Evaluation of Aviguard, a Commercial Competitive Exclusion Product for Efficacy and After-Effect on the Antibody Response of Chicks to Salmonella

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ABSTRACT

The competitive exclusion (CE) action of Aviguard (AG) and its effects on the antibody response of chicks were evaluated in this study. We observed that AG protected the chicks from overwhelming colonization. Fourteen days after infection, fewer AG-pretreated than nonpretreated chicks shed salmonellae from their cloaca in both infected groups, although much less from SE-infected chicks. Antibody titers of sera produced to Salmonella typhimurium (ST) and SE in pretreated and nonpretreated chicks were not significantly different.

(Key words: competitive exclusion, Salmonella enteritidis, Salmonella typhimurium)

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INTRODUCTION

Nontyphoid salmonellae are common to human bacterial gastroenteritis worldwide, particularly with respect to food safety (Lax et al., 1995; CDC, 1998; Fierer and Swancutt, 2000). Contaminated food of animal origin, especially poultry, remains the major source of these pathogens (Pivnick and Nurmi, 1982; D’Aoust, 1994; Solano et al., 1998). Contamination of poultry with salmonellae results from infected breeders, contaminated feed, and environmental factors (e.g., rodents, insects, wild birds, humans) (Pivnick and Nurmi, 1982). There is thus a need to control salmonellae in poultry. This has been largely achieved by the use of competitive exclusion (CE), a concept originally described by Nurmi and Rantala (1973).

In competitive exclusion, adult intestinal content (Rantala, 1974) or cultures of such material (Schneitz et al., 1981; Pivnick and Nurmi, 1982) are administered to protect newly hatched chicks (Lloyd et al., 1974) against subsequent infection by salmonellae. The concept has attracted the interest of several researchers, who developed and tested numerous CE cultures to reduce enteric pathogen colonization or infection in commercial-type poultry (Snoeyenbos et al., 1978; Rigby and Pettit, 1980; Blanchfield et al., 1984; Wierup et al., 1988; Nuotio et al., 1992; Corrier et al., 1994; Abu-Ruwaida et al., 1995; Palmu and Camelin, 1997; Hume et al., 1998; Barbour et al., 1999; Fukata et al., 1999; Mead, 2000; Seo et al., 2000; Methner et al., 2001; Stern et al., 2001). CE is now widely accepted, even in some countries’ national control programs, as an effective means of reducing the incidence and severity of chick infections with salmonellae and other intestinal pathogens (Hirn et al., 1992; Wireup et al., 1992; Cadwell et al., 2000; Methner, 2000). The first commercially available CE product, Broilact, was developed in Finland as a suspension, and has been in use in northern Europe since 1987 (Wierup et al., 1992). This product has also been used in combination with enrofloxacin in eradicating salmonellae from broiler breeder farms (Koehler and Poeppel, 1994). The short shelf-life of this CE product, however, had limited its use. Several other commercial CE cultures have been developed and studied to reduce the severe contamination of salmonellae in poultry (Salvat et al., 1992; Corrier et al., 1995; Primm et al., 1997; Cadwell et al., 2000).

Aviguard (AG) was developed as a novel product with longer shelf life than other CE products. It is a freeze-dried fermentation product for use in chickens and turkeys as spray treatment or drinking water application. This product contains a mixture of live, commensal bacteria, partially characterized, which is representative of the ma-
ture bacterial populations in the cecum of adult chickens. Existing reports on the CE efficacy of AG are few and short of details, particularly in regard to invasive *Salmonella* serotypes (e.g., ST or SE) and the effect of its use on the antibody response of pretreated chickens to infecting salmonellae. In this study, we tested the CE efficacy of AG against ST and SE, common zoonotic serotypes in poultry (Carraminana et al., 1997; Salano et al., 1998; Schwartz, 1999; Seo et al., 2000) and its effect on the antibody response of chicks under laboratory conditions.

**MATERIALS AND METHODS**

### Test Chicks

White Leghorn chicks (n = 50; 1-d-old; BW = 53.08 g ± 2.12) were obtained from the National Institute of Animal Industry. The chicks were housed in a quiet room previously fumigated with formaldehyde, and given antibiotic-free starter feed and tap water ad libitum.

### Test Strains

*Salmonella typhimurium* L1338 (ST) and SE L1225 of nalidixic acid-resistant salmonellae (NA⁺) were used as test strains that were previously cloned (Hegazy and Adachi, 2000). ST and SE are common pathogens in human food poisoning due to consumption of contaminated poultry meat and eggs (Carraminana et al., 1997; Solano et al., 1998; Schwartz, 1999), hence their use for our study. One colony of each test strain was inoculated into 100 mL of trypticase soy broth (TSB) and incubated overnight at 37°C. Each culture was then centrifuged for 15 min at 10,000 rpm and the sediment was inoculated on deoxycholate hydrogen sulfide lactose (DHL) agar supplemented with 25 µg/mL of NA. After overnight incubation at 37°C, colonies were subcultured onto fresh NA-supplemented DHL (DHL-NA) agar and incubated overnight at 37°C. The resulting cultures were then used for the study.

### Preparation of Bacterial Suspension

One colony of ST or SE from the DHL-NA agar culture was transferred into 100 mL of TSB, and incubated for 18 h at 37°C. The culture was then centrifuged 100 times with sterile TSB and 0.5 mL of the diluted culture was inoculated orally. Thereafter, the inocula were diluted in saline (0.85% NaCl), spread on DHL agar and after 24 h incubation at 37°C, the colony-forming units were counted (ST = 4.8 × 10⁷; SE = 7.5 × 10⁶).

### Cecal Content

Freeze-dried avian cecal content, AG⁷ (Batch No. 071296) was used. It is recommended by the manufacturer for use in poultry either as spray application or powder (suspended in normal drinking water of the chicks). In this study, we used it in a different way, by gavaging directly into the crop to ensure complete dose delivery. AG (2.5 g) was suspended in 100 mL of sterile distilled water, and 0.5 mL of it was administered to all pretreated chicks.

### Treatment

The chicks were randomly allocated into five groups of 10 chicks each, according to the following protocol: Groups 1 and 3 were pretreated with AG and 6 h later given ST and SE, respectively; Groups 2 and 4 were given ST and SE, respectively; Group 5 received AG only. Both AG and test salmonellae were administered orally, and over a 22-d period the chicks were monitored for mortality, cloacal shedding, BW changes, tissue colonization, and antibody responses to the infecting salmonellae. The test was repeated two times using the same protocol to measure reproducibility.

### Tissue Colonization by Salmonellae

A cloacal swab was taken from each chick on Days 1, 4, 7, and 14 after infection. The swab was spread onto the DHL-NA agar plate, and colonies were counted after incubation for 24 h at 37°C. After infection (22 d) chicks that survived were bled, and the counts of salmonellae from their liver, kidney, and spleen were determined. Cross-section of each organ was stamped five times (Yoshida and Minato, 1986) on the DHL-NA agar plate and spread. Colonies on the plate were counted after 24 h incubation at 37°C.

### Serum Sample

Blood samples from chicks were taken (Hegazy and Adachi, 2000) and left to stand overnight at room temperature. The resulting serum was collected by centrifugation (3,000 rpm × 30 min), and the supernatant was then stored at 5°C until needed.

### Agglutination Test

One colony of salmonellae on DHL-NA agar was transferred into TSB and incubated overnight at 37°C. Cells were harvested by centrifugation (10,000 rpm × 15 min at 4°C) and then resuspended in 25 mL of formalized saline (0.85% NaCl in 1% formaldehyde) and stored at room temperature. Part (0.25 mL) of the formalized cell suspension was mixed with an equal volume of serial twofold-diluted serum, and after incubation for 1 h at 50°C, results were observed. Saline (0.85% NaCl in distilled water) was used as negative control.

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³Wako Pure Chemical Industries, Chyuu-ku Osaka, Japan.
⁴All Japan Agriculture Coop., Chiyoda-ku, Japan.
⁵Becton Dickinson, Cockeysville, MD.
⁶Nissui Pharmaceutical Co. Ltd., Toshima-ku, Tokyo, Japan.
Electrophoresis

The SDS-PAGE was conducted following the method described by Laemmli (1970). One colony of the test salmonellae prepared on DHL agar was incubated overnight in 100 mL of TSB at 37 C. The resulting culture was centrifuged for 15 min (15,000 rpm at 4 C), and the sediment was resuspended in 10 mL of saline. One milliliter of the suspension was transferred into 1.5-mL microcentrifuge tubes and centrifuged for 5 min (15,000 rpm at 4 C). The sediment was suspended in 10 µL of 10% SDS and an equal volume of loading buffer [0.125 M Tris (hydroxymethyl) aminomethane (Tris), 4% SDS, 10% 2-mercaptoethanol, 0.2% bromophenol blue, pH 6.8] was added. The prepared sample was boiled for 10 min at 100 C, centrifuged for 1 min (15,000 rpm at 20 C) and 2 µL of the supernatant was electrophoresed with 5 µL of molecular weight reference marker on 10% acrylamide gel for 3 h at 10 mA using a small electrophoresis chamber. Separated proteins were stained in 0.25% Coomassie Brilliant Blue R250 in methanol:acetic acid:distilled water (5:1:5) and destained in methanol:acetic acid:distilled water (2:3:35).

Immunoblotting

Immunoblotting was conducted following the method described by Towbin et al. (1979). Charged protein was transferred on nitrocellulose membrane (Hybond-C11) by applying current of 0.8 mA, for 4 h in the semi-dry blotter described by Towbin et al. (1979). Charged protein was overlaid on the membrane and probed for 1 h. The membrane was again washed with the buffer three times, 30 min each and three times with PBS (pH 7.2). Antigen-antibody reaction on membrane was detected with a detection substrate mixture [magnesium sulfate, 1-naphthylisodisodium phosphate nH2O2, Fast Blue BB salt] in alkaline phosphatase-buffered boric acid (pH 10.0). When sufficient visualization was observed, reaction was stopped by removal of substrate with tap water.

Statistical Analyses

Statistical analyses were chi-squared test for mortality and ANOVA for tissue colonization, BW, and antibody titer using WebStat 2.0. Significance was determined at P = 0.05.

RESULTS

Mortality and Body Weight

Number of deaths after the inoculation of salmonellae is presented in Table 1. Because of the high challenge, death occurred in Groups 1, 2, and 3. However, there was no difference (P > 0.05) between AG-treated and nontreated chicks.

Body weight of chicks at the end of the observation period are presented in Table 2. Infection with ST resulted in chicks with lower BW than SE-infected chicks. The BW of chicks pretreated with AG was not different (P > 0.05) than BW of the nonpretreated ones (Groups 2 and 4).

Tissue Colonization by Salmonellae

Isolation of salmonellae from cloacal swab is presented in Table 3. A day after infection with the appropriate test strain, large numbers of salmonellae were isolated from most of the chicks, but 14 d later this was observed only in the chicks that were not pretreated. The pretreated ones were either free of salmonellae or excreting only a small number. Salmonellae were not found in AG-pretreated uninfected control chicks during the period of observation.

Colonization of some organs by salmonellae at necropsy is as presented in Table 4. Fairly large numbers of salmonellae were isolated from liver, kidney, and spleen a few days after infection, but fewer were isolated later in the observation period.

<table>
<thead>
<tr>
<th>Test group</th>
<th>Test strains</th>
<th>AG</th>
<th>Mortality (n died/total treated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ST</td>
<td>+</td>
<td>3/10</td>
</tr>
<tr>
<td>2</td>
<td>ST</td>
<td>−</td>
<td>2/10</td>
</tr>
<tr>
<td>3</td>
<td>SE</td>
<td>+</td>
<td>1/10</td>
</tr>
<tr>
<td>4</td>
<td>SE</td>
<td>−</td>
<td>0/10</td>
</tr>
<tr>
<td>5</td>
<td>TSB</td>
<td>+</td>
<td>0/10</td>
</tr>
</tbody>
</table>

1Mortalities Aviguard (Microbial Developments Ltd., Worcestershire, England) pretreated and nonpretreated chicks were insignificantly (X2; P > 0.05) different.

2AG = Aviguard; ST = Salmonella typhimurium; TSB = trypticase soy broth.

<table>
<thead>
<tr>
<th>Group</th>
<th>Strains</th>
<th>AG</th>
<th>Body Weight (g; mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ST</td>
<td>+</td>
<td>114.6 ± 21.7</td>
</tr>
<tr>
<td>2</td>
<td>ST</td>
<td>−</td>
<td>98.0 ± 33.5</td>
</tr>
<tr>
<td>3</td>
<td>SE</td>
<td>+</td>
<td>135.5 ± 24.9</td>
</tr>
<tr>
<td>4</td>
<td>SE</td>
<td>−</td>
<td>110.2 ± 22.5</td>
</tr>
<tr>
<td>5</td>
<td>TSB</td>
<td>+</td>
<td>112.1 ± 25.2</td>
</tr>
</tbody>
</table>

1The final body weights of control and treated chicks at end of the 22-d observation period were insignificantly (ANOVA; P < 0.05) different.

2AG = Aviguard (Microbial Developments Ltd., Worcestershire, England); ST = Salmonella typhimurium; TSB = trypticase soy broth.
Serology

Antibody titers are presented in Table 5. Antibody titers of the chicks infected with ST were higher than those infected with SE. No difference, however, was observed between AG-pretreated and nonpretreated chicks. Sera with high antibody titers were tested for cross-reactivity with antigens. There was no detectable cross-reaction between the sera of ST-infected chicks and SE antigens as well as between the sera of SE-infected chicks and ST antigens at 1:25 dilution.

SDS-PAGE

Result of SDS-PAGE of SDS-solubilized bacterial proteins is presented in Figure 1. Molecular weights of the envelope proteins of salmonellae were determined by comparison with the molecular weight marker. Results showed similarity in the protein profiles of ST and SE. Both strains yielded thick bands at 71.4, 67.7, 44.0, and 30.3 kDa.

Immunoblotting

Figure 2 presents the reaction between ST and antisera. Lanes 1 to 3 correspond to chick Nos. 3 to 5 of Group 2, and Lanes 4 to 10 correspond to chick Nos. 4 to 10 of Group 1. Sera from chicks reacted strongly with proteins of molecular weights 71.4, 67.7, and 44.0 kDa. Figure 3 presents the reaction between ST and the sera of ST-infected chicks. In Group 2, a strong reaction was observed with proteins of molecular weights 71.4, 67.7, and 44.0 kDa. No significant difference was observed between Groups 1 and 2. Figures 4 and 5 present reactions with SE. In Group 3, sera reacted strongly with proteins of molecular weights 71.4, 67.7, 44.0, and 30.3 kDa. Sera from chicks in Group 4 also reacted with 71.4, 67.7, 44.0, and 30.3 kDa proteins. Sera from infected chicks reacted with 71.4, 67.7, 44.0, and 30.3 kDa proteins yielding four major bands at positions corresponding to their molecular weights. Cross-reaction between the antigens and the sera from chick Nos. 4 (Group 1), 5 (Group 2), 2 (Group 3), and 8 and 10 (Group 4) is shown in Figure 6. Sera from ST-infected chicks reacted strongly with proteins of molecular sizes of 71.4, 44.0, and 30.3 kDa in ST and the sera from SE-infected chicks reacted with 71.4, 67.7, 44.0, and 30.3 kDa proteins in SE. Those sera also reacted strongly with 71.4, 44.0, and 30.3 kDa proteins in SE and ST, while the band with the protein with molecular size of 67.7 kDa was confirmed by the reaction between the sera from the SE-infected chicks and SE.

DISCUSSION

The use of CE cultures as means of controlling infection or colonization of the gastrointestinal tract by bacte-

![Table 3](https://academic.oup.com/ps/article-abstract/81/11/1653/1545922/Evaluation-of-aviguard-a-commercial-competitive/1656)

<table>
<thead>
<tr>
<th>Group</th>
<th>Test strains</th>
<th>AG</th>
<th>Average score for isolation of salmonellae (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>1</td>
<td>ST</td>
<td>+</td>
<td>1.80 (1.32)</td>
</tr>
<tr>
<td>2</td>
<td>ST</td>
<td>-</td>
<td>1.60 (1.27)</td>
</tr>
<tr>
<td>3</td>
<td>SE</td>
<td>+</td>
<td>1.30 (0.95)</td>
</tr>
<tr>
<td>4</td>
<td>SE</td>
<td>-</td>
<td>1.10 (0.32)</td>
</tr>
<tr>
<td>5</td>
<td>TSB</td>
<td>+</td>
<td>1.00 (0.00)</td>
</tr>
</tbody>
</table>

1Scores were assigned according to the following scheme: Death, 5; colony-forming units > 100 cfu/g; 4; 10 to 100 cfu/g; 3; <10 cfu/g; 2; 0 cfu/g; 1; differences between the treatment groups and control (TSB) were insignificant (ANOVA; P > 0.05); 0 isolation implies no growth on plates even when inoculation on selective media was preceded by incubation in Hajna Tetrathionate enrichment broth (Nissui Pharmaceutical Co., Tokyo).

2AG = Aviguard (Microbial Developments Ltd., Worcestershire, England); ST = Salmonella typhimurium; TSB = trypticase soy broth.
tissue colonization and death as evidenced from their low mortality and rate of colonization. In agreement with the expected nonspecificity of undefined CE cultures (Mead, 2000), AG effectively protected against both SE and ST. This action is of particular importance in view of ubiquitous existence of these pathogens in poultry, and also because chicks infected within a couple of days post-hatch are usually highly susceptible to 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). Interestingly, the chicks were protected by AG from the much higher inoculum challenge used in this study than the minimum bacterial dose of 10 cells required to cause salmonellae disease in day-old chicks (Milner and Shaffer, 1952; Seuna, 1979).
FIGURE 5. Immunoblotting reaction of test chick sera against SE L1225. M = molecular weight marker. Lanes 1 to 5 correspond to Chicks 6 to 10 of Group 3; Lanes 6 to 10 correspond to Chicks 6 to 10 of Group 4. Major bands are indicated by arrows.

Newly hatched chicks are even more susceptible to becoming infected than older chickens (Cox et al., 1990). Undefined natural cultures have been shown to markedly protect chicks against Salmonella more than defined cultures (Mead and Impey, 1985).

The significantly higher antibody titers of sera from nonpretreated chicks infected with SE compared with those of ST-infected chicks are consistent with the greater tendency of SE to cause systemic infection consistent with ST (whose infections are usually localized to the intestine). Our results of a significant antibody immunity developed against SE are in contrast with a previous report (Holt et al., 1999). However, the nonsignificant difference in antibody titer between ST- and SE-infected pretreated chicks may suggest that antibody response was not interfered with by AG. The negative reaction with heterologous antigen suggests that it was serotype-specific.

TABLE 5. Comparison of the agglutination titers of sera from test chicks

<table>
<thead>
<tr>
<th>Group</th>
<th>Test strains</th>
<th>AG</th>
<th>Mean agglutination titer (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ST</td>
<td>+</td>
<td>60.71 (28.35)</td>
</tr>
<tr>
<td>2</td>
<td>ST</td>
<td>-</td>
<td>46.88 (8.84)</td>
</tr>
<tr>
<td>3</td>
<td>SE</td>
<td>+</td>
<td>108.33 (58.63)</td>
</tr>
<tr>
<td>4</td>
<td>SE</td>
<td>-</td>
<td>125.00 (46.29)</td>
</tr>
<tr>
<td>5</td>
<td>TSB</td>
<td>+</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

1Differences between the treatment groups and control (TSB) were significant (ANOVA; P > 0.05). Titers of sera from nonpretreated chicks infected with SE were significantly (ANOVA; P < 0.05) higher than those from ST-infected chicks, but chicks pretreated with Aviguard and infected with ST and SE did not differ significantly (ANOVA; P > 0.05) in their antibody titers. Agglutination reaction not detectable at 1:10 dilution was designated <10.

2AG = Aviguard (Microbial Developments Ltd., Worcestershire, England); ST = Salmonella typhimurium; TSB = trypticase soy broth.

Infected chicks, whether or not pretreated with AG, produced similar antibodies, suggesting further that the CE action of AG did not alter the antigen composition of salmonellae. Cross-reactions of sera from ST-infected chicks with SE antigens and of sera from SE-infected chicks with ST antigens yielded only a few and very weak bands of doubtful significance. However, that the sera from SE-infected chicks did not cross-react with the 67.7 kDa antigenic protein of ST suggests that this protein is SE-specific and the antibody to it could be a diagnostic marker of SE-infection.

In conclusion, this study has shown that AG is capable of protecting chicks from overwhelming colonization by salmonellae, without interfering with normal antibody response of chicks to infection by salmonellae. We also observed a significant antibody response to SE infection. Our study shows AG is effective as a CE product, for prophylactic control of salmonellae, particularly ST and SE, in poultry. A field trial may further prove the usefulness of AG for prophylactic control of salmonellae in poultry. This study also adds to our current understanding of antibody immunity against SE in poultry.

REFERENCES


