg/ml); CAZ, ceftazidime; CPD, cefpodoxime; CRO, ceftriaxone; ATM, aztreonam; FEP, cefepime; FOX, cefoxitin; CMZ, cefmetazole; MEM, meropenem; Clin iso. clinical isolate.

engaged in food handling work in 5 prefectures. The remaining 5 isolates were from facal specimens of diarrheic patients from 4 different hospitals located in the same prefecture. The origins of these strains are shown in Table 1.

Minimum inhibitory concentrations (MICs) of β-lactams, quinolones, fosfomycin, and sulfamethoxazole-trimethoprim for each isolate were determined by a broth microdilution method with a WalkAway-96 SI system (Beckman Coulter, Brea, CA, USA), according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Phenotypic screening of β-lactamases was performed using inhibitors: clavulanic acid (CLA) for ESBL and 3-aminophenylboronic acid (APB, 300 μg / disk) and cloxacillin (750 μg /disk) for AmpC β-lactamase. ESBL genes were analyzed by PCR. Full-length *bla* genes were amplified using primers specific for $bla_{\text{TEM}}$, $bla_{\text{SHV}}$, or $bla_{\text{CTX-M-2}}$. Then, purified PCR products were sequenced directly on both strands with a BigDye Terminator Cycle Sequencing Ready Reaction Kit and an ABI 3130xl genetic analyzer (Applied Biosystems, Foster City, CA, USA). The nucleotide and deduced amino acid sequences were analyzed with basic local alignment search tool (BLAST) programs <http://www.ncbi.nlm.nih.gov/blast>. pAmpC β-lactamase genes were detected by multiplex PCR, followed by direct sequencing using sequence primers (5). The presence of IS*Ecp1* upstream of the *bla* genes was investigated by PCR and sequencing with a combination of IS*Ecp1* forward primer (5'-TGCTCTGTGGATAACTTGCAG-3') and CTX-M/R1 (5'- CTCCGCTGCCGGTTTTATC-3') or CITMR reverse primers (5). Plasmid transferability was examined by conjugation using a broth mating method with the sodium azide-resistant *Escherichia coli* J53. Plasmid incompatibility (Inc) groups were determined by PCR-based plasmid replicon typing (6).

As shown in Table 1, MIC values of cefotaxime (CTX) and ceftazidime (CAZ) for the 10 isolates ranged from 4 to > 128 μg/ml and from 8 to 128 μg/ml, respectively. In particular, the presence of CLA reduced the CTX MICs of strains, nos. 3, 4, 5, 8, and 10 (CTX MIC of 64 to > 128 μg/ml), indicating the production of ESBLs. ESBL production was also confirmed by CLA inhibition of CTX, CAZ, and aztreonam. For those 5 strains, high MICs of the forth-generation cephalosporin (cefepime) were found, but MIC values of cephamycins (cefoxitin and cefmetazole) were low. The remaining 5 strains, nos. 1, 2, 6, 7, and 9 (CTX MIC from 4 to 32 μg/ml) showed low MIC values of cefepime and were thus suspected to be pAmpC β-lactamase producers. The inhibitory effect of APB and cloxacillin on CAZ MIC was also suggestive of pAmpC β-lactamase production. All of the 10 isolates were susceptible to piperacillin-tazobactam, levofloxacin, ciprofloxacin, and fosfomycin (data not shown), in addition to meropenem.

PCR and sequencing revealed that $bla_{\text{CTX-M-2}}$ identified in strain nos. 3 (serotype Stanley), 8 and 10 (serotype Muenchen) was the predominant type among ESBL

producers, which was followed by $bla_{TEM-52}$ (strain no. 4, serotype Manhattan) and $bla_{SHV-12}$ (strain no. 5, serotype Infantis). The $bla_{CMY-2}$ was identified in all 5 pAmpC β-lactamase producers: strain nos. 1 (serotype Infantis), 2 (serotype Rissen), 6 (serotype Newport), 7 (serotype Infantis), and 9 (serotype Saintpaul) (Tables 1 and 2). CMY-2 is a widely disseminated enzyme in *Salmonella* spp. of animal origin. The enzymes CTX-M-15 and CTX-M-14 are the most widely distributed ESBL variants in both domestic and global settings among the third-generation cephalosporin-resistant *Enterobacteriaceae* clinical isolates. Those 2 ESBLs are still rare among NTS isolates derived from humans and animals in Japan, although they have been increasingly detected in Europe and Asia (7,8). Our finding of a high frequency of $bla_{CMY-2}$ or $bla_{CTX-M-2}$ genes was consistent with other studies that focused on chicken products in Japan (9,10). However, it is notable that $bla_{CTX-M-15}$ and $bla_{CTX-M-14}$ were not detected in this study.

Analysis of the upstream region of the *bla* genes identified that $bla_{CMY-2}$ in strain nos. 1, 2, 6, and 7, and $bla_{CTX-M-2}$ in strain no. 3 were preceded by IS*Ecp1*, which provides promoter sequences that enhance *bla* expression and may be associated with mobilization and dissemination. Sequences of the spacer region between the right inverted repeat of IS*Ecp1* and $bla_{CMY-2}$ (116 bp) or $bla_{CTX-M-2}$ (49 bp) were identical to those described previously (11,12).

Strain nos. 1, 3, 4, 5, 7, and 9 yielded *Escherichia coli* J53 transconjugants with *bla* genes, whereas strain nos. 2, 6, 8, and 10 failed to yield transconjugants, despite repeated attempts. PCR-based replicon typing results of resistance plasmids in either parental strains or transconjugant strains are shown in Table 2. IncA/C, IncP, and IncI1 plasmids were present in strains harboring $bla_{CMY-2}$ which were detected predominantly in this study. Strains carrying $bla_{CTX-M-2}$ had IncN, IncY, and IncHI2 plasmids, and those carrying $bla_{TEM-52}$ and $bla_{SHV-12}$ had IncFIB and IncP, respectively. IncA/C and IncI1 plasmids are the most common carriers of $bla_{CMY-2}$ in human *Salmonella* isolates (13). IncA/C is a broad-host-range plasmid conferring multidrug resistance to *Salmonella*, *E. coli*, and other *Enterobacteriaceae* isolates, and contributes to successful dissemination of $bla_{CMY}$ as well as metallo-β-lactamase genes such as $bla_{NDM}$. Narrow-host-range IncI1 plasmids are commonly associated with the spread of $bla_{CMY}$, $bla_{CTX-M}$, or $bla_{SHV-12}$. These 2 Inc group plasmids are also associated with the spread of $bla_{CMY}$ and $bla_{CTX-M}$ in food-producing animals. In Japan, IncA/C, IncI1, and IncP plasmids carrying $bla_{CMY-2}$ have been identified in *Salmonella* isolates from cattle and broilers (14,15). Though limited in number, those Inc plasmids were detected among NTS isolates harboring $bla_{CMY-2}$ from food handlers in addition to diarrheic patients: these findings raise concerns about future increases in third-generation cephalosporin-resistance among invasive NTS, causing an important clinical and public health issue. Thus, our results underscore the necessity of more systematic nationwide investigations including continuous monitoring of antimicrobial resistance in *Salmonella* spp.

Table 2. Genetic features associated with *bla* genes

| Strain no. | MIC (μg/ml) | | *bla* gene | IS*Ecp1* | Incompatibility type in transconjugants |
|---|---|---|---|---|---|
| | CTX | CAZ | | | |
| 1 | 4 | 16 | $bla_{CMY-2}$ | + | P |
| 2 | 32 | 64 | $bla_{CMY-2}$ | + | (P, A/C)[1)] |
| 3 | > 128 | 16 | $bla_{CTX-M-2}$ | + | N |
| 4 | > 128 | 32 | $bla_{TEM-52}$ | − | FIB |
| 5 | 64 | 128 | $bla_{SHV-12}$ | − | P |
| 6 | 32 | 64 | $bla_{CMY-2}$ | + | (A/C) |
| 7 | 4 | 8 | $bla_{CMY-2}$ | + | P |
| 8 | > 128 | 32 | $bla_{CTX-M-2}$ | − | (Y) |
| 9 | 32 | 64 | $bla_{CMY-2}$ | − | I1 |
| 10 | > 128 | 16 | $bla_{CTX-M-2}$ | − | (HI2) |

[1)]: Incompatibility types of plasmids derived from parental strain are indicated in parentheses.
CTX, cefotaxime; CAZ, ceftazidime.

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**Conflict of interest** None to declare.

## REFERENCES

1. Izumiya H, Mori K, Higashide M, et al. Identification of CTX-M-14 β-lactamase in a *Salmonella enterica* serovar Enteritidis isolate from Japan. Antimicrob Agents Chemother. 2005;49:2568-70.
2. Osawa K, Shigemura K, Shimizu R, et al. Antimicrobial resistance in *Salmonella* strains clinically isolated in Hyogo, Japan (2009–2012). Jpn J Infect Dis. 2014;67:54-7.
3. Imoto A, Ooi Y, Edogawa S, et al. Liver abscess caused by CTX-M-55-type extended-spectrum β-lactamase (ESBL)-producing *Salmonella* Enteritidis. Intern Med. 2014;53:1699-703.
4. Ishiguro F, Kyota Y, Mochizuki M, et al. Comparison of multidrug-resistant *Salmonella enterica* serovar Newport isolates from a patient and sewages in Fukui Prefecture. Kansenshougaku Zasshi. 2005;79:270-5. Japanese.
5. Perez-Perez FJ, Hanson ND. Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. J Clin Microbiol. 2002;40:2153-62.
6. Carattoli A, Bertini A, Villa L, et al. Identification of plasmids by PCR-based replicon typing. J Microbiol Methods. 2005;63:219-28.
7. Burke L, Hopkins KL, Meunier D, et al. Resistance to third-generation cephalosporins in human non-typhoidal *Salmonella enterica* isolates from England and Wales, 2010–12. J Antimicrob Chemother. 2014;69:977-81.
8. Tamang MD, Nam H-M, Kim T-S, et al. Emergence of extended-spectrum β-lactamase (CTX-M-15 and CTX-M-14)-producing nontyphoid *Salmonella* with reduced susceptibility to ciprofloxacin among food animals and humans in Korea. J Clin Microbiol. 2011;49:2671-5.
9. Noda T, Murakami K, Etoh Y, et al. Increase in resistance to extended-spectrum cephalosporins in *Salmonella* isolated from retail chicken products in Japan. PLoS One. 2015;10:e0116927.

10. Matsumoto Y, Izumiya H, Sekizuka T, et al. Characterization of *bla*$_{\text{TEM-52}}$-carrying plasmids of extended-spectrum-β-lactamase-producing *Salmonella enterica* isolates from chicken meat with a common supplier in Japan. Antimicrob Agents Chemother. 2014;58:7545-7.
11. D'Andrea MM, Literacka E, Zioga A, et al. Evolution and spread of a multidrug-resistant *Proteus mirabilis* clone with chromosomal AmpC-type cephalosporinases in Europe. Antimicrob Agents Chemother. 2011;55:2735-42.
12. Harada S, Ishii Y, Saga T, et al. Chromosomal integration and location on IncT plasmids of the *bla*$_{\text{CTX-M-2}}$ gene in *Proteus mirabilis* clinical isolates. Antimicrob Agents Chemother. 2012;56:1093-6.
13. Hopkins KL, Liebana E, Villa L, et al. Replicon typing of plasmids carrying CTX-M or CMY β-lactamases circulating among *Salmonella* and *Escherichia coli* isolates. Antimicrob Agents Chemother. 2006;50:3203-6.
14. Sugawara M, Komori J, Kawakami M, et al. Molecular and phenotypic characteristics of CMY-2 β-lactamase-producing *Salmonella enterica* serovar Typhimurium isolated from cattle in Japan. J Vet Med Sci. 2011;73:345-9.
15. Chuma T, Miyasako D, Dahshan H, et al. Chronological change of resistance to β-lactams in *Salmonella enterica* serovar Infantis isolated from broilers in Japan. Front Microbiol. 2013;4:113.