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REVIEW

Molecular Epidemiological Analysis of *Salmonella enterica* subsp. *enterica* Serovar Typhimurium Isolated from Cattle in Hokkaido, Japan

Yukino TAMAMURA[1]* and Ikuo UCHIDA[2]

[1] Bacterial and Parasitic Diseases Research Division, National Institute of Animal Health, NARO (Tsukuba, Ibaraki 305-0856, Japan)
[2] Veterinary Bacteriology, Department of Pathology, School of Veterinary Medicine, Rakuno Gakuen University (Ebetsu, Hokkaido 069-8501, Japan)

Abstract

Salmonella enterica subsp. *enterica* serovar Typhimurium is one of the most common causative agents of bovine salmonellosis. This review summarizes the molecular epidemiology of *S.* Typhimurium isolated from cattle in Hokkaido, Japan. The molecular epidemiology of 545 isolates collected between 1977 and 2009 from cattle in Hokkaido was investigated using pulsed-field gel electrophoresis (PFGE). Nine main clusters (I-IX) were identified by PFGE. Clusters I and VII were dominant in 1992-2002 and 2002-2009, respectively. Next, we analyzed the features of cluster I and VII isolates. Cluster I isolates produced the ADP-ribosyltransferase toxin, ArtA/ArtB which were fatal in mice. Cluster VII isolates were multidrug resistant and shared a virulence-resistance plasmid. Thus, epidemiological surveillance enables early recognition of epidemic *Salmonella* clones.

Discipline: Animal health
Additional key words: bovine salmonellosis, molecular epidemiology

Introduction

Salmonella enterica serovar Typhimurium is a common cause of salmonellosis in humans and animals. *S.* Typhimurium often involves rapid dissemination of the predominant epidemic strains over a large geographic area. One exemplary definitive phage type 104 (DT104), which has multidrug resistance, has emerged and spread to many countries (Glynn et al. 1998, Sameshima et al. 2000, Threlfall et al. 1994, Villar et al. 1999). The severity of clinical illness in *S.* Typhimurium DT104 outbreaks suggests that this strain possesses enhanced virulence. To avoid the dissemination of salmonellosis, a detailed characterization of *S.* Typhimurium is required for studying the epidemiology of outbreaks and determining the source of contamination. Pulsed-field gel electrophoresis (PFGE) is the gold standard for discriminating among strains at the DNA level (Swaminathan et al. 2001). Furthermore, multiple-locus variable number tandem repeat analysis (MLVA), based on amplification of a variable number of tandem repeat areas, is thought to have greater discriminatory power than PFGE and has been proposed as an alternative for genotyping highly clonal groups of bacteria (Lindstedt et al. 2003, Lindstedt et al. 2004). Molecular subtyping of *S.* Typhimurium isolates by standard procedures would assist in identifying *Salmonella* epidemics and enable the monitoring of changes in epidemic patterns. Therefore, a surveillance system based on molecular subtyping techniques, such as PFGE and MLVA, is needed for early recognition of such epidemics.

This review summarizes the molecular epidemiology of *S.* Typhimurium isolated from cattle in Japan's northern island of Hokkaido where dairy farming is a primary agro-industry, as determined by PFGE and MLVA.

1. PFGE, MLVA, and antimicrobial resistance patterns of 545 *S.* Typhimurium isolates

The molecular characterization of 545 *S.*

*Corresponding author: e-mail ytamamura@affrc.go.jp
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Typhimurium isolates obtained from cattle from 1977 to 2009 in Hokkaido, Japan was carried out by PFGE analysis. We observed 116 PFGE patterns among the 545 isolates after digestion of the DNA with XbaI (Tamamura et al. 2011). A dendrogram was generated using UPGMA algorithms with Dice coefficients, and nine distinct clusters (I-IX) with greater than 74% similarity were designated. Cluster I consisted of 248 isolates. From 1993 to 2003, cluster I was the dominant cluster in terms of the number of isolates, although the numbers declined after 2003 (Table 1). In contrast, cluster VII consisted of 165 isolates and became more prevalent since 2002 (Table 1). Representative isolates of the 116 PFGE patterns were subjected to MLVA based on five variable tandem repeat (VNTR) loci and then distinguished into 68 MLVA profiles. The MLVA profiles were used for categorical clustering in BioNumerics software, and a minimum-spanning tree was constructed (Fig. 1). MLVA clustering was achieved when neighbors differed in no more than one of the five VNTR loci. As shown in Figure 1, the MLVA profiles were classified into four main clusters (A, B, C, and D). Eighteen of the 21 isolates belonging to PFGE cluster I were classified into MLVA cluster A, and 19 of the 21 isolates belonging to PFGE cluster VII were assigned to MLVA cluster D. Of the 248 isolates belonging to PFGE cluster I, 243 (98%) possessed the internal segment of the 16S-23S spacer region of bacterial rRNA genes (162-bp amplicon), which is specific to DT104 (Pritchett et al. 2000), suggesting that almost all of the cluster I isolates were related to DT104. In contrast, cluster VII isolates did not possess the 162-bp amplicon and were identified as phage type untypable. Taken together, these analyses showed that PFGE cluster I including DT104 was dominant in 1992-2002, whereas PFGE cluster VII increased since 2002. The increase of bovine origin DT104 in 1990s has been reported in a previous study (Sameshima et al. 2000). We also observed an apparent increase in the incidence of bovine salmonellosis in adult cattle in Hokkaido since 1992, although the relationship between DT104 and salmonellosis in adult cattle was unknown.

We surveyed the antimicrobial resistance patterns of 545 *S*. Typhimurium isolates. A total of 423 isolates (78%) exhibited resistance to at least five of the tested antimicrobials. Most of the PFGE cluster I isolates (91%) exhibited resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline, which is the typically observed DT104 pentaresistance pattern.

Table 1. PFGE clusters identified from 545 *S*. Typhimurium isolates from cattle between 1977 and 2009

	No. of isolates by PFGE cluster									
Year of isolation	I	II	III	IV	V	VI	VII	VIII	IX	Total no. of isolates
1977-1991	2	8	0	30	3	20	0	12	1	76
1992	4	1		5	1	6				17
1993	27					3				30
1994	38			1	1					40
1995	22				2					24
1996	12				1					13
1997	21									21
1998	19	1	2					1		23
1999	28									28
2000	9	2				1	1			13
2001	13	1			1	1	1			17
2002	13	3			1		10			27
2003	18	5					12			35
2004	6	3					19			28
2005	3	1			2		42			48
2006		5					32			37
2007	10	3					44			57
2008	1	2					3			6
2009	2	2					1			5
Total	248	37	2	36	12	31	165	13	1	545

Whereas an antibiotic resistance type showing resistance to ampicillin, streptomycin, sulfonamides, tetracycline, and kanamycin was found in 125 out of 165 cluster VII isolates. A total of 162 of the 165 isolates from cluster VII showed ampicillin resistance, and 26 isolates showed cefazolin resistance. Three isolates with PFGE profiles VII-17 and VII-21 were sensitive to all of the antibiotics and did not belong to MLVA cluster D. These results indicated that cluster VII, a novel multidrug resistant clone emerged since 2002.

2. Characterization of pertussis-like toxin from isolates belonging to PFGE cluster I

PFGE cluster I consisting of DT104 or relatives increased since 1992. DT104 was reported to produce pertussis-like toxin, ArtA/ArtB (ArtAB), which catalyzes ADP-ribosyltransfer to bovine brain G-proteins (Saitoh et al. 2005, Uchida et al. 2009). We surveyed the prevalence of the *artAB* gene among 545 *S*. Typhimurium isolates, and 98% of cluster I isolates were positive for *artAB*, whereas only five of the 297 isolates belonging to other clusters (1.6%) were positive for *artAB*. We induced the production of ArtAB by mitomycin C, and purified ArtAB by sequential Affi-Gel Blue, hydroxyapatite, and hydrophobic interaction chromatography. In sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), we observed two major bands at 27 and 13.8 kDa, consistent with the expected molecular weights of ArtA and ArtB, respectively (Fig. 2 A) (Tamamura et al. 2017). The purified proteins catalyzed ADP-ribosylation of the 41-kDa α subunit of the pertussis toxin (Ptx)-sensitive heterotrimeric G protein from bovine brain. Purified ArtAB dissociated into ArtA and ArtB during Mono Q chromatography, and the subunits were separated (Fig. 2 B). The molar ratio of ArtA to ArtB was approximately 1:5, suggesting that ArtAB is a member of the AB_5 toxin family. ArtAB comprises a single A subunit and pentamer of B subunits similar to cholera toxin, *E. coli* heat-labile enterotoxins, Shiga toxin, and subtilase cytotoxin (Fig. 2 C) (Beddoe et al. 2010). To examine the *in vivo* toxicity of ArtAB, mice (n = 5-8) were administered dilutions of the purified ArtAB by intraperitoneal (IP) injection. The 50% lethal dose of ArtAB in mice was 0.21 μg/mouse. Survival time was inversely related to injection dose (Fig. 2 D). As we still lack the evidence for ArtAB expression *in vivo*, further studies on the regulation and expression of the toxin are required to clarify the importance of ArtAB in the virulence of cluster I isolates.

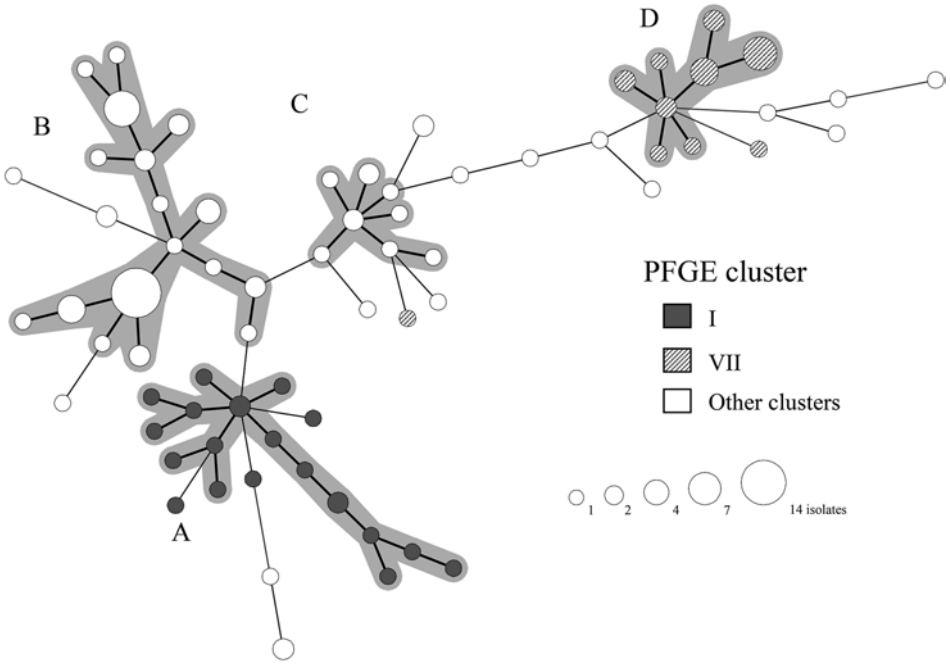

Fig. 1. Minimum-spanning tree based on MLVA allelic profiles showing phylogenetic relationships between representative isolates of the 116 PFGE patterns. Adapted from Tamamura et al. 2011.

Each node represents a unique MLVA profile. The nodal size is proportional to the number of isolates per MLVA. The patterns in the nodes indicate PFGE clusters (I, VII, and others). The different lines indicate distances between the circles, where a thick line represents a closer distance than a thin line. The MLVA clusters differing by zero or one variable-number tandem-repeat loci are regarded as a group and highlighted (MLVA clusters A–D).

3. Characterization of multidrug-resistance plasmids from isolates belonging to PFGE cluster VII

S. Typhimurium carries a serovar-specific virulence plasmid containing the spv locus, which is thought to enhance mouse virulence (Guiney & Fierer 2011). Recently, serovar-specific virulence plasmids of various

Table 2. Antimicrobial resistant patterns and PFGE clusters of 545 *S.* Typhimurium isolates

	PFGE cluster									
Antimicrobial resistance pattern[a]	I	II	III	IV	V	VI	VII	VIII	IX	Total
AMP, CHL, STR, SUL, TET, KAN, CFZ, SXT							23			23
AMP, CHL, STR, SUL, TET, KAN, CFZ, CTX							1			1
AMP, CHL, STR, SUL, TET, KAN, CFZ							1		1	2
AMP, CHL, STR SUL, TET, KAN, NAL						3				3
AMP, STR, SUL, TET, KAN, CFZ, SXT							1			1
AMP, CHL, STR, SUL, TET, KAN, SXT	1									1
AMP, STR, SUL, TET, KAN, CFZ								1		1
AMP, CHL, STR, SUL, TET, KAN	1	1		5	1	9	2			19
AMP, CHL, STR, SUL, TET, NAL	5			1		1				7
AMP, STR, SUL, TET, KAN, GEN							1			1
AMP, STR, SUL, TET, KAN, SXT							4			4
AMP, CHL, SUL, TET, NAL				1						1
AMP, STR, TET, CFZ, NAL								1		1
AMP, CHL, STR, SUL, KAN				1						1
AMP, CHL, STR, SUL, TET	218			6		2				226
AMP, CHL, SUL, TET, KAN				3		1				4
AMP, STR, SUL, TET, KAN						1	125			126
AMP, CHL, SUL, TET, SXT				1						1
AMP, CHL, SUL, TET	3			3						6
AMP, STR, SUL, TET	2	1	1				1			5
AMP, STR, TET, KAN		1								1
AMP, SUL, TET, KAN							1			1
CHL, SUL, TET, KAN				1						1
STR, SUL, TET, NAL				1				2		3
SUL, TET, KAN, NAL				1						1
AMP, CHL, SUL					1					1
AMP, STR, TET		1								1
AMP, TET, KAN							1			1
STR, SUL, TET		5	1			1		1		8
STR, TET, KAN								1		1
CHL, SUL, TET				1						1
AMP, SUL	1	1				1	1			4
AMP, TET, KAN						1				1
CHL, SUL						1				1
STR, SUL	13									13
STR, TET		1						1		2
SUL, KAN				1						1
SUL, NAL				1	2					3
SUL, TET		3		1						4
NAL								1		1
STR		1			1					2
SUL		4		5		5		3		17
TET				1						1
Sensitive	4	18		2	7	5	3	2		41
Total	248	37	2	36	12	31	165	13	1	545

[a]Abbreviations: AMP: ampicillin; CHL: chloramphenicol; STR: streptomycin; SUL: sulfonamides; TET: tetracycline; KAN: kanamycin; SXT: sulfamethoxazole–trimethoprim; CFZ: cefazolin; CTX: cefotaxime; Nal: nalidixic acid; GEN: gentamicin

sizes have been found in *Salmonella*; some of these virulence-resistance plasmids, each encoding different resistance genes, have been detected in *Salmonella* isolates collected in various countries (Chu & Chiu 2006, Chu et al. 2001, Guerra et al. 2002, Llanes et al. 1999). The cluster VII isolates harbored plasmids ranging in size from approximately 78 kb to 130 kb. We determined the complete sequences of plasmids extracted from cluster VII. Plasmids pYT1 and pYT2 (GenBank accession numbers AB576781 and AB605179, respectively) were isolated from the representative isolates of cluster VII, and were 112,670 bp and 132,842 bp, respectively (Tamamura et al. 2013). These plasmids were closely related to pSLT, a 94-kb virulence-associated plasmid of S. Typhimurium LT2 strain (Mcclelland et al. 2001). The pYT1 and pYT2 sequences consist of segments derived from pSLT DNA and single large inserts (32,495 bp in pYT1 and 52,666 bp in pYT2). The inserted DNA in

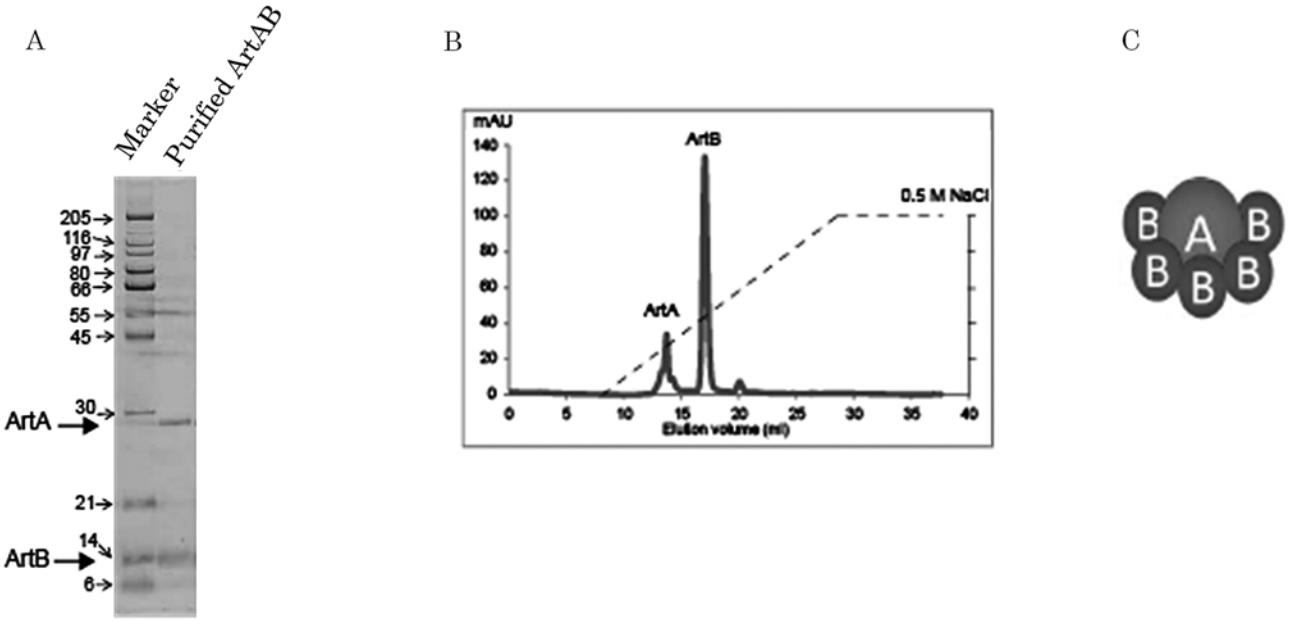

Fig. 2. Purification, structure and in vivo toxicity of ArtAB. Adapted from Tamamura et al. 2017.
(**A**) SDS-PAGE analysis of purified ArtAB from *S.* Typhimurium DT104. The gel was stained using a silver staining kit. (**B**) Separation of ArtA and ArtB by Mono-Q anion exchange chromatography. The line shows the increasing salt gradient (0 to 0.5 M NaCl in 30 mM Tris-HCl, pH 8.8). (**C**) Schematic illustration of ArtAB subunit structure. (D) Percentage of surviving mice following challenge with indicated amounts of ArtAB over time. BALB/c mice (n = 5-8) were challenged with graded doses of ArtAB and observed daily over a two-week period.

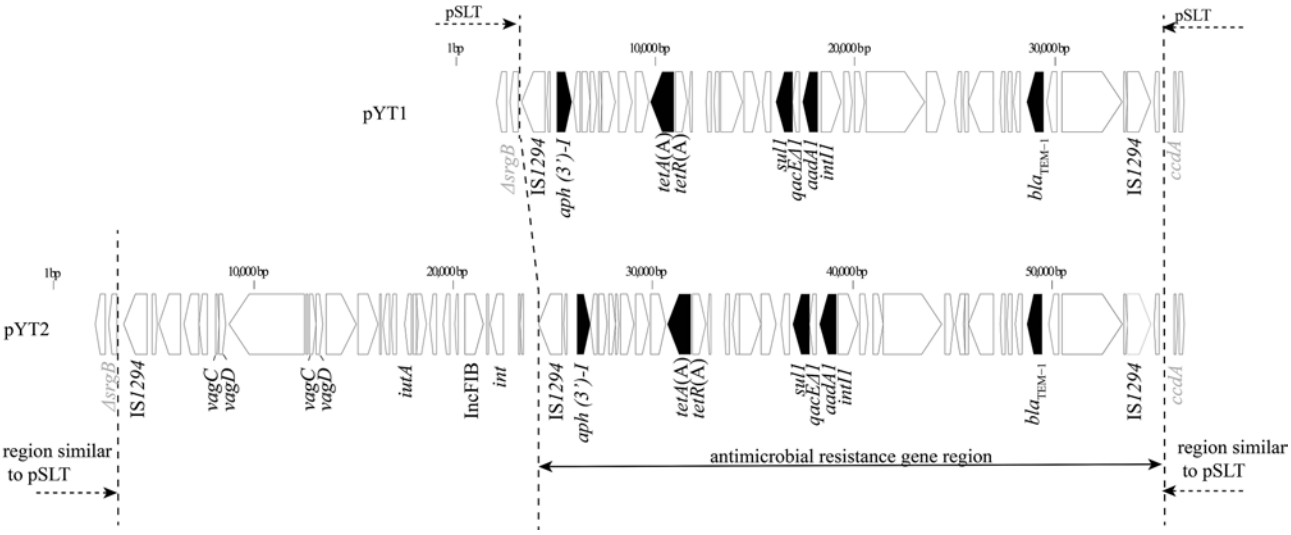

Fig. 3. Schematic representation of the inserted segment in pYT1 and pYT2. Adapted from Tamamura et al. 2013.
Genes encoding antibiotic resistance (black block arrow) and other functions (white block arrow) are shown.

both plasmids consists of a region surrounded by two copies of IS*1294* in opposite orientations and some flanking sequences (Fig. 3). The pYT1 and pYT2 plasmids shared the same antimicrobial resistance gene region, identical to a part of plasmid from S. Dublin pSD_88 (GenBank accession number JF267652), and include the genes *aph(3')-I*, *tetA*, *aadA1*, *qacEΔ1*, *sul1*, and $bla_{\text{TEM-1}}$ (resistance to kanamycin, tetracycline, streptomycin-spectinomycin, ammonium antiseptics, sulfonamides, and ampicillin, respectively; Fig. 3). In addition to the resistance region, pYT2 possesses a region that is nearly identical to the segments of plasmids *S*. Typhimurium pU302L (GenBank accession number NC 006816) and *S*. Dublin pSD88, and includes genes encoding plasmid maintenance (*vagC* and *vagD*), the IncFIB replicon, and the iron acquisition-associated virulence gene (*iutA*; Fig. 3). Sequence analysis suggested that pSD88 and pU302-L may be sources of the regions inserted in pYT1 and pYT2. Therefore, pYT1 and pYT2 may be derived from the virulence plasmid pSLT by acquiring IS*1294* and adjacent sequences of pSD88 and/or pU302-L or those of related plasmids through the integration of IS*1294*, followed by inversion and recombination. Therefore, the virulence plasmid pSLT may act as a gene-exchange platform, facilitating the sampling of genes collected from other bacteria in the environment as suggested by Kingsley et al. (Kingsley et al. 2009). The presence of resistance and virulence determinants on the same plasmid allows coselection of both properties by antimicrobial agents; more virulent and antibiotic-resistant bacteria may subsequently arise.

Conclusion

Our results show that the proportion of PFGE cluster I isolates, including DT104 isolates, increased since 1992 and decreased since 2004. DT104 produces a pertussis-like toxin (ArtAB) having lethal activity for mice. On the other hand, we observed an increase beginning in 2002 in bovine-origin *S*. Typhimurium isolates of PFGE cluster VII, an emerging multidrug resistance clone carrying a virulence-resistance plasmid, suggesting that clonal replacement is occurring. The use of standardized subtyping methods such as PFGE and MLVA allows a comparison of isolates from different areas, and routine and long-term epidemiological surveillance with such methods enables early recognition of epidemic *Salmonella* clones.

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