Assessing the Prevalence of Salmonella enterica in Poultry Hatcheries by Using Hatched Eggshell Membranes

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ABSTRACT Salmonella enterica causes a number of significant poultry diseases and is also a major pathogen in humans. Most poultry infected by Salmonella become carriers; infection may also be fatal, depending on the particular serovar and the age of the bird at infection. Younger birds are more susceptible to infection by Salmonella, so it is critical that hatcheries monitor birds. We developed a method to use hatched eggshell membranes (HEM) to assess contamination by Salmonella in poultry hatching cabinets and to evaluate the prevalence of Salmonella in a goose hatchery and rearing farm. Comparison of the Salmonella isolation rate in hatching cabinets using 3 sampling methods showed that the highest Salmonella contamination was detected in HEM, and that these results differed significantly from those obtained from fluff samples and cabinet swab samples ($P < 0.05$). Analysis of HEM was also used to evaluate Salmonella contamination in goose, chicken, and duck hatcheries. The lowest Salmonella-positive rate was found for the chicken hatchery, followed by the goose and the duck hatcheries ($P < 0.05$). Six serogroups of Salmonella were detected in the 3 hatcheries: A, B, C1, C2, D, and E. The distribution of these serogroups differed among the hatcheries. Salmonella serogroup C1 was the major serogroup found in geese, compared with serogroup B in chickens and ducks. However, Salmonella Typhimurium was dominant in 1 goose hatchery and also in geese from this hatchery that had been transferred to a farm. Antimicrobial susceptibility analysis showed that Salmonella Typhimurium strains isolated from the farm geese with diarrhea showed significantly higher resistance to doxycycline, colistin, sulfamethoxazole-trimethoprin, and cephalothin than those isolated from the hatchery ($P < 0.05$). Therefore, HEM as a detection target can be used to monitor Salmonella contamination in hatching cabinets and also be used to assess Salmonella prevalence in poultry hatcheries and rearing farms.

Key words: Salmonella enterica, hatched eggshell membrane, hatching cabinet, hatchery

INTRODUCTION More than 2,500 Salmonella serovars have been identified, most of which belong to the species Salmonella bongori and Salmonella enterica. Based on pathogenesis, S. enterica can be divided into 2 broad groups. Group 1 consists of a large number of serovars, including Salmonella enterica serovars Typhimurium and Enteritidis, which can cause paratyphoid in infected animals. This group can colonize the alimentary tract of food animals, and can also cause gastrointestinal disease in a broad range of hosts, including humans. Group 2 comprises a small number of serovars that cause systemic typhoid-like disease in a restricted range of host species. Examples include Salmonella pullorum and Salmonella gallinarum, which cause pullorum disease and fowl typhoid in poultry. In poultry, pullorum disease, fowl typhoid, and paratyphoid may result in a high mortality rate in young birds, but affected adults typically have a nonlethal chronic or carrier status. In a carrier bird, Salmonella always exists in the alimentary tract and the reproductive system, and can thus be transmitted to humans through contaminated eggs and meat. Humans consuming the contaminated eggs or meat may contract salmonellosis (Gast, 1997). Salmonella can be introduced into eggs by both vertical and horizontal transmission paths. In poultry, Salmonella can persist in both the spleen and the reproductive tract for a long time. During birds’ sexual maturation, Salmonella colonizes both the ovary and the oviduct of hens, and then infects eggs directly (Cox et al., 2000; Wigley et al., 2001). Among the serovars, both Salmonella Typhimurium and Salmonella Enteritidis can bind to isthmal secretions and be incorporated into the egg during formation; the Salmonella is localized on the inner side of the eggshells, where it is protected from the antimicrobial factors in egg white (Buck et al., 2003). In addition, the outer

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surface of the eggshell may be contaminated by *Salmonella* from feces. Moreover, *Salmonella* can also efficiently penetrate into the interior of eggs, especially in incubators and hatcheries (Cason et al., 1993; Bailey et al., 1994; Schoeni et al., 1995). Hatchery-acquired *Salmonella* substantially reduces the effectiveness of subsequent competitive exclusion treatments to prevent *Salmonella* from colonizing young chicks. Control of *Salmonella* contamination in hatchings is critically important for controlling *Salmonella* infection in broilers (Bailey et al., 1998). Thus, development of an efficient method to monitor *Salmonella* contamination in hatchings would be beneficial for *Salmonella* surveillance and control. Bailey et al. (1996) determined the effect of hatchling contamination treatment on *Salmonella* contamination by analyzing hatched eggshell fragments. In this study, hatched eggshell membranes (HEM) were collected and assayed for *Salmonella* contamination to assess hatchery contamination. The association of *Salmonella* prevalence in hatcheries with *Salmonella* prevalence in a rearing farm was also examined.

**MATERIALS AND METHODS**

**Sample Collection**

In the study, 3 tests (denoted test A, test B, and test C) were designed. In test A, *Salmonella* contamination in a hatchin cabinet was evaluated using HEM as the detection target. Five trials were performed in goose hatcheries. Each trial was performed using 3 to 6 hatchling cabinets, with 10 hatched eggshell halves, 1 fluff sample, and 1 cabinet swab per cabinet. When any sample in each cabinet tested positive, this cabinet was counted as positive. In test B, HEM analysis was used to evaluate *Salmonella* prevalence in goose, duck, and chicken hatcheries. The hatched eggshell halves were taken from 2 goose hatcheries (denoted GA and GB), 2 duck hatcheries (DA, DB), and 3 chicken hatcheries (CA, CB, CC). It should be noted that formaldehyde fumigation of eggs was done in chicken hatcheries but not in the other hatcheries. In test C, after *Salmonella Typhimurium* was identified as the dominant *Salmonella* serotype in GA, a goose farm rearing the goslings from GA was visited and cloacal swabs were collected from geese ~4 wk old with diarrhea. The association of *Salmonella* contamination in the poultry hatchery with *Salmonella* infection in the associated rearing farms was investigated.

**Bacterial Media and Antiseras**

All media and antisera used were purchase from Difco & BBL of Becton Dickinson and Company (Franklin Lakes, NJ). Gram-negative broth (GN, Difco 0486) and Rappaport-Vassiliadis broth (RV, Difco 1858) were used to enrich gram-negative bacteria and *Salmonella*. However, cloacal swabs were cultured in selenite CYS (SC, Difco 0687) broth. Xylose Lys deoxycholate agar (XLD, Difco 0788), sugar iron agar (TSI, Difco 0265), and Lys iron agar (LIA, Difco 0849) were used to differentiate *Salmonella* from other bacteria. *Salmonella* isolates were routinely grown on brain heart infusion agar (BHIA, Difco 0418) plates. Further, O antiserum (O antigen: 1, 4, 5, 12, Difco 2948) and H antiserum (H antigen: i, 1, 2, 7; Difco 2824, 265, 2266, 2477) were used to identify the serotype of each *Salmonella* isolate.

**Isolation of Salmonella from Hatchery Samples**

Samples from different sources were treated separately. Collected HEM were separated from the eggshell halves with sterile forceps, and the fluff samples were immersed in aseptic distilled water. Next, the HEM and the fluff samples were each put in tubes containing 5 mL of GN broth. The inner wall surfaces of hatchling cabinets were sampled by vigorously swabbing an area of approximately 30 cm² with dragging moist swabs that had been autoclaved within bottles of GN broth. The swabs were returned to the bottles after sampling. The above-mentioned samples were incubated at 37°C for 24 h.

If the initial isolation was *Salmonella*-negative, a delayed secondary enrichment was performed as described by Waltman and Mallinson (1995). The negative broth was kept at room temperature for 5 to 7 d. One milliliter of the broth was then transferred into 9 mL of RV broth and incubated at 37°C for 24 h. Selectively enriched samples from GN and RV broth were streaked onto XLD plates. These plates were incubated at 37°C for 24 h, and typical *Salmonella* colonies were selected as recommended by the manufacturer (Difco). In addition, at least 2 colonies of each plate were positively identified by TSI and LIA.

Serology was performed using *Salmonella* O and H antisera and *Salmonella* grown on fresh (18 to 24 h) BHI broth. All isolates were serogrouped by the slide agglutination test with the use of O antiserum to differentiate serogroup B, and were serotyped by the tube agglutination test with the use of H antiserum to identify the Typhimurium serovar.

**Isolation of Salmonella from Cloacal Swabs**

Cloacal swabs taken from 4-wk-old geese with diarrhea were transferred to 9 mL of SC broth and incubated at 37°C for 24 h. The methods used to identify typical *Salmonella* colonies were as described above.

**Antimicrobial Susceptibility Test**

The antimicrobial susceptibility test was performed by a standard disk diffusion method (National Committee for Clinical Laboratory Standards, 2000). The antimicrobial agents used were as follows: chloramphenicol (30 μg), trimethoprim/sulfamethoxazole (25 μg), tetracycline (30 μg), doxycycline (30 μg), ampicillin (10 μg), amoxicillin/clavulanic (30 μg), spectinomycin (100 μg), colistin (10 μg), gentamicin (10 μg), cephalothin (30 μg), flumequine (15 μg), and enrofloxacin (5 μg). Susceptible
and resistant strains were named according to the criteria suggested by the National Committee for Clinical Laboratory Standards (2000). If the diameter of inhibition zones yielded by a tested strain was larger than that of the resistant strain, this strain was reported as a resistant strain. *Escherichia coli* ATCC 25992 was used as a reference strain for the disc control.

**Statistical Analysis**

The chi-squared test was used to analyze differences in the *Salmonella* isolation rate among samples isolated from HEM, fluff samples, and cabinet swabs as well as in *Salmonella* contamination among the poultry species. In addition, differences in the resistance status of *Salmonella* Typhimurium isolates among samples collected from HEM of GA and the diarrheal geese originating from GA were analyzed.

**RESULTS**

**Evaluation of HEM as the Detection Target for Monitoring Salmonella Contamination**

To establish suitable methods to monitor *Salmonella* contamination in the hatchery cabinets, samples were collected from 3 different sources: HEM, fluff, and cabinet swabs. The *Salmonella* isolation rate ranged from 27.3 to 36.4% without any significant difference among the 3 sampling methods (Table 1). When any sample in each cabinet tested positive, this cabinet was counted as positive. Therefore, the positive *Salmonella* isolation rate was significantly different among sampling methods by using cabinet as a unit (Table 2). That is, the *Salmonella* contamination rate of HEM was significantly higher than that of the fluff and cabinet swab (P < 0.05; Table 2), suggesting that HEM may be a more sensitive marker to evaluate the *Salmonella* contamination of hatching cabinets.

**Prevalence of Salmonella in Chicken, Duck, and Goose Hatcheries**

Having established that HEM analysis was an effective method for detecting *Salmonella* contamination, we then used this method to evaluate and compare contamination in chicken, duck, and goose hatcheries. In test B, significant differences in *Salmonella* contamination were observed in HEM samples from the 3 types of hatcheries.

**Prevalence of Salmonella in a Goose Hatchery and Its Associated Rearing Farm**

*Salmonella* Typhimurium was dominant in both hatchery GA and its associated rearing farm. Four-week-old geese originating from GA showed signs of diarrhea, and infection by *Salmonella* Typhimurium was observed in their alimentary tracts. The percentage of *Salmonella* Typhimurium in *Salmonella* was 81.25% (26/32), and the

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**Table 1. Isolation of *Salmonella enterica* of the samples from 3 different sources in goose hatcheries**

<table>
<thead>
<tr>
<th>Sources</th>
<th>Samples tested</th>
<th>Positive samples</th>
<th><em>Salmonella</em>-positive rate1 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hatched egg membranes</td>
<td>220</td>
<td>63</td>
<td>28.6</td>
</tr>
<tr>
<td>Fluff samples</td>
<td>22</td>
<td>8</td>
<td>36.4</td>
</tr>
<tr>
<td>Cabinet swabs</td>
<td>22</td>
<td>6</td>
<td>27.3</td>
</tr>
</tbody>
</table>

1*Salmonella*-positive rate = (positive samples/samples tested) × 100%.

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**Table 2. Isolation of *Salmonella enterica* from the hatched eggshell membranes, fluff samples, and cabinet swab samples in different hatch cabinets in goose hatcheries**

<table>
<thead>
<tr>
<th>Sources</th>
<th>Hatched eggshell membranes</th>
<th>Fluff samples</th>
<th>Cabinet swabs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial</td>
<td>66.72 (2/3)</td>
<td>33.3 (1/3)</td>
<td>33.3 (1/3)</td>
</tr>
<tr>
<td>First</td>
<td>100.0 (6/6)</td>
<td>50.0 (3/6)</td>
<td>66.7 (4/6)</td>
</tr>
<tr>
<td>Second</td>
<td>100.0 (5/5)</td>
<td>0 (0/5)</td>
<td>0 (0/5)</td>
</tr>
<tr>
<td>Third</td>
<td>60.0 (3/5)</td>
<td>20.0 (1/5)</td>
<td>0 (0/5)</td>
</tr>
<tr>
<td>Fourth</td>
<td>100.0 (3/3)</td>
<td>100.0 (3/3)</td>
<td>33.3 (1/3)</td>
</tr>
<tr>
<td>Fifth</td>
<td>86.36a (19/22)</td>
<td>36.36b (8/22)</td>
<td>27.27b (6/22)</td>
</tr>
</tbody>
</table>

a,bData in the same row with different superscripts are different (P < 0.05).

1*Salmonella*-positive rate = (positive cabinets/cabinets tested) × 100%.

2When any hatched eggshell membrane sample tested positive in each cabinet, the tested cabinet was counted as positive.

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**Table 3. The prevalence of *Salmonella* in poultry hatcheries assessed by *Salmonella* isolated from hatched eggshell membranes**

<table>
<thead>
<tr>
<th>Trial</th>
<th>Duck</th>
<th>Goose</th>
<th>Chicken</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>60 (18/30)</td>
<td>24.7 (41/166)</td>
<td>13.33 (6/45)</td>
</tr>
<tr>
<td>Second</td>
<td>20 (6/30)</td>
<td>24.46 (68/278)</td>
<td>0 (0/60)</td>
</tr>
<tr>
<td>Third</td>
<td>—</td>
<td>—</td>
<td>0 (0/60)</td>
</tr>
<tr>
<td>Total</td>
<td>40P (24/60)</td>
<td>24.55P (109/444)</td>
<td>4.44P (6/135)</td>
</tr>
</tbody>
</table>

a,bData in the same row with different superscripts are different (P < 0.05).

1*Salmonella*-positive rate = (positive samples/samples tested) × 100%.
Salmonella Typhimurium isolation rate was 22.61% (26/115). The antibiotic susceptibility test was performed for the isolates from GA and from the rearing farm. Salmonella Typhimurium isolates from geese with diarrhea showed significantly higher resistance to doxycycline, colistin, sulfamethoxazole-trimethoprin, and cephalothin than isolates from GA (P < 0.05; Figure 1).

**DISCUSSION**

Hatching cabinets contaminated by Salmonella are correlated with outbreaks of salmonellosis in poultry. The assessment of Salmonella contamination in hatching cabinets is critical for the control of Salmonella infection in broilers (Bailey et al., 1998). In a hatchery, there is no difference in the rate of isolation of Salmonella from eggshells vs. day-of-hatch chick rinse samples (17 vs. 21%), but there is a lower rate of Salmonella isolation from intestinal samples (Bailey et al., 1994). A sensitive detection target is important for a correct assessment. Hence, the intestinal samples from day-of-hatch are not appropriate for Salmonella detection in a hatchery. In addition, the need to monitor every hatching cabinet between replications is critical for the evaluation of Salmonella contamination in a hatchery. An outbreak of Salmonella Typhimurium occurred in 1- to 2-wk-old broiler flocks. The bacteria were also isolated from the breeder’s source flock, and the disease was thought to be transmitted from the eggs at hatching. However, the offspring from some hatches were not infected (Mario, 1990). Salmonella infection rates of growing chickens do not differ among broiler hatcheries, but do show significant differences among replications within hatching cabinets (Bailey et al., 1994). This study evaluated Salmonella contamination from different sources (Tables 1 and 2). Although the Salmonella isolation rates were similar among the 3 sample sources, HEM was more sensitive, more reliable, and the easiest method for analyzing the Salmonella contamination in hatching cabinets while using the total cabinet number to calculate the Salmonella contamination rate (Table 2). Eggshells and associated membranes have been found to be a frequent site of Enteritidis and Typhimurium contamination after naturally and experimentally induced Salmonella infections (Cason et al., 1993; Bailey et al., 1994; Limawong-
pranee et al., 1999). *Salmonella* has been isolated from cecal samples of 7-d chicks near isolation from their eggshells (Bailey et al., 1994, 1996, 1998). Bailey et al. (1996) used eggshell fragments to evaluate the spread of *Salmonella Typhimurium* in poultry hatching cabinets. Here, we separated the inner membranes from hatched egg halves to obtain cultures that avoided the contamination on eggshells during sampling. In other words, the HEM method detected contamination only from the inner surface of the eggshell. Therefore, HEM are a good detection target for monitoring *Salmonella* contamination.

Using HEM analysis to compare the prevalence of *Salmonella* in poultry hatcheries, we obtained different levels and serogroup distributions among ducks, geese, and chickens (Tables 3 and 4). Positive *Salmonella* isolation rates were lowest in chickens, medial in geese, and highest in ducks. According to our observations, hatching cabinet sanitation plays a major role in controlling infection. The best sanitation, in particular formaldehyde fumigation of eggs, was found in the chicken hatcheries. In contrast, sanitation in the duck hatcheries was the worst. In Taiwan, the isolation rate of *Salmonella* spp. was 4.6% for ducks and 20% for duck farms, and the highest isolation rate was found in ducklings under 2 wk of age (Tsai and Hsiang, 2005).

*Salmonella Typhimurium*, as a broad-host-range pathogen, may be a major pathogen of salmonellosis in ducks and geese. Most cases (93%) of salmonellosis in ducks were caused by *Salmonella Typhimurium* (Price et al., 1962). In market-ready geese, 47% of *Salmonella*-positive flocks were infected by *Salmonella Typhimurium* (Mann and McNabb, 1984). In addition, *Salmonella Typhimurium* caused salpingitis in mature ducks and geese (Bisgaard, 1995). In ducks and geese, the disease was thought to be transmitted from the eggs at hatching as chickens. Here, we found that *Salmonella Typhimurium* was the dominant species in 1 of 2 goose hatcheries (GA) and on the goose farm in geese with diarrhea that originated from GA. The *Salmonella Typhimurium* strains isolated from the goose farm were multidrug resistant and specifically showed significantly higher resistance to doxycycline, colistin, sulfamethoxazole-trimethoprin, and cephalothin than isolates from GA (Figure 1). Therefore, *Salmonella Typhimurium* appears to be a causative agent of diarrhea in goslings, and the higher drug resistance suggests the potential application of these antibiotics in animal feed.

In conclusion, this study demonstrates the feasibility and value of using HEM analysis to examine the prevalence of *Salmonella* in poultry hatcheries and farms. This method is superior to other methods because of the easy collection, high sensitivity, and low contamination during sampling. We showed that use of this method in hatcheries to monitor infection could help identify affected animals before they go on to farms.

### REFERENCES


