Elimination of Early *Salmonella enteritidis* Infection After Treatment with Competitive-Exclusion Culture and Enrofloxacin in Experimentally Infected Chicks

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ABSTRACT The effect of normal avian gut flora (NAGF) and enrofloxacin administration on the early infection of young chicks by *Salmonella enteritidis* (SE) was determined using day-old White Leghorn chicks. Day-old chicks were divided into two groups, untreated control and NAGF-treated, and then infected with $10^6$ cfu of SE per chick by oral gavage. The untreated, infected chicks were further divided into two groups and were either left untreated or medicated with a regimen of 10 mg/kg of enrofloxacin in drinking water daily for 10 d, followed by two doses of NAGF beginning at 10 and 8 wk of age in Trial 1 and Trial 2, respectively. Liver, spleen, and cecum samples were tested for the presence of SE, and immunological responsiveness was investigated up to 12 wk of age. Compared with the untreated group, the cecal colonization of SE was significantly ($P < 0.05$) decreased in the NAGF-treated group in Trials 1 and 2. No significant differences in organ infection were observed in the NAGF-treated vs. untreated birds. Although a significant effect of the combined treatment of enrofloxacin treatment and NAGF on the early infection was not shown in Trial 1, compared with enrofloxacin only or the untreated group, a significant reduction ($P < 0.05$) in the number of infected chickens and in the number of SE in the cecal contents was observed at 10 wk of age in Trial 2. The enrofloxacin treatment did not increase opportunistic colonization by SE due to the use of the antibiotic in either trial. The plasma and intestinal immunological responses were not significant at the early age (up to 12 wk) of the birds. The use of enrofloxacin, followed by NAGF, could aid the elimination of SE from young chicks persistently infected at an early age. The combined treatment, compared with enrofloxacin alone, protected chickens from reinfection by 40%.

(Key words: *Salmonella enteritidis*, enrofloxacin, normal avian gut flora)

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INTRODUCTION

*Salmonella enteritidis* (SE) continues to be a major concern for the layer industry, both nationally and internationally, due to the implication of eggs or egg products in a number of outbreaks of food-borne human SE infections. The organism can infect chickens at various stages in their life cycle, but there are times when the susceptibility of chickens to infection is dramatically increased. It has been shown previously that chicks infected within a couple of days posthatch are highly susceptible to an SE infection, and many birds infected at this time remain persistently infected and cannot mount an effective immune response against the invading organism (Gast and Holt, 1998; Holt et al., 1999). Agents that would reduce the susceptibility of the birds to infection, as well as minimize the severity of the infection, would be a boon to an industry struggling to deliver a safe and wholesome product to the consumer.

Competitive exclusion (CE) culture is a probiotic agent derived from the intestinal flora of pathogen-free adult chickens. It is used to augment the minimal intestinal flora normally found in newly hatched chicks and thereby help protect against infections by intestinal pathogens such as *Salmonella*. Numerous CE cultures have proven to be a successful means of excluding *Salmonella* from day-old chicks (Stavric, 1987, 1992; Abu-Ruwida et al., 1995). Competitive exclusion treatment has been shown to allow the early establishment of a normal avian gut flora (NAGF) derived from a pathogen-free adult. Various commercial CE cultures have been developed and studied to reduce the severe contamination of *Salmonella* in poultry (Salvat et al., 1992; Corrier et al., 1995; Primm et al., 1997). Competitive exclusion also applies to situations in

Abbreviation Key: BGR = brilliant green agar containing rifampicin; CE = competitive exclusion; NAGF = normal avian gut flora; PI = postinoculation; SE = *Salmonella enteritidis*; SERR = rifampicin-resistant *Salmonella enteritidis*.
which the flora have been destroyed or seriously disturbed by antibiotic treatment or stress. Combined antibiotic therapy and CE treatment can be recommended, especially when breeding or laying flocks are infected with invasive Salmonella serotypes (e.g., SE or Salmonella typhimurium) (Seuna and Nurmi, 1979; Nurmi, 1992).

In our previous study (Seo et al., 2000), enrofloxacin was shown to be a candidate for removing precolonized Salmonella, and could also be followed by the administration of a CE culture to allow the establishment of NAGF in molted birds. Enrofloxacin is a fluoroquinolone antibiotic that has been shown to be very effective against Salmonella infections in livestock animals (Redman et al., 1989; Rutgers et al., 1994). The combined action of enrofloxacin followed by reconstitution of intestinal flora with CE culture was shown previously to be efficacious in eliminating SE from flocks (Johnson et al., 1992; Goren, 1993; Istvan et al., 1994; Boldet et al., 1995; Humbert et al., 1997; Reynolds et al., 1997). However, no studies to date have shown enrofloxacin and CE culture to be effective in eliminating persistent colonization of Salmonella in chickens in a highly controlled laboratory situation.

The ability of NAGF to prevent the establishment of a persistent SE infection in 1-d-old chicks was examined. Furthermore, the efficacy of enrofloxacin in clearing SE from young chicks persistently infected at an early age and the usefulness of NAGF in reconstituting intestinal flora and preventing reintroduction of SE into the birds were examined.

MATERIALS AND METHODS

Salmonella enteritidis, Competitive Exclusion Product, and Antimicrobial Agent

The challenge organism was SE phage type 13a, originally obtained from Dr. C. E. Benson at the University of Pennsylvania (accession code #19299-52-1). A rifampicin-resistant SE (SERR) mutant was obtained from the stock cultures dispersed by the method explained in a previous study (Seo et al., 2000). Three days prior to infection, one of the stock cultures was thawed and sub cultured for 2 consecutive d on nutrient agar, then transferred to tryptic soy broth and incubated overnight at 37 C. Cultures were serially diluted (10-fold) in PBS (pH 7.2) to 10^7 cfu/mL, and viable cell counts in colony-forming units were determined by the plating of dilutions on brilliant green agar with 200 µg/mL of rifampicin. An NAGF product derived from specific pathogen-free chickens, and enrofloxacin were used as a CE culture and an antimicrobial agent in the current studies.

Chickens and Treatments

Two trials were conducted using 150 specific pathogen-free White Leghorn chicks hatched in a pathogen-free environment. Groups of 11 to 12, day-old-hatch layer chicks were housed on wire floors in formaldehyde gas-sterilized Horsfall-Bauer isolation cabinets and administered water (in a cup watering system) and starter feed ad libitum under continuous lighting at 37 C. Ten chicks per treatment were sacrificed at four sampling periods unless otherwise specified. Chicks were randomly allocated to NAGF-treated and untreated control groups. Chicks in two cabinets received 0.5 mL NAGF each, reconstituted in sterile water according to the manufacturer’s direction, by oral gavage as they were initially placed into the cabinet. The birds in these two NAGF treatment cabinets and the birds in the nine cabinets containing the untreated controls were challenged with 0.1 mL of a 10^{-2} dilution (1.5 x 10^9 cfu per bird) of an overnight broth culture of SERR (1.5 x 10^9 cfu/mL) at 2 d of age. In each trial, 12 chicks in one isolation cabinet were left uninfected, and three chicks were sacrificed at each of four sampling periods. The chicks remained in the isolation cabinets until 4 wk, at which time they were transferred into battery cages (10 to 15 birds per cage). Chicks in the untreated control group (nine cabinets), at 8 wk postinoculation (PI) in Trial 1 and 6 wk PI in Trial 2, were divided into three equal subgroups as follows: 1) administered 10 mg/kg enrofloxacin for 10 d followed by two doses of NAGF, 2) enrofloxacin only (10 mg/kg for 10 d), and 3) untreated. In Trial 2, eight chicks in Subgroups 1 and 2 were challenged again with 1 mL of 10^{-2} dilution of SERR 1 d after the last NAGF administration.

Sampling for Bacterial Analysis

Ten infected, untreated control chicks, 10 NAGF-infected chicks, and three negative control chicks were bled and sacrificed to test immunological responsiveness and SE organ persistence at 4 and 8 wk PI. Ten enrofloxacin-treated chicks, 10 enrofloxacin and NAGF-treated chicks, and 10 untreated chicks were sampled for the same purpose above at 10 and 12 wk PI in Trial 1 and at 8 and 10 wk PI in Trial 2. Ten enrofloxacin and NAGF-reinfected chicks and 10 enrofloxacin-reinfected chicks were sacrificed at 9 wk of age (1 wk postreinfecion). The liver, spleen, and one cecum were aseptically harvested into weighed stomacher bags. The jejunum, ileum, colon, and remaining cecum were removed, placed into 50-mL tubes, and frozen until use for intestinal immunity sampling. The organ samples were diluted 1:10 (wt:vol) in cold (7 C) tetrazionate enrichment broth and stomached for 60 s. A 0.1-mL amount of the stomached samples was plated onto brilliant green agar containing 0.2 mg/mL rifampicin (BGR). The cecum samples were serially diluted (10-fold) in PBS, and 0.1 mL of each dilution was spread onto BGR. The plates were incubated overnight at 37 C, and SE colony-forming units were determined. Samples stom-
achieved in tetrathionate were enriched by incubation at 37 C overnight. For any negative plates, 0.1 mL of the respective tetrathionate enrichment was plated onto BGR that was then incubated overnight at 37 C. The identity of presumptive SE colonies was confirmed biochemically and serologically using triple sugar iron agar, lysine iron agar, and Salmonella O antisera group D. Because the enumeration method had a minimum detection threshold of 1.0 × 10² cfu/g, samples that were negative for direct enumeration, but positive after tetrathionate enrichment, were arbitrarily assigned a value of 50 cfu/g. The numbers of SE colony-forming units per gram in each treatment group were transformed to log₁₀, and then means were calculated. Samples that had no growth on the primary culture plates and in the tetrathionate enrichment were considered as containing no S. enteritidis.

ELISA Test for Antibody Responses

The birds were bled at each sampling time from the wing veins into 2-cc syringes. The intestinal samples were flushed out using 10 mL 0.1 M glycine buffer (pH 8, 0.05% Tween 20). The blood and intestinal contents were centrifuged at 15,000 × g for 5 min, and the supernatants were frozen at −20 C until time of assay. One-hundred microliters of serum (diluted to 1:250 in PBS, pH 7.2, 1% Tween 20, 0.1% BSA) or 0.1 mL of intestinal flushing samples (diluted to 1:10 in PBS, Tween, and BSA, as above) were tested for the presence of specific antibodies against SE flagella using an ELISA described by Holt and Porter (1993). Samples with absorbance or optical density at a wavelength of 405 nm two times higher than negative controls were considered as immune positive.

Statistical Analysis

The numbers of SE in each treatment group were transformed to log₁₀ cfu/g and analyzed statistically by one-way ANOVA. Data were analyzed using Fisher’s exact test for percentages of SE-positive birds and an unpaired t test for log number of bacteria in the tissues to determine significant differences.

RESULTS

Salmonella enteritidis Numbers in Chicks Previously Treated with Normal Avian Gut Flora

All samples from uninoculated control chickens were negative for SE. The challenge organism was successfully established in groups of NAGF-untreated chicks up to 10 wk in this study. Results from the two replicate trials that were conducted to determine the effect of NAGF on persistent infection in 1-d-old chicks are shown in Table 1. There were no significant differences in the treated vs. the untreated birds for the numbers of SE or percentages of SE-positive samples of liver or spleen, and SE was not detected in these organs by 8 wk PI in either trial. Compared with untreated controls, however, the number of SE from cecal material at 4 and 8 wk PI was significantly reduced ($P < 0.05$) in the chicks provided with NAGF in both trials. In Trial 2, a 6.5 log reduction in SE cfu was observed in NAGF-treated chicks at 4 wk PI. Significant reductions ($P < 0.05$) in percentage culture-positive and number of colony-forming units at 8 wk PI were seen in chicks treated with NAGF culture in both trials.

Combined Treatment of Enrofloxacin and Normal Avian Gut Flora

No SE were detected in liver and spleen tissue samples at 8 wk PI (Table 2). Persistent infection of SE in the cecum

### TABLE 1. Effect of normal avian gut flora (NAGF) on early infection of Salmonella enteritidis in 1-d-old chicks

<table>
<thead>
<tr>
<th>Samples</th>
<th>4 wk postinfection</th>
<th>8 wk postinfection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>NAGF</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td>1.2 ± 1.0 (60)</td>
<td>0.4 ± 0.8 (20)</td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td>1.0 ± 1.1 (5)</td>
<td>1.0 ± 0.8 (60)</td>
</tr>
<tr>
<td><strong>Cecum</strong></td>
<td>7.7 ± 0.7 (100)</td>
<td>4.1 ± 2.2* (80)</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td>0.8 ± 1.0 (40)</td>
<td>0.4 ± 0.8 (20)</td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td>0.8 ± 1.0 (40)</td>
<td>0.7 ± 0.9 (40)</td>
</tr>
<tr>
<td><strong>Cecum</strong></td>
<td>8.0 ± 0.3 (100)</td>
<td>1.5 ± 0.5* (90)</td>
</tr>
</tbody>
</table>

1Chicks received NAGF on the first day of hatch and were challenged with 1.5 × 10⁷ cfu per bird of S. enteritidis at 2 d of age.
2Values are presented as mean ± SD with percentage positive in parentheses. Ten birds were randomly selected for culturing at each sampling interval.
3Samples that had no growth on the primary culture plates and in the tetrathionate enrichment were considered as containing no S. enteritidis.
4Values that had no growth on the primary culture plates and in the tetrathionate enrichment were considered as containing no SE (0 cfu/g) and assigned to 0.1 log cfu/g for statistical analysis.
5GraphPad Software, San Diego, CA 92121.
was shown in chicks at 10 wk PI in Trial 1, with a 30% reduction in culture-positive birds in enrofloxacin-treated chicks (Table 2). No detectable SE was found in any group of chicks by 12 wk postchallenge in Trial 1. The study was repeated, but treatment was performed 2 wk earlier to take advantage of the higher SE counts that would make the evaluation of treatment effects easier. The combined treatment of enrofloxacin and NAGF significantly reduced the SE number in the cecum compared with the untreated group at 8 wk postchallenge ($P < 0.05$) and with both the untreated and enrofloxacin-alone groups at 10 wk postchallenge (Table 2). A significant reduction ($P < 0.05$) in percentage SE-positive birds occurred in the enrofloxacin and NAGF group compared with the enrofloxacin-alone group at 8 wk postchallenge, and the untreated group at 10 wk postchallenge (Table 2). Enrofloxacin did not increase the opportunistic colonization by SE due to the use of the antibiotic in Trials 1 or 2. A numerical reduction in the amount of SE and the percentage SE-positive birds occurred in the group treated with the combination of enrofloxacin and NAGF compared with the enrofloxacin-alone group. Although a significant reduction was not observed, the combined treatment protected chickens from reinfection by approximately 40%, compared with chicks reinfected after being treated with only enrofloxacin in Trial 2 (Table 3).

### Immune Responses

Immune responses were investigated in all stages of chicks in Trials 1 and 2, and the combined ELISA results are presented in Table 4. No plasma or intestinal responses were detected in any of the uninfected control birds (data not shown). In general, there was no notable development of immunity observed for IgG or IgA, and there was no significant difference among groups. Few positive serum samples (8%) were detectable at 4 wk PI (Table 4), but by Week 10, 45% of the samples contained detectable antibody levels. The percentage of samples with antibody titers increased slightly at 10 wk, and these percentages never surpassed 45% over the next 12 wk. Intestinal IgA levels increased from 8% positive samples at 4 wk PI to 18% positive at 10 wk PI (Table 5). The intestinal immune response dropped dramatically to 0 at 12 wk PI. No differences among treatment groups were observed for the IgA response.

### DISCUSSION

Newly hatched chicks are extremely susceptible to *Salmonella* infection, requiring 100- to 1,000-fold fewer salmonellae to become infected compared with older birds (Cox et al., 1990). The consequence of infections by SE in the hatchery can persist through egg production (Gast and Holt, 1998). Therefore, special attention needs to be paid

### TABLE 2. Effect of enrofloxacin (enro) and normal avian gut flora (NAGF) treatment on the early infection of *Salmonella enteritidis* (SE) in 1-d-old chicks

<table>
<thead>
<tr>
<th>Samples</th>
<th>Untreated</th>
<th>Enro</th>
<th>NAGF + Enro</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trial 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.9 ± 1.2 (40)</td>
<td>0.2 ± 0.5 (10)</td>
<td>0.2 ± 0.5 (10)</td>
</tr>
<tr>
<td>Cecum</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Trial 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Spleen</td>
<td>2.8 ± 2.0 (80)</td>
<td>2.4 ± 1.4 (100)</td>
<td>1.0 ± 1.6 (40)</td>
</tr>
<tr>
<td>Cecum</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Counts and percentage positive within rows with different letters are significantly different ($P < 0.05$).

Chicks that were challenged with $1.5 \times 10^6$ cfu per bird of SE at 2 d of age, at 8 wk postinoculation in Trial 1 and 6 wk postinoculation in Trial 2, were divided into three equal subgroups (20 birds per group) as follows: 1) administered 10 mg/kg enrofloxacin for 10 d followed by two doses of NAGF, 2) enrofloxacin only, and 3) untreated. Culture tests were performed at 10 and 12 wk, and at 8 and 10 wk postchallenge in Trial 1 and Trial 2, respectively.

Values are presented as mean ± SD with percentage positive in parentheses. Ten birds were randomly selected for culturing at each sampling interval.
to reducing contact of the chicks with the pathogen at this crucial stage of life. The results presented here indicate that early treatment of chickens with NAGF could not only reduce intestinal colonization by SE at this early stage, but could also reduce subsequent persistent infections. Gast and Holt (1998) observed very persistent fecal shedding of SE for at least 24 wk after oral infection of 1-d-old chicks. Because commercial laying hens usually begin egg production at 21 wk, one potential effect of early NAGF treatment could be the prevention of SE-contaminated eggs. The CE treatment could also be useful in situations in which the NAGF has been destroyed or seriously disturbed by antibiotic treatment and severe stress, as seen when birds are induced to molt (Seo et al., 2000).

When infected birds only received NAGF treatment, infection with *Salmonellae* was not prevented (data not shown) or was, at best, only partially prevented (Seuna et al., 1980). Seuna and Nurmi (1979) found that antimicrobials commonly used in experimental *Salmonella* therapy could not permanently eliminate colonization 1 wk after antibiotic withdrawal. The use of antibiotics could provide a niche for pathogens ready to colonize the chicken gut while eliminating total intestinal microorganisms. Research on CE and intestinal microecology may verify the suspected association between widespread use of subtherapeutic antibiotics and the proliferation of gram-negative, food-borne pathogens in the gut (Nurmi et al., 1992). In fact, Seuna et al. (1980) observed that the use of certain antibiotics without further combined therapy increased the *Salmonella* infection rate in chickens when cultured 1 wk posttreatment. They also found that the effect of the cecal culture was best when the abolition of *Salmonella* by the preceding antimicrobial treatment had been the most complete. In the current study, enrofloxacin did not reduce the infection rate in the treated groups lacking cecal culture, but did decrease the infection rate when it was combined with cecal culture. The current study, therefore, demonstrates that enrofloxacin has potential for use with commercial NAGF products to reduce persistent SE infection in chickens prior to the start of egg lay. Treating infected layers with antibiotics followed by CE culture before moving them into cages for egg production could reduce egg contamination by SE.

Chickens contaminated with *Salmonella* directly after hatching can remain infected until maturity without developing significant immunity against the organism (Holt et al., 1999). Our study showed similar results, in that antibody responses in plasma and intestinal samples were minimal over a 12-wk period. However, SE usually persisted beyond 4 wk PI only in the intestinal tract, which is coincidental with the development of detectable humoral antibody levels in the majority of sampled chicks (Holt et al., 1999).

Increasing concern about using antibiotics arises from the fact that consistent use of one kind of drug may allow the development of drug-resistant bacteria in livestock animals. Colonies isolated from enrofloxacin-treated

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### TABLE 4. Serological detection of anti-*Salmonella enteritidis* antibodies in plasma (IgG) at various time periods from birds infected at 1 d of age; the combined ELISA results in Trials 1 and 2 are presented

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Number exhibiting positive immune response/total number of birds tested (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 wk postinfection</td>
</tr>
<tr>
<td>NAGF1</td>
<td>3/20 (15)</td>
</tr>
<tr>
<td>NAGF + enrofloxacin</td>
<td>ND</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>ND</td>
</tr>
<tr>
<td>Untreated</td>
<td>1/20 (5)</td>
</tr>
<tr>
<td>Average, %</td>
<td>8</td>
</tr>
</tbody>
</table>

1NAGF = normal avian gut flora.  
2ND = not determined.
chickens were sent to a commercial laboratory in order to compare their resistance to enrofloxacin with original strains used for inoculation. The SE isolates were not found to have developed in our study (data not shown).

In conclusion, the use of NAGF as part of a CE regimen may provide 1-d-old chicks with a good defense against SE infection. Once a persistent infection is established, judicious use of antibiotics like enrofloxacin may provide a basis for treatment.

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REFERENCES


