Combined Effect of Antibiotic and Competitive Exclusion Treatment on *Salmonella* Enteritidis Fecal Shedding in Molted Laying Hens

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**ABSTRACT**

*Salmonella* Enteritidis is an important pathogen for the layer industry, primarily because of its ability to infect hens and ultimately contaminate egg contents. Studies have shown that stress situations, such as flock recycling (induced molting), can increase *Salmonella* Enteritidis problems in the flock. The present study examined the effect of antibiotic treatment and competitive exclusion (CE) on *Salmonella* Enteritidis shedding in the period following molt and 14-day feed withdrawal. In two separate trials, 48 birds after molt and feed withdrawal were divided into one group that was treated for 10 days with enrofloxacin in water followed by administration of CE culture and a group that was left untreated. *Salmonella* Enteritidis shedding was significantly reduced in the antibiotic-CE group. The *Salmonella* Enteritidis shedding rate was 33 and 25% in untreated birds versus 4 and 0% in the enrofloxacin-CE group on the two test days. These results indicate that treatment of *Salmonella* Enteritidis–positive laying hens after molting with enrofloxacin and CE culture can substantially reduce *Salmonella* Enteritidis problems due to molting and would be a possible alternative to diverting eggs for pasteurization or slaughtering the infected flock. Possible development of bacterial resistance in conjunction with antibiotic use is also discussed.

Foodborne illness due to *Salmonella* Enteritidis has been attributed to the consumption of poultry and egg products (1). By 1990, *Salmonella* Enteritidis appeared as the most common serotype in the United States, and it prevailed from 1993 to 1996 in human foodborne illness due to salmonellae. Eggs are an important source of *Salmonella* Enteritidis infections in humans, and consuming undercooked or raw eggs is directly associated with human salmonellosis (1).

Induced molting is commonly used by the layer industry in the United States to stimulate multiple egg-laying cycles in hens (3) along with a recuperation of eggshell quality and egg production (23). However, this production practice has been identified as an important risk factor for *Salmonella* Enteritidis infection in laying hens (14). Methods are therefore sought that allow producers to recycle their flocks without exacerbating the *Salmonella* Enteritidis problem on the farm.

Competitive exclusion (CE) has been used in the poultry industry around the world to eliminate *Salmonella* from chickens. CE cultures, obtained from a preparation of chicken intestinal microflora or defined mixtures of adult chicken gut bacteria, have shown efficacy in reducing *Salmonella* infection in broilers and layers, especially in newly hatched individuals (7, 11). Older birds, such as breeding stock, and recycled birds that are known *Salmonella* carriers require alternative therapies to reduce or eliminate the infection, such as combined antibiotic therapy and CE treatment (25).

Although substantial scientific evidence indicates that antibiotic use may actually enhance enteric colonization by some antibiotic-resistant strains of salmonellae, anecdotal evidence suggests that antibiotic prophylaxis and treatment for salmonellosis are occasionally attempted in the United States (19). Antimicrobial treatment followed by CE treatment has been shown to be effective in eliminating *Salmonella* from chickens (8, 17, 18, 22). However, limited information exists regarding anti- *Salmonella* therapy in hens following a molt procedure. With the recent implementation of zero tolerance of some foodborne pathogens on poultry products, *Salmonella* Enteritidis prevention techniques are urgently needed. Previous work by Goren demonstrated that combined treatment of chickens with Baytril followed by the administration of CE culture was effective in terminating *Salmonella* Enteritidis carriage (10). In the current study, we examined the efficacy of enrofloxacin combined with a CE culture against *Salmonella* Enteritidis infection in the induced molted birds.

**MATERIALS AND METHODS**

**CE culture and antibiotics.** Normal avian gut flora (NAGF; Aviguard, Bayer, Shawnee Mission, Kan.) is a lyophilized, par-
tially defined, mixed culture derived from the whole cecal material of adult pathogen-free chickens. Enrofloxacin (Baytril, Bayer) is a quinolone carboxylic acid derivative and has a broad spectrum of activity against gram-positive and gram-negative bacteria in various animals (24).

Test animals. Two replicate trials were conducted, each using single-comb, specific pathogen-free white leghorn chickens, approximately 56 weeks old. They were obtained from two laying flocks maintained at the Southeast Poultry Research Laboratory. Chickens were housed in individual layer cages and allowed to acclimate for 1 week before the experiment, during which time they were exposed to a 16-h light and 8-h dark regimen (on at 0100) and allowed water and an antibiotic-free, pelleted, layer-breeder ration ad libitum before the molt procedure began.

Salmonella Enteritidis. The challenge organism was a Salmonella Enteritidis phage type 13a, originally obtained from Dr. C. E. Benson at the University of Pennsylvania (accession code 19299-52-1). Rifampicin-resistant Salmonella Enteritidis (Salmonella Enteritidis rif-R) was generated by plating the original strain onto rifampicin gradient plates (9). There was no loss of virulence confirmed by the comparison of the mutant strain and the original strain using 1-day-old chicks. The chicks challenged with 10⁶ CFU/ml of Salmonella Enteritidis rif-R or the original strain exhibited equivalent intestinal and organ levels of Salmonella Enteritidis at 7 days after challenge. Stocks of the mutant cells were grown overnight in tryptic soy broth (Difco Laboratories, Detroit, Mich.) and harvested by aseptic centrifugation. The pellet was resuspended with sterile fresh medium containing 50% (vol/vol) glycerol, and aliquots of 0.4 ml of the cell suspension containing at least 10⁸ cells/ml were placed into each sterile 1.5-ml tube and stored at −20°C in a freezer. Three days before infection, one of the stocks was thawed and subcultured for 2 consecutive days on nutrient agar (Difco) then transferred to tryptic soy broth and incubated overnight at 37°C.

Molt procedure and treatment. Chickens were molted using a previously described procedure (16). Seven days before feed deprivation, birds were exposed to an 8-h light and 16-h dark photoperiod, which continued throughout the experiments. Fifty-six birds were divided into two main experimental groups, molt (48 birds) and unmolted (eight birds), and transferred into every other individual layer cage. Feed was removed for 14 days from the molted group, and on day 4 of feed removal, all birds were infected with a 1 ml 10⁻² dilution of Salmonella Enteritidis rif-R (approximately 1 × 10⁷ cell/ml) by gavage. Following the readministration of feed, the molted group was divided into two subgroups, with subgroup 1 receiving 10 mg/kg of enrofloxacin in water for 10 days via a proportioner attached to the nipple water system and subgroup 2 left alone as controls. On completion of enrofloxacin administration, the subgroup 1 birds received NAGF 24 and 96 h following the final day of enrofloxacin administration, and then all molted birds were tested for Salmonella Enteritidis shedding. The NAGF-only group was not included in this study, since a preliminary experiment showed no significant difference when compared with the untreated group.

Intestinal shedding of Salmonella Enteritidis. Chickens were assayed for intestinal shedding of Salmonella Enteritidis on day 10 after challenge for eight molted and unmolted chickens and 4 and 10 days after completion of NAGF treatment for the molting groups. The birds were sampled using a modification of a previous procedure (16). Fecal samples were collected on 23 by 28-cm food-grade polyurethane trays (Genpack Corp., Glens Falls, N.Y.) using an intraperitoneal injection of 0.5 ml of 5% pilocarpine (Sigma Chemical Co., St. Louis, Mo.) to induce hypersecretion of the intestine. The trays were placed 10 cm beneath each individual layer cage for approximately 1 h, and about 10 ml of the alimentary secretions were collected from the trays into 50-ml conical tubes. Two 1-ml aliquots were taken from each alimentary secretion sample. The first aliquot was serially diluted (10-fold) in phosphate-buffered saline (pH 7.2), and 100 µl of each dilution, including the undiluted sample, was spread onto brilliant green agar containing 200 µg/ml of rifampicin. The second 1-ml aliquot was added to 9 ml of tetrathionate brilliant green broth. All plates and broths were incubated overnight at 37°C, at which time the plates were examined and Salmonella Enteritidis colonies were enumerated. For samples with no detectable Salmonella Enteritidis, 100 µl of the original tetrathionate enrichment broth was spread plated onto the brilliant green agar containing 200 µg/ml of rifampicin. Following incubation (overnight at 37°C), these plates were examined for the presence of Salmonella Enteritidis colonies. The numbers of Salmonella Enteritidis per ml shed in each treatment group were transformed to log₁₀, and then means were calculated. Because the theoretical threshold of detection of the tetrathionate enrichment was one Salmonella Enteritidis organism per ml, samples that had no growth on the primary culture plates and in the tetrathionate enrichment were considered as containing no Salmonella Enteritidis and assigned to 0.1 log CFU/ml for statistical analysis. Samples that had no growth on the primary culture plates but were culture positive following tetrathionate enrichment were presumed to contain 9 CFU/ml of Salmonella Enteritidis, the theoretical maximum number of organisms that could be present in the sample and detectable by tetrathionate enrichment but not detectable by direct plating.

Statistical analysis. Data were analyzed using Fisher’s exact test for shed rate and the unpaired t test for log number of bacteria to determine significant differences in the number of Salmonella Enteritidis shed and Salmonella Enteritidis shed rate (GraphPad Software, San Diego, Calif.).

RESULTS AND DISCUSSION

The molting procedure had a significant (P < 0.05) effect on the severity of the Salmonella Enteritidis infection. The numbers of Salmonella Enteritidis found in fecal samples from molted and unmolted hens after 14 days of feed removal are shown in Table 1. The Salmonella Enteritidis challenge organism could be readily detected in fecal samples from molted and unmolted hens. However, molted birds shed 5 log₁₀ CFU/ml more Salmonella Enteritidis than unmolted counterparts in trial 1 and 3.7 log₁₀ CFU/ml more.

### Tables

**Table 1. Effect of 14-day feed removal (induced molt) on the fecal numbers and shedding rate of Salmonella Enteritidis in hens**

<table>
<thead>
<tr>
<th>Group</th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmolted</td>
<td>1.5 ± 1.3 b</td>
<td>2.1 ± 1.0 b</td>
<td>1.9 ± 1.1 b</td>
</tr>
<tr>
<td>Molted</td>
<td>6.5 ± 0.6 b</td>
<td>5.8 ± 0.9 b</td>
<td>6.1 ± 0.8 b</td>
</tr>
</tbody>
</table>

*Values are presented as mean ± SD log₁₀ CFU/ml with shedding rate percentages in parentheses. Fecal material was obtained from 56-week-old, retired white leghorn chickens infected at 10⁷ CFU per bird on 10 days after infection.*

*Values are significantly different from unmolted groups (P < 0.05).*
TABLE 2. Comparison of the amount of Salmonella Enteritidis shed by molted birds and shedding rate after treatment with Baytril and Aviguarda

<table>
<thead>
<tr>
<th></th>
<th>Salmonella Enteritidis, fecal contents</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>10 days after treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treated</td>
<td>Untreated</td>
<td>Treated</td>
<td>Untreated</td>
</tr>
<tr>
<td>Trial</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>1</td>
<td>0.1 ± 0.5 (4)</td>
<td>0.6 ± 0.9* (33*)</td>
<td>0</td>
<td>0.4* ± 1.0 (25*)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.2 ± 0.6 (13)</td>
<td>0.6 ± 1.0 (33*)</td>
<td>0.2* ± 0.5 (20*)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combined</td>
<td>0.1 ± 0.4 (6)</td>
<td>0.5 ± 0.8* (33*)</td>
<td>0.3* ± 0.3 (21*)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are presented as mean ± SD log_{10} CFU/ml with shedding rate percentages in parentheses. Shedding rate: (number of positive birds/number of total birds) × 100.

Values are significantly different between treated and untreated groups (P < 0.05).

Salmonella Enteritidis in trial 2 at day 10 after challenge (P < 0.05). All the molted birds analyzed before the start of antibiotic treatments were Salmonella Enteritidis culture positive.

Four days after treatment in trial 1 (Table 2), only 4% of the enrofloxacin and NAGF–treated hens were still culture positive for Salmonella Enteritidis compared with 33% of the untreated group (P < 0.05). The treated hens shed an average 0.1 log_{10} CFU/ml of Salmonella Enteritidis compared with 0.5 log_{10} CFU/ml of Salmonella Enteritidis shed by the untreated group. Six days later, no Salmonella Enteritidis shedding could be detected with treated group, whereas 25% of the untreated group were still shedding an average 0.4 log_{10} CFU/ml of Salmonella Enteritidis (P < 0.05). Similar results were observed in trial 2, with shedding rates of 33% and 17% in the untreated group versus 13 and 0% in the treated group on days 4 and 10 after treatment, respectively. An examination of the combined totals for trials 1 and 2 show that 8.5% of the treated hens shed Salmonella Enteritidis at 4 days after treatment compared with 33% of the untreated hens, and at 10 days no treated hens were shedding Salmonella Enteritidis compared with 21% of the untreated group.

Flock recycling, commonly known as induced molting, became a major economic tool for the layer industry. However, studies showed that feed removal could compromise the immune system and change normal gut flora, resulting in birds being more vulnerable to Salmonella infection (5, 13). Several intervention schemes against Salmonella Enteritidis in molted hens have been applied, including molt diets and modified drinking water during a molt (6, 15). Slaughtering Salmonella Enteritidis–infected (positive) flocks would be a good way to remove the potential source of Salmonella, but it would be an expensive method of Salmonella Enteritidis eradication and also cause other problems (8). In the current study, birds receiving NAGF–only treatment after the 14 days of feed withdrawal were not protected against the Salmonella infection (data not shown). Studies to date have not demonstrated any protection in molting hens receiving CE cultures, possibly because the primary benefit of CE treatment is to populate areas where an intestinal microflora has not been established, such as in day-old chicks. In adult hens, a mature microflora is already present, so administration of additional cecal microbes will only provide minimal protective effect (6). An alternative method consisting of a sequential treatment using antibiotic and CE culture during the molt procedure was investigated under controlled experimental conditions and found to be efficacious for reducing Salmonella infection in the present study. Such techniques may provide producers with a way to eliminate Salmonella Enteritidis infection in vulnerable chickens following a long period of feed removal. However, the use of antibiotics on the farm has come under close scrutiny, since antibiotic-resistant Salmonella strains were increasingly recovered from human infections (12). Because salmonellae are notoriously resistant to many antibiotics and are capable of rapidly developing resistance when exposed, antibiotic prophylaxis or treatment may actually increase the frequency and severity of Salmonella Enteritidis infection in hens (19). Furthermore, some antibiotics augment the chickens’ susceptibility to salmonellae (2, 4, 21). Oral administration of antibiotics could also facilitate the proliferation of other resistant enterobacterial organisms and result in the transmission of antibiotic resistance between species residing in the intestinal tract (20). Therefore, it is imperative that optimal therapeutic regimens be developed that maximize the effective treatment of the birds while reducing the antibiotic dose necessary to clean the Salmonella Enteritidis infection.

It can be concluded that the use of antibiotics combined with CE culture may be an effective therapy against Salmonella Enteritidis in hens exposed to the stress of molting. Results of the current study are consistent with previous studies that reported that a combined treatment of antibiotics and CE culture eliminates the carriage of Salmonella in chickens (25, 26).

However, considering the increased worldwide incidence of antibiotic resistance observed in enteric bacteria, the widespread administration of antibiotics to molted flocks cannot be recommended. Rather, the judicious application of these therapeutic agents based on elimination of the Salmonella Enteritidis infections in the flocks would be more appropriate and would allow producers to recycle their flocks.

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REFERENCES