Immune Response to Recombinant Escherichia coli Iss Protein in Poultry

Aaron M. Lynne
Iowa State University

Steven L. Foley
Marshfield Clinic Research Foundation

Lisa K. Nolan
Iowa State University, lknolan@iastate.edu

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Abstract
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.

Keywords
APEC, avian colibacillosis, immunization, Iss

Disciplines
Veterinary Microbiology and Immunobiology | Veterinary Preventive Medicine, Epidemiology, and Public Health

Comments
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Research Note—

Immune Response to Recombinant *Escherichia coli* Iss Protein in Poultry

Aaron M. Lynne,\textsuperscript{A} Steven L. Foley,\textsuperscript{B} and Lisa K. Nolan\textsuperscript{AC}

\textsuperscript{A}Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA 50010
\textsuperscript{B}Marshfield Clinic Research Foundation, Marshfield, WI 54449

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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\textsubscript{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

\textsuperscript{C}Corresponding author.
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. *Escherichia 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) as previously described (11). Using pLN330, Iss protein, fused to glutathione-S-transferase (GST), was used in these studies. pLN330 is composed of pGEX-6P-3 with the iss gene (GenBank accession number AF0422279) without the coding region for the signal sequence (the first 72 nucleotides of AF0422279) (11).

**Expression and solubilization of Iss fusion proteins.** Expression of GST-Iss from pLN330 was induced with isopropyl-beta-D-thiogalactopyranoside (IPTG) as previously described (11). GST-Iss expression was induced with IPTG; the fusion was solubilized with commercial kits, purified by affinity chromatography, and its identity confirmed by immunosorbant assays (ELISA) to determine the presence of antibodies specific for GST-Iss and purified Iss using methods previously described (10).

**Protein purification.** Purification of GST-Iss from pLN330 was induced with isopropyl-beta-D-thiogalactopyranoside (IPTG) as previously described (11). Reduced cells were lysed with B-PER bacterial protein extraction reagent (Pierce, Rockford, IL) according to manufacturer’s instructions. Inclusion bodies containing GST-Iss were solubilized with inclusion body solubilization reagent according to manufacturer protocols (Pierce). Protein was refolded by dialysis against decreasing concentrations of urea and finally against Tris buffer (25 mM Tris-HCl, 150 mM NaCl, pH 7.5).

**Protein purification.** Purification of GST-Iss and Iss was achieved through use of an affinity matrix. The refolded protein was combined with a 50% slurry of glutathione sepharose 4B beads (Pharmacia) and incubated for 1 hr at 4 C. The mixture was added to a 10-cm² column, and the “flow-through” was collected. The column was washed three times with phosphate-buffered saline (PBS). For GST-Iss purification, the fusion protein was eluted with glutathione elution buffer (10 mM glutathione, 50 mM Tris, pH 8.0). For Iss purification, sepharose beads with bound fusion protein were resuspended in PreScission cleavage buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol) (Pharmacia). The fusion protein was cleaved using PreScission protease (Pharmacia). The mixture was added to a 10-cm² column, and Iss was eluted with cleavage buffer. Purification of the protein was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (18). Protein samples were separated on 15% acrylamide Tris-HCl gels (Bio-Rad, Hercules, CA) and stained with Blue-Bandit protein stain (Amresco, Solon, OH).

**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.

<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>

**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
Immune response to Iss

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 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 (1,5,8,9,12,15,16,17,24). Many of these have only been effective against homologous challenge. Kwaga et al. (17) attenuated a strain of APEC O2 serogroup by mutating the carAB operon that encodes genes for carbamoyl-phosphophatase 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 Peighambari 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

### Table 3. Serum antibody titers.

<table>
<thead>
<tr>
<th>Group (dose)</th>
<th>Anti-GST-Iss serum antibody titers</th>
<th>Anti-Iss serum antibody titers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average titer preimmunization</td>
<td>Average titer postimmunization</td>
</tr>
<tr>
<td>1A (50 µg)</td>
<td>10^2</td>
<td>10^3</td>
</tr>
<tr>
<td>2A (50 µg)</td>
<td>10^2</td>
<td>10^3</td>
</tr>
<tr>
<td>3A (2 µg)</td>
<td>10^2</td>
<td>10^3</td>
</tr>
<tr>
<td>4A (none)</td>
<td>10^2</td>
<td>0^2</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.

**Four birds dead postchallenge; three culture-positive for E. coli with severe systemic lesions; and one culture-negative with no macroscopic lesions.

**Two birds dead postchallenge and culture-positive for E. coli with severe systemic lesions.

*Significantly different from nonvaccinated controls (P ≤ 0.05).

**Significantly different from nonvaccinated controls (P ≤ 0.05). (The Mann-Whitney U-test was used to determine the statistic.)

Fig. 1. SDS-PAGE of the proteins used in this study. (A) SDS-PAGE of GST-Iss obtained after expression of pLN330 in E. coli BL21, followed by lysis of the cells with B-PER, solubilization of the protein with inclusion-body solubilization reagent, and purification with glutathione sepharose 4B. Lane S = molecular size standard; lane 1 = E. coli BL21 cell lystate; lane 2 = E. coli BL21, containing pLN330, following induction with IPTG; and lane 3 = purified GST-Iss. (B) SDS-PAGE of purified protein (Iss) obtained after cleavage of GST-Iss with PreScission protease. Lane S = molecular size standard; lane 1 = E. coli BL21 cell lystate; lane 2 = E. coli BL21, containing pLN330, following induction with IPTG; and lane 3 = purified Iss.

SDS-PAGE (Fig. 1). Purified Iss was obtained by cleaving Iss from the GST fusion partner and confirmed by SDS-PAGE (Fig. 1).

Chickens immunized with GST-Iss were able to generate a humoral immune response against GST-Iss and purified Iss as compared to controls. Birds that received a higher dose of GST-Iss had average antibody titers of 10,000 to 100,000 against GST-Iss, whereas birds that received a lower dose had average titers of 1000 (Table 3). Sera from birds also recognized purified Iss. Birds that received a high dose of fusion protein had average antibody titers of 10,000 against purified Iss, whereas birds that received lower doses had lower average titers of 1000 (Table 3).

During the observation period following challenge, no birds died that were challenged with APEC-C-O2. For birds challenged with APEC-C-O78, no birds died in group 1B; four birds died in group 2B (three of which cultured positive for E. coli); one bird, which was culture-positive for E. coli, died in group 3B; and two birds, both of which were culture-positive for E. coli, died in group 4B. Birds had lower lesion scores when immunized with GST-Iss, regardless of the challenge strain used, as compared to controls. Among the vaccinated birds, those that received smaller doses of GST-Iss had lower lesions scores when challenged with APEC of the O2 or O78 serogroups (Table 4).
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


