The Journal of Veterinary Medical Science

NOTE

Public Health

Genetic characteristics of emerging *Salmonella enterica* serovar Agona strains isolated from humans in the prior period to occurrence of the serovar shift in broilers

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J. Vet. Med. Sci.
81(8): 1117–1120, 2019
doi: 10.1292/jvms.18-0522

Received: 30 August 2018
Accepted: 2 June 2019
Advanced Epub: 21 June 2019

ABSTRACT. Our previous studies found that a dominant serovar of *Salmonella enterica* isolates from three farms raising broilers in 2014 and 2015 was serovar Agona and the number of Infantis isolates decreased (the serovar shift). In this study, 52 *S.* Agona strains which isolated between 1993 and 2008, were compared to the serovar shift clone by molecular epidemiology and phylogenetic analyses, using pulsed field gel electrophoresis and whole genome sequence analyses. Of the 52 strains, one strain isolated from a human case in 1995 was genetically identical to the serovar shift clone, even though it was isolated prior to the serovar shift. These results suggested that the *S.* Agona serovar shift clone had existed in a source other than chicken penetrated chicken population.

KEY WORDS: pulsed field gel electrophoresis, *Salmonella* Agona, serovar shift clone, whole genome sequencing

Salmonella enterica has been causing food-borne outbreaks of gastroenteritis around the world. The Ministry of Health, Labour and Welfare of Japan reported that *S. enterica* was the third leading cause of bacterial food-borne infections in Japan in 2017 [9]. Among its serovars, *S. enterica* serovar Agona has been a public health concern since 1970s because many large outbreaks have been reported in several countries [1], including Japan [4–6].

Our previous study [8] revealed that the dominant *S. enterica* serovar had changed at a company's broiler chicken-raising farms located in Yamanashi and Nagano prefectures in Japan since 2014. The change made *S.* Agona dominant instead of *S.* Infantis. In addition, in another study, we showed that *S.* Agona strains isolated from broiler chickens had highly similar genetic profiles, as determined by pulsed field gel electrophoresis (PFGE) analyses and whole genome sequence (WGS) analyses [12]. These results suggested that the serovar shift clone had emerged in the broiler-raising farms, and such a shift could change the incidence of *Salmonella* infections in humans [12].

Generally, emerging diseases are new diseases that appear in a population, or existing diseases that rapidly increase in incidence or geographic range [7]. The emerging disease can arise mainly in two ways. First, a pathogen in the environment or in another species of host simply penetrates and disseminates in a new population. Second, a genetic variation of a pathogen arises while existing in a population and disseminate in the same population [7]. The reason that the serovar shift clone was able to emerge in a broiler population needs to be determined to control further similar emergence and dissemination. However, at present, it is not known how *S.* Agona emerged as the serovar shift clone. The research on whether serovar shift clone was detected in many areas

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(Supplementary material: refer to PMC https://www.ncbi.nlm.nih.gov/pmc/journals/2350/)

or limited in one area may be able to assess the epidemic tendency of future serotype shift clones in broiler.

In this study, *S*. Agona strains isolated prior to the emergence of the serovar shift were investigated by molecular epidemiology and phylogenic analyses. It was reported that the biased distribution of a particular *Salmonella* serovar clone could be seen [11]. We selected Chiba prefecture because it is a municipality near Yamanashi and Nagano prefectures, and chicken meat from broiler-raising farms with the serovar shift has not been consumed. We researched broiler-raising farms in Chiba prefecture in 2015, and only *S*. Infantis was detected and no serovar shift clone of *S*. Agona was detected [8]. This result indicated that the serovar shift clone was not from the broiler breeders. In this study, we investigated whether the serovar shift clone was present in strains isolated before 2015. Fifty-two *S*. Agona strains isolated in Chiba prefecture during 1993 to 2008 were analyzed in this study (Supplementary Table 1). Two strains isolated from sporadic human cases in Yamanashi prefecture were also investigated (Supplementary Table 1). The molecular epidemiological analysis of the strains in this study was done by PFGE analyses with XbaI or BlnI DNA digests as previously described [12]. Minimum spanning tree (MST) analysis was carried out using the PFGE pattern data obtained in this study and the PFGE data of the serovar shift clone in our previous study [12] using BioNumerics Ver. 5.1 (Applied Maths, Saint-Martens-Latem, Belgium) and the same conditions as previously described [12]. In addition, strains were selected for WGS analysis based on the MTS analysis of the PFGE data (Supplementary Figs. 1 and 2, Supplementary Table 1). If a strain did not form a complex with other strains in an MST reconstructed from PFGE analysis of DNA digests with XbaI and/or BlnI, the strain was selected for further analysis. Also, if several strains formed a complex in an MST reconstructed from the PFGE analysis with both XbaI and BlnI, at least one of the strains was arbitrarily selected for further analysis.

WGS analysis was done as previously described with slight modification [12]. Briefly, DNA libraries were prepared using a TruSeq Nano DNA HT Sample prep Kit (Illumina, San Diego, CA, U.S.A.), and 150 or 151 cycles of dual-index paired-end sequencing were done using NextSeq500 (Illumina). If the sufficient amount of read data was not obtained from a strain, it was reanalyzed using Miseq (Illumina) systems. The Illumina analysis pipeline (bcl2fastq v2.17.1.14 or v2.18.0.12) was used for exporting FASTQ files. Raw read data were deposited in the Sequence Read Archive (SRA) of the DNA Data Bank of Japan (DDBJ, Submission in progress). The FASTQ files were analyzed using CLC Genomics Workbench software version 7.5 (CLC bio, Aarhus, Denmark). Read data were mapped to a reference genome (*S*. Agona strain SL483, GenBank Accession Number CP001138) using the same conditions as in our previous study [12], and single nucleotide polymorphisms (SNPs) were identified. SNPs in the core-genes of *S*. Agona strains [13] were further selected using an in-house Ruby (ISO/IEC 30170) script. These SNPs were checked by the Φ_w test for significant recombination using Splits Tree Ver. 4.11.3 [3]. Phylogenetic analysis of these SNPs was carried out using MEGA Ver. 6.06 [10] with the same conditions as previously described [12].

These SNPs were analyzed using our previous reported WGS data for the serovar shift clone (DDBJ Accession No. DRA006655) by the maximum likelihood method with the same conditions as previously described [12]. The WGS data were also analyzed by population genetic analysis. Linkage disequilibrium of the separated groups of strains was evaluated by the standardized index of association (I^S_A) [2] using LAIN software Ver. 3.6. The significance of the I^S_A value was determined by a Monte Carlo simulation with 10^3 resamplings.

When tested strains were analyzed by PFGE analyses with XbaI digests, one strain isolated from a human in Chiba (CS95128) formed a complex with the strains derived from the serovar shift clone, which were designated in our previous study [12]. Also, one of the two strains isolated from human cases in Yamanashi (09-29) formed a complex with the strains derived from the serovar shift clone (Fig. 1, Supplementary Table 1). On the other hand, in an MST reconstructed using data from PFGE analyses of DNA digests with BlnI, none of the strains isolated in Chiba formed a complex with the strains derived from the serovar shift clone (Supplementary Table 1).

The WGS data in this study did not show significant recombination by the Φ_w test (P=0.3284). Phylogenetic analysis using the WGS data showed a similar result to PFGE analysis of DNA digests with XbaI; i.e., only one of the strains isolated in Chiba (CS95128) and one of the two strains isolated in Yamanashi (09-29) formed a cluster (designated as Cluster 2) with the serovar shift clone (Fig. 2). The other strains isolated in Chiba and the remaining strain isolated in Yamanashi (06-25) also formed cluster (designated as Cluster 1). Population genetics showed significant linkage disequilibrium among the strains in Cluster 1 (I^S_A=0.005) and in Cluster 2 (I^S_A=0.1603).

These results suggested that the serovar shift clone had already existed in the environment or in another host species and it has penetrated a new population; i.e., broiler chickens. This was supported by the following two observations. First, one strain isolated in 1995 in Chiba (CS95128) could possess the almost identical DNA to the serovar shift clone, because PFGE analysis of XbaI digests and phylogenetic analysis of the WGS data in this study showed the genetic identity between the serovar shift clone and strain CS95128. Second, the population genetic analysis in this study showed that strain CS95128 and the serovar shift clone were probably derived from the same recent common ancestor, because significant linkage disequilibrium was found among the Cluster 2 strains [2].

Our results could rule out the possibility that the serovar shift clone was originated from a *S*. Agona strain, which had already existed in broiler chickens, and was disseminated after a genetic variation arose in the strain. In this study, strain 09-29 was clustered in Cluster 2, so it could be also surmised that the strain is the ancestor of the serovar shift clone that had been in chicken population. However, the results of molecular epidemiology and phylogeny in this study could not detect any significant genetic variation between strain 09-29 and other serovar shift clone strains Also, the Yamanashi Meat Hygiene Inspection Laboratory surveys [8] reported that *S*. Agona strains have been isolated from broilers in Yamanashi prefecture since 2008. Therefore, it was suggested that the serovar shift clone had already penetrated before 2009.

Our results also indicated that penetration of a pathogen into a new host may be a key factor in a pathogen becoming a serious

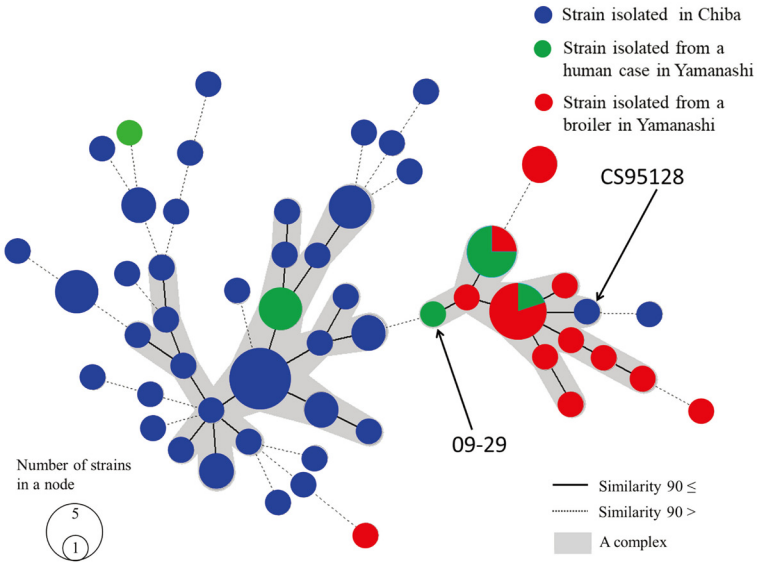

Fig. 1. Minimum spanning tree reconstructed from pulsed field gel electrophoresis (PFGE) patterns of *Salmonella* Agona strains' DNAs digested with XbaI. Black arrows indicate strains that formed a complex with the serovar shifted clone (see Supplementary Table 1).

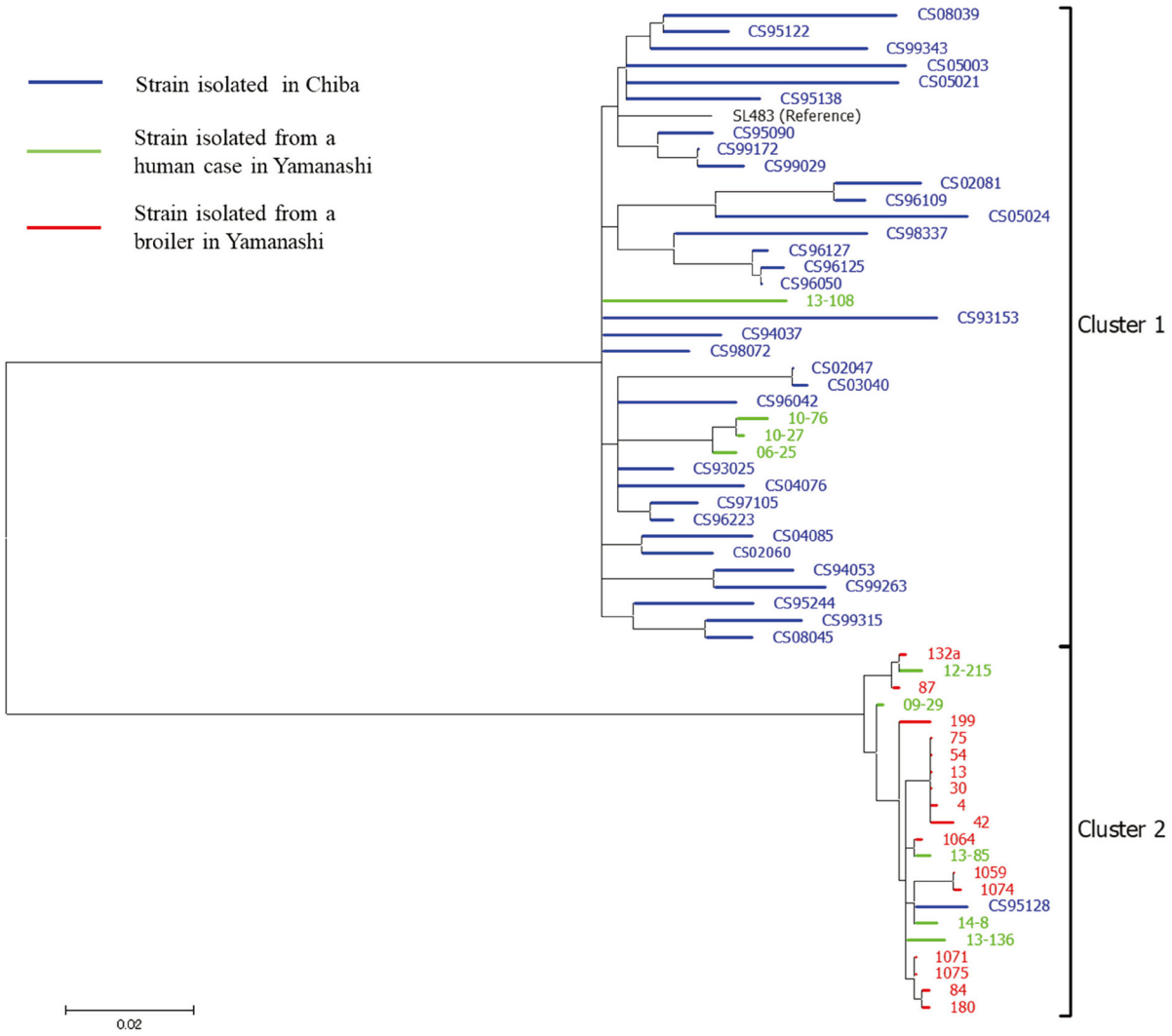

Fig. 2. Phylogenetic tree reconstructed from whole genome sequence data of *Salmonella* Agona strains using the maximum likelihood method.

public health concern. Before this emerging occurred, the incidence of this serovar shift clone in humans may have been quite low, since only one strain was isolated in 16 years in Chiba. Our previous study indicated that four strains of the serovar shift clone were isolated from humans for 3 years in the area where chicken meat was consumed from the broiler-raising farms in which the serovar shift strains were isolated [12]. Although the increase of human *S*. Agona infections was too small for statistical analysis, there was a large difference between the incidence of human cases before (0.06 times per year) and after (1.33 times per year) emergence of the serovar shift clone. Therefore, the spread of the serovar shift clone to other farms should be prevented in the future.

ACKNOWLEDGMENTS. Part of this study was supported by the Ito Foundation Grant in fiscal 2016, MEXT-Supported Program for the Strategic Research Foundation at Private Universities, 2013–2017 (S1311017) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and JSPS KAKENHI Grant number 26460540 and 16K08031.

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