Antimicrobial Resistance and Genetic Characteristics of *Salmonella Typhimurium* Isolated from Horses in Hokkaido, Japan

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**ABSTRACT.** In this study, we examined the antimicrobial susceptibility of 16 *Salmonella Typhimurium* isolates obtained from horses, and applied several genetic methods, namely polymerase chain reaction (PCR) for detecting class 1 integrons, multiplex PCR for detecting multidrug resistant *S. Typhimurium* definitive phage type 104 (MR–DT104), and fluorescent amplified-fragment length polymorphism (FAFLP). Seven isolates with an ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline (ACSSuT) type resistance pattern, harbored two class 1 integrons with sizes of 1.2 and 1.0 kb, and were identified as DT104 by bacteriophage typing. These isolates also showed a typical MR–DT104 amplification pattern, which was positive for *floR*, *sprC*, *invA*, and *int*, in multiplex PCR. In the FAFLP analysis, the equine DT104 isolates and the previously reported ACSSuT-type resistant bovine isolates, which were also isolated in Hokkaido were included in the same genetic cluster. Our results retrospectively indicate that MR–DT104 infection has existed in horses in Japan at least since 1996, and it was suggested that there is a highly epidemiological relationship between the equine MR–DT104 isolates and certain multidrug resistant bovine isolates in the same area.

**KEY WORDS:** DT104, equine, multidrug resistance, *Salmonella Typhimurium*.

Equine salmonellosis is commonly observed at breeding farms and veterinary hospitals in many countries [4, 10, 24, 25]. More than 2,500 serotypes of salmonellae have already been recognized, and the most common serotypes isolated from horses include Typhimurium, Agona, Anatum, Newport, and Krefeld [21]. In Japan, most cases of equine salmonellosis have been caused by *S. Abortusequi*. *S. Typhimurium* infections have occasionally been observed as epidemics, outbreaks, or sporadic cases [2]. Recently, there have been reports of multidrug resistant *S. Typhimurium* infections in horses [2, 28]. Multidrug resistant *S. Typhimurium* definitive phage type DT104 (MR–DT104) is recognized as an important cause of enteric infection in humans and domestic animals throughout the world [12, 13, 19]. Many MR–DT104 isolates are resistant to ampicillin (A), chloramphenicol (C), streptomycin (S), sulfonamide (Su), tetracycline (T), and this is denoted as ACSSuT-type resistance [12, 14, 23]. MR–DT104 isolates have been obtained from horses in Canada and The Netherlands [26, 27]. Although this phage type has frequently been isolated from livestock in Japan [9, 20], there have been no reports describing its isolation from horses. Anzai et al. reported [2] several *S. Typhimurium* isolates from foals in Japan that had a similar antimicrobial resistance profile, and that were resistant at least to ampicillin, chloramphenicol, streptomycin, and tetracycline. It has been reported that ACSSuT-type DT104 generally has distinctive genetic characteristics including resistance genes and an integron structure [15, 16]. In our study, we characterized *S. Typhimurium* isolates obtained from horses in Japan by phage typing, antimicrobial susceptibility testing, multiplex polymerase chain reaction (PCR) testing developed for detecting ACSSuT-type MR–DT104 [15], and PCR testing to detect class 1 integrons [5], in order to determine the presence and characteristics of MR–DT104 and other multidrug resistant phage types in equine isolates. We also examined the genetic relatedness between equine isolates and bovine isolates that were obtained in Hokkaido, which is the main dairy farming and horse breeding area in Japan, by fluorescent amplified-fragment length polymorphism (FAFLP) fingerprinting.

Sixteen *S. Typhimurium* isolates were used in this study. The isolates were obtained from clinical specimens taken from horses between 1981 and 2004. Fifteen isolates were obtained in Hokkaido. One isolate was obtained in Tokyo. The biochemical properties of these isolates were examined using an API 20E system (bioMerieux, Marcy l’Etoile, France) and serotyped using antisera against *Salmonella* spp. (Denka Seiken, Tokyo, Japan). Bacteriophage typing was performed at the National Institute of Infectious Diseases according to the method prescribed by the Health Protection Agency (HPA), London, United Kingdom [1]. In this study, the susceptibilities of the isolates to 10 antimicrobial agents (ampicillin, chloramphenicol, streptomycin, gentamicin, sulfamethoxazole, sulfamethoxazole/trimethoprim, tetracycline, minocycline, ciprofloxacin, and enrofloxacin) were examined using Etest\(^{®}\) (AB BIODISK, Solna,
Sweden). Etest® was performed according to the manufacturer’s instructions. The breakpoint for each antimicrobial agent was set according to the CLSI recommendation [6], except as regards streptomycin. Sixteen μg/ml was used as the resistance breakpoint for streptomycin, in accordance with a previous report [8]. Escherichia coli ATCC 25922 and Staphylococcus aureus ATCC 29213 were used as quality control strains with this method.

We used the DNA of the 16 equine isolates and the DNA of 15 S. Typhimurium isolates, which was obtained from cattle as described previously [22]. The DNA of the equine isolates was extracted using an InstaGene matrix (Nippon Bio-Rad Laboratories, Tokyo, Japan) according to the manufacturer’s instructions. DNA from the bovine isolates was provided by the National Institute of Animal Health. We used 2 PCR methods for the isolates in this study. The first was a multiplex PCR technique developed for detecting ACSSuT-type MR-DT104 [15]. PCR products of 584, 392, 321, and 265 bp amplified by the multiplex PCR and the class 1 integron PCR were electrophoresed using 2% and 1.2% of agarose gel at 100 V for 30 min, respectively, and they were visualized with ethidium bromide.

FAFLP fingerprinting was used to examine the genetic relatedness between the bovine and equine isolates. The DNA from the equine isolates for FAFLP was prepared using Easy-DNA kit (Invitrogen Corp., Carlsbad, CA, U.S.A.), and the DNA from the bovine isolates was provided by the National Institute of Animal Health. FAFLP was performed with the AFLP™ Microbial Fingerprinting Kit (Applied Biosystems) according to the manufacturer’s instructions. Preselective PCR was performed with preselective primers, namely EcoRI primer (5’GACTGGTAC-CAATT-3’) and Msel primer (5’-GATGAGTCTGAGTAA-3’). Selective PCR was performed with selective primers, namely FAM-labeled EcoRI-0 primer (same sequence as EcoRI primer) and MselA primer (Msel primer plus A). These PCRs were performed with a GeneAmp 9700 apparatus, and electrophoresis of the PCR products was performed with an ABI Prism 3130 genetic analyzer (Applied Biosystems). GeneMapper software® (Applied Biosystems) was used to size and quantify individual PCR fragments automatically using the internal size standards (GeneScan™-500 ROXTM Size standard; Applied Biosystems) and also to produce discrete (0, 1) data. The RESTDIST and NEIGHBOR programs in the PHYLIP package were used to analyze the discrete data. The dendrogram was drawn by NJplot [18] using the analyzed data.

The definitive phage type and antimicrobial susceptibility of the 16 isolates used in this study for 10 antimicrobial agents are shown in Table 1. Seven isolates (Sal-7, Sal-8, Sal-229, Sal-231, Sal-234, Sal-238, and Sal-240) from two outbreaks in 1996 and 2004 were identified as DT104 by the bacteriophage typing. In the antimicrobial susceptibility test, 4 isolates (ST-11, ST-12, ST-13, and ST-15) were

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Phage type</th>
<th>MIC (μg/ml)</th>
</tr>
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<tbody>
<tr>
<td>ST-1 RDNC(1)</td>
<td>&gt;256</td>
<td>4</td>
</tr>
<tr>
<td>ST-5 RDNC(1)</td>
<td>&gt;256</td>
<td>4</td>
</tr>
<tr>
<td>ST-9 RDNC(1)</td>
<td>&gt;256</td>
<td>4</td>
</tr>
<tr>
<td>ST-11 UT(2)</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>ST-12 UT(2)</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>ST-13 UT(2)</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>ST-14 U208</td>
<td>1.0</td>
<td>4</td>
</tr>
<tr>
<td>ST-15 UT(3)</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Sal-7 104</td>
<td>&gt;256</td>
<td>128</td>
</tr>
<tr>
<td>Sal-8 104</td>
<td>&gt;256</td>
<td>128</td>
</tr>
<tr>
<td>Sal-228 193</td>
<td>1.0</td>
<td>4</td>
</tr>
<tr>
<td>Sal-229 104</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Sal-231 104</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Sal-234 104</td>
<td>&gt;256</td>
<td>&gt;256</td>
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<tr>
<td>Sal-238 104</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Sal-240 104</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
</tbody>
</table>

Abbreviations: AMP, ampicillin; CHL, chloramphenicol; STR, streptomycin; GEN, gentamicin; SX, sulfamethoxazole; SXT, sulfamethoxazole/trimethoprim; TET, tetracycline; MIN, minocycline; CIP, ciprofloxacin; ENR, enrofloxacin.

a) The value in parentheses is a breakpoint for resistance.
b) RDNC, reacted but did not conform to any known Anderson's phage types [1].
c) UT, Untypable.

Table 1. Antimicrobial susceptibility of 16 S. Typhimurium isolates obtained from horses in Japan for 10 antimicrobial agents
resistant to 7 antimicrobial agents. Ten isolates were resistant to 5 antimicrobial agents, and these isolates were of 2 types, the minocycline-resistant type (ST-1, ST-5, and ST-9) and the chloramphenicol-resistant type (Sal-7, Sal-8, Sal-229, Sal-231, Sal-234, Sal-238, and Sal-240). Minocycline-resistant isolates were epizootic strains in 1981, and chloramphenicol-resistant isolates were outbreak strains in 1996 and 2004. One isolate (Sal-228) was only resistant to streptomycin. The remaining isolate (ST-14) was susceptible to all the antimicrobial agents used in this study. All of the isolates were susceptible to gentamicin, ciprofloxacin, and enrofloxacin.

The multiplex PCR results for 16 equine isolates and 15 bovine isolates are shown in Fig. 1A. Four PCR products were amplified from all of the equine isolates (Sal-7, Sal-8, Sal-229, Sal-231, Sal-234, Sal-238, and Sal-240) identified as DT104 by the bacteriophage typing, as were three bovine isolates (KT11, NET-8, and NET-57). The PCR product of floT was not amplified from the non-DT104 equine isolates and the remaining bovine isolates. In contrast, the PCR product of invA was amplified from all the isolates. The results of the PCR for detecting class 1 integrons are shown in Fig. 1B. Two PCR products, 1.2 and 1.0 kb in size, were amplified from the equine and bovine isolates, which were identified as MR-DT104 by the multiplex PCR. A 1.6 kbp-PCR product was amplified from 4 equine isolates (ST-11, ST-12, ST-13, and ST-15), all of which were resistant to 7 antimicrobial agents. A 2.0 kb-PCR product was amplified from 5 bovine isolates (NET21, NET30, N48, N54, and N57). No product was amplified from the remaining isolates. A complete correspondence was observed between the class 1 integron PCR results and the multiplex PCR results for int.

The result of a FAFLP analysis of 16 equine isolates and 15 bovine isolates is shown in Fig. 2. One hundred and forty seven discrete letters generated by the amplified fragments of FAFLP were used for genetic analysis. Six clusters were observed in the dendrogram. A close genetic similarity was observed between the DT104 equine isolates and three of the bovine isolates (KT11, NET-8, and NET-57). These isolates had same profile of the multiplex PCR and the class 1 integron.
integron PCR. Although the remaining equine isolates were included in cluster B with the bovine isolate N49, 2 subclusters were generated by the 3 equine isolates (ST-1, ST-5, and ST-9) obtained in 1981 and the 4 equine isolates (ST-11, ST-12, ST-13, and ST-15) obtained from 1990 to 1993, respectively. ST-14 was independent of these subclusters, and was the most similar to N49.

It is reported that, in Japan, DT104 has existed in livestock since 1990 and in humans since 1986 [13, 14, 20]. In this study, 7 isolates obtained from 2 outbreaks at horse farms in 1996 and 2004 were identified as ACSSuT-type DT104 by antimicrobial susceptibility testing, bacteriophage typing, the multiplex PCR for ACSSuT-type DT104, and the PCR for class 1 integrons. To the best of our knowledge, the 1996 outbreak appears to be the first MR-DT104 infection in horses in Japan. These DT104 isolates displayed an ACSSuT-type antimicrobial resistance pattern, and harbored integrons with sizes of 1.2 and 1.0 kb. These results indicate that the isolates possess typical MR-DT104 characteristics as described previously [7, 19]. Although all four PCR products of the multiplex PCR were observed in the equine DT104 isolates, at least 2 PCR fragments were not amplified in all the non-DT104 equine isolates, and a lack of the floR gene was common in these non-DT104 isolates. These results support a previous study showing that the multiplex PCR is useful for the identification of DT104 in diagnostic laboratories without a method for bacteriophage typing. The phage types of the isolates, which were resistant to 7 antimicrobial agents, were designated as UT. A PCR product 1.6 kb in size was detected in these isolates. It is likely that there are 2 genes, defI and aadA, which contribute to the trimethoprim and aminoglycoside resistance, respectively, in the 1.6-kb PCR product according to the resistance pattern of the isolates and the data provided in a previous report [17]. A PCR product 2.0 kb in size was detected in the 5 bovine isolates. There were 3 kinds of antimicrobial resistant patterns in the isolates, namely the ACT-type (NET21 and NET30), the ACSSuT-type (N57), and the ACSSuT-type (N48 and N81) [22]. Tamada et al. reported [11] that a 2.0 kb integron was found in ACT-type or ACT plus kanamycin resistant-type S. Typhimurium isolates. Further analyses will be needed to examine the integron structures of the isolates. No integron was detected from the equine isolates obtained in 1981, although these isolates were resistant to 5 antimicrobial agents. Further studies are required for determining the mechanisms of the multidrug resistance in these isolates.

Although it is controversial, antimicrobial therapy for salmonellosis in horses may be needed for severe infections including septicaemia, bacterial translocation and the presence of widespread epithelial damage [21]. The use of trimethoprim-sulfonamide combinations and/or chloramphenicol is generally advocated for the treatment of salmonellosis in horses [21]. For the treatment of strains that are resistant to these agents, the application of third-generation cephalosporins or fluoroquinolones has been suggested [21]. In this study, all of the isolates were susceptible to 2 fluoroquinolones and gentamicin. Most of the recent Salmonella isolates in Japanese livestock were also susceptible to them [3]. Fluoroquinolones may become a powerful candidate for use in treating severe salmonellosis in horses, although gentamicin is not recommended as a candidate for such treatment because of its low activity against intracellular microorganisms such as Salmonella spp.

In this study, a high relatedness was observed between the equine DT104 isolates and 3 bovine isolates in the FALP fingerprinting. Both types of isolate had the same characteristics including the PCR product of the multiplex PCR, the class 1 integron PCR, and ACSSuT type antimicrobial resistance. Tamada et al. reported that the bovine isolates, which was belonged a particular genetic cluster as determined by FAFLP and PEGE, including KT11, NET-8, and NET-57 caused an epidemic in cattle and became a majority of S. Typhimurium isolates obtained from cattle in Hokkaido in 1990s [22]. They also reported that all of the DT104 isolates obtained from animals fell into the same genetic cluster [22]. Although the phage types of the isolate KT11, NET-8, and NET-57 have not been defined yet, phage types of these three bovine isolates probably are DT104 or related phage types such as U302 from the result of the multiplex PCR and the class 1 integron PCR. Our results suggest that The DT104 isolates in horses were transmitted from an area of epidemic S. Typhimurium infection in cattle in Hokkaido.

In conclusion, it is retrospectively indicated that MR-DT104 infections have existed in horses at least since 1996. Molecular characterization suggests an epidemiological relationship between the equine MR-DT104 isolates and some bovine MR-S. Typhimurium isolates obtained in the same area. In addition, other multidrug resistant S. Typhimurium isolates had already infected horses before 1996. Our results suggest that there is a risk of transmitting multidrug resistant S. Typhimurium from horses to farmers and/or veterinarians in that area, and also suggest that there is a need to routinely collect epizootic information about S. Typhimurium infections in livestock and wildlife in order to prevent the transmission of this organism from a contaminated environment to a neighboring horse farm.

REFERENCES