

Change in Antimicrobial Resistance Pattern in *Salmonella enterica* serovar Typhimurium Isolates Detected in a Beef Cattle Farm

Masaru SUGAWARA¹⁾, Francis SHAHADDA²⁾, Hidemasa IZUMIYA³⁾, Haruo WATANABE³⁾, Ikuo UCHIDA⁴⁾, Yukino TAMAMURA⁴⁾, Masahiro KUSUMOTO²⁾, Taketoshi IWATA²⁾ and Masato AKIBA^{2)*}

¹⁾Aizu Livestock Hygiene Service Center, 90 Muramae, Kamikoya, Koyomachi, Aizuwakamatsu, Fukushima 965-0077, Japan

²⁾Safety Research Team, National Institute of Animal Health, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan

³⁾Department of Bacteriology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

⁴⁾Hokkaido Research Station, National Institute of Animal Health, 4 Hitsujigaoka, Toyohira, Sapporo 062-0045, Japan

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ABSTRACT. Multidrug-resistant *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) isolates with four different antimicrobial resistance patterns obtained from a beef cattle farm were characterized to determine their clonality. Macrorestriction analysis of genomic DNA revealed that these four isolates are closely related to each other and can be classified as a newly emerged pulsed-field gel electrophoresis type among cattle: cluster VII. Three of the four isolates showed resistance to extended-spectrum cephalosporins (ESCs), and this resistance was mediated by AmpC β -lactamase encoded by the *bla*_{CMY-2} gene in a 190-kbp IncA/C plasmid. Results of restriction analysis and IncA/C backbone PCR suggest that the three 190-kbp plasmids are identical and that a 70-kbp IncA/C plasmid of the ESC-susceptible isolate is derived from the 190-kbp plasmid by a deletion event. Three isolates harboured a virulence-resistance plasmid (165 or 180 kbp), and restriction analysis revealed that these plasmids were identical or closely related to each other. These results suggest that the four *S. Typhimurium* cluster VII isolates originate from a common ancestor that probably invaded the farm prior to the salmonellosis outbreak. Antimicrobial resistance patterns may not necessarily reflect the relationships of the isolates.

KEY WORDS: antimicrobial resistance, extended-spectrum cephalosporins, farm, *Salmonella enterica* serovar Typhimurium.

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Salmonella enterica serovar Typhimurium (*S. Typhimurium*) is a common cause of salmonellosis in humans and animals [6, 14]. The replacement of penta-resistant *S. Typhimurium* DT104 by a novel group of genetically related multidrug-resistant *S. Typhimurium* showing pulsed-field gel electrophoresis (PFGE) pattern cluster VII (ST-VII) has been reported in Hokkaido, Japan [10]. The ST-VII isolates were detected for the first time in 2000 and have been dominant since 2002 among bovine isolates in this area. Among 165 ST-VII isolates tested, 158 showed resistance to five or more antimicrobials, including ampicillin, streptomycin, sulfonamides, tetracyclines and kanamycin. Moreover, 26 of the 158 isolates showed resistance to extended-spectrum cephalosporins (ESCs) in addition to these five antimicrobials. The resistance to ESCs was attributed to CMY-2 β -lactamase, encoded by the *bla*_{CMY-2} gene located on a chromosome or plasmid. Plasmid-mediated CMY-2 β -lactamase-producing *S. Typhimurium* isolates belonging to ST-VII were also isolated from cattle in Fukushima prefecture in 2007 [9].

These ESC-resistant ST-VII isolates obtained from cattle in Japan were collected from different farms and were epidemiologically unrelated to each other. The stability of the resistance pattern of the isolates implicated in the disease

outbreak is not clear. In the process of molecular epidemiological investigation of bovine salmonellosis caused by ESC-resistant ST-VII isolates, we observed multiple antimicrobial resistance patterns among the isolates detected in a beef cattle farm. This study was conducted to determine the clonality of these ST-VII isolates.

The *S. Typhimurium* isolates used in this study are listed in Table 1. Isolates L-3694, L-3695, L-3706 and L-3708 were derived from different calves in a beef cattle farm in Fukushima prefecture. Isolate L-3695 is the same as 19-1823 described elsewhere [9]. Usually, up to 300 crossbred (Holstein and Japanese Black) beef cattle are raised, and about 10 calves are introduced from other prefectures into the farm once a fortnight. In November 2007, salmonellosis occurred in an infant-feeding stall which raised about 50 calves. Isolates L-3694, L-3695 and L-3706 originated from initial samples obtained in November 2007, and L-3708 was detected 2 months later in the same stall. *S. Typhimurium* was not isolated from any other site on the farm. Cefazolin was administered to the affected animals before obtaining the results of antimicrobial susceptibility testing. Administration of cefazolin was replaced by fosfomycin after knowing the results.

These isolates were identified as *Salmonella* spp. based on colony morphology on selective media and biochemical testing, as previously described [3]. Serovar identification was performed by microtitre and slide agglutination methods according to the latest version of the Kaufmann and White scheme [8] using antisera (Denka Seiken, Tokyo,

*CORRESPONDENCE TO: Dr. AKIBA, M., Safety Research Team, National Institute of Animal Health, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan.
e-mail: akiba@affrc.go.jp

Table 1. Antimicrobial resistance patterns and plasmid profiles of *S. Typhimurium* isolates used in this study

Strain	Antimicrobial resistance pattern ^{a)}	Plasmid profile (kbp)
L-3694	AMP, CFZ, CTX, STR, TET, CHL, SUL	190, 100
L-3695	AMP, CFZ, CTX, KAN, STR, TET, CHL, SUL	190, 165
L-3706	AMP, KAN, STR, TET, CHL, SUL	165, 70
L-3708	AMP, CFZ, CTX, KAN, STR, TET, CHL, SUL, SXT	190, 180, 120

a) AMP, ampicillin; CFZ, cefazolin; CTX, cefotaxime; KAN, kanamycin; STR, streptomycin; TET, tetracycline; CHL, chloramphenicol; SUL, sulfamethizole; SXT, trimethoprim-sulfamethoxazole.

Japan). Macrorestriction analysis using *Xba*I endonuclease (Takara Bio, Shiga, Japan) was performed by PFGE according to the PulseNet protocol and interpreted as described previously [10].

Digestion of genomic DNA of isolates L-3694, L-3695, L-3706 and L-3708 with *Xba*I produced 18–20 fragments (Fig. 1). Three polymorphic fragments were found (165, 155 and 95 kbp). These polymorphisms can be explained by one or two genetic differences, suggesting a close relationship among the isolates [11]. These isolates were classified in cluster VII based on the macrorestriction patterns (data not shown) [10].

Bacteriophage typing was performed according to the methods of the Public Health Laboratory Service, London, United Kingdom (1). Isolates L-3694, L-3695, L-3706 and L-3708 were untypable with the standard Colindale panel of phages.

To investigate the antimicrobial susceptibilities of the isolates, the Kirby–Bauer disc diffusion test was performed using Mueller–Hinton agar plates (Becton, Dickinson and Company, Sparks, MD, U.S.A.) according to the Clinical and Laboratory Standards Institute (CLSI) standards (formerly National Committee for Clinical and Laboratory Standards) [7] using the following antimicrobials: ampicillin (10 µg), cefazolin (30 µg), cefotaxime (30 µg), kanamycin (30 µg), streptomycin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), fosfomycin (50 µg), sulfamethizole (250 µg), trimethoprim–sulfamethoxazole (1.25/23.75 µg), nalidixic acid (30 µg), ofloxacin (5 µg) and norfloxacin (10 µg) (Becton, Dickinson and Company). Susceptibility testing results in this study were interpreted according to the criteria established by CLSI [2, 7].

As shown in Table 1, the antimicrobial resistance patterns of isolates L-3694, L-3695, L-3706 and L-3708 were different from each other. Isolate L-3695 showed resistance to ampicillin, cefazolin, cefotaxime, kanamycin, streptomycin, tetracycline, chloramphenicol and sulfamethizole. Isolate L-3708 showed additional resistance to trimethoprim–sulfamethoxazole, while isolates L-3694 and L-3706 did not show resistance to kanamycin and cephalosporins, respectively. Cephalosporinase was detected in isolates L-3694, L-3695 and L-3708 by the P/Case test (Nissui Pharmaceutical, Tokyo, Japan), and the *bla*_{CMY-2} gene was detected in the same isolates by PCR using the primer pairs listed in Table 2.

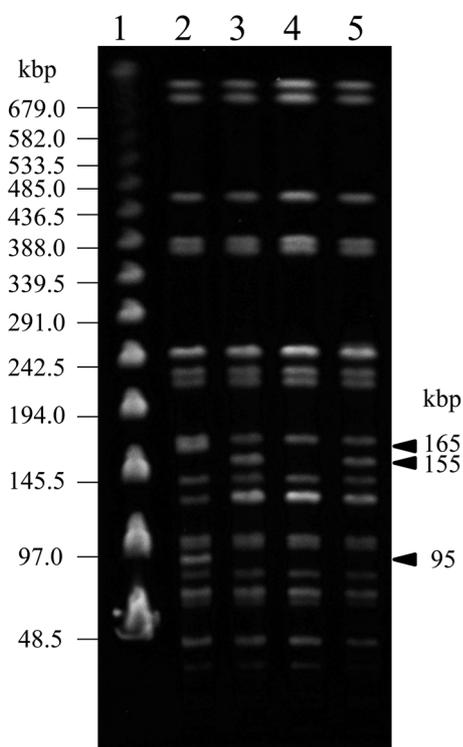


Fig. 1. Macrorestriction analysis of genomic DNA of the *S. Typhimurium* isolates digested with *Xba*I by PFGE. Lane 1, lambda ladder PFG marker; lane 2, L-3694; lane 3, L-3695; lane 4, L-3706; lane 5, L-3708. Black arrowheads at the right of each panel indicate polymorphic bands.

Plasmid DNA was isolated by the method described by Kado and Liu [(4) followed by phenol–chloroform extraction. Transformation of *E. coli* JM109 was performed by electroporation using a MicroPulser (Bio-Rad Laboratories, Hercules, CA, U.S.A.) according to the manufacturer's instructions. The transformants were selected on Luria–Bertani agar plates (Becton, Dickinson and Company) containing cefazolin (100 µg/ml), kanamycin (25 µg/ml) or streptomycin (25 µg/ml). Related genes in each plasmid were localized by Southern blot analysis using the primer pairs listed in Table 2. DNA probe labelling, hybridization and detection were performed using DIG-PCR and the DIG

Table 2. Primers used in this study

Primer name	Sequence (5'→3')	Target	Amplicon size	Reference
CMY-2F	TTTATCCAATCCGCGCTCGA	<i>bla</i> _{CMY-2}	470	This study
CMY-2R	TTCCCGCCGTTGAGGTAAAC			
A/C FW	GAGAACCAAAGACAAAGACCTGGA	<i>repA</i> (IncA/C)	465	[9]
A/C RV	ACGACAAACCTGAATTGCCTCCTT			
spvBF	AGCAGTTTTTATCGCCTGGA	<i>spvB</i>	526	This study
spvBR	GGTGGAACATCAGGACTTGG			
TEM-1F	AGCATCTTACGGATGGCATG	<i>bla</i> _{TEM-1}	520	This study
TEM-1R	AGTGAGGCACCTATCTCAGC			
II FW	CGAAAGCCGGACGGCAGAA	RNAI (IncII)	139	[9]
II RV	TCGTCGTTCCGCCAAGTTCGT			

Table 3. Antimicrobial resistance patterns and results of hybridization experiments with each plasmid

Plasmid (kbp)	Origin	Antimicrobial resistance pattern ^{a)}	Hybridization with the gene-specific probe ^{b)}				
			<i>bla</i> _{CMY-2}	<i>bla</i> _{TEM-1}	IncA/C	IncII	<i>spvB</i>
190	L-3694	AMP, CFZ, CTX, STR, TET, CHL, SUL	+	-	+	-	-
190	L-3695	AMP, CFZ, CTX, STR, TET, CHL, SUL	+	-	+	-	-
165	L-3695	AMP, KAN, TET, SUL	-	+	-	-	+
70	L-3706	STR, TET, CHL, SUL	-	-	+	-	-
165	L-3706	AMP, KAN, TET, SUL	-	+	-	-	+
190	L-3708	AMP, CFZ, CTX, STR, TET, CHL, SUL	+	-	+	-	-
180	L-3708	AMP, KAN, TET	-	+	-	-	+
120	L-3708	STR, TET, SUL, SXT	-	-	-	+	-

a) Data obtained from *E. coli* transformants; AMP, ampicillin; CFZ, cefazolin; CTX, cefotaxime; KAN, kanamycin; STR, streptomycin; TET, tetracycline; CHL, chloramphenicol; SUL, sulfamethizole; SXT, trimethoprim-sulfamethoxazole.

b) +, hybridization signal was observed; -, hybridization signal was not observed.

nucleic acid detection kits (Roche Diagnostics, Basel, Switzerland).

As shown in Table 3, the genes *bla*_{CMY-2} and *repA* (replicon type IncA/C) were located on the 190-kbp plasmids isolated from L-3694, L-3695 and L-3708. These transformants showed resistance to ampicillin, cefazolin, cefotaxime, streptomycin, tetracycline, chloramphenicol and sulfamethizole. The transformant containing a 70-kbp plasmid isolated from L-3706 did not show resistance to ampicillin, cefazolin and cefotaxime, while a *repA* gene (replicon type IncA/C) signal was detected in the 70-kbp plasmid. The transformant with the 165-kbp plasmid showed resistance to ampicillin, kanamycin, tetracycline and sulfamethizole, while that with the 180-kbp plasmid did not show resistance to sulfamethizole. The *bla*_{TEM-1} and *spvB* genes were located on a 180-kbp plasmid isolated from L-3708 in addition to the 165-kbp plasmids isolated from L-3695 and L-3706, suggesting that these plasmids are virulence-resistance plasmids. The transformant with the 120-kbp plasmid isolated from L-3708 showed resistance to streptomycin, tetracycline, sulfamethizole and trimethoprim-sulfamethoxazole, and the *repA* gene (replicon type IncII) was detected in the plasmid.

To investigate the genetic relatedness of these plasmids, restriction analysis was performed using endonucleases *Hind*III and *Sal*I. The IncA/C plasmids isolated from L-3694, L-3695 and L-3708 showed identical restriction patterns after *Hind*III digestion (Fig. 2A). Addition of a frag-

ment and loss of a fragment larger than 23.1 kbp were observed in the 70-kbp plasmid isolated from L-3706 compared with other IncA/C plasmids. The *Sal*I restriction patterns of all IncA/C plasmids were indistinguishable from each other (Fig. 2B). To compare plasmid structure, IncA/C backbone PCR was performed using the method described by Welch *et al.* [13]. All 13 IncA/C backbone regions were successfully amplified from the 190-kbp plasmids isolated from L-3694, L-3695 and L-3708, while consecutive 6 regions (nos.4–9) were not amplified from the 70-kbp plasmid isolated from L-3706. In addition, the 190-kbp IncA/C plasmids were all self-transmissible, while the 70-kbp IncA/C plasmid was not (data not shown). These data suggest that the 190-kbp IncA/C plasmids are genetically identical, and that the 70-kbp plasmid was derived from the 190-kbp IncA/C plasmid because of deletion of genetic regions including the *bla*_{CMY-2} and conjugative transfer-mediating genes.

Among the virulence-resistance plasmids, the 165-kbp plasmids isolated from L-3695 and L-3706 showed identical restriction patterns after *Sal*I digestion (Fig. 2B). Addition of a fragment of approximately 12 kbp was observed in the 180-kbp plasmid isolated from L-3708 compared with the 165-kbp plasmid. The *Hind*III restriction patterns of three virulence-resistance plasmids were indistinguishable (Fig. 2A). These data suggest that the 165-kbp plasmids are genetically identical and that the 180-kbp plasmid was derived from the 165-kbp plasmid by insertion of genetic

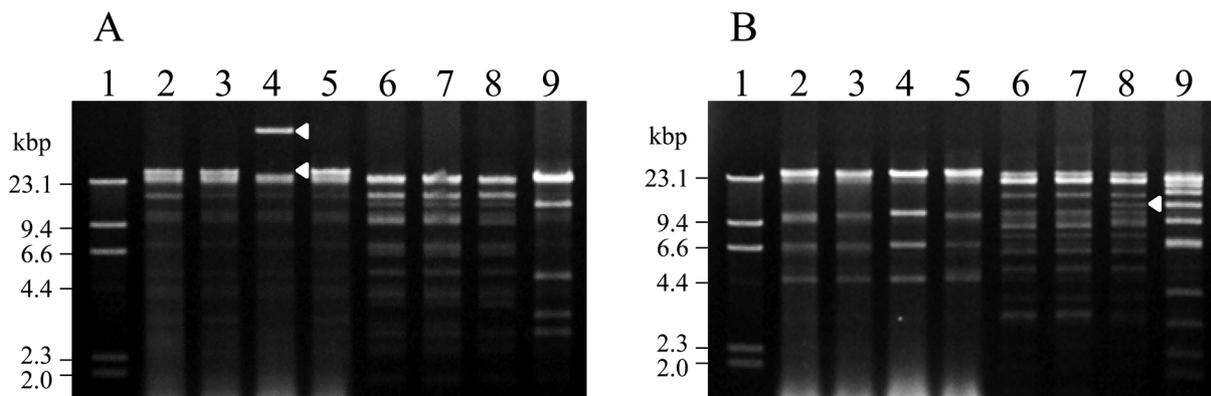


Fig. 2. Restriction analysis of plasmid DNA of the *S. Typhimurium* isolates digested with *Hind*III (A) and *Sal*I (B). Lane 1, λ DNA *Hind*III digest marker; lane 2, 190-kbp plasmid of L-3694; lane 3, 190-kbp plasmid of L-3695; lane 4, 70-kbp plasmid of L-3706; lane 5, 190-kbp plasmid of L-3708; lane 6, 165-kbp plasmid of L-3695; lane 7, 165-kbp plasmid of L-3706; lane 8, 180-kbp plasmid of L-3708; lane 9, 120-kbp plasmid of L-3708. White arrowheads shown to the right of a lane indicate the addition or loss of a band.

material. The insertion event may disrupt a gene conferring sulfamethizole resistance.

These data suggest that isolates L-3694, L-3695, L-3706 and L-3708 originate from a common ancestor that invaded the beef cattle farm. Insertions or deletions of genes in plasmids or conjugative transfer of the *IncII* plasmid may generate diversity of antimicrobial resistance patterns. Although isolates L-3694, L-3695 and L-3706 were detected when there was selection pressure from cefazolin administration, L-3708 was detected after changing the antimicrobial from cefazolin to fosfomycin. The isolate L-3708 seems to have acquired the *IncII* plasmid, which confers trimethoprim resistance, in the absence of selection pressure from trimethoprim. These data suggest that antimicrobial selection pressure is not necessary for the transfer of resistance plasmids, as reported elsewhere [5, 12].

Taken together, these results show the diversity of antimicrobial resistance patterns based on genetic changes in four ESC-resistant ST-VII isolates obtained from a beef cattle farm. These isolates seem to have originated from a common ancestor that invaded the farm prior to the disease outbreak. This phenomenon should be considered when conducting epidemiological surveys of salmonellosis caused by ESC-resistant ST-VII isolates. Antimicrobial resistance patterns may not necessarily reflect the relationships of the isolates.

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