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SUMMARY. Colibacillosis accounts for significant losses to the poultry industry, and control efforts are hampered by limited understanding of the mechanisms used by avian pathogenic Escherichia coli (APEC) to cause disease. We have found that the presence of the increased serum survival gene (iss) is strongly associated with APEC but not with commensal E. coli, making iss, and the protein it encodes (Iss), candidate targets of colibacillosis control procedures. To assess the potential of Iss to elicit a protective response in chickens against APEC challenge, Iss fusion proteins were produced and administered subcutaneously to four groups of 2-wk-old specific-pathogen-free leghorn chickens. At 4 wk postimmunization, birds were challenged with APEC from serogroups O2 and O78 via intramuscular injection. At 2 wk postchallenge, birds were necropsied, and lesions consistent with colibacillosis were scored. Also, sera were collected from the birds pre- and postimmunization, and antibody titers to Iss were determined. Immunized birds produced a humoral response to Iss, and they had significantly lower lesion scores than the unimmunized control birds following challenge with both APEC strains. Birds that received the smallest amount of immunogen had the lowest lesion scores. Although further study will be needed to confirm the value of Iss as an immunoprotective antigen, these preliminary data suggest that Iss may have the potential to elicit significant protection in birds against heterologous E. coli challenge.

KEY words: APEC, avian colibacillosis, immunization, Iss

Abbreviations: APEC = avian pathogenic E. coli; CAMP = cyclic adenosine monophosphate; CFU = colony-forming unit; ELISA = enzyme-linked immunosorbant assays; GST = glutathione S-transferase; GST-Iss = Iss protein fused to glutathione S-transferase; IPTG = isopropyl-beta-D-thiogalactopyranoside; LB = Luria–Bertani; PBS = phosphate-buffered Saline; PBS-t = phosphate-buffered saline with 0.05% Tween 20; SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Colibacillosis, caused by avian pathogenic Escherichia coli (APEC), is a major problem for the poultry industry in the United States resulting in significant annual losses (2). One of the problems in colibacillosis control is that no single bacterial trait has been identified that can be used as an identifier of virulent avian isolates (2) or as a target of control strategies. Previous work showed that complement resistance may play an important role in APEC virulence (14,19,20,28), and the increased serum survival gene or iss (4), which is associated with E. coli complement resistance (4,6,7), was found significantly more often in APEC than it was in the E. coli isolates of apparently healthy birds (22,23). This strong association between iss and APEC suggested that iss-centric strategies might be useful in colibacillosis control.

For example, Iss, the protein encoded by iss (3,13), might be useful as an immunogen capable of eliciting a protective response against APEC infection in birds. If Iss could stimulate an immunoprotective response in birds, it might have wide-ranging benefits, because iss is found in APEC of many serogroups and in APEC isolated from various lesion types, avian host species, and forms of colibacillosis (22,23). This widespread distribution of iss among APEC suggests that an Iss-based vaccine could provide broad protection to birds against heterologous APEC challenge. Computer analysis of Iss’ predicted amino acid sequence has suggested that many portions of Iss are antigenic (13), and Iss is thought to be exposed on the bacterial surface in intact E. coli (3), meaning that it is accessible to the host’s immune system. Such observations suggest
that Iss may have the ability to evoke an immunoprotective response in birds against APEC that would have wide application. Consequently, in the present study, Iss was expressed and purified, and its potential to elicit an immunoprotective response against heterologous APEC challenge in chickens was assessed.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** Strains used were *E. coli* BL21 (DE3) (Stratagene, La Jolla, CA), *E. coli* APEC-C-O2, and *E. coli* APEC-C-O78. *Esherichia coli* BL21 was used for expression of GST-Iss and *E. coli* APEC-C-O2 and APEC-C-O78 were used in experimental challenges. APEC-C-O2 is an APEC of the O2 serogroup, and APEC-C-O78 is an APEC of the O78 serogroup. The O2 and O78 serogroups are among the most common ones causing disease in birds (25). All strains were maintained on Luria–Bertani (LB) agar (Difco, Detroit, MI) with ampicillin (100 μg/ml; Amresco, Solon, OH) where appropriate.

pGEX-6P-3 (Pharmacia Biotech, Piscataway, NJ), a plasmid expression vector designed for production of proteins fused to glutathione-S-transferase (GST), was used in these studies. pLN330 is composed of a vector designed for production of proteins fused to glutathione-S-transferase (GST-Iss), was used in these studies. pLN330 (10). Briefly, coating antigen (either GST-Iss or Iss) was diluted to 2–10 μg/ml in coating buffer (0.02 M carbonate, 0.03 M bicarbonate, pH 9.6). The wells were blocked with 3% solution of bovine serum albumin, dissolved in coating buffer. To determine antibody titers, sera were serially diluted in phosphate-buffered saline with 0.05% Tween 20 (PBSt). Following incubation with primary antibody, the antibody was removed and plates were washed with PBSt and incubated with goat anti-chicken alkaline-phosphatase–conjugated IgG (Sigma, St. Louis, MO). The plates were washed with PBSt and developed with P-nitrophenyl phosphate. Color development was recorded at 405 nm using an automated ELISA plate reader (model ELx808; Biotek, Winooski, VT.).

**Vaccine preparation and immunization procedure.** Immunization protocol. Each bird was given 0.5 ml of a water-in-oil emulsion containing either 50 μg, 10 μg, or 2 μg of GST-Iss per dose. At 3 wk of age, each chicken in groups 1A, 1B, 2A, 2B, 3A, and 3B received a 0.5-ml dose of the vaccine (Table 1). Birds in groups 4A and 4B were not vaccinated (nonvaccinated controls). The injections were given subcutaneously in the back of the neck at the midpoint between the head and body.

**Challenge infection.** Four weeks following vaccination, each bird was subjected to challenge with an APEC strain. Each bird in each group was given a 1.0-ml intramuscular injection of either 10⁸ or 10⁷ colony-forming units (CFUs) of APEC-C-O2 or 10⁸ CFUs of APEC-C-O78, as indicated in Table 1. Birds were observed for 14 days following challenge. Birds that died were necropsied and observed for lesions consistent with colibacillosis; cultures of bone marrow were taken for bacterial isolation on eosin methylene blue agar. Presumptive isolates were verified with API 20 strips (bioMerieux, Durham, NC). After the observation period, birds that survived were euthanatized, necropsied, and observed for lesions consistent with colibacillosis. Lesions consistent with colibacillosis, including mild or severe airsacculitis, pericarditis, perihepatitis, and/or death were scored according to a system outlined in Table 2.

**Biostatistics.** Efficacy of the vaccine against challenge was evaluated by average lesion scores of vaccines, as compared to unvaccinated birds. Data were analyzed by the chi-square 2-by-2 test with the significance at the P ≤ 0.05 level. For a valid test, the average lesion score of the positive control group must have been ≥2.0.

**RESULTS**

As previously described, iss was cloned into pGEX-6P-3 to produce a GST-Iss fusion protein (11). GST-Iss expression was induced with IPTG; the fusion was solubilized with commercial kits, purified by affinity chromatography, and its identity confirmed by

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of birds</th>
<th>Vaccine level (μg/dose)</th>
<th>Challenge serogroup</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>16</td>
<td>50</td>
<td>O2</td>
</tr>
<tr>
<td>1B</td>
<td>16</td>
<td>50</td>
<td>O78</td>
</tr>
<tr>
<td>2A</td>
<td>16</td>
<td>10</td>
<td>O2</td>
</tr>
<tr>
<td>2B</td>
<td>16</td>
<td>10</td>
<td>O78</td>
</tr>
<tr>
<td>3A</td>
<td>16</td>
<td>2</td>
<td>O2</td>
</tr>
<tr>
<td>3B</td>
<td>16</td>
<td>2</td>
<td>O78</td>
</tr>
<tr>
<td>4A</td>
<td>16</td>
<td>0</td>
<td>O2</td>
</tr>
<tr>
<td>4B</td>
<td>16</td>
<td>0</td>
<td>O78</td>
</tr>
</tbody>
</table>

**Score and Lesions**

0. No lesion
1. Cloudy air sacs (mild airsacculitis)
2. Fibrinous airsacculitis (severe airsacculitis)
3. Fibrinous airsacculitis and pericarditis
4. Fibrinous airsacculitis, pericarditis, perihepatitis, or death (must reisolate *E. coli* from the bone marrow of the femur for confirmation if dead)

Birds were divided into eight groups of 16 as indicated in Table 1. Birds were placed in stainless steel HEPA-filtered negative-pressure isolators 1 wk before vaccination. Birds were fed and watered *ad libitum* and were handled according to Institution Animal Care and Use protocols.

**Test animals.** One hundred twenty-eight 2-wk-old specific-pathogen-free leghorn chickens were obtained from Charles River Laboratories (Boston, MA). All chickens were from the same hatch.
sedimentation at this time with limitations being placed on the use of antimicrobial agents in animal production is being given close scrutiny in the past may not be as effective in the future. Also, use of certain therapeutic agents in poultry production (26,27). Consequently, control of avian colibacillosis using vaccines in certain situations may prove desirable.

Thus far, vaccines designed to prevent avian colibacillosis have been met with mixed results. Vaccines against APEC of various serogroups have been produced (1,5,8,9,12,15,16,17,21,24). Many of these have only been effective against homologous challenge. Kwaga et al. (17) attenuated a strain of APEC O2 serogroup by mutating the cyaA operon that encodes genes for carbamoylphosphatase synthetase, essential for arginine and pyrimidine biosynthesis. This vaccine was effective in turkeys against a homologous APEC challenge but not a heterologous challenge. In a study by Peighambadi and colleagues (21), APEC strains of O2 and O78 serogroups were attenuated by deletion of cya and crp, which encode 3',5'-cyclic adenosine monophosphate (cAMP) and cAMP receptor protein, respectively. The Δcya and Δcrp mutant of the E. coli O2 strain provided protection in chickens challenged with a homologous strain, but again, not against heterologous challenge. This failure to protect against heterologous challenge may prove a critical limitation of colibacillosis vaccines because APEC are quite diverse in terms of serogroup (23).

Targeting a surface-exposed structure found in most APEC, regardless of serogroup, would seem to be a logical approach to the development of a colibacillosis vaccine. It is thought that the widespread distribution of iss/Iss across APEC may avoid the limitations of some of these other vaccines. The iss gene is strongly associated with APEC of various serogroups but not with fecal E. coli isolates from apparently healthy birds (22,23), making iss, and the protein it encodes, a potential marker for virulent avian E. coli. iss was cloned into the plasmid pGEX-6P-3, and the resulting construct was used to vaccinate birds, those that received smaller doses of GST-Iss had lower lesion scores when immunized with GST-Iss, regardless of serogroup (23).

As previously stated, avian colibacillosis is a costly disease to the poultry industry, accounting for multimillion-dollar losses annually (2), making efficacious colibacillosis control measures highly desirable. Colibacillosis control frequently focuses on management approaches designed to reduce predisposing conditions among production birds, such as viral or mycoplasma infections (2). However, management strategies that have controlled colibacillosis in the past may not be as effective in the future. Also, use of antimicrobial agents in animal production is being given close scrutiny at this time with limitations being placed on the use of vaccines due to concerns about the development of antibiotic resistance (26,27).

In this study, we aimed to develop a colibacillosis vaccine using the iss gene as a potential marker for virulent avian E. coli. The iss gene encodes a potential marker for virulent avian E. coli (22,23), making iss a promising vaccine candidate. The iss gene has been shown to be associated with APEC of various serogroups (23).

To investigate the potential of iss as a vaccine candidate, we developed a vaccine that was able to protect turkeys against colibacillosis. The vaccine was made using the iss gene along with a vaccine delivery system (Table 3).

Table 3. Serum antibody titers.

<table>
<thead>
<tr>
<th>Group (dose)</th>
<th>Average titer preimmunization</th>
<th>Average titer postimmunization</th>
<th>Average titer preimmunization</th>
<th>Average titer postimmunization</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A (50 µg)</td>
<td>10^2</td>
<td>10^3</td>
<td>10^2</td>
<td>10^3</td>
</tr>
<tr>
<td>2A (50 µg)</td>
<td>10^2</td>
<td>10^3</td>
<td>10^2</td>
<td>10^3</td>
</tr>
<tr>
<td>3A (2 µg)</td>
<td>10^2</td>
<td>10^3</td>
<td>10^2</td>
<td>10^3</td>
</tr>
<tr>
<td>4A (none)</td>
<td>10^2</td>
<td>10^3</td>
<td>0^2</td>
<td>10^3</td>
</tr>
</tbody>
</table>

Table 4. Average lesion scores.

<table>
<thead>
<tr>
<th>Vaccine level (µg/dose)</th>
<th>Challenge</th>
<th>O2 challenge serogroup</th>
<th>O78 challenge serogroup</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Yes</td>
<td>0.38*</td>
<td>0.56*</td>
</tr>
<tr>
<td>10</td>
<td>Yes</td>
<td>0.75*</td>
<td>1.87*</td>
</tr>
<tr>
<td>50</td>
<td>Yes</td>
<td>1.31</td>
<td>1.63**</td>
</tr>
<tr>
<td>None</td>
<td>Yes</td>
<td>2</td>
<td>2.88*</td>
</tr>
<tr>
<td>None</td>
<td>No</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*One bird dead postchallenge and culture-positive for E. coli with no macroscopic lesions.

**Significantly different from nonvaccinated controls (P ≤ 0.05). (The Mann-Whitney U-test was used to determine the statistic.)
to produce a GST-Iss fusion protein. Because Iss alone is quite small, there was concern that it might not elicit a strong immune response. Therefore, GST was selected as a fusion partner in an effort to elicit a stronger immune response. There was also some concern that the majority of antibodies elicited by GST-Iss would be directed against GST and not Iss, because GST is approximately three times the size of Iss. However, birds immunized with GST-Iss were able to produce antibody titers against GST-Iss and Iss that were significantly different from unimmunized controls. Also, Iss did stimulate an immunoprotective response against heterologous challenge. Paradoxically, lower doses seemed to offer better protection than did higher doses, a result we cannot account for at this time. Much more research will be needed to determine if use of iss/Iss based vaccines and other control strategies are practical. Based on the results of this study, we feel that an iss/Iss based control strategy is worthy of future consideration and study.

REFERENCES