Public Health Assessment of *Salmonella enterica* Serovar Enteritidis Inactivated-Vaccine Treatment in Layer Flocks

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Although there have been several reports on the efficacy assessment of a *Salmonella enterica* serovar Enteritidis vaccine against intestinal and parenchymatous organ diseases of laying hens, no public health risk characterization of its long-term effect on eggs has been reported. In this study, we attempted to assess the public health effect of an inactivated *S. enterica* serovar Enteritidis vaccine against serovar Enteritidis contamination of chicken eggs. We analyzed serovar Enteritidis isolation test results from four windowless farms in which inactivated-vaccine administration was initiated based on the sanitary monitoring program of a farm. When flocks with and without *S. enterica* serovar Enteritidis vaccine treatments were mixed, the application of an inactivated serovar Enteritidis vaccine decreased the most probable number (MPN) of bacteria by at least 100-fold in broken (liquid) egg samples positive for serovar Enteritidis, although a statistical difference between those MPNs could not be obtained. The isolation frequency after the vaccine application was less than 1/10 (P < 0.01). No *S. enterica* serovar Enteritidis bacteria were isolated approximately 1 year after all of the chickens had received the inactivated serovar Enteritidis vaccine. It was suggested that an adequate administration of an inactivated serovar Enteritidis vaccine reduced the contamination risk of eggs (the number of isolated serovar Enteritidis cells and detection frequency) compared to the contamination risk of eggs laid by nonvaccinated hens.

The microorganism risk of food and food materials has been studied in order to be characterized (http://www.who.int/foodsafety/micro/en/, http://www.fao.org/DOCREP/005/Y4392E/y4392e0j.htm), and *Salmonella enterica* serovar Enteritidis is the most serious human health risk factor associated with chicken eggs. The major causative food material of *S. enterica* serovar Enteritidis food poisoning is serovar Enteritidis-contaminated eggs (1, 2, 3, 5, 6, 13). In trace-back surveys of patients with serovar Enteritidis food poisoning, the causative dishes were vegetable salad, cakes, and various other dishes which had been cross-contaminated with serovar Enteritidis-contaminated eggs. Serovar Enteritidis proliferates in contaminated eggs in the distribution step depending on storage conditions, such as duration and temperature, increasing the risk of serovar Enteritidis-induced food poisoning (6, 12). Many epidemiological surveys have revealed that a few serovar Enteritidis-contaminated eggs were included in shipped eggs from serovar Enteritidis-infected flocks (13). However, experimentally infected hens rarely lay serovar Enteritidis-contaminated eggs. It is well known that the risk of food poisoning by serovar Enteritidis-contaminated eggs varies among each step of egg processing, from farms to distribution, processing, and cooking (1, 9).

Risk variation during the course of distribution and cooking processes, from farms to dining tables, has been assessed with regard to the prevention of *S. enterica* serovar Enteritidis-induced food poisoning, but there has been no characterization study concerning the effect of an inactivated serovar Enteritidis vaccine on each process, especially concerning the output of farming over the long term. Therefore, we attempted to characterize the risk of serovar Enteritidis contamination for eggs taken from serovar Enteritidis-contaminated layer farms with and without treatment with the *S. enterica* serovar Enteritidis inactivated vaccine.

MATERIALS AND METHODS

Experimental design. The study was performed at four layer farms managed by one owner in the Western part of Japan (Table 1). Before this study, the administration of *S. enterica* serovar Enteritidis vaccination had already been initiated in some flocks as a part of the government control of serovar Enteritidis. The vertical integration system of the four farms included parent stocks, a feed mill, growing and layer farms, and an egg washing, grading, and packing (GP) center that showed no evidence of serovar Enteritidis contamination (15). The four farms had tried to prepare a sanitation system for each process of the vertical integration system, from the introduction of parent stocks and the purchasing of feed materials in the feed mill to supermarket storage facilities. This serovar Enteritidis measurement program contained a standard sanitation operation procedure and inspection program for each production process, as previously reported (15). In each process of the vertical integration system, the 25 most critical control points (CCP) had been established for serovar Enteritidis control, and serovar Enteritidis isolation tests were included in the 25 CCPs as a part of the sanitary program. Then, the isolation test results were analyzed. A detailed monitoring program regarding this part of layer farming is described in Table 1. We previously reported that positive cases were noted only in samples from the egg production processes involving laying hens (only CCP 16). The serovar Enteritidis-positive samples were from chicken feces, broken (liquid) eggs, and environmental swabs. However, the frequency of serovar Enteritidis...
isolation from feces and environmental swabs was low, and the number of bacteria was below the measurement limits. Thus, these samples were not analyzed in this study.

About 50% of the flocks from the four farms had received an S. enterica serovar Enteritidis vaccine at the time of initiation of this study. New S. enterica serovar Enteritidis vaccine-treated flocks were introduced after culled chickens were shipped, and all flocks were vaccinated 3 years after the initiation of vaccination.

Chicken flocks were monitored. Four farms (farms A, B, C, and D) were investigated. Farm A consisted of nine bird houses maintaining about 620,000 birds. Farms B and C consisted of 10 houses maintaining about 430,000 birds and 8 houses maintaining about 360,000 birds, respectively, and the houses had a raised-floor structure in both farms. Farm D consisted of 10 houses, with a straight cage structure, maintaining about 890,000 birds. Farms A and B introduced chickens that had been raised by their own raising farms. The main breed maintained during the study period was Dekalb. Each farm had an in-line GP center. All farms introduced chicks from one hatchery and used feeds prepared by one feed mill. On initiation of this study, S. enterica serovar Enteritidis was isolated from all four farms. An egg breaker had reported the presence of serovar Enteritidis-contaminated broken egg samples from farms A, B, and C before this study. When the farms were informed, a new bird house was under construction in farm D and was completed several years before this study. However, the first S. enterica serovar Enteritidis contamination was confirmed in farm D upon initiation of this study. An inactivated serovar Enteritidis vaccine (Layermune SE; Biomune Co.) was subcutaneously administered twice at 45 to 50 days of age. The vaccine was administered to the chickens at the time of initiation of this study. For the determination of the most probable number (MPN) of bacteria, samples were separately cultured using the method previously reported (6). When S. enterica serovar Enteritidis was isolated by the quadruplicate method, the number of isolated bacteria was presented as <2. All materials for serovar Enteritidis isolation were stored at room temperature. In the calculation of the mean MPN, values of <2 and ≥1,600 were regarded as 1 and 2,000, respectively. The statistical analysis used in this study is the chi-square test for incidence of serovar Enteritidis isolation.

TABLE 1. Outline of hen groups included in this study

<table>
<thead>
<tr>
<th>Farm</th>
<th>Description of breeding hen location</th>
<th>Description of feed mill</th>
<th>Ownership of growing farm</th>
<th>No. of birds</th>
<th>No. of laying houses</th>
<th>Method of poultry manure disposal</th>
<th>Structure of laying house</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Shared</td>
<td>Shared</td>
<td>Owned by the same farm</td>
<td>620,000</td>
<td>9</td>
<td>Stocked until removal</td>
<td>Raised flooring, type A</td>
</tr>
<tr>
<td>B</td>
<td>Shared</td>
<td>Shared</td>
<td>Owned by the same farm</td>
<td>360,000</td>
<td>8</td>
<td>Stocked until removal</td>
<td>Raised flooring, type A</td>
</tr>
<tr>
<td>C</td>
<td>Shared</td>
<td>Shared</td>
<td>Owned by the same farm</td>
<td>430,000</td>
<td>10</td>
<td>Stocked until removal</td>
<td>Raised flooring, type A</td>
</tr>
<tr>
<td>D</td>
<td>Shared</td>
<td>Shared</td>
<td>Owned by the same farm</td>
<td>890,000</td>
<td>10</td>
<td>Removed every 3 to 5 days</td>
<td>Low floor, straight levels</td>
</tr>
</tbody>
</table>

TABLE 2. Farm sanitary monitoring program (only for laying flocks)*

<table>
<thead>
<tr>
<th>Check stage</th>
<th>Time or location of checked items</th>
<th>Source of sample</th>
<th>Test period</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>Adult chicken, before introduction of young hens</td>
<td>Environmental swab of laying house</td>
<td>After cleaning and disinfection</td>
</tr>
<tr>
<td>16</td>
<td>During laying period</td>
<td>Feces</td>
<td>Once every 2 months in the laying house</td>
</tr>
<tr>
<td></td>
<td>During laying period</td>
<td>Dust</td>
<td>Once every 3 months in the laying house</td>
</tr>
<tr>
<td></td>
<td>During laying period</td>
<td>10 kg or more of liquid eggs</td>
<td>Once per month in the laying house</td>
</tr>
<tr>
<td>17</td>
<td>Workers</td>
<td>Stool</td>
<td>Once per month</td>
</tr>
<tr>
<td>18</td>
<td>Water and environment</td>
<td>Potable water</td>
<td>Once every 3 months</td>
</tr>
<tr>
<td></td>
<td>Water and environment</td>
<td>Rat</td>
<td>When captured</td>
</tr>
</tbody>
</table>

* Twenty-five important monitoring points were set, beginning with the parent flock and feed plants through to the transportation of eggs. Of these 25 points, 16 to 18 points were conducted at the egg-collecting farm. Excluding these points, no S. enterica serovar Enteritidis isolation was observed during this study. All tests took place on an adult chicken farm.

RESULTS

S. enterica serovar Enteritidis isolation on four farms. As shown in Fig. 1 to 4, serovar Enteritidis was isolated from several broken (liquid) egg samples. In the first year, for farm A, S. enterica serovar Enteritidis was isolated from houses 4 (vaccinated; an MPN of 8/100 ml in May and <2 in August and September), 5 (nonvaccinated; an MPN of >1,600 in April), and 7 (nonvaccinated; an MPN of <2 in October) (Fig. 1). In the following year, serovar Enteritidis was isolated from houses 4 (vaccinated; an MPN of 7 in July and <2 in September) and 5 (vaccinated; an MPN of <2 in August and September). In the third year, serovar Enteritidis was isolated from houses 2 (vaccinated; an MPN of <2 in April and June) and 3 (vaccinated; an MPN of <2 in April and June). In the fourth year, S. enterica serovar Enteritidis was continuously isolated during a limited period in the summer; for this part of the study, a research team was established.

For farm B, in the first year, S. enterica serovar Enteritidis was isolated only from house 4 (vaccinated; an MPN of 8 in
May), but serovar Enteritidis was not isolated from any broken egg sample thereafter (Fig. 2).

In farm C, in the first year, *S. enterica* serovar Enteritidis was isolated from houses 2 (vaccinated; an MNP of 6 in October) and 3 (nonvaccinated; no MNP determined in August; an MPN of 39 in November) (Fig. 3). In the second year, serovar Enteritidis was isolated only from house 3 (an MPN of \( \geq 2 \) in November). *S. enterica* serovar Enteritidis was not isolated from any liquid egg sample thereafter.

For farm D, in the first year, *S. enterica* serovar Enteritidis was isolated from house 1 (nonvaccinated; an MPN of >1,600 in March; an MPN was not determined in September and November) (Fig. 4). *S. enterica* serovar Enteritidis was also isolated from a liquid egg sample from house 3 during the same period (the MPN was not determined). Although the data are not shown, a weak hen in house 1 (nonvaccinated) was autopsied after forced molting in February in the second year, and serovar Enteritidis was isolated from the chicken. *S. enterica* serovar Enteritidis was isolated from a broken egg sample of this flock in the second year, but serovar Enteritidis was not isolated from this farm thereafter.

Excluding results without bacterial counting, the MPNs (per 100 ml) were compared. In nonvaccinated flocks, the MPN was 2 in two samples, 39 in one sample, and >1,600 in two samples, with a mean (± the standard error) MPN of 674.8 ±

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**FIG. 1.** Feeding records and *Salmonella enterica* serovar Enteritidis isolates from egg samples in flocks of serovar Enteritidis-contaminated layer farm A. +, age (in days) of flocks at the beginning of each year; ○, egg samples negative for serovar Enteritidis; ●, egg samples positive for *S. enterica* serovar Enteritidis at <10 MPN/100 ml; ● ●, egg samples positive for serovar Enteritidis at >1,000 MPN/100 ml, with attached figures under circle symbols showing serovar Enteritidis bacterial counts (MPN/100 ml); ▼, replaced with new flock; ◄, out of flock; dashed line, nonvaccinated flock; thin black line, flock vaccinated once with serovar Enteritidis bacterin; thick black line, flock vaccinated twice with serovar Enteritidis bacterin.

**FIG. 2.** Feeding records and *S. enterica* serovar Enteritidis isolates from egg samples in flocks of serovar Enteritidis-contaminated layer farm B. For a description of the figure, see legend to Fig. 1.
In contrast, in vaccinated flocks, the MPN was <2 in eight samples, 6 in one sample, 7 in two samples, and 8 in one sample, with a mean (± the standard error) of 2.5 ± 0.1. In a comparison between the vaccinated and nonvaccinated flocks, the mean MPN of the vaccinated flock was 1/269.9 of that of the nonvaccinated flock, and the highest MPN was 1/100 or less, with no statistical difference. In a comparison of the isolation frequencies throughout the period from the first to the third year, the positivity rates were 25.0% (10/40, number of positive samples/number of tested samples) and 2.45% (14/571) in the nonvaccinated and vaccinated flocks, respectively (with a P value of <0.01 for the nonvaccinated flocks).

Relationship between house proximity to GP center and incidence of serovar Enteritidis isolates. There was a characteristic pattern regarding the relationship between serovar Enteritidis isolation and the location of the GP center. Excluding several exceptions, *S. enterica* serovar Enteritidis was isolated from samples taken from houses near the GP center. In contrast, serovar Enteritidis was not isolated from samples taken from the house farthest from the GP center. The common

![FIG. 3. Feeding records and *S. enterica* serovar Enteritidis isolates from egg samples in flocks of serovar Enteritidis-contaminated layer farm C. For a description of the figure legend, see legend to Fig. 1.](image)

![FIG. 4. Feeding records and *S. enterica* serovar Enteritidis isolates from egg samples in flocks of serovar Enteritidis-contaminated layer farm D. For a description of the figure, see legend to Fig. 1.](image)
point between farms A, B, and C was the type of bird house structure, raised flooring with a type A frame, whereas a low-floor, straight-cage structure was employed in farm D. Poultry feces were stocked until the flocks were sent to a chicken meat processing plant in farms A, B, and C, whereas feces were removed every 3 to 5 days in farm D, suggesting that the vector was mice.

Seasons with a high rate of *S. enterica* serovar Enteritidis isolation. Seasons with a high frequency of *S. enterica* serovar Enteritidis isolation were analyzed. The findings indicated that serovar Enteritidis was frequently isolated during warm seasons, and 90% of all isolates were isolated between April and October.

Summary. As the number of introductions of vaccinated flocks increased, the frequency of serovar Enteritidis isolation from the poultry environment decreased. No isolation of serovar Enteritidis was observed after the introduction of the third vaccinated flock. In raised poultry houses on a farm with an in-line GP center, the incidence of serovar Enteritidis isolation was presumed to be higher in poultry houses near the GP center (this may be due to serovar Enteritidis contamination mediated by rats). Finally, the incidence of serovar Enteritidis isolation from eggs is likely to increase during warmer seasons.

**DISCUSSION**

*Salmonella enterica* serovar Enteritidis is still one of the important food-borne diseases to be analyzed and measured, and shell eggs have been strongly suggested as a main source of *S. enterica* serovar Enteritidis outbreaks (1, 2, 5, 13). For example, in Japan (Ministry of Health, Labor, and Welfare, Infectious Agents Surveillance Report, August 2006), incidents of the outbreaks caused by *Salmonella* infections peaked at 825 with 11,888 patients, and the decreased tendency continued until 2007, during which time there were 126 outbreaks with 3,603 patients. The outbreaks caused by *Salmonella* infection are due to serovar Enteritidis infection at over 60%, and the source has been thought to have originated mainly from serovar Enteritidis-circulated layer farms.

In this study, we investigated the degree of prevention of *S. enterica* serovar Enteritidis contamination of eggs provided by the administration of inactivated serovar Enteritidis vaccine to laying hens. For serovar Enteritidis infection control in chickens, several sanitation programs, including HACCP (hazard analysis and CCP) programs, have been introduced in the last decade, and the serovar Enteritidis vaccine is employed by some programs but not by others. However, the use of the serovar Enteritidis vaccine to reduce the risk of contamination of shell eggs for long-term farming is not fully understood (2, 3, 4, 8, 10, 11, 14, 15), and the vaccine has been administered at an inadequate dose, half dose or lower, in some farms. These findings outline the current food culture concerning eggs and the state of inactivated-vaccine application in Japan.

In this study, *S. enterica* serovar Enteritidis was isolated from many samples from vaccinated and nonvaccinated flocks, but the isolation frequency and bacterial count decreased with the use of the *S. enterica* serovar Enteritidis vaccine. At the beginning of the study (with vaccinated and nonvaccinated flocks mixed), the number of isolated serovar Enteritidis (MPN) bacteria and the isolation frequency from liquid eggs obtained from vaccinated flocks were at least 100-fold and 10-fold lower (*P < 0.01*) than those from nonvaccinated flocks, respectively. In the fourth year, no *S. enterica* serovar Enteritidis contamination occurred, excluding the cross-contamination that occurred in an egg-breaking plant in the summer (data not shown). Regarding the time of isolation, 90% of isolation-positive samples were noted during warm seasons, from April to November. As for the type of houses, *S. enterica* serovar Enteritidis was isolated from broken egg samples derived from hens maintained in raised-floor houses near the GP center, whereas serovar Enteritidis was not isolated from samples derived from hens maintained in houses far from the GP center, suggesting that horizontal *S. enterica* serovar Enteritidis infection mediated by mice and human movement occurred.

*S. enterica* serovar Enteritidis contamination of eggs laid by vaccinated and nonvaccinated flocks was assessed. The serovar Enteritidis isolation test in the monitoring program used liquid egg samples prepared from 20 kg of eggs or more. It may have been possible that *S. enterica* serovar Enteritidis proliferated during the period between the collection of eggs in houses and the isolation test. However, more than 1,600 serovar Enteritidis cells per 100 ml (MPN) were isolated from the nonvaccinated flock, and this MPN exceeded the range in which food poisoning induced by serovar Enteritidis-contaminated eggs does not definitely occur. In other words, no food poisoning incidentally occurred during the study period, but it was possible. For the flocks treated with the inactivated serovar Enteritidis vaccine, the maximum MPN/100 ml was 8 (about 4 bacterial cells/egg, regarding the volume of the edible portion of an egg as 50 ml), showing that the risk of serovar Enteritidis-induced food poisoning was markedly reduced by the inactivated serovar Enteritidis vaccine. Even if the inhibitory effect of vaccination-induced antibodies transferred to egg yolk on cross-contamination during transport and cooking of eggs is negligible, the number of serovar Enteritidis bacteria necessary to cause food poisoning is considered to vary depending on age. The inhibitory effect of an inactivated serovar Enteritidis vaccine on the risk of serovar Enteritidis-induced food poisoning may be higher than the effects of reducing the isolation frequency to less than 1/9 and the number of *S. enterica* serovar Enteritidis bacteria to less than 1/100.

In conclusion, although it is not possible to estimate the likelihood that serovar Enteritidis vaccine-induced antibodies in egg yolk will reduce the risk of serovar Enteritidis-induced food poisoning during the transport and cooking of eggs, administration of an inactivated *S. enterica* serovar Enteritidis vaccine to domestic fowl reduced the number of serovar Enteritidis bacteria isolated from liquid egg samples and the isolation frequency to less than 1/100 (no statistical difference) and 1/10 (*P < 0.01*), respectively, showing that the vaccination was beneficial for reducing the risk of *S. enterica* serovar Enteritidis-induced food poisoning.

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