IMMUNOLOGY, HEALTH, AND DISEASE

Comparison of the Association of Age with the Infection of *Salmonella* and *Salmonella enterica* Serovar Typhimurium in Pekin Ducks and Roman Geese

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ABSTRACT Nontyphoid *Salmonella* have a broad host range in poultry and mammals, and serovar Typhimurium is a threat to public health. In this study, normal and sick ducks and geese were collected from 12 farms in Taiwan to investigate the age-associated infection of *Salmonella* and *Salmonella enterica* Serovar Typhimurium in Roman geese (*Anser anser domesticus*) and Pekin ducks (*Anas platyrhynchos domesticus*). In normal birds, the prevalence of *Salmonella* differed between species, and with age [e.g., 1-wk group, 37.5% (30/80) for ducks and 5.2% (6/116) for goslings (*P* < 0.05) vs. 4-wk group, 1% (1/96) for ducks and 12.1% (21/174) for geese]. *Salmonella Typhimurium* was identified from the visceral organs of moribund young geese suffering with colibacillosis and rieperellosis isolated from 2 goose farms (farm A and B, respectively). At farm B, 22.9% (27/118) of 4-wk geese with diarrhea were *Salmonella Typhimurium*-positive compared with 4.6% (8/174) of 4-wk normal geese. All *Salmonella* Typhimurium strains except one harbored a 94.7-kb virulence plasmid. Subcutaneous injection of *Salmonella Typhimurium* isolate 91NGL1 resulted in different clinical signs and pathogenesis between ducks and geese. In addition, the mean infectivity dose ratios of ducks to geese were 3.2 and 85.0 for 4- and 12-d birds, respectively, suggesting that goslings were more susceptible to *Salmonella Typhimurium*, and resistance to *Salmonella Typhimurium* increased with age, especially for ducks. Therefore, *Salmonella Typhimurium* infection should be more common in goose farms than in duck farms, especially in the younger birds.

Key words: *Salmonella*, *Salmonella Typhimurium*, age, infection, waterfowl

INTRODUCTION

The known 2,500-plus *Salmonella* serovars, all but *Salmonella* Bongori belong to the species *Salmonella enterica*, which can be divided into 2 categories (Gast, 1997). One group that causes paratyphoid in infected animals consists of a large number of serovars including the *S. enterica* serovars Typhimurium and Enteritidis. As broad-host-range pathogens, this group can colonize the alimentary tract and cause gastrointestinal disease in animals and humans. The occurrence of salmonellosis is determined by host factors, including animal species and age, and pathogenic factors that include the bacterial dose, infection route, serovars, and strains (Poppe, 2000). A severe mortality in 1- to 2-wk-old infected broilers was found to be associated with *Salmonella Typhimurium* (Padron, 1990), and several virulence factors play a major role in mortality [e.g., 94.7-kb virulence plasmid (pSTV) encoding *spv* operon is involved in *Salmonella Typhimurium* survival in macrophage facilitating systemic infection (Hoeft et al., 1989)]. Another group that causes systemic typhoid-like disease in a restricted range of host species comprises a small number of serovars, including *Salmonella Pullorum* and *Salmonella Gallinarum*, which cause pullorum disease and fowl typhoid, respectively, in poultry and may result in a high mortality rate in young chicks and poultry (Gast, 1997). However, ducks only experience a short subclinical symptom period after *S. Pullorum* infection (Buchholz and Fairbrother, 1992). Unlike the acute disease seen in young birds after infection, adult birds typically have a nonlethal chronic or carrier status, especially in ducks (Henry, 2000). In a carrier bird, paratyphoid salmonellae may exist in the alimentary tract and the reproductive system and can be transmitted to humans through contaminated eggs and meat. In ducks reared in the field or in open houses, *Salmonella* in the intestinal tract may contribute to the spread of *Salmonella*...
infection (Bisgaard, 1981). As one of the most predominant Salmonella serotypes that affect poultry, Salmonella Typhimurium has been found in 60% of carcass rinses and 18.4% cloacal swabs in market-ready geese (Mann and McNabb, 1984) and is involved in 93% of the Salmonella infections in ducklings. An infected duckling may be dehydrated, emaciated, and experiencing difficulty breathing, and dying from opisthotonus (Price et al., 1962). Moreover, in mixed infection with other pathogens, Salmonella Typhimurium can cause septicaemia in young ducklings (Henry, 2000) and chronic salpingitis in geese and ducks (Bisgaard, 1995). Recently, our group of investigators identified Salmonella Typhimurium in the alimentary tracts of 4-wk-old diseased geese (Chao et al., 2007), and the association of Salmonella Typhimurium infection in goslings with its hatchery (Yu et al., 2008). The objective of this study was to evaluate the effect of age with Salmonella and Salmonella Typhimurium in waterfowl.

MATERIALS AND METHODS

Sample Collection

The tested samples taken for salmonellae culture were from 12 farms in Taiwan including 6 normal goose farms, 4 normal duck farms, and 2 diseased goose farms (farm A and farm B) where animals suffered from colibacillosis and riemerelliosis. The moribund and culled goslings from farm A and B were examined by necropsy and microbiology. Then Salmonella was isolated from each animal’s duodenum, liver, heart, brain, blood, and lung. At farms A and B, 5 to 10% of geese exhibited diarrhea, growth retardation, twisted necks, lameness, dehydration, and labored breathing, and some were moribund. In normal or nonaffected farms, the rates of culling were under 1%. Cloacal swabs were taken from 1-wk-old and 4-wk-old birds at the normal farms and from 4-wk-old geese with diarrhea at farm B.

Bacterial Media and Antisera

All media and antisera were purchased from Difco & BBL of Becton Dickinson and Company (Franklin Lakes, NJ). The samples of viscer al organs were streaked on MacConkey’s agar (Difco 0075) plates. Selenite cysteine (SC, Difco 0687) broth and Rappaport-Vassiliadis broth (RV, Difco 1858) were used to enrich gram-negative bacteria and Salmonella in the cloacal swabs. Xylose lysine deoxycholate agar (XLD, Difco 0788), triple sugar iron agar (TSI, Difco 0265), and lysine iron agar (LIA, Difco 0849) were used to differentiate Salmonella from other bacteria. Salmonella isolates were routinely grown on brain heart infusion agar (Difco 0418) plates. Further characterization of recovered salmonellae was performed using O antiserum (Difco 2947, 2948, 2949, 2950, 2951, 2952, 1953, 2954, 2973, and 3029) and H antiserum (H antigen: i; 1; 2; 7, Difco 2824; 265; 2266; 2477).

Isolation of Salmonella from Visceral Organ and Cloacal Swabs

The samples were taken aseptically with sterile swabs from each tissue and then streaked onto MacConkey’s and XLD plates. These plates were incubated at 37°C for 24 h, and typical Salmonella colonies were selected as recommended by the manufacturer (Difco). At least 2 colonies of each plate were positively identified by TSI and LIA. Cloacal swabs taken from birds were transferred into 9 mL of SC broth and 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). After incubating at room temperature for 5 to 7 d, 1 mL of the negative broth was then transferred into 9 mL of RV broth and incubated at 37°C for 24 h. Selectively enriched samples from SC 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). Furthermore, at least 2 colonies on each plate were positively identified by TSI and LIA. All isolates were serogrouped by the slide agglutination test using O antiserum and serotyped to identify Salmonella Typhimurium by the tube agglutination test using H antisera.

Identification of Virulence Plasmid of Salmonella Typhimurium by DNA–DNA Hybridization

Plasmid DNA of Salmonella Typhimurium was subjected to gel electrophoresis, as described previously (Kado and Liu, 1981). The plasmid DNA was then transferred onto a Zeta-Probe membrane (Bio-Rad, Hercules, CA) as recommended by the manufacturer. The virulence plasmid was further confirmed by Southern blotting hybridization with a probe of spvC DNA fragment amplified by PCR, as described previously (Chiu and Ou, 1996; Southern, 1975). PCR products of spvC were purified by Wizard SV gel and the PCR Clean-up System (Promega, Madison, WI), labeled with digoxigenin-11-dUTP (Roche, Indianapolis, IN), and used as the DNA probe. After hybridization of DIG-labeled probe and addition of anti-DIG antibody conjugated with peroxidase, the membrane was reacted with CSPD (Roche) chemiluminescent substrate and then exposed to x-ray film. If both PCR and DNA hybridization were positive for a Salmonella Typhimurium isolate, it was concluded that the isolate harbored the virulence plasmid.

Determination of LD50 and ID50 Value of Salmonella Typhimurium Isolate 91NGL1 to Ducks and Geese

The method of determining LD50 and ID50 values was modified from the report of Ishibashi and Arai (1996). The tested Roman geese (Anser anser domesticus) and Pe-
<table>
<thead>
<tr>
<th>Bird</th>
<th>Prevalence of each serogroup, 1 % (no.)</th>
<th>Prevalence of Salmonella, 2 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sick</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 wk goose</td>
<td>B: 42.9 (9/21) C1: 28.6 (6/21) D: 28.6 (6/21) E: — G: —</td>
<td>12.1% (21/174)</td>
</tr>
<tr>
<td>1 wk duck</td>
<td>B: 3.3 (1/30) C1: 96.7 (29/30) D: — E: — G: —</td>
<td>37.5% (30/80)</td>
</tr>
<tr>
<td>4 wk duck</td>
<td>B: — C1: 100 (1/1) D: — E: — G: —</td>
<td>1.0% (1/96)</td>
</tr>
</tbody>
</table>

1Isolation rate = (number of each serogroup Salmonella /total number of Salmonella), %.

2Prevalence = (total number of Salmonella /total sample number), %.

In normal birds, the prevalence of Salmonella with different superscripts differed significantly (P < 0.05).
Table 3. The percentage of the main clinical signs in each group of ducks and geese after subcutaneous injection with Salmonella Typhimurium isolate 91NGL1

<table>
<thead>
<tr>
<th>Clinical sign</th>
<th>4-d-old group, %</th>
<th>12-d-old group, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Duck</td>
<td>Goose</td>
</tr>
<tr>
<td>Lameness</td>
<td>15.5 (11/71)</td>
<td>0* (0/71)</td>
</tr>
<tr>
<td>Torticollis</td>
<td>0</td>
<td>0 (0/71)</td>
</tr>
<tr>
<td>Stunting</td>
<td>2.82 (2/71)</td>
<td>5.6 (4/71)</td>
</tr>
<tr>
<td>Death within 48 h PF1</td>
<td>22.5 (16/71)</td>
<td>36.6* (26/71)</td>
</tr>
<tr>
<td>Death within 24 h PI</td>
<td>7.04 (5/71)</td>
<td>23.9 (17/71)</td>
</tr>
</tbody>
</table>

1Percentage was calculated by the total number of birds developing clinic sign over the total number of 4 subcutaneously injected groups in each age category for each species.

2PF = postinoculation.

*The percentage of clinical signs differed significantly (P < 0.05) between goose and duck at the same age.

RESULTS AND DISCUSSION

The Prevalence of Salmonella and Salmonella Typhimurium in the Duck and Goose Farms

Prevalence of Salmonella differed between species, their age, and infectious condition of waterfowls (Table 1). In normal birds, the highest prevalence of Salmonella was observed in 1-wk-old ducklings with a 37.5% (30/80) isolation rate compared with 1-wk-old goslings (5.2%, 6/116) or 4-wk-old ducks (1%, 1/96), respectively (P < 0.05).

In comparing Salmonella prevalence among the 4-wk-old animals, the rate in ducks (1%, 1/96) was significantly lower than that in geese (12.1%, 21/174; P < 0.05). A similar prevalent trend with a rate of 24% was previously reported for 2-wk-old ducklings with only 0.5% found in 4-wk-old ducks (Tsai and Hsiang, 2005), and the highest Salmonella isolations reported by Price et al. (1962) were found in ducks less than 3 wk of age.

Further analysis in the current work revealed that the prevalence of Salmonella in 1-wk-old goslings was significantly lower than that of 4-wk-old goslings (P = 0.05; Table 1). This rapid decrease in Salmonella incidence associated with age was observed for ducks but not for geese (Table 1). In adult ducks, Salmonella incidence was 8.7% for cecal samples in Vietnam (Tran et al., 2004), a rate higher than that reported in this study (1.0%, Table 1) and the 0.5% reported by Tsai and Hsiang (2005) for 4-wk-old ducks in Taiwan. These data suggest that waterfowl species and the variable environmental conditions of different farms influence Salmonella incidence.

In the present study, diverse Salmonella serogroups (B, C1, C2, D, E, and G) were isolated from the sick geese, but not from the healthy geese and ducks (Table 1). Although serogroup C1 Salmonella was the most prevalent serogroup in healthy ducklings in this study, serogroup B Salmonella was the most common serogroup found in duck hatcheries by Chao et al. (2007). Data in the current study suggests that ducklings are sensitive to serogroup C1 Salmonella. Among serogroup B Salmonella, which was the most common serogroup in healthy and sick geese, Salmonella Typhimurium was the predominant serovar isolated from birds necropsied on farm A and farm B, with isolation rates of 50% (3/6) and 37.6% (6/16), respectively (Table 2).

With its high ability to invade intestinal and lymphoid tissues of the bursa and cecum (Aabo et al., 2002, Chaffee et al., 2003) and its broad host range, Salmonella Typhimurium has been identified from young chickens, ducklings, and goose throughout the world (Price et al., 1962; Mann and McNabb, 1984; Padron, 1990; Bisgaard, 1995; Henry, 2000; Chao et al., 2007). In addition, it causes chronic salpingitis in geese (Bisgaard, 1995) as well as invasion of the visceral organs of 2- to 5-wk-old geese with mixed infection. Salmonella Typhimurium may not be the primary cause of a disease in young ducklings; however, it might cause septicemia with complications with other infectious diseases (Henry, 2000). When complicated with colibacillosis and riemerellosis, Salmonella

Table 4. The tested animal number, the percentage of mortality and infectivity at 10-d postinoculation, and the colony-forming units (cfu) of the original bacterial dose in each species

<table>
<thead>
<tr>
<th>Bird</th>
<th>Age (cfu of OBD1)</th>
<th>Mortality, % (dead no./test no.)</th>
<th>Infectivity, % (infected no./test no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B2</td>
<td>C</td>
</tr>
<tr>
<td>Goose</td>
<td>4-d (3.2 × 106)</td>
<td>94 (17/18)</td>
<td>83 (15/18)</td>
</tr>
<tr>
<td></td>
<td>12-d (1.5 × 109)</td>
<td>100 (13/13)</td>
<td>73 (11/15)</td>
</tr>
<tr>
<td>Duck</td>
<td>4-d (9.5 × 109)</td>
<td>95 (18/19)</td>
<td>37 (7/19)</td>
</tr>
<tr>
<td></td>
<td>12-d (4.4 × 1010)</td>
<td>67 (14/21)</td>
<td>45 (9/20)</td>
</tr>
</tbody>
</table>

1The original bacterial dose (OBD) was used for treatment A and was 3.2 × 10⁶, 1.5 × 10⁹, 9.5 × 10⁹, and 4.4 × 10¹⁰ for goose 4, and 12, and duck 4, and 12 d, respectively. Treatments B, C, and D were serial dilutions (10⁻¹, 10⁻², and 10⁻³) of this original bacterial dose, respectively, and treatment E was the unchallenged (control) treatment.

2The B, C, and D groups were subcutaneously injected with OBD 10-fold serially diluted to 10⁻¹, 10⁻², and 10⁻³, respectively, and the E group was injected with PBS.
Typhimurium was associated with septicemia and diarrhea in young geese in farm A and farm B in the current work (Table 2). In normal farms, 0.86 and 4.6% rates of subclinical infection with Salmonella Typhimurium were found in the alimentary tract of 1- and 4-wk-old geese, respectively (Table 2), suggesting that carriers may carry this intracellular pathogen surviving in immune cells without any symptoms of disease in the duck and goose. Therefore, a study using high doses of Salmonella Typhimurium was conducted to evaluate the histopathological changes and lethality of Salmonella Typhimurium on duck and goose.

**Different Response of Duck and Goose to Salmonella Typhimurium Isolate 91NGL1**

Characterization of Salmonella Typhimurium. Virulence plasmids are involved in survival of Salmonella Typhimurium in macrophage that facilitate bacterial systemic infection (Hoertt et al., 1989), and all examined Salmonella Typhimurium isolates harbored a 94.7-kb virulence plasmid (Table 2), except one strain in a 1-wk-old goose from a normal goose farm. This finding confirms an earlier report that most human Salmonella Typhimurium isolates harbor pSTV (Chiu et al., 2006), suggesting that this plasmid might be associated with infection in geese. Therefore, we further used isolate 91NGL1 with pSTV to infect young geese and ducks to observe the development of clinical signs after Salmonella Typhimurium infection.

**Different Pathological Effects of Salmonella Typhimurium on Duck and Goose.** After injections with isolate 91NGL1, the symptoms and mortality differed between ducks and geese (Table 3). Lameness was seen in sick ducks, but not in sick geese and reversely higher mortality was found in geese at 24 and 48 h postinoculation (Table 3). The major gross lesions of the dead birds

<table>
<thead>
<tr>
<th>Age</th>
<th>LD$_{50}$ ($\times 10^7$)</th>
<th>ID$_{50}$ ($\times 10^7$)</th>
<th>LD$<em>{50}$/ ID$</em>{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Duck (D)</td>
<td>Goose (G)</td>
<td>D/G$^1$</td>
</tr>
<tr>
<td>4 d</td>
<td>113.8</td>
<td>21.8</td>
<td>5.3</td>
</tr>
<tr>
<td>12 d</td>
<td>1,779.5</td>
<td>23.3</td>
<td>76.5</td>
</tr>
</tbody>
</table>

$^1$D/G represents the LD$_{50}$ of duck over the LD$_{50}$ of goose and the ID$_{50}$ of duck over the ID$_{50}$ of goose.
included enlarged and congested livers, spleens, and hearts. In subacute cases, congested lungs and thickened pericardia were found (Figure 1). Some survivors showed leg weakness, diarrhea, and poor growth; however, most survivors were clinically normal. The percentages of mortality and infectivity at 10 d postinoculation with different dose in the geese and ducks were presented in Table 4, and these data were used to determine LD50 and ID50. The distribution of Salmonella Typhimurium varied among tissues. Salmonella Typhimurium was isolated from liver and heart for all the birds that died during the testing period. For 16 geese survivors at 10 d postinoculation, Salmonella Typhimurium was also isolated from the visceral organs and the isolation rates were 50.0% for gallbladder, 31.3% for lung, 31.3% for brain, 12.5% for bone marrow, and 6.3% for liver. However, no Salmonella Typhimurium was isolated from heart samples. Salmonella Typhimurium caused histopathological changes including perihepatitis (6/10 for geese vs. 1/6 for ducks, Figure 1A); fibrinoid pericarditis (9/10 for geese vs. 2/6 for ducks, Figure 1D); and air sacculitis (8/10 for geese vs. 0/6 for ducks) to geese. Riemerella anatipestifer and Coenonia anatina can also cause fibrinous serositis in the liver, air sac, and heart to the ducks and geese with acute or chronic septicemia (Vandamme et al., 1999; Huang et al., 2002). Therefore, earlier and rapid differentiation of these various infectious pathogens should be made in goose farms. In addition, the ID50 ratios of ducks/geese were 3.2 and 85.0 for 4-d and 12-d birds, respectively (Table 5). These data indicate that goslings are more susceptible to Salmonella enterica and that the resistance to Salmonella Typhimurium was associated with an increase in age, especially a rapid increase in such resistance for the ducklings.

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