**Molecular typing of *Salmonella enterica* serovar 4,[5],12:i:- isolates from humans, animals and river water in Japan by multilocus variable-number tandem repeat analysis and pulsed-field gel electrophoresis**

Noriko IDO[1*], Kaori IWABUCHI[2], Yusuke SATO’O[3,4], Yasuo SATO[1], Masaru SUGAWARA[5], Gakuji YAEGASHI[1], Masaru KONNO[6], Masato AKIBA[7], Kiyoshi TANAKA[8], Katsuhiko OMOE[3,4] and Ikuo UCHIDA[4,8]

[1] Iwate Prefecture Central Livestock Hygiene Service Center, Takizawa, Japan
[2] Research Institute for Environmental Sciences and Public Health of Iwate Prefecture, Morioka, Japan
[3] Department of Veterinary Medicine, Faculty of Agriculture, Iwate University, Morioka, Japan
[4] The United Graduate School of Veterinary Sciences, Gifu University, Gifu, Japan
[5] Iwate Prefecture North Livestock Hygiene Service Center, Karumai, Japan
[6] Iwate Agricultural Research Center, Taneyama livestock laboratory, Oshu, Japan
[7] National Institute of Animal Health, Tsukuba, Japan
[8] Hokkaido Research Station, National Institute of Animal Health, Sapporo, Japan

(Received 8 September 2014/Accepted 13 January 2015/Published online in J-STAGE 25 January 2015)

**ABSTRACT.** Fifty-one *Salmonella enterica* serovar 4,[5],12:i:- (S. 4,[5],12:i:-) isolates (14 human strains, 34 animal strains and 3 river water strains) which are assumed to be monophasic variants of S. Typhimurium were analyzed using pulsed-field gel electrophoresis (PFGE) and multiple-locus variable-number tandem repeat analysis (MLVA) in order to investigate their genetic diversities and relationships. PFGE, MLVA and combination of them identified 28, 27 and 34 profiles (Simpson’s diversity indices [DI]=0.94, 0.96 and 0.97), respectively. No correlations were detected between MLVA clustering and PFGE clustering or phage typing. These results suggested that S. 4,[5],12:i:- originated from multiple S. Typhimurium ancestors. Two cattle and one pig isolates showing identical phage types as well as PFGE and MLVA profiles to human isolates S. 4,[5],12:i:- suggested the existence of the links between human infections and animal reservoirs.

**KEYWORDS:** MLVA, monophasic variant, PFGE, *Salmonella* 4,[5],12:i:-, S. Typhimurium

doi: 10.1292/jvms.14-0465; *J. Vet. Med. Sci.* 77(5): 609–613, 2015
in a 1% megabase agarose gel using the CHEF DR III apparatus (Bio-Rad Laboratories, Hercules, CA, U.S.A.) in 0.5× TBE (89 mM Tris, 89 mM boric acid and 2 mM EDTA) at 14°C, 6 V/cm. The pulse time was increased from 2.2 to 63.8 sec for 19 hr. A photograph of representative PFGE profiles was scanned and saved in TIFF format to be analyzed using Fingerprinting II software (Bio-Rad Laboratories). Digestion profiles of the isolates were compared each other by using Dice similarity coefficient. The cluster analysis was done using the hierarchic unweighted pair arithmetic average algorithm (optimization, 0.5%; tolerance, 1.0%), and a dendrogram was prepared. Profiles with minor variations (three or fewer band differences) were designed as different subtypes (indicated by different Arabic numerals) within a type (indicated by the same uppercase letters), which corresponded to similarities of approximately 80% or more [20]. Otherwise, profiles were designated as different genotypes (indicated by different uppercase letters) when rates of homology were less than 80%.

MLV A was performed using 5 loci: STTR-9, STTR-5, STTR-6, STTR-10pl and STTR-3, as described previously [13]. DNA for PCR was isolated from cells scraped from Luria-Bertini (LB) agar plates, as described previously [19]. PCR amplification was performed using an initial denaturation cycle of 95°C for 5 min, fol-
lowed by 30 cycles of 30 sec of denaturation at 94°C, 30 sec of annealing at 55°C and 1 min of extension at 72°C. Samples were then incubated for 3 min at 72°C to complete the extension. All PCR products were sequenced to confirm the number of tandem repeats. The amplified products were purified with QIAquick PCR purification columns (Qiagen, Hilden, Germany) and sequenced in both directions using Big Dye Terminator v3-1 chemistry (Applied Biosystems, Foster City, CA, U.S.A.). The number of tandem repeats at each locus was manually determined using Genetyx version 10.0 (Genetyx, Tokyo, Japan). The motif copy numbers present in the tandem array were imported into the BioNumerics software version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium), and a minimum-spanning 3 (MST) was generated using the categorical coefficient. To evaluate the diversities of MLVA loci, Nei’s diversity indices were calculated using POPGENE version 1.32 (http://www.ualberta.ca/~fyeh/popgene.html).

To investigate the genetic relationship between human and animal isolates in detail, a combination usage of PFGE and MLVA was attempted. The isolates showing identical profiles both PFGE and MLVA were typed the same combination type indicated by Arabic numerals. To compare the discriminatory powers of PFGE, MLVA and combination of them, Simpson’s diversity index (DI) and 95% confidence intervals (CI) were calculated using Epicompare version 1.0 (http://www3.ridom.de/epicompare/).

Figure 1 summarizes PFGE and MLVA typing of the 51 S. 4,[5],12:i:- isolates. Twenty-eight different PFGE profiles (DI, 0.94; 95% CI, 0.91–0.98) were identified by BlnI digestion. A cluster analysis of PFGE identified seven major clusters (C, D, G, H, J, L and M) and seven minor profiles (A, B, E, F, I, K and N). The dominant cluster C included 5 PFGE profiles (C1–C5) representing 20 isolates. The other clusters included between 2 and 4 PFGE profiles composed of between 2 and 7 isolates. MLVA identified 27 profiles (DI, 0.96; 95% CI, 0.93–0.98), and five MLVA loci varied in their degree of polymorphism, which was reflected by Nei’s diversity indices; STTR3, 0.52; STTR5, 0.74; STTR6, 0.83; STTR9, 0.58; STTR10pl, 0.85. A cluster analysis using MST revealed eight major clusters (I–VIII) and 5 minor profiles (IX–XIII), as shown in Fig. 2. The dominant cluster II included 2 MLVA profiles composed of 13 isolates. Cluster III included 7 MLVA profiles composed of 11 isolates. The other clusters included between 1 and 5 MLVA profiles composed of between 2 and 8 isolates. Three MLVA clusters, II, VII and VIII, included only one phage type. The other MLVA clusters included between 2 and 5 phage types, respectively.

Combining the PFGE and MLVA yielded 34 combination types (DI, 0.97; 95% CI, 0.94–0.99) (Fig. 1). The dominant combination type 3 (PFGE; C1, MLVA; IIa) was composed of the 8 bovine isolates obtained from different farms located in the same town. Combination type 6 (PFGE; C3, MLVA; IIIc) included 1 human and 2 cattle isolates derived from different towns. Combination type 7 (PFGE; C3, MLVA; IIb) included 2 isolates from different human sporadic cases, 1 isolate from a pig and 1 isolate from the sewage water of a different town. Combination type 18 (PFGE; G1, MLVA; IIIf) included 4 human isolates obtained from different sporadic cases in the same town. Combination type 30 (PFGE; L1, MLVA; VII) and 32 (PFGE; M1, MLVA; VIII) included 2 isolates each, which were obtained from different samples in the same town.

In the present study, various profiles were detected by PFGE and MLVA, respectively. The discriminatory power of combination typing was greater than single usage. These data suggest that combination typing of PFGE and MLVA is useful for discrimination of S. 4,[5],12:i:- isolates, as Kurosawa et al. reported previously [11]. On the other hand, the discriminatory power of phage typing (DI, 0.82; 95% CI, 0.74–0.91) of these isolates was lower than that of PFGE and MLVA [10]. Biased distribution of phage types seemed to reduce the DI value, and thus, we didn’t utilize phage typing data in this study.

Although the correlations between MLVA cluster, PFGE cluster and phage type were previously reported by the analyses of S. Typhimurium [13, 16], no correlation was found in this study. For example, MLVA cluster III included 5 distinct PFGE profiles (C2, C3, D2, G1 and G2). PFGE profile C3 was found in MLVA clusters II and IX. These results indicated that the isolates we examined consisted of
multiple distinct clones. Previous studies suggested that S. 4,[5],12:i:- strains from several countries seem likely to be clonal. Most of S. 4,[5],12:i:- isolates from Germany were typed as DT193 [8]. In Spain and Thailand, U302 was the most prevalent phage type [1, 4]. However, Zamperini et al. [22] concluded that S. 4,[5],12:i:- isolates from Georgia were originated from multiple S. Typhimurium, since some monophasic isolates showed identical or similar PFGE profiles with S. Typhimurium. We identified that the deletion or point mutations in the fljAB operon and hin gene were the bases of the monophagic phenotype of the S. 4,[5],12:i:- isolates in the previous study [10]. In this study, 42 isolates lacking fljAB operon were divided into 15 clusters and 5 minor profiles by PFGE. MLVA divided these isolates into 6 clusters and 2 minor profiles. Eight isolates having the point mutations in the genes fljA and hin showed five PFGE profiles (D4, L1, L2, M1 and M2) and six MLVA profiles (V, VII, VIII, X, XI and XII). These results supported the hypothesis that S. 4,[5],12:i:- isolates in Japan were originated from multiple S. Typhimurium ancestors.

Best et al. [3] reported the presence of identical MLVA types across isolates from poultry, pigs and humans, suggesting that animals may be involved in the dissemination of S. Typhimurium through the food chain. Furthermore, several studies [8, 14] reported S. 4,[5],12:i:- isolates from pig, pork and humans showed identical or closely related profiles by PFGE or MLVA and suggested pig or pork might be a source of infection. In this study, two bovine isolates and one pig isolate belonging to combination types 6 and 7 showed identical phage types as well as PFGE and MLVA profiles to human isolates. Although, these strains were collected from the towns scattered throughout the prefecture, and there was no direct contact between these human and animals, these results suggest a possibility that food-producing animal is one of the reservoirs of S. 4,[5],12:i:- in Japan.

In conclusion, the present study demonstrated the usefulness of combined usage of PFGE and MLVA for S. 4,[5],12:i:- typing. The various profiles resulting from PFGE and MLVA suggested that S. 4,[5],12:i:- spreading in Japan may have originated from multiple S. Typhimurium ancestors. The presence of identical phage types as well as PFGE and MLVA profiles across human and animal isolates of S. 4,[5],12:i:- suggests the existence of the links between human infections and animal reservoirs.

ACKNOWLEDGMENTS. We gratefully acknowledge the members of the prefectural livestock hygiene service centers and prefectural institutes of public health for kindly providing the Salmonella isolates used in this study. This work was partly supported by the Ministry of Health, Labour and Welfare of Japan (H24-Shokuhin-Ippan-008).

REFERENCES

1. Amavisit, P., Boonyawiwat, W. and Bangtrakulchont, A. 2005. Characterization of Salmonella enterica serovar Typhimurium and monophasic Salmonella serovar 4,[5],12:i:- isolates in Thailand. J. Clin. Microbiol. 43: 2736–2740. [Medline] [CrossRef]

2. Arbeit, R. D. 1995. Laboratory procedures for the epidemiologic analysis of microorganisms, pp. 190–208. In: Manual of Clinical Microbiology, 6th ed. (Murray, P. R., Baron, E. J., Pfaller, M. A., Tenover, F. C. and White, C. T., eds.), American Society for Microbiology, Washington, D.C.

3. Best, E. L., Lindstedt, B. A., Cook, A., Clifton Hadley, F. A., Threlfall, E. J. and Liebana, E. 2003. Multiple-locus variable-number tandem repeat analysis of Salmonella enterica subsp. enterica serovar Typhimurium: comparison of isolates from pigs, poultry and cases of human gastroenteritis. J. Appl. Microbiol. 103: 565–572. [Medline] [CrossRef]

4. de la Torre, E., Zapata, D., Tello, M., Mejia, W., Frias, N., Garcia Pena, F. J., Mateu, E. M. and Torre, E. 2003. Several Salmonella enterica subsp. enterica serotype 4,5,12:i:- strains in Spain. J. Clin. Microbiol. 41: 2395–2400. [Medline] [CrossRef]

5. Echeita, M. A., Aladuen, A., Cruchaga, S. and Usera, M. A. 1999. Emergence and spread of an atypical Salmonella enterica subsp. enterica serotype 4,5,12:i:- strain in Spain. J. Clin. Microbiol. 37: 3425. [Medline]

6. Echeita, M. A., Herrera, S. and Usera, M. A. 2001. Atypical, fljB-negative Salmonella enterica subsp. enterica strain of serovar 4,5,12:i:- appears to be a monophasic variant of serovar Typhimurium. J. Clin. Microbiol. 39: 2981–2983. [Medline] [CrossRef]

7. Garaizar, J., Porwollik, S., Echeita, A., Rementeria, A., Herrera, S., Wong, R. M., Frye, J., Usera, M. A. and McClelland, M. 2002. DNA microarray-based typing of an atypical monophasic Salmonella enterica serovar. J. Clin. Microbiol. 40: 2074–2078. [Medline] [CrossRef]

8. Hauser, E., Tietze, E., Helmuth, R., Junker, E., Blank, K., Prager, R., Rabbsch, W., Appel, B., Fruth, A. and Malorny, B. 2010. Pork contaminated with Salmonella enterica serovar 4,[5],12:i:-, an emerging health risk for humans. Appl. Environ. Microbiol. 76: 4601–4610. [Medline] [CrossRef]

9. Hopkins, K. L., Maguire, C., Best, E., Liebana, E. and Threlfall, E. J. 2007. Stability of multiple-locus variable-number tandem repeats in Salmonella enterica serovar Typhimurium. J. Clin. Microbiol. 45: 3058–3061. [Medline] [CrossRef]

10. Ido, N., Lee, K., Ibawuchi, K., Izumiya, H., Uchida, I., Kusumoto, M., Iwata, T., Ohsinski, M. and Akiba, M. 2014. Characteristics of Salmonella enterica serovar 4,[5],12:i:- as a monophasic variant of serovar Typhimurium. PLoS ONE 9: e104380. [CrossRef]. [Medline]

11. Kurosawa, T., Tanaka, K., Tanaka, Y., Uchida, I., Kobayashi, A., Hata, E., Kanno, T., Akiba, M., Yukawa, S. and Tamura, Y. 2012. Molecular typing of Salmonella enterica serotype Typhimurium and serotype 4,5,12:i:- isolates from cattle by multiple-locus variable-number tandem-repeats analysis. Vet. Microbiol. 160: 264–268. [Medline] [CrossRef]

12. Laorden, L., Herrera-Leon, S., Martinez, I., Sanchez, A., Kromidas, L., Bikandi, J., Rementeria, A., Echeita, A. and Garaizar, J. 2010. Genetic evolution of the Spanish multidrug-resistant Salmonella enterica serovar 4,5,12:i:- monophasic variant. J. Clin. Microbiol. 48: 4563–4566. [Medline] [CrossRef]

13. Lindstedt, B. A., Vardund, T., Aas, L. and Kapperud, G. 2004. Multiple-locus variable-number tandem-repeats analysis of Salmonella enterica subsp. enterica serovar Typhimurium using PCR multiplexing and multicolor capillary electrophoresis. J. Microbiol. Methods 59: 163–172. [Medline] [CrossRef]

14. Mousong, J., Marques, P., Ragimbeau, C., Huberty-Krau, P., Losch, S., Meyer, G., Moris, G., Strottner, C., Rabbsch, W. and Schneider, F. 2007. Outbreaks of monophasic Salmonella en-
terica serovar 4,[5],12:i:- in Luxembourg, 2006. Euro Surveill. 12: E11–E12. [Medline]

15. Murphy, T. M., McNamara, E., Hill, M., Rooney, N., Barry, J., Egan, J., O’Connell, A., O’Loughlin, J. and McFaddyn, S. 2001. Epidemiological studies of human and animal Salmonella typhimurium DT104 and DT104b isolates in Ireland. Epidemiol. Infect. 126: 3–9. [Medline]

16. Ngoi, S. T., Lindstedt, B. A., Watanabe, H. and Thong, K. L. 2013. Molecular characterization of Salmonella enterica serovar Typhimurium isolated from human, food, and animal sources in Malaysia. Jpn. J. Infect. Dis. 66: 180–188. [Medline] [CrossRef]

17. Powell, N. G., Threlfall, E. J., Chart, H. and Rowe, B. 1994. Subdivision of Salmonella enteritidis PT 4 by pulsed-field gel electrophoresis: potential for epidemiological surveillance. FEMS Microbiol. Lett. 119: 193–198. [Medline] [CrossRef]

18. Switt, A. I., Soyer, Y., Warnick, L. D. and Wiedmann, M. 2009. Emergence, distribution, and molecular and phenotypic characteristics of Salmonella enterica serotype 4,5,12:i. Foodborne Pathog. Dis. 6: 407–415. [Medline] [CrossRef]

19. Tamamura, Y., Uchida, I., Tanaka, K., Okazaki, H., Tezuka, S., Hanyu, H., Kataoka, N., Makino, S., Kishima, M., Kubota, T., Kanno, T., Hatama, S., Ishihara, R., Hata, E., Yamada, H., Nakaoka, Y. and Akiha, M. 2011. Molecular epidemiology of Salmonella enterica serovar Typhimurium isolates from cattle in Hokkaido, Japan: evidence of clonal replacement and characterization of the disseminated clone. Appl. Environ. Microbiol. 77: 1739–1750. [Medline] [CrossRef]

20. Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H. and Swaminathan, B. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. 33: 2233–2239. [Medline]

21. Yatsuyanagi, J., Waguri, A., Sakuraba, M., Aoki, T., Kaneko, N., Fujii, S., Ohta, M., Takahashi, M., Kobayashi, T., Ozawa, N. and Sugama, K. 2008. Serovars and antimicrobial susceptibilities of Salmonella isolates from sporadic diarrheal cases in Tohoku district, April 2006-March 2007. Infect. Agents Surveillance Rep. 29: 164–166 (in Japanese).

22. Zamperini, K., Soni, V., Waltman, D., Sanchez, S., Theriault, E. C., Bray, J. and Maurer, J. J. 2007. Molecular characterization reveals Salmonella enterica serovar 4,[5],12:i:- from poultry is a variant Typhimurium serotype. Avian Dis. 51: 958–964. [Medline] [CrossRef]