**Introduction**

Human infections by foodborne bacterial pathogens are a critical public health problem. Among the foodborne bacterial pathogens that cause human infections in the United States, the main source of Salmonella Enteritidis (SE) diseases associated with the consumption of raw or undercooked chicken eggs. Salmonella Enteritidis is the only serotype that routinely contaminates eggs. The transovarian transmission of SE and subsequent contamination of the eggs before egg shell formation is considered to be the main route of egg contamination by SE. To evaluate whether invasion of ovarian follicles is an important step during the production of eggs contaminated by SE, we used an in vitro invasion assay to determine ovarian follicle invasion by 5 SE strains. After inoculating the freshly collected ovarian follicles, all 5 SE strains were able to invade into the follicles after 2 h of incubation at 37°C. The mean percentage of SE invasion ranged from 0.016 to 0.034% and no significant difference was found among the SE strains. For Escherichia coli K-12 strain, which was used as a negative control, the mean percentage of invasion was 0.0003%. The in vitro follicle invasion by SE strains demonstrated in this study may reflect the ability of the strains to invade ovarian follicles in laying hens once SE cells reach ovaries through various routes.

**Key words:** Salmonella Enteritidis, laying hen, ovarian follicle, invasion, egg

**ABSTRACT**

Salmonella is the major foodborne bacterial pathogen worldwide. Among numerous serotypes, Salmonella Enteritidis (SE) is one of the most common Salmonella serotypes responsible for human infections in the United States. The main source of SE outbreaks is foods associated with raw or undercooked chicken eggs. Salmonella Enteritidis is the only serotype that routinely contaminates eggs. The transovarian transmission of SE and subsequent contamination of the eggs before egg shell formation is considered to be the main route of egg contamination by SE. To evaluate whether invasion of ovarian follicles is an important step during the production of eggs contaminated by SE, we used an in vitro invasion assay to determine ovarian follicle invasion by 5 SE strains. After inoculating the freshly collected ovarian follicles, all 5 SE strains were able to invade into the follicles after 2 h of incubation at 37°C. The mean percentage of SE invasion ranged from 0.016 to 0.034% and no significant difference was found among the SE strains. For Escherichia coli K-12 strain, which was used as a negative control, the mean percentage of invasion was 0.0003%. The in vitro follicle invasion by SE strains demonstrated in this study may reflect the ability of the strains to invade ovarian follicles in laying hens once SE cells reach ovaries through various routes.
ly cooked hens’ eggs (Ward et al., 2000; Velge et al., 2005). In fact, among more than 2,500 serotypes SE is the only serotype that routinely contaminates chicken eggs, suggesting that SE possesses unique genetic and phenotypic characteristics that enable routine egg contamination by this serotype.

The modes of transmission are considered to be both vertical (transovarian) and horizontal (shell egg penetration) routes. The vertical route of transmission is a result of infection in the reproductive tract that causes the contamination of the eggs by SE before egg shell is formed (Gast and Beard, 1990; Okamura et al., 2001). In the horizontal route, SE penetrates the egg shell and multiplies within the egg after the shell is contaminated with SE from the external environment (Cox et al., 2000; Messens et al., 2006). Although experimental evidence supports transovarian transmission through the vertical route, it is still uncertain which steps in the process are associated with the unique ability of SE to contaminate eggs routinely (Gast et al., 2004). One possible mechanism is that SE cells, once transmitted to the reproductive tract, invade ovarian follicles at different stages of development before egg shell formation.

In this study, we investigated the ability of SE strains and the EC strain to invade laying hens’ ovarian follicles by in vitro invasion assays in an effort to evaluate the importance of follicle invasion in the process leading to the production of SE-contaminated eggs.

**MATERIALS AND METHODS**

**Bacterial Strains and Preparation of Bacterial Cultures**

Five different strains of SE and Escherichia coli (EC) K-12 strain were used in this study (Table 1). All frozen bacterial cultures were resuscitated by transfer into Luria-Bertani (LB) broth media (Difco, Sparks, MD) for 2 successive cycles of 24 h of incubation at 37°C. All SE strains and the EC strain were maintained and cultured in LB media. The bacterial cultures for ovarian follicle invasion assay were prepared by incubating the cultures until optical densities at 600 nm reached 0.2, which corresponds to a midlog phase.

**Collection and Preparation of Ovarian Follicles**

Ovarian follicles were collected from a flock of Single Comb White Leghorn hens maintained in the poultry science research facility at the University of Arkansas (Fayetteville) and other local farms. Birds used for follicle collection were between 60 and 80 wk of age and had not been subjected to a forced molting. After the hens were killed, ovarian follicles were removed from the ovaries of laying hens aseptically. All of the follicles obtained from each hen were rinsed with sterile 1× PBS and distributed evenly into 6 groups, with follicles in different stages of maturation in each group. Then the follicles in each group were placed in 90 mL of sterile culture media, Hank’s balanced saline solution (HBSS) supplemented with sodium bicarbonate (EMD Chemicals Inc., Gibbstown, NJ) to a final concentration of 4.2 mM.

**Ovarian Follicle Invasion Assay**

The ovarian follicle invasion assay previously devised by Howard et al. (2005) was used in our study with some modifications. After ovarian follicles were prepared as described above, bacterial cultures were added to 1× HBSS cell medium containing the collected ovarian follicles to a final concentration of $10^6$ cfu/mL. Immediately after inoculation, an aliquot of the medium was collected and used to determine the total colony-forming units in the medium postinoculation of bacteria.

Follicles were then incubated with bacteria for 2 h at 37°C. The follicles were subsequently removed from the inoculated medium, rinsed with sterile 1× PBS, and then placed in sterile 100 mL of 1× HBSS supplemented with gentamicin sulfate (Omnipur, EMD Chemicals Inc.) at a final concentration of 500 μg/mL. The follicles were incubated for an additional 4 h at 37°C. Treatment with gentamicin (500 μg/mL) was used to kill all free-living bacteria present in the culture media, selecting for those bacteria that were able to invade the ovarian follicles. Subsequent to 4 h of incubation with gentamicin, each group of follicles was removed from the culture media, rinsed, and stomached separately with 10 mL of sterile 1× PBS. These stomached samples were then used to determine the total colony-forming units recovered from the ovarian follicles.

For enumeration of bacterial cells at $t_0$ and $t_{inv}$, each sample was serially diluted into 1× PBS and plated in triplicate sets onto brilliant green agar (Difco) plates supplemented with novobiocin (25 μg/mL) or LB agar (Difco) plates supplemented with novobiocin (25 μg/mL) for enumeration of SE and EC strains, respectively. Inoculated plates were incubated for 24 h at 37°C and colony-forming units were counted. The average colony-forming units of $t_0$ and $t_{inv}$ samples from triplicate sets were used for calculation of the percentage invasion using the following equation: % invasion = $[cfu_t/t_{inv} × cfu_{t_0}] × 100$, where $cfu_t$ is the total colony-forming units in the media postinoculation of bacteria and $cfu_{t_0}$ is the total colony-forming units recovered from the ovarian follicles contents.

Four separate trials of ovarian follicle invasion assays were performed for each strain.

**Statistical Analysis**

Each trial consisted of 6 groups, and each group was inoculated with a different bacterial strain (5 SE and 1 EC). The percentage invasion from 4 separate trials was analyzed using JMP 7 statistical software (SAS.
Institute, Cary, NC). The ANOVA test for significance was used, with the statistical significance set at $P < 0.05$.

**RESULTS AND DISCUSSION**

After incubating the ovarian follicles with bacterial strains, all strains were able to penetrate into the ovarian follicles during the 2 h of incubation at 37°C. The mean percentage invasion of the ovarian follicles by SE and EC strains determined in this study is shown in Figure 1. The mean percentage invasion of the SE strains ranged from 0.016 to 0.034%, whereas it was 0.0003% for EC, which was used as the negative control. The percentage invasion was closely similar among the 5 SE strains. The percentage invasion of SE13076 (0.016%), which showed lowest invasion among all 5 SE strains, was 53-fold higher than that of EC K-12 strain (0.0003%). However, no significant ($P > 0.05$) difference was found in percentage invasion among all strains tested.

The concentration of gentamicin sulfate used in this study (500 μg/mL) was determined previously to ensure that all bacteria in the media were killed and bacterial counts inside the follicles were not affected (Howard et al., 2005). As expected, when we plated culture media after 4 h of incubation with gentamicin sulfate at this final concentration, no viable bacteria were detected.

The goal of this study was to determine and compare the capability of different SE strains to invade laying hens’ ovarian follicles. Our study showed through the in vitro invasion assay that all tested SE strains were able to penetrate the ovarian follicles under the assay conditions used in this study. Also, the result indicated that all SE strains invaded the follicles consistently without significant variation among all strains.

Developing eggs have been found to be contaminated with SE in the reproductive tract of laying hens after both natural and experimental infections (Humphrey et al., 1989; Bichler et al., 1996). Transovarian transmission of SE has been confirmed by others following experimental infection of SE-free laying hens. In the study by Gast et al. (2004), approximately 66.7% of ovary and oviduct samples from experimentally infected hens were contaminated with SE. In another study, SE was recovered from 71% of egg contents following experimental infection of laying hens (Braden, 2006).

In the study by Howard et al. (2005), developing small white follicles were invaded more efficiently by SE strain PT 19A than were follicles at different stages of maturity. The attachment of SE to ovarian follicles was observed in follicular granulosa cells and the level of attachment varied among the ovarian follicles at different developmental stages (Thiagarajan et al., 1994). Mizumoto et al. (2005) also showed that SE was associated with follicular explants.

Our study demonstrated that different SE strains have the ability to invade ovarian follicles with insignificant variation. Our results, combined with those from previous studies, suggest that SE strains can efficiently adhere to and penetrate hens’ ovarian follicles in a manner dependent on follicle maturity. The ovarian colonization by SE may lead to invasion of ovarian follicles, contributing to the production of contaminated eggs. However, the factors responsible for the higher prevalence of SE in eggborne infections compared with other *Salmonella* serotypes are still unclear. Future work should compare follicle invasion capacity between SE strains and that of other *Salmonella* serotypes.

**ACKNOWLEDGMENTS**

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### Table 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Strain</th>
<th>Reference or source</th>
</tr>
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<tbody>
<tr>
<td>SE14</td>
<td><em>Salmonella</em> Enteritidis LK5 PT 8</td>
<td>Edwards et al., 2000</td>
</tr>
<tr>
<td>SE45</td>
<td><em>Salmonella</em> Enteritidis PT 13A</td>
<td>National Veterinary Services Laboratories, Ames, IA</td>
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<td>SE134</td>
<td><em>Salmonella</em> Enteritidis PT 4 NCTC13349</td>
<td>National Collection of Type Cultures, Porton Down, Salisbury, UK</td>
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<td>SE266</td>
<td><em>Salmonella</em> Enteritidis BM4246</td>
<td>Billy Hargis, University of Arkansas, Fayetteville, AR</td>
</tr>
<tr>
<td>SE13076</td>
<td><em>Salmonella</em> Enteritidis ATCC 13076</td>
<td>American Type Culture Collection, Manassas, VA</td>
</tr>
<tr>
<td>EC-K12</td>
<td><em>Escherichia coli</em> K-12</td>
<td>The Coli Genetic Stock Center, Yale University, New Haven, CT</td>
</tr>
</tbody>
</table>

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**Figure 1.** The mean percentage invasion of *Salmonella* Enteritidis (SE) strains and *Escherichia coli* (EC) K-12 strain into ovarian follicles.
REFERENCES


