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Note

Isolation of *Salmonella enterica* serovar Agona strains and their similarities to strains derived from a clone caused a serovar shift in broilers

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ABSTRACT

Salmonella enterica serovar Agona strains isolated from human cases were compared to strains that were derived from a clone caused a serovar shift in broilers. Pulsed field gel electrophoresis (PFGE) analysis with XbaI or BlnI digestion showed that three of seven strains from human case strains and most of the 81 strains from broilers were clustered in single complex in a minimum spanning tree (MST) reconstructed from the PFGE data. All the strains from human cases and 22 randomly selected strains from broilers were also analyzed by whole genome sequencing (WGS). Analysis of single nucleotide polymorphism (SNP) in the *S.* Agona core genes showed that four strains from human cases and all the strains from broilers were clustered in a maximum likelihood phylogenetic tree (ML tree) and an MST. These results indicated that the strains derived from the clone caused the serovar shift had already spread to humans. PFGE analysis with XbaI showed that four strains from broilers did not cluster with the other strains in an MST, though all those strains clustered in an ML tree and an MST reconstructed from SNP data. Moreover, three strains from broilers did not cluster in an MST reconstructed from PFGE with BlnI digestion, though those strains clustered in an ML tree and an MST reconstructed from SNP data. Therefore, it was suggested that *S.* Agona strains derived from a particular clone could not be traced by PFGE analysis but can be investigated by WGS analysis.

Salmonella enterica is a well-known foodborne pathogen. Although *S.* Infantis has been prevalent in broilers in Japan, our previous study found that the frequency of *S.* Infantis isolation had greatly decreased and that *S.* Agona was predominant serovar isolated in three out of four farms raising broilers [1]. Thus far, this serovar shift has only been reported in those farms and the selective pressure for that shift is still not known. Several molecular epidemiological studies have indicated that *S.* Infantis strains isolated from humans are similar to strains isolated from broilers, suggesting that humans might be infected by consumption of contaminated chicken meat [2,3]. Therefore, human infections by *S.* Agona strains, which were derived from the clone involved in the serovar shift (the serovar shift clone), would occur in the geographic area where chicken meat from those broiler-raising farms was retailed. *S.* Agona has become a public health issue since the 1970s because of large outbreaks during that time [4]. Moreover, the emergence of multidrug resistant *S.* Agona strains due to integrons has been concerned in several countries [5], so the dynamics of a particular clone such as the serovar shift clone needs to be investigated.

However, it is difficult to determine whether *S.* Agona strains isolated from human cases were derived from the serovar shift

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clone, even if the human cases were in an area where chicken meat from the *S*. Agona-infected broiler farms was retailed. Pulsed field gel electrophoresis (PFGE), the gold standard method for the molecular epidemiology of bacterial infections, lacks sufficient resolution to differentiate bacterial strains. This makes it difficult to determine whether the strains isolated from human cases were derived from a common source without additional data from epidemiological contact surveys [6]. However, epidemiological contact surveys of sporadic human diarrhea cases are not usually carried out by local health centers in Japan.

Several recent studies indicated that whole genome sequencing (WGS) analysis using next generation sequencers is effective for analyzing the molecular epidemiology of some pathogens [3,7]. In this study, we collected *S*. Agona strains isolated from sporadic human diarrhea cases during 2010–2014 in Yamanashi prefecture of Japan, where chicken meat from the *S*. Agona-infected broiler farms was retailed. Those strains were compared to *S*. Agona strains previously isolated from broilers in 2014 [1] by PFGE and WGS analyses. All of the strains in this study ($n = 88$) are listed in Supplementary Table 1.

PFGE analyses with XbaI and BlnI digestion were done as previous described [8], and the PFGE pattern data were imported into BioNumerics software version 5.1 (Applied Maths). The similarities of PFGE patterns were calculated previously described [8]. Minimum spanning tree (MST) analysis was carried out using a similarity matrix with the following options: hypothetical types were not allowed, similarity bin sizes were 2.0 for XbaI and 2.5 for BlnI, the priority rule was that first link types had maximum numbers of SLVs', and the minimum size was three types for formation of complexes. A complex was formed by strains with up to five changes when XbaI was used for PFGE analysis, which indicated those strains had more than 90% similarity [8]. A complex was also formed by strains with up to two changes when BlnI was used for PFGE analysis, which indicated that those strains had more than 95% similarity [8].

WGS analysis was carried out as previously described [7]. Briefly, sequence libraries were prepared with Nextera XT DNA Sample Prep Kits (Illumina). A hundred cycles of dual-index paired-end sequencing were done using the Illumina HiSeq2500 System (Illumina). The Illumina analysis pipeline (CASAVA 1.6.0) was used for image analysis, base calling, and quality score calibration. Read data were sorted and exported to FASTQ files. Raw read data were deposited in the Sequence Read Archive (SRA) in the DNA Data Bank of Japan (DDBJ, DRA 006655). The FASTQ files were analyzed using CLC Genomics Workbench software version 7.5 (CLC bio). Read data were mapped to a reference genome (*S*. Agona strain SL483, GenBank Accession Number CP001138) with the “Non-specific match was ignored” option. a fixed ploidy variant detection method with the “a coverage and count filter with minimum coverage was 30” option was used to detect single nucleotide polymorphism (SNP). SNP in the core-genes of *S*. Agona strains [9] was selected for further studies using an in-house Ruby (ISO/IEC 30170) script.

The data of selected SNP were concatenated and phylogenetic analysis was done by the maximum likelihood method with the same conditions as in our previous study [3]. The SNP data were also analyzed using BioNumerics software (Applied Maths), and an MST was reconstructed with the same conditions as described previously [7]. The SNP data were imported into Splits Tree software version 4.11.3 and the Φ_w test, a statistical test for recombination, was used to determine the occurrence of recombination [10].

MSTs reconstructed by PFGE analysis with XbaI or BlnI digestion showed that most of the strains in this study clustered in each single complex (Supplementary Figs. 1 and 2). Three strains isolated from human cases (strain 12-215, 13-85 and 14-8) were in one complexes in these MSTs. When all of the seven strains from human cases and 22 randomly selected strains from broilers were analyzed by WGS analyses, the result of Φ_w test indicated no significant recombination-related SNPs in the core genes in this study ($P = 0.1065$). The ML tree reconstructed from the SNP data in the core genes showed that these strains were divided into two clusters designated cluster 1 and 2. Cluster 1 contained the genome mapping reference strain (SL483 strain) and three strains from human cases, and cluster 2 contained all of the strains isolated from broilers and the other isolated from human cases (Fig. 1). The MST reconstructed using the SNP data in the core genes showed that all the cluster 2 strains had relatively short branch lengths, while all the cluster 1 strains had relatively long branch lengths (Fig. 2).

These results indicated that the *S*. Agona strains derived from the serovar shifting clone have already spread to humans. Unfortunately, epidemiological contact surveys were not done for the human cases in this study, so it is unclear whether those cases were infected by the consumption of contaminated food or by another route of infection. The spread of a foodborne pathogen was speculated by an increase in the numbers of people infected by the organism [11,12]. However, it is uncommon that the spread of a new type of a bacterial pathogen was detected prior to the occurrence of a large outbreak by the organism as shown in this study. Strain 12-215 in this study, which was isolated in 2012, was clustered with strains derived from the serovar shift clone, suggesting that the spread of these strains had already started before we elucidated the serovar shift in broilers [1]. Further studies are needed to determine when and how strains derived from the serovar shift clone started to spread in this area.

In this study, SNP data in *S*. Agona core genes [9] were used to investigate the relationship between *S*. Agona strains from human cases and broilers. We previously reported that, using WGS analysis, Shiga toxin-producing *Escherichia coli* O157 strains from a common source could be differentiated from unrelated strains [7]. In that study, strains from a common source were clustered within 10 pairwise distances on an MST. The results of this study showed four out of seven *S*. Agona strains from human cases were clustered with those from broilers within 10 pairwise distances on an MST (Fig. 2). This result suggested the possible transmission of *S*. Agona strains from chicken meat to human. However, it has not been evaluated whether the same threshold value could be used to detect the transmission by any kinds of pathogens, though such a value was evaluated using a pathogen other than *S*. Agona [7]. Further studies are needed to corroborate the validity of the threshold value.

We previously reported that many SNPs were observed in descendant strains derived from one strain of enterohemorrhagic *Escherichia coli* serovar O157:H7/H- by sub-culturing [13]. Therefore, not all the detected SNPs should be used to analyze the relationships among bacterial strains. Despite that, there is no information on which SNPs should be excluded from comparisons of *S*. Agona strains. We believe that the SNP data in the core genes used in this study do not include such culture-derived SNPs, since the SNP data in the core genes used here were stable enough to be used for phylogenetic analysis of *S*. Agona strains [9]. This hypothesis was supported by the result of Φ_w test, which indicated no significant recombination-related SNPs in the core genes in this study.

WGS analysis indicated that there may be other routes of human infection by *S*. Agona strains in the geographic area of this study. Thus, it would have been difficult to detect the onset of spread of the strains derived from the serovar shift clone even if the number of human *Salmonella* cases had been continuously monitored.

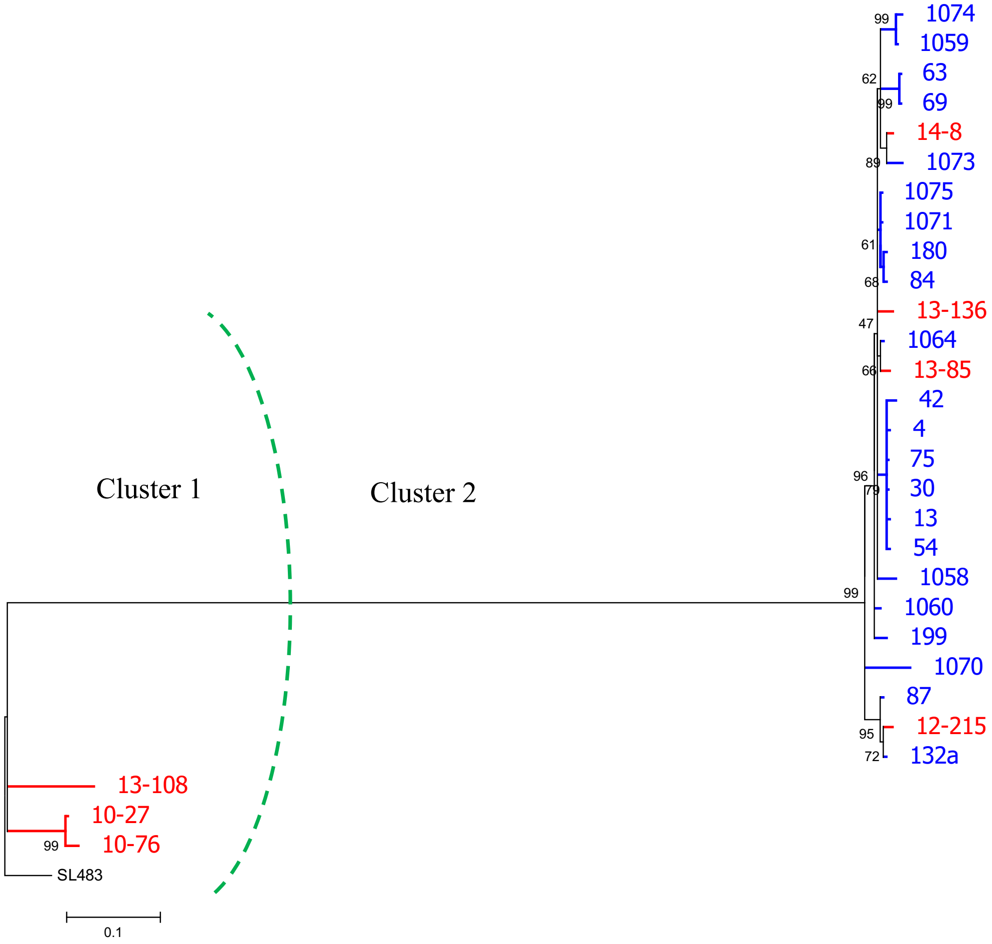

Fig. 1. A maximum likelihood tree reconstructed using SNP data in the core genes of the seven *S.* Agona strains from human cases and 22 randomly selected strains from broilers in this study. In this tree, strains in blue font were isolated from broilers and strains in red font were isolated from human cases.

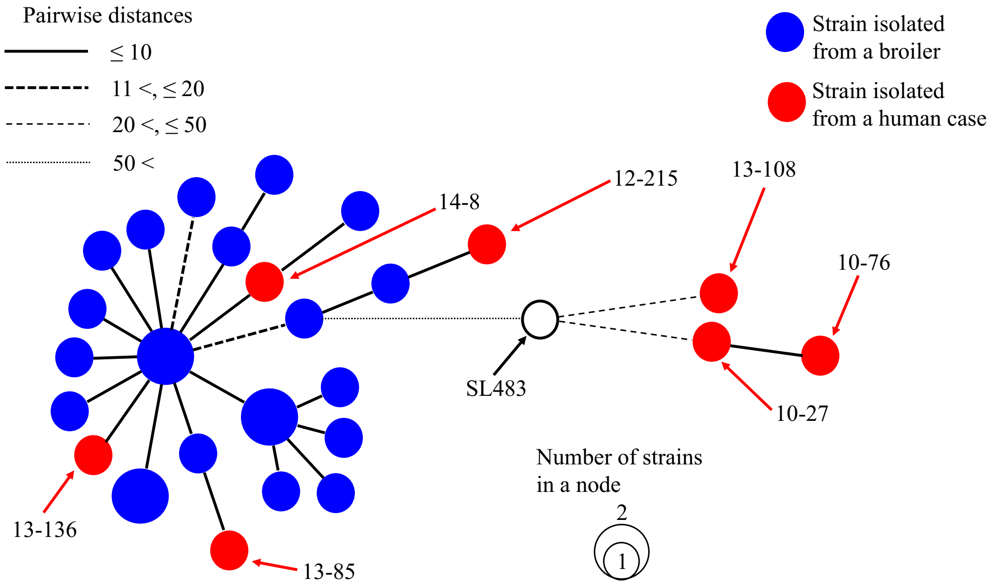

Fig. 2. A minimum spanning tree of the seven *S.* Agona strains from human cases and 22 randomly selected strains from broilers in this study reconstructed using SNP data in the *S.* Agona core genes.

According to the data of the Agriculture and Livestock Industries Cooperation of Japan, the amount of imported chicken meat has been increasing in Japan, and Brazil and Thailand are major sources of the importation. There are some reports indicating the *S*. Agona contamination in broilers or chicken meat in those countries [14,15], which would confound efforts to monitor the onset of the spread of a particular clone. In this study, most of the strains derived from the serovar shift clone isolated from broilers were also clustered by PFGE analysis, indicating the efficacy of PFGE analysis to demonstrate the derivation of *S*. Agona strain in a certain extent. However, this study also indicated a limitation of PFGE analysis. Although one strain (i.e., strain 13-136), which was isolated from a human case, did not cluster with the other strains derived from the serovar shift clone by PFGE analysis using BlnI, that strain did cluster with these strains by WGS analysis. There were three PFGE bands differences between that strain (strain 13-136) and a strain derived from the serovar shift clone (strain4), indicating that strain 13-136 was also one of the strains derived from the serovar shift clone (Supplementary Fig. 3). These results also suggested that some genetic changes would have already occurred among the strains derived from the serovar shift clone.

Such genetic changes can be also seen in other PFGE analysis data. Among the strains used for WGS analysis in this study, strain 30, 42, 54, and 63 did not cluster with the large complex of the strains derived from the serovar shift clone when XbaI was used for PFGE analysis, though these strains did cluster when BlnI was used. In contrast, strain 87, 132, and 1064 did not cluster when BlnI was used, but all these strains did cluster when XbaI was used (Supplementary Table 1, Supplementary Figs. 1 and 2). These results suggested that the longer the period of the spread of the organism will be, the more difficult by PFGE analysis the surveillance of *S*. Agona strains derived from a particular clone would gradually become. On the other hand, there are some difficulties associated with WGS analysis. First, the cost of WGS analyses is expensively compared to that of PFGE analysis, so WGS analysis should be done after screening of tested strains is done by PFGE analysis. Second, the analysis of WGS data is more complex than that of PFGE analysis data as shown in our previous studies [3,7,13]. In particular, SNPs and small insertions or deletions due to recombination are troublesome [7,13], so the occurrence of recombination-related variations must be tested.

In conclusion, this study successfully demonstrated that the serovar shift in broilers affected the prevalence of *Salmonella* serovar isolated from human cases and that WGS analysis was useful for tracing strains derived from a particular clone. If chicken meat contaminated with *S*. Agona strains derived from the serovar shift clone is widely distributed, there would be more human infections caused by these strains in other geographic areas. Further surveillance of *S*. Agona strains derived from the serovar shift clone should be done using WGS analysis.

Conflicts of interest

The authors declare that they have no conflict of interest to disclose.

Authorship declaration

All the authors have substantially contributed to this study and meet the ICMJE authorship criteria.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jiac.2018.07.003.

References

[1] Murakami S, Seki M, Suzuki Y, Takano A, Suzuki K, Okazaki M, et al. Investigation of *Salmonella* Agona colonized in broiler chickens and *Salmonella* contamination in minced chicken. J Jpn Soc Poult Dis 2016;52:180–4 [in Japanese].

[2] Murakami K, Horikawa K, Otsuki K. Genotypic characterization of human and environmental isolates of *Salmonella choleraesuis* subspecies *choleraesuis* serovar Infantis by pulsed-field gel electrophoresis. Microbiol Immunol 1999;43:293–6.

[3] Yokoyama E, Murakami K, Shiwa Y, Ishige T, Ando N, Kikuchi T, et al. Phylogenetic and population genetic analysis of *Salmonella enterica* subsp. *enterica* serovar Infantis strains isolated in Japan using whole genome sequence data. Infect Genet Evol 2014;27:62–8.

[4] Crump JA, Griffin PM, Angulo FJ. Bacterial contamination of animal feed and its relationship to human illness. Food Safety 2002;35:859–65.

[5] Doublet B, Butaye P, Imberechts H, Collard J-M, Chaslus-Dancla E, Cloeckaert A. *Salmonella* Agona harboring genomic island 1-A. Emerg Infect Dis 2004;10:756–8.

[6] Davis MA, Hancock DD, Besser TE, Call DR. Evaluation of pulsed-field gel electrophoresis as a tool for determining the degree of genetic relatedness between strains of *Escherichia coli* O157:H7. J Clin Microbiol 2003;41:1843–9.

[7] Yokoyama E, Hirai S, Ishige T, Murakami S. Application of whole genome sequence data in analyzing the molecular epidemiology of Shiga toxin-producing *Escherichia coli* O157:H7/H-. Int J Food Microbiol 2018;264:39–45.

[8] Yokoyama E, Maruyama S, Kabeya H, Hara S, Sata S, Kuroki T, et al. Prevalence and genetic properties of *Salmonella enterica* serovar Typhimurium definitive phage type 104 isolated from *Rattus norvegicus* and *Rattus rattus* house rats in Yokohama City, Japan. Appl Environ Microbiol 2007;73:2624–30.

[9] Zhou Z, McCann A, Litrup E, Murphy R, Cormican M, Fanning S, et al. Neutral genomic microevolution of a recently emerged pathogen, *Salmonella enterica* serovar Agona. PLoS Genet 2013;9. e1003471.

[10] Huson DH, Bryant D. Application of phylogenetic networks in evolutionary studies. Mol Biol Evol 2006;23:254–67.

[11] Rabsch W, Prager R, Koch J, Stark K, Poggentin P, Bockenühl J, et al. Molecular epidemiology of *Salmonella enterica* serovar Agona: characterization of a diffuse outbreak caused by antiseed-fennel-caraway infusion. Epidemiol Infect 2005;133:837–44.

[12] Yokoyama E, Etoh Y, Ichihara S, Horikawa K, Konishi N, Kai A, et al. Emergence of enterohemorrhagic *Escherichia coli* serovar O157 with highly similar pulsed-field gel electrophoresis patterns. J Food Prot 2011;74:1324–7.

[13] Yokoyama E, Hirai S, Ishige T, Murakami S. Single-nucleotide polymorphisms in the whole-genome sequence data of Shiga toxin-producing *Escherichia coli* O157:H7/H- strains by cultivation. Curr Microbiol 2017;74:425–30.

[14] Bangtrakulnonth A, Pornreongwong S, Pulsrikan C, Sawanpanyalert P, Hendriksen RS, Wong DMALF, et al. Salmonella serovars from humans and other sources in Thailand, 1993–2002. Emerg Infect Dis 2004;10:131–6.

[15] Palmeira A, des Santos LR, Borsoi A, Rodrigurs LB, Calasans M, do Nascimento VP. Serovars and antimicrobial resistance of *Salmonella* spp. isolated from Turkey and broiler carcasses in southern Brazil between 2004 and 2006. Rev Inst Med Trop Sao Paulo 2016;58:19.