

Epidemiological Characteristics of *Salmonella enterica* Serovar Typhimurium from Healthy Pigs in Japan

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(Received 26 January 2009/Accepted 20 September 2009/Published online in J-STAGE 13 November 2009)

ABSTRACT. We characterized 53 *Salmonella enterica* ser. Typhimurium strains recovered from healthy pigs during 1998–1999 (n=12) and 2004–2005 (n=41) as to their carriage of DT104 spacer region, class 1 and 2 integrons, virulence genes (*spvC*, *rck*, and *pefA*), and *XbaI*- and *BlnI*-Pulsed-Field Gel Electrophoresis (PFGE) profiles. No DT104 strain was detected in 1998–1999, whereas 65.9% (27/41) of the strains in 2004–2005 were DT104 showing resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, tetracycline, and cephaloridine (R-type ACSSuT+). Class 1 integron-associated genes, *aadA2* (1.0-kbp amplicon) and *pse1* (1.2-kbp amplicon), were found in all the DT104 strains (27/27). One strain showing resistance to streptomycin, sulfonamides, tetracycline, and trimethoprim (R-type SsuTTm) harbored another class 1 integron-associated gene (*dhfrXII-orfF-aadA2*) on 1.9-kbp amplicon. Virulence gene *spvC* was found in 92.5% (49/53) and *rck* and *pefA* were found in 88.7% (47/53) of the strains, whereas *spvC*, *rck*, and *pefA* were found in all the DT104 strains. Ser. Typhimurium strains were categorized into four clusters (X1, X2, X3, and X4a/X4b) by *XbaI*-PFGE, or into nine clusters (B1, B2, B3a/B3b, B4, B5, B6, B7, B8, B9a/B9b) by *BlnI*-PFGE analyses. DT104 strains were restricted into X2, or into B2, B3a/B3b, and B6 clusters, indicating that our multidrug-resistant DT104 strains from healthy pigs might have derived from at least three independent clones, with the most widespread clone being the cluster B6 strains isolated in Kanto, Tokai, Chugoku, and Kyushu regions.

KEY WORDS: epidemiology, *Salmonella*, swine.

J. Vet. Med. Sci. 72(1): 61–66, 2010

Salmonella enterica serovar Typhimurium is one of the most common serovars causing food-borne illness in human and often exhibits resistance to multiple antimicrobial agents. Ser. Typhimurium reportedly carries integron, which is strongly linked to increased antimicrobial resistance (27, 36), and several virulence genes, such as *spvC* (*Salmonella* plasmid virulence), *rck* (resistance to complement killing), and *pefA* (plasmid-encoded fimbria) [6, 16, 17, 30]. Moreover, multidrug-resistant ser. Typhimurium definitive phage type 104 (DT104) has emerged and spread over the world (21). DT104 has been isolated from pigs and pork products in many countries [9, 11, 25, 26, 28, 38].

In Japan, ser. Typhimurium DT104 was first reported from human sources in the late 1980s, and from livestock and environment in the 1990s [22, 34]. The prevalence of this phage type in cattle, pigs and poultry has also been reported [10, 23, 25]. However, the strains in most of these studies were from clinical specimens [10, 23]. Moreover, the genotypic characteristics, such as pulsed-field gel electrophoresis (PFGE) types, and the carriage of integrons and virulence genes, of isolates from healthy pigs are largely unknown [10, 25].

In our previous study, we reported the serotype diversity and antimicrobial susceptibility of *Salmonella enterica* iso-

lates from Japanese healthy pigs [12]. Notably, ser. Typhimurium, the second most prevalent serovar (12/67; 17.9%) among the isolates in 1998–1999, became the most prevalent (41/126; 32.5%) in 2004–2005 with 65.8% (27/41) of them exhibiting resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, tetracycline, and cephaloridine (R-type ACSSuT+) [12].

The aim of the present study was to elucidate the epidemiological characteristics of those ser. Typhimurium strains based on the carriage of DT104 spacer region, integrons, virulence genes, and *XbaI*- and *BlnI*-PFGE profiles.

MATERIALS AND METHODS

Bacterial strains: A total of 53 ser. Typhimurium strains (1 representative isolate from clinically healthy individual pigs), including 12 strains isolated in 1998–1999 and 41 strains in 2004–2005 from 10 pig farms in 5 regions (Tohoku, Kanto, Tokai, Chugoku, and Kyushu), were included in this study. These strains were studied previously for their antimicrobial susceptibility [12].

Determination of ser. Typhimurium DT104: PCR amplification of an internal segment of the 16S-23S spacer region of bacterial rRNA genes was used to identify ser. Typhimurium DT104 as previously described [32]. The PCR products, detected as 243-bp and 162-bp DNA bands, were confirmed by sequencing.

Identification of integrons: The presence of class 1 and 2

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integrons was investigated by PCR method as described by DeLappe *et al.* [8]. The sequences of PCR products were used for BLAST search against GenBank database.

Detection of virulence genes: Detection of virulence genes, *spvC*, *rck*, and *pefA*, was performed by PCR method as described previously [19], and their identity was confirmed by sequencing.

Pulsed-field gel electrophoresis (PFGE): PFGE was performed according to the PulseNet standardized protocol [33]. Briefly, plugs were digested for 3 hr with 25U of *Xba*I or *Bln*I (Roche). DNA fragments were separated by agarose gel electrophoresis in a 1% SeaKem Gold agarose gel (Cambrex Bio Science) with CHEF DR-III PFGE system (Bio-Rad). *S. enterica* ser. Braenderup CCUG50923 (H9812) was used as a molecular size marker.

DNA fingerprint analysis: DNA fingerprints were analyzed with BioNumerics software (Version 5.0, Applied Maths, Belgium). After an automatic band search and a band-based analysis using Dice coefficient with 1.5% band position tolerance, cluster analysis was performed by using the unweighted pair-group method with arithmetic averages (UPGMA).

RESULTS

Prevalence of ser. Typhimurium DT104: Among the ser. Typhimurium strains examined, 50.9% (27/53) were positive for DT104 spacer region by PCR method. Specifically, all the twelve strains isolated in 1998–1999 were negative for that DNA fragment, but 65.9% (27/41) of isolates in

2004–2005, which exhibited ACSSuTCe resistance (R-type ACSSuT+), were positive. The farm-level prevalence of DT104-positive strains in 2004–2005 was 66.7% (6/9).

Prevalence of integrons: Class 1 integron-associated genes, streptomycin-spectinomycin resistance gene *aadA2* (1.0-kbp amplicon) and ampicillin resistance gene *pse1* (1.2-kbp amplicon), were found in 50.9% (27/53) of total ser. Typhimurium strains or in all ser. Typhimurium DT104 strains (27/27) (Table 1). One strain of R-type SSuTTm harbored another class 1 integron-associated genes, dihydrofolate reductase, putative protein, and streptomycin/spectinomycin adenyltransferase (*dhfrAXII-orfF-aadA2*), on 1.9-kbp amplicon (Table 1).

Class 2 integron-associated genes (*dfrA1-sat1-aadA1*, *sat1-ereA-aadA1*, or *sat-sat1-aadA1*) [2, 5, 21] were not detected in any of ser. Typhimurium examined.

Carriage of virulence genes: Virulence gene *spvC* was found in 92.5% (49/53) and *rck* and *pefA* were found in 88.7% (47/53) of ser. Typhimurium strains. Alternatively, all ser. Typhimurium DT104 strains were positive for *spvC*, *rck*, and *pefA*.

Cluster analysis: The cluster analyses of *Xba*I- and *Bln*I-PFGE patterns of 52 ser. Typhimurium strains are shown in Figs. 1 and 2. One strain was excluded from the analysis due to its unreadable PFGE pattern.

By *Xba*I analysis, all ser. Typhimurium strains fell into four clusters (X1, X2, X3, and X4) at the 80% cut off value. The X4 cluster was further divided into X4a and X4b at the 85% cut off value.

By *Bln*I analysis, all strains fell into nine clusters (B1–B9)

Table 1. Characteristics of ser. Typhimurium strains from healthy pigs

PFGE		Resistance phenotype ^{a,b}	No. of isolates	Pig farm ^d	DT104	Class 1 integron	Virulence genes		
<i>Xba</i> I	<i>Bln</i> I					Gene (amplicon size in kbp)	<i>spvC</i>	<i>rck</i>	<i>pefA</i>
X3	B1	–	1	A	–	1	1	1	1
X2	B6	ACSSuTCe	10	B,C,D,E	10	<i>aadA2</i> (1.0), <i>pse1</i> (1.2)	10	10	10
	B2	ACSSuTCe	14	F	14	<i>aadA2</i> (1.0), <i>pse1</i> (1.2)	14	14	14
	B3a	ACSSuTCe	1	E	1	<i>aadA2</i> (1.0), <i>pse1</i> (1.2)	1	1	1
	B3b	ACSSuTCe	2	G	2	<i>aadA2</i> (1.0), <i>pse1</i> (1.2)	2	2	2
X4a	B7	SSuTTm	2	H	–	–	2	2	2
X4b	B5	T, –	12 ^c	I	–	–	12	12	12
X1	B4	SSuT	3	E	–	–	–	–	–
	B8	SSuT	4	J	–	–	4 ^e	2 ^e	2 ^e
	B9a	SSuT	1	J	–	–	1	1	1
	B9b	SSuT	2	J	–	–	2	2	2
ND	ND	SSuTTm	1	E	–	<i>dhfrXII-orfF-aadA2</i> (1.9)	–	–	–

ND, not determined.

a) Refer to reference Futagawa-Saito *et al.*, 2008.

b) A, ampicillin; C, chloramphenicol; S, streptomycin; Su, sulfonamide; T, tetracycline; Ce, cephaloridine; Tm, trimethoprim.

c) Isolated during 1998–1999.

d) Regions at which the farms are localized: Pig farm A, Tohoku; B, Kanto; C, Tokai; D, Chugoku; E, Kyushu; F, Kanto; G, Kyushu; H, Chugoku; I, Tohoku; J, Kanto.

e) Two strains were *spvC*-positive, and two strains were *spvC-rck-pefA*-positive.

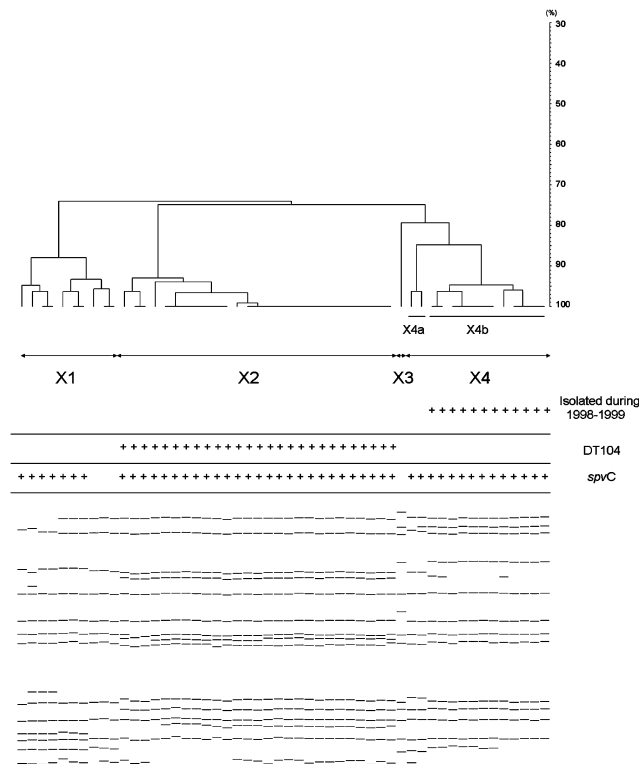
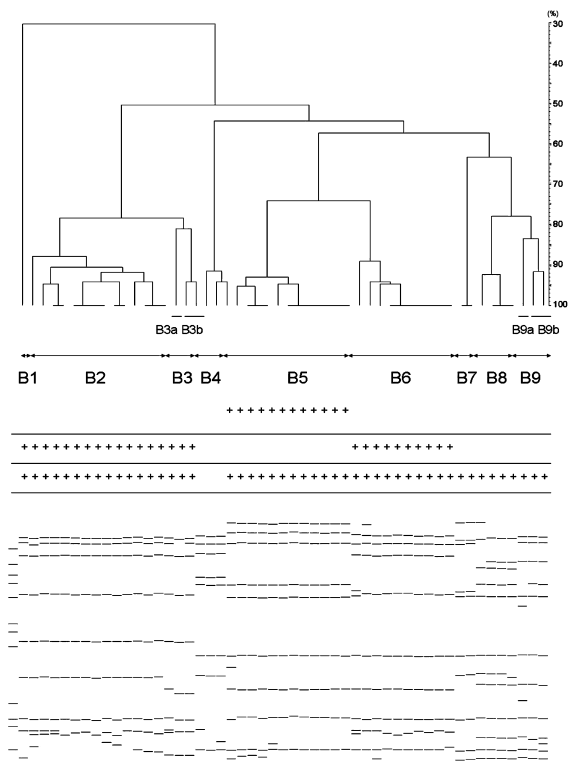
A. *Xba*I-PFGEB. *Bln*I-PFGE

Fig. 1. Dendrogram of *Xba*I-PFGE (A) and *Bln*I-PFGE (B) of 52 ser. Typhimurium strains analyzed with BioNumerics software (Version 5.0, Applied Maths, Belgium).

at the 80% cut off value. And two clusters (B3 and B9) were further divided into two subclusters (B3a and B3b, and B9a and B9b) at the 85% cut off value.

All DT104 strains fell into the unique cluster X2 by *Xba*I, or into clusters and subclusters B2, B3a/B3b, and B6 by *Bln*I.

DISCUSSION

Among the ser. Typhimurium strains examined, 50.9% (27/53) were determined as DT104 because 1) they were positive for DT104 spacer region by PCR method [32], which has been demonstrated to be a highly reliable DT104 detection method [1, 2], 2) they exhibited R-type ACSSuT+, and 3) they were positive for the class 1 integron-associated 1.0- and 1.2-kb amplicons. Class 1 integron-associated gene cassettes, commonly identified on 1.0-kb and 1.2-kb amplicons, have been reported in DT104 strains with R-type ACSSuT from human, animal, and environment in many countries [14, 29, 41]. In addition, our DT104 strains carried the class 1 integron-associated antimicrobial resistance genes, *aadA2* and *pse1* [7].

We observed a high prevalence (65.9%; 27/41) of ser. Typhimurium DT104 in clinically healthy pigs, during 2004–2005 investigation period. Since this observation is in

agreement with a similar report (61.5%; 16/26) from Japan in 2003–2005 [25], DT104 strains seem to be more prevalent on the pig farms in Japan, compared to other countries such as Denmark (with the distribution rate of 3.3%, 1997–2006) [9], Iceland (25.5%, 2001–2005) [38], UK (41.5%, 1997–2004) [4], and Canada (49.4%, 2003) [31]. Notably, we did not observe any DT104 strains among our 1998–1999 isolates.

One strain carries *dhfr*XII-*orfF*-*aadA2* on 1.9-kb amplicon, which has been found in gram-negative bacteria [3, 13] as well as in *Staphylococcus* [35, 40]. In *Staphylococcus*, this amplicon was reportedly implicated in intergeneric horizontal transfer in hospital settings [35]. But in this study, this amplicon might not be associated with horizontal transfer as it was not detected in other strains from the same farm (i.e., E farm in Table 1).

As we observed in this study, most ser. Typhimurium strains (88.7%; 47/53) carry the three virulence genes *spvC*, *rck*, and *pefA*, which are known to be located on a virulence plasmid [6, 16, 17, 30] and to be serovar-specific [6, 18]. This prevalence rate is not much different from 73.1% (19/26) of Japanese healthy pigs in a previous report [25]. The *spvC* has been reported to be stably maintained in all DT104 strains [24]. Similarly, *spvC* and two other virulence genes were found in all DT104 strains. But, these virulence genes

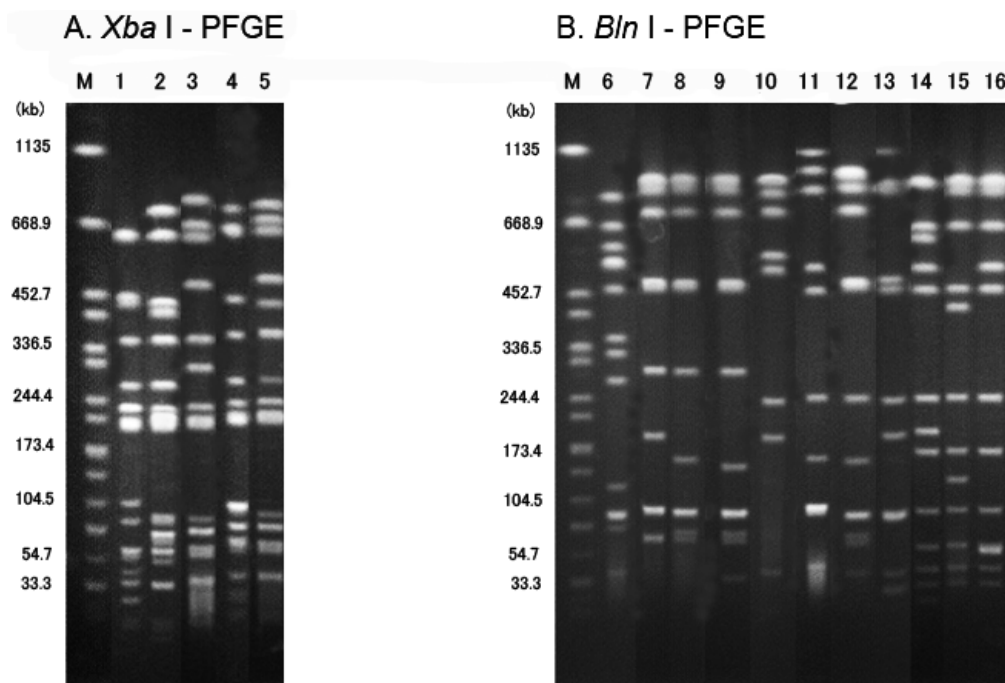


Fig. 2. Patterns of *Xba*I-PFGE (A) and *Bln*I-PFGE (B) of representative ser. Typhimurium strains with their cluster assignment from Fig. 1. Marker of *S. enterica* ser. Braenderup CCUG50923 (H9812) strain was digested with *Xba*I. Lanes M, Marker; 1, X1; 2, X2; 3, X3; 4, X4a; 5, X4b; 6, B1; 7, B2; 8, B3a; 9, B3b; 10, B4; 11, B5; 12, B6; 13, B7; 14, B8; 15, B9a; 16, B9b.

were not detected in the 1.9-kb- amplicon (*dhfrXII-orfF-aadA2*) positive-strain, which is in agreement with the finding of the absence of the *spvC* and *rck*, and this 1.9-kb-amplicon in a previous report [39].

We found a strong link between the resistance phenotype and *Xba*I-PFGE profiles. All DT104 strains with R-type ACSSuT+ were restricted to cluster X2 and share similar *Xba*I-PFGE profile of the previously identified DT104 strains [15, 37](data not shown). Other ser. Typhimurium strains with R-type SSuT were also restricted to cluster X1 (Fig. 1A and Table 1). The strains belonging to X4b cluster, which were isolated in 1998–1999 from the same pig farm, were resistant only to tetracycline.

*Bln*I-PFGE was more powerful than *Xba*I-PFGE in distinguishing our strains. There was a link between the pig farms and *Bln*I-PFGE profiles. Ser. Typhimurium strains from 8 pig farms (A, B, C, D, F, G, H, and I) were restricted to single individual cluster. However, strains from pigs kept in the same building in J farm fell into three clusters B8, B9a, and B9b. But cluster B9a/B9b strains have more than 80% similarity and have the same class 1 integron and virulence gene profile, indicating that they belong to a single clone (Fig. 1B and Table 1). On the other hand, E farm strains belonging to three clusters B3a, B4, and B6 were isolated from three buildings (Fig. 1B and Table 1). These results indicate the presence of either monoclonal or plural clones of ser. Typhimurium in Japanese pig farms (as observed in the J and E farms, respectively).

All DT104 strains were clustered into the unique cluster X2 by *Xba*I, or into clusters and subclusters B2, B3a/B3b, and B6 (Fig. 1A, B). Cluster B2 strains were found only in one farm of Kanto region, and subcluster B3a and B3b strains in farms E and G, respectively, of Kyushu region. Cluster B6 strains were isolated from the farms B, C, D, and E, which are located at Kanto, Tokai, Chugoku, and Kyushu regions, respectively. Besides, they share similar *Bln*I-PFGE patterns with the DT104 strains isolated from patients from a large-scale outbreak in Japan in Sep. 2003 [37] (data not shown). It is plausible that the cluster B6 strains have spread into the environment and animals in Japan. But the origin of outbreak was never confirmed and therefore transmission route could not be established.

Taken together, this study shows that multidrug-resistant ser. Typhimurium DT104 strains spread among healthy pigs in Japan might be originated from at least three independent clones, with the cluster B6 strains being the most widely spread. And, though number of human outbreaks due to DT104 remains low in Japan, more detailed studies on the epidemiology of this phage type for identifying the sources of contamination in pig farms and determining efficient methods to control and eliminate those strains, are of great importance.

ACKNOWLEDGMENTS. This research was partly supported by The Promotion and Mutual Aid Corporation for Private Schools of Japan and a Grant-in-Aid for Matching

Fund Subsidy for Private Universities.

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