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SUMMARY. Avian pathogenic *Escherichia coli* (APEC) cause colibacillosis, a disease which is responsible for significant losses in poultry. Control of colibacillosis is problematic due to the restricted availability of relevant antimicrobial agents and to the frequent failure of vaccines to protect against the diverse range of APEC serogroups causing disease in birds. Previously, we reported that the increased serum survival gene (*iss*) is strongly associated with APEC strains, but not with fecal commensal *E. coli* in birds, making *iss* and the outer membrane protein it encodes (Iss) candidate targets for colibacillosis control procedures. Preliminary studies in birds showed that their immunization with Iss fusion proteins protected against challenge with two of the more-commonly occurring APEC serogroups (O2 and O78). Here, the potential of an Iss-based vaccine was further examined by assessing its effectiveness against an additional and widely occurring APEC serogroup (O1) and its ability to evoke both a serum and mucosal antibody response in immunized birds. In addition, tissues of selected birds were subjected to histopathologic examination in an effort to better characterize the protective response afforded by immunization with this vaccine. Iss fusion proteins were administered intramuscularly to four groups of 2-wk-old broiler chickens. At 2 wk postimmunization, chickens were challenged with APEC strains of the O1, O2, or O78 serogroups. One week after challenge, chickens were euthanatized, necropsied, any lesions consistent with colibacillosis were scored, and tissues from these birds were taken aseptically. Sera were collected pre-immunization, postimmunization, and post-challenge, and antibody titers to Iss were determined by enzyme-linked immunosorbent assay (ELISA). Also, air sac washings were collected to determine the mucosal antibody response to Iss by ELISA. During the observation period following challenge, 3/12 nonimmunized chickens, 1/12 chickens immunized with 10 μg of GST-Iss, and 1/12 chickens immunized with 50 μg of GST-Iss died when challenged with the O78 strain. No other deaths occurred. Immunized chickens produced a serum and mucosal antibody response to Iss and had significantly lower lesion scores than nonimmunized chickens following challenge, regardless of the challenge strain. This study expands on our previous report of the value of Iss as an immunoprotective antigen and demonstrates that immunization with Iss can provide significant protection of chickens against challenge with three different *E. coli* strains.

RESUMEN. La cepa recombinante Iss como una posible vacuna contra la colibacilosis aviar.
La *Escherichia coli* patogénica aviar (APEC) causa la colibacilosis, una enfermedad que es responsable de importantes pérdidas en la avicultura. El control de la colibacilosis es problemático debido a la limitada disponibilidad de agentes antimicrobianos relevantes y a la frecuente falla de las vacunas para proteger contra el diverso rango de serogrupos de APEC que causan enfermedades en las aves. Anteriormente, se reportó que el gene de la supervivencia aumentada contra suero (Iss) está fuertemente asociado con las cepas APEC, pero no con las cepas de *E. coli* comensales en materia fecal de las aves, favoreciendo que el gene *iss* y la proteína de membrana externa que codifica este gene (Iss) como los candidatos adecuados para procedimientos de control contra la colibacilosis. Los estudios preliminares en las aves mostraron que la inmunización con proteínas de fusión Iss protegieron contra el desafío con dos de los serogrupos APEC que más comúnmente se presentan (O2 y O78). En este caso, el potencial de una vacuna basada en Iss se examinó mediante la evaluación de su eficacia contra un serogrupo de APEC adicional y que se presenta ampliamente, (O1) y también se analizó su capacidad para evocar una respuesta de anticuerpos en el suero y en las mucosas en las aves inmunizadas. Además, los tejidos de las aves seleccionadas fueron sometidos a examen histopatológico con el fin de caracterizar mejor la respuesta de protección proporcionada por la inmunización con esta vacuna. Las proteínas de fusión Iss fueron administradas por vía intramuscular a cuatro grupos de pollos de engorde de dos semanas de edad. A las dos semanas postinmunización, los pollos fueron desafiados con cepas APEC de los serogrupos O1, O2, ó O78. Una semana después del desafío, se practicó la eutanasia de los pollos y se realizó la necropsia, las lesiones compatibles con colibacilosis fueron registradas, y los tejidos de estas aves se recolectaron en forma aséptica. Muestras de suero fueron recolectadas antes de la vacunación, postinmunización, y después del desafío, y los títulos de anticuerpos contra la ISS se determinaron mediante un ensayo de inmunoabsorción con enzimas ligadas (ELISA). Además, se recolectó el aire lavado de los sacos para determinar la respuesta de anticuerpos contra Iss en la mucosa por ELISA. Durante el período de observación posterior al desafío, 3 de 12 gallinas inmunizadas, 1 de 12 pollos inmunizados con 10 μg de GST-Iss, y 1 de 12 pollos inmunizados con 50 μg de GST-Iss murieron después de ser desafiados con la cepa O78. No se presentó más mortalidad. Los pollos vacunados produjeron una respuesta de anticuerpos en suero y en las mucosas contra Iss y mostraron puntuaciones más bajas en las lesiones, en comparación con los pollos no inmunizados y que fueron desafiados, independientemente de la cepa de desafío. Este estudio amplía el reporte previo sobre el valor de la proteína Iss como un antígeno inmunoprotector y demuestra que la inmunización con la Iss puede proporcionar una importante protección en los pollos contra el desafío con tres cepas diferentes de *E. coli*.

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Avian pathogenic *Escherichia coli* (APEC), the causative agent of colibacillosis, is a major problem for the poultry industry in the United States, resulting in significant losses annually (3). Additionally, recent reports, providing evidence of a link between APEC-contaminated poultry (21) and extraintestinal pathogenic *E. coli* (ExPEC)-caused neonatal meningitis (24,44) and urinary tract infections in human beings (24,39), suggest that control of avian colibacillosis may have a beneficial impact on both avian and human health. Control of colibacillosis is problematic, with the increased restrictions on the use of antimicrobial agents in poultry making vaccine-based control, where appropriate, increasingly desirable. Unfortunately, vaccine-based control has proven difficult, to date, as APECs are serotypically diverse with vaccines frequently failing to protect against all but homologous challenge. Thus, identification of vaccine targets, occurring among APECs, is an important step in the development of useful colibacillosis vaccines that will have widespread utility. Here, we evaluate an outer membrane protein (Iss)-based vaccine for its ability to elicit protection in chickens against heterologous challenge with APEC of three of the most prominent serogroups implicated in avian colibacillosis, including serogroups O1, O2, and O78 (40).

The increased serum survival gene *iss* (5,7,8,9,10,12,17,34,35,48), described for its contribution to *E. coli*'s complement resistance (5,8,9), was significantly more likely to occur in APEC than it was in *E. coli* isolated from the feces of apparently healthy chickens (37,40). The gene is found in most APEC regardless of its host type of origin, lesion of origin, or serogroup (40) and is linked to large, transmissible virulence plasmids which appear to be the defining trait of the APEC pathotype (23,40). Iss, the protein it encodes (4,16), occurs in APEC's outer membrane where it is readily accessible to the host defenses (31). This strong association between *iss* and APEC, along with outer membrane location of Iss, suggested that *iss*-centric strategies might be useful in colibacillosis control.

Previously, we evaluated the potential of a recombinant Iss fusion protein to elicit a protective response in chickens against APEC challenge (30). In this pilot study, chickens immunized with glutathione-S-transferase-Iss (GST-Iss) were able to produce serum antibody titers against GST-Iss and Iss that were significantly different from nonimmunized controls. Also, this Iss-based vaccine elicited a significant immunoprotective response to challenge with two different APEC strains, as compared to nonimmunized controls, with lower vaccine doses offering better protection than higher doses. Such results suggested that Iss vaccines might be useful in protecting against APEC infections in birds. However, this pilot study did have limitations. The previous study did not take into account any vaccine-associated immunopathology that could have contributed to the lesions observed. Such pathologic changes might be dose related, providing an explanation for the 'apparent' inverse relationship between immunoprotection and vaccine dose. Also, in the initial pilot study, mucosal antibody responses were not assessed. Here, we sought to further study this vaccine and the protection it provides against colibacillosis by challenging vaccinates and control chickens with one of three common APEC serogroups implicated in

disease and by monitoring the mucosal and serum anti-Iss antibody levels and histopathologic changes of the challenged animals. In this way, we hope to better characterize Iss' utility as an anticolibacillosis vaccine.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains used were *E. coli* BL21 (DE3) (Stratagene, La Jolla, CA), APEC strains APEC O1 (O1:K1) (19), APEC O2 (O2:K2) (22), and χ7122 (O78:K80) (38). *Escherichia coli* BL21 was used for expression of GST-Iss while APEC O1, APEC O2, and χ7122 were used in experimental challenges. All strains maintained on Luria-Bertani 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 GST, was used in these studies. pLN330 is composed of pGEX-6P-3 with the *iss* gene sequence (GenBank accession number AF0422279) without the coding region for the signal sequence (the first 72 nucleotides of *iss*) (13). Using pLN330, Iss protein fused to glutathione S-transferase (=GST-Iss) can be expressed in *E. coli* BL21.

Expression and purification of Iss fusion proteins. Expression of GST-Iss from pLN330 was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) as previously described (13). Induced 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 (Pierce) according to manufacturer protocols. The protein was refolded by dialysis against decreasing concentrations of urea and finally against Tris buffer (25mM Tris-HCl, 150 mM NaCl, pH 7.5). 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, Waukesha, WI) and incubated for 1 hr at 4 C. The mixture was added to a 10-ml column and the "flow through" 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, 1mM dithiothreitol [Pharmacia]). The fusion protein was cleaved using PreScission protease (Pharmacia). The mixture was added to a 10-ml column and Iss was eluted with cleavage buffer.

Chickens. Five, 2-wk-old specific-pathogen-free (SPF) leghorn chickens and 240 1-day-old broiler chickens were obtained from Charles River Laboratories (Charles River SPAFAS, North Franklin, CT) and a commercial farm, respectively. SPF chickens were used to obtain hyperimmune and negative control sera for enzyme-linked immunosorbant assay (ELISA) experiments while broilers were used for challenge studies. All chickens were housed in the Laboratory Animal Resources building (Iowa State University, Ames, IA), given access to food and water *ad libitum*, and handled according to Institution of Animal Care and Use protocols. SPF chickens were placed in stainless steel HEPA-filtered negative-pressure isolation rooms prior to vaccination.

Immunization of broiler chickens. Broiler chickens were randomized, banded, and divided into four groups of 60 chickens with each bird

receiving a unique number. At 2 wk of age, each bird was given 0.5 ml (100 µg/ml) of QuilA[TM] adjuvant (Superfos Biosector a/s, Frederikssund, Denmark) in PBS, containing either 50 µg, 10 µg, 2 µg, or 0 µg (PBS control) of GST-Iss, intramuscularly into the thigh.

Challenge experiment. Two weeks following immunization, 12 broiler chickens from each group were separated into four different rooms and were subjected to challenge with an APEC strain. Each chicken in the same room was challenged with the same strain. Chickens in rooms 1, 2, and 3 received 0.1 ml (10^7 colony-forming units) of APEC O1, APEC O2, or χ7122, respectively, into the left caudal thoracic air sac. As a negative control, chickens in room 4 received 0.1 ml of PBS, mixed with the adjuvant, via the same route. Chickens were observed for signs of disease for 7 days following challenge. Chickens that died were necropsied and examined for lesions consistent with colibacillosis. In addition, samples from air sacs, pericardium, and liver were collected for bacterial isolation on MacConkey agar and in brain heart infusion broth. After the observation period, survivors were euthanatized with Sleepaway® (Fort Dodge Laboratories, Fort Dodge, IA), necropsied, examined for lesions consistent with colibacillosis, and samples were taken as before. Lesions consistent with colibacillosis, including mild or severe airsacculitis, perihepatitis, pericarditis, or death, were scored according to the established system outlined in Table 1 (36). In an effort to keep lesion scoring as objective as possible, the researchers who were performing the necropsy and scoring lesions were not told to which groups the birds belonged. Each bird was scored by two people independently and then scores were compared for consistency. Any scores that were not the same were scored by a third individual. If the third score matched one of the first two scores, that score was recorded.

Collection of sera. Blood was collected from chickens before immunization, 1 wk postimmunization, 2 wk postimmunization, and 1 wk postchallenge. Sera were collected from blood samples and stored at −20 C for later evaluation using ELISA.

Collection of air sac washings. Air sacs from the immunized and control chickens were washed with 1 ml of sterile PBS, centrifuged, and the supernatant from each was stored at −20 C for later evaluation using ELISA.

Production of control sera for ELISA. Hyperimmune chicken sera (HICS) were raised against Iss in five SPF chickens. At the age of 2 mo, each chicken was given a 0.5-ml suspension, intramuscularly, containing 50 µg of QuilA adjuvant and 50 µg of GST-Iss. Two booster injections were given 2 wk apart via the same route. Chickens were bled 10 days after the last immunization to collect HICS. Sera collected before immunization served as the negative control in the ELISA assays.

ELISA. Sera collected from the challenge experiment were used to perform the antigen-specific ELISA to determine the presence of

Table 1.   Lesion scoring.[A]

Location and description	Score
Air sacs	
Normal	0
Mild cloudiness and thickness	1
Moderate cloudiness and thickness accompanied by serous exudates or fibrin spots	2
Extensive cloudiness and thickness accompanied by muco- or fibrinopurulent exudate	3
Heart and pericardium	
Normal	0
Turbid with extensive or cloudy fluid in the pericardial cavity	1
Marked pericarditis	2
Liver	
Normal	0
Slight amount of fibrinous exudate	1
Marked perihepatitis	2
Recovery of *E. coli* from either air sacs, pericardial sac, or liver	1

[A]Maximum possible score = 8.

antibodies specific for Iss using methods previously described (30). Iss coating antigen was diluted to 2–10 µg/ml in coating buffer (0.02 M carbonate, 0.03 M bicarbonate, pH 9.6); 100 µl of the coating antigen was added to each of the wells of a 96-well plate. The plate was covered and incubated overnight at 4 C. The plate was then washed three times with coating buffer. Wells were blocked with 200 µl of a 3% solution of bovine serum albumin, dissolved in coating buffer, and incubated for 3 hr at room temperature to prevent non-specific binding. The blocker was removed and the trays were washed three times with PBS with 0.05% Tween 20 (PBSt).

To determine antibody titers, sera were serially diluted in PBSt. HICS was used as a positive control and PBSt was used as a negative control. One-hundred microliter samples of each dilution were dispensed into the wells of the plate. For each ELISA, trays were incubated for 1 hr at room temperature. Following incubation with primary antibody, the antibody was removed and the plates were washed five times with PBSt. One-hundred microliters of alkaline phosphatase-conjugated goat anti-chicken IgG (Sigma, St. Louis, MO) was added to each well. Plates were washed seven times with PBSt and developed with *p*-nitrophenyl phosphate in diethanolamine buffer. Color development was recorded at 405 nm using an automated ELISA plate reader (Biomek Plate Reader; Beckman, Fullerton, CA). To determine the presence of mucosal antibodies specific for Iss, the ELISA was performed in the same manner except that undiluted air-sac washings were used. However, total protein concentrations of each washing were determined and samples were normalized to relatively equal protein concentrations for use in ELISA.

Histopathology. Samples from air sacs, pericardium, and liver of necropsied chickens were collected and processed for light microscopy. Tissues were fixed in 10% buffered formalin embedded in paraffin and sectioned at 5 µm. Sections were stained with hematoxylin and eosin and examined with light microscopy.

Biostatistics. Efficacy of the vaccine against challenge was evaluated by the number of deaths and average lesion scores of vaccinates as compared to unvaccinated chickens. Data were analyzed by the Fisher's least significant difference multiple comparison test with the significance level set at $P \le 0.05$.

RESULTS

GST-Iss was expressed from *E. coli* BL21 (DE3) containing pLN330 by induction with IPTG. Cells were then harvested and lysed. Inclusion bodies were solubilized, and the fusion protein was purified by affinity chromatography (30) and used as an immunogen. Also, recombinant Iss was produced for use with ELISA to determine serum titers. Iss was cleaved from its fusion partner with PreScission protease and the fusion partner was removed by affinity chromatography (30).

Chickens immunized with GST-Iss were able to generate an antibody immune response against Iss as compared to controls (Fig. 1). All chickens that were immunized with GST-Iss had a significant increase ($P < 0.05$) in serum IgG antibody titer compared to chickens that received PBS. After challenge with APEC, all chickens showed an increase in serum anti-Iss IgG titers, regardless of the challenge strain. No significant difference was observed in titer levels of chickens challenged with different APEC strains. Chickens that were immunized with GST-Iss also showed a significantly greater ($P < 0.05$) anti-Iss IgA and IgG response in air sac mucosa samples (Fig. 2). Also, no significant difference was observed in titer levels of chickens challenged with the different *E. coli* strains.

During the observation period following challenge, 3/12 nonimmunized chickens, 1/12 chickens immunized with 10 µg of GST-Iss, and 1/12 chickens immunized with 50 µg of GST-Iss died when challenged with O78 strain χ7122. No other deaths occurred. Chickens had significantly lower lesion scores when immunized with
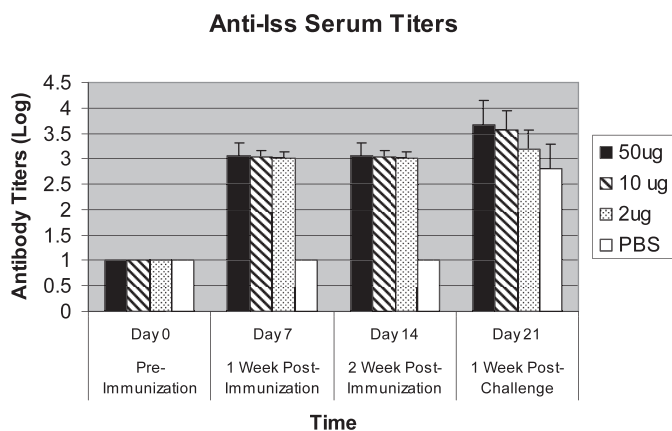
Anti-Iss Serum Titers

Fig. 1.   Immunogenicity of Iss in chickens. Columns represent the means for each group, bars represent standard deviations.

GST-Iss, regardless of dose level or challenge strain used, when compared to controls (Table 2).

Macroscopic and microscopic lesions (or both) in the air sacs, liver, and pericardium consistent with colisepticemia were evident in some of the chickens that were challenged with *E. coli*. These lesions were detected in chickens challenged with each of the different APEC strains. Macroscopic lesions observed included thickened air sacs with caseous exudate and yellowish, fibrinous membranes covering the liver and the heart (Fig. 3). Histopathologic lesions included heterophil and mononuclear cell infiltration, hyperplasia of the epithelium, and the presence of necrotic foci in the air sacs, liver, pericardium, and myocardium in varying combinations (Fig. 4). One bird vaccinated with 50 µg of GST-Iss, but not challenged with any of the challenge strains, showed lesions consistent with colisepticemia. Bacterial cultures of the air sac, pericardium, liver, and blood were all positive when cultured for *E. coli*.

DISCUSSION

Avian colibacillosis is a costly disease to the poultry industry, accounting for multimillion-dollar losses annually (3). There is a need for improved control of avian colibacillosis because current control strategies, which focus on reducing predisposing conditions among production chickens, have proven largely ineffective (3). In addition, strategies that have controlled colibacillosis in the past may not be as effective in the future, as recent studies in our lab show that
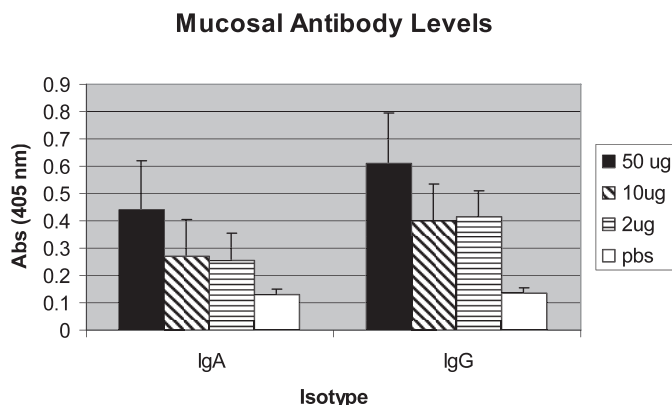
Mucosal Antibody Levels

Fig. 2.   Mucosal antibody levels as determined by ELISA of air sac washings 2 wk postimmunization. Columns represent the means for each group, bars represent standard deviations.

APECs are becoming more resistant to antimicrobials over time (unpubl. data). Also, the use of antimicrobial agents in animal production is under close scrutiny, with limitations being placed on the use of some therapeutic agents in poultry production (10). Thus, it seems likely that future control of colibacillosis by using vaccines may prove increasingly desirable.

To date, vaccines designed to prevent avian colibacillosis have been met with mixed results. Vaccines against various *E. coli* serogroups have been produced (1,2,6,7,11,12,14,15,25,27,29,30,32,36,41,45,46,47). The composition of these vaccines has been quite variable and has included live, attenuated *E. coli* strains (1,25,29,36), inactivated *E. coli* strains (11,12,32), active immunization with various *E. coli* antigens (7,30,41,46,47), and passive immunization (2,6,26). While many of these vaccines were effective against homologous challenge, they did not offer broad-range protection against heterologous challenge. Failure to protect against heterologous challenge may prove a critical limitation of colibacillosis vaccines because of the diversity among APECs (40).

Previously, we evaluated the potential of a recombinant Iss fusion protein to elicit a protective response in chickens against APEC challenge with O2 and O78 strains (30). Chickens immunized with GST-Iss were able to produce antibody titers against GST-Iss and Iss that were significantly different from nonimmunized controls. Also, Iss did stimulate an immunoprotective response against challenge with the different APEC strains. Paradoxically, lower doses seemed to offer better protection than higher doses, a result we could not explain adequately due to the limited scope of the study. However, at that time, we felt that this result could be due to immunologic unresponsiveness or immunopathology associated with excessively high doses of antigen (28). In this study, we immunized broiler chickens and challenged them with one of three different APEC strains of the O1, O2, or O78 serogroups. These isolates were different than those used in our previous study. Our results showed that chickens immunized with GST-Iss at 2 wk of age were able to mount a strong antibody response against the Iss portion of the fusion protein that was significantly different from nonimmunized controls. Also, when nonimmunized controls were subsequently challenged with an iss^+ APEC strain, anti-Iss antibody titers increased to the levels of those seen in immunized chickens, confirming our previous observation that Iss is surface-exposed in APEC, where it interacts with the immune system during infection. Immunized chickens also showed a mucosal antibody response in the air sacs with both IgG and IgA isotypes, directed against Iss, corresponding to results seen in other studies (27). As colibacillosis often begins as a respiratory disease, an effective immune response in the mucosa of the air sacs is desirable. Histopathologic examination of challenged birds revealed lesions consistent with colibacillosis. These data, plus the fact that the challenge strains were cultured from the lesions, indicate that the lesions observed were due to APEC infection.

The average lesion scores for chickens immunized with GST-Iss were significantly lower than nonimmunized controls for all dose levels regardless of challenge strain. In our pilot study, lower doses of the immunogen provided better protection against subsequent challenge than did higher doses. The higher lesion score associated with the higher doses of immunogen could be due to immunopathology caused by the large levels of immunogen, thereby increasing the lesions scores in those chickens that received the higher doses. In the present study, while all doses significantly lowered lesion scores compared to controls, there was no consensus as to which dose level offered the best protection. For the three groups of immunized chickens that were given PBS instead of a challenge strain, no lesions

Table 2.   Average lesion score after immunization and challenge.[A]

Vaccine level (μg/dose)	Challenge strain			
	O1	O2	O78	PBS
50	0.167 (0.389)[B]	0.33 (0.65)[B]	1.27 (2.37)[BC]	0.2 (0.63)
10	0.167 (0.389)[B]	0.00 (0.00)[B]	1.27 (2.10)[BD]	0.0 (0.0)
2	0.90 (2.50)[B]	0.167 (0.58)[B]	1.27 (2.00)[BD]	0.0 (0.0)
PBS	2.38 (2.10)	1.36 (1.03)	4.09 (3.30)	0.0 (0.0)

[A]Values are means (standard deviations) with 12 chickens per group.
[B]Significantly different ($P < 0.05$) from lesion score of chickens vaccinated with PBS and challenged with same strain.
[C]Three deaths occurred.
[D]One death occurred.

consistent with colibacillosis were seen for chickens immunized with 2 μg and 10 μg, while one bird that received 50 μg of immunogen showed mild lesions consistent with colibacillosis, resulting in a small average lesion score that was not significantly different ($P < 0.05$) than controls. The average lesion scores are interesting, as they do not confirm the results of our previous study in regard to vaccine efficacy and its relationship to dosage. We had hypothesized that the higher lesions scores associated with the higher Iss doses were due to immunization-caused pathology; we were unable to confirm that hypothesis in the current study.

While this study was unable to account for the results obtained previously, the data are useful in further characterization of Iss as a potential vaccine candidate. Here, immunization with a GST-Iss fusion was shown to provide significant protection against three different APEC isolates of different serogroups. The ability to protect against heterologous challenge is critical, as APECs are very diverse (37,40). Thus, the fact that the Iss fusion protein was effective against all three serotypes tested is promising, as the challenge isolates used in this study are quite diverse in their content of virulence factors and antigens (19,20,22,38). Also, the histopathology found in nonimmunized, challenged chickens shows that the gross lesions seen in these chickens were due to APEC infection.
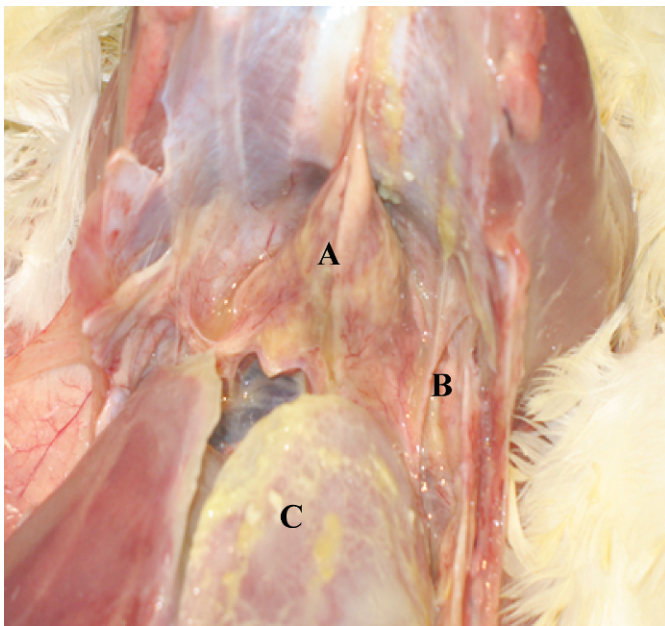

Fig. 3.   Colisepticemic lesions of an unvaccinated chicken challenged with *E. coli* O78. (A) pericarditis; (B) airsacculitis; (C) perihepatitis. A yellowish, fibrinous covering is present on the serosal surfaces of the air sacs, heart, and liver.

This histopathology was not seen in immunized chickens, suggesting that immunization with the Iss fusion protein not only provided protection against challenge, it lacked an evident adverse effect. Finally, because the Iss fusion protein provided a mucosal antibody response in the air sac, we suspect that an Iss-based vaccine might prove effective in preventing avian colibacillosis. Future field trials will be beneficial in assessing this supposition.

Overall, our current study demonstrates that the Iss fusion protein is capable of eliciting protection of chickens against challenge with different APEC strains. While these results are encouraging, our study did have limitations. While vaccinates demonstrated protection against three of the more common serogroups occurring in APEC (43), many other serogroups are known to cause disease in chickens (40). Thus, future studies will need to assess the vaccine for its ability to evoke protection against multiple APEC strains. However, because the O1, O2, and O78 serogroups are among the more commonly occurring serogroups causing colibacillosis, significant and important protection of birds might follow Iss vaccination whether or not it protected against other serogroups. Also, while *iss* is one of the most widely occurring traits among APECs (37,40), a small subset of *E. coli* isolated from avian colibacillosis lack *iss* (37,40), suggesting that an Iss vaccine would not protect chickens from infections with all APECs. However, coverage need not be total to provide significant benefit to flock health.

Therefore, an efficacious and cost-effective Iss-based vaccine, protective against APEC O1, O2, and O78 serogroups, could prove beneficial to the poultry industry. It might also, in turn, be beneficial to human health. Recently, researchers have explored the possibility that there is a link between APEC and human ExPEC, including uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC). When Rodriguez-Siek *et al.* (39) compared traits of APEC and human UPEC, they found that these two groups overlapped in their serogroups, phylogenetic groups, and virulence genotypes including in their possession of *iss*. Johnson *et al.* (19,22) showed that *iss* occurs on a conserved virulence cluster found on conjugative ColV and ColBM plasmids of APEC isolates. Skyberg *et al.* (42) found that transfer of these virulence plasmids to commensal *E. coli* increased the recipient strains' ability to grow in human urine and to colonize the kidney of mice. In order to follow up on this observation, Johnson *et al.* (20) sequenced the entire genome of APEC O1 (an O1:K1:H7 strain) and compared it to other known *E. coli* genomes. The APEC O1 genome was greater than 95% similar to the genomes of human ExPEC (20). Also, the APEC genome was found to be very closely related to ExPEC strains UPEC UTI89 and NMEC RS218, based on *in silico* multilocus sequence typing (20). Others findings have confirmed these similarities. For instance, when Moulin-Schouler *et al.* (33) compared O18:K1 ExPEC strains of human and avian origin for common virulence factors and genetic relationship, they found that APEC and NMEC
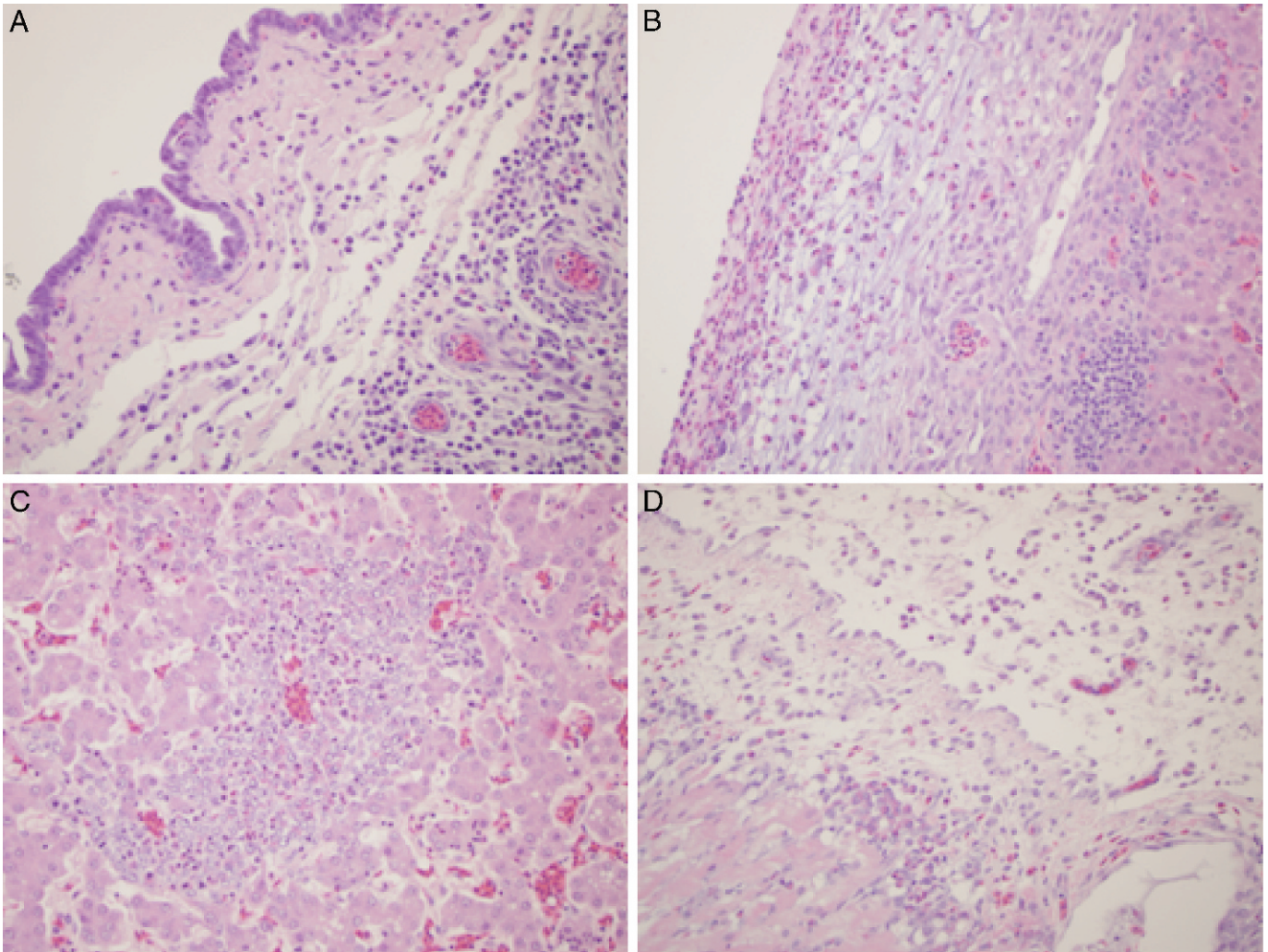
Fig. 4.   Histopathologic lesions: (A) Airsacculitis in an unvaccinated chicken challenged with APEC O78. Epithelial hyperplasia, vascularization, and infiltration with heterophils and lymphocytes. Fibrinous material is present on the serosal surface ($\times$40). (B) Perihepatitis in an unvaccinated chicken challenged with APEC O78. Fibrinopurulent material on the liver surface and infiltration with lymphocytes and heterophils ($\times$40). (C) Hepatic lesions in an unvaccinated chicken challenged with APEC O78. Necrosis of hepatocytes and subsequent infiltration with lymphocytes ($\times$40). (D) Cardiac lesions in an unvaccinated chicken challenged with APEC O78. Infiltration of pericardium and epicardium with lymphocytes and heterophils. Fibrinopurulent exudate and giant cells can be seen in the pericardium ($\times$40).

share several virulence factors and overlap in their phylogenetic grouping. Also, in a study of over 1,000 ExPEC from human and avian sources, Johnson *et al.* (24) found a mixed cluster of ExPEC subpathotypes (APEC, NMEC, and UPEC) that shared much overlap in their virulence genotypes and belonged to serogroups, MLST types, and phylogenetic groups that typify human pathogens. They regarded the isolates of this mixed cluster as being potential zoonotic pathogens. In addition to their similarities in chromosomal virulence genes, most of these isolates harbored *iss*-containing plasmids, suggesting that Iss-based vaccines might be efficacious in protecting against infection with these organisms. Interestingly, Tivendale *et al.*'s (44) further evaluation of members of this cluster in avian and mammalian models of disease appears to have confirmed their zoonotic potential. Thus, development of Iss-based vaccines might prove beneficial to both avian and human health. Indeed, development of a universal ExPEC vaccine could find a broad spectrum of use, protecting against such human diseases as septicemia, urinary tract infection, and meningitis and such avian diseases as septicemia, airsacculitis, cellulitis, and peritonitis. Also,

such a vaccine might reduce the occurrence of ExPEC-caused diseases in many other hosts such as felines, canines, swine, and bovines, as ExPEC-caused diseases occur widely among various animal hosts (18). Thus, future research to evaluate the protective potential of Iss-based vaccines, as described here against ExPEC infections in nonavian hosts, seems warranted.

REFERENCES

1. Amoako, K. K., T. Prysliak, A. A. Potter, S. K. Collinson, W. W. Kay, and B. J. Allan. Attenuation of an avian pathogenic Escherichia coli strain due to a mutation in the rpsL gene. Avian Dis. 48:19–25. 2004.

2. Arp, L. H. Effect of passive immunization on phagocytosis of blood-borne Escherichia coli in spleen and liver of turkeys. Am. J. Vet. Res. 43:1034–1040. 1982.

3. Barnes, H. J., L. K. Nolan, and J. P. Vaillancourt. Colibacillosis. In: Diseases of poultry, 12th ed. Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan, and D. E. Swayne, eds. Blackwell Publishing, Ames, IA. pp. 691–732. 2008.

4. Barondess, J. J., and J. Beckwith. Bor gene of phage lambda, involved in serum resistance, encodes a widely conserved outer membrane lipoprotein. J. Bacteriol. 177:1247–1253. 1995.

5. Binns, M. M., D. L. Davies, and K. G. Hardy. Cloned fragments of the plasmid ColV,I-K94 specifying virulence and serum resistance. Nature 279:778–781. 1979.

6. Bolin, C. A., and A. E. Jensen. Passive immunization with antibodies against iron-regulated outer membrane proteins protects turkeys from Escherichia coli septicemia. Infect. Immun. 55:1239–1242. 1987.

7. Chaffer, M., B. Schwartsburd, and E. D. Heller. Vaccination of turkey poults against pathogenic *Escherichia coli*. Avian Pathol. 26:377–390. 1997.

8. Chuba, P. J., M. A. Leon, A. Banerjee, and S. Palchaudhuri. Cloning and DNA sequence of plasmid determinant iss, coding for increased serum survival and surface exclusion, which has homology with lambda DNA. Mol. Gen. Genet. 216:287–292. 1989.

9. Chuba, P. J., S. Palchaudhuri, and M. A. Leon. Contributions of traT and iss genes to the serum resistance phenotype of plasmid ColV2-K94. FEMS Microbiol. Lett. 37:135–138. 1986.

10. Crawford, L. Animal drugs, feeds, and related products; enrofloxacin for poultry; withdrawal of approval of new animal drug application. Docket No. 2000N-1571, OC 2005194. Final Rule. pp. 44048–44049. 2005.

11. Deb, J. R., and E. G. Harry. Laboratory trials with inactivated vaccines against Escherichia coli (O78 K80) infection in fowls. Res. Vet. Sci. 20:131–138. 1976.

12. Deb, J. R., and E. G. Harry. Laboratory trials with inactivated vaccines against Escherichia coli (O2:K1) infection in fowls. Res. Vet. Sci. 24:308–313. 1978.

13. Foley, S. L., S. M. Horne, C. W. Giddings, M. Robinson, and L. K. Nolan. Iss from a virulent avian Escherichia coli. Avian Dis. 44:185–191. 2000.

14. Frommer, A., P. J. Freidlin, R. R. Bock, G. Leitner, M. Chaffer, and E. D. Heller. Experimental vaccination of young chickens with a live, non-pathogenic strain of *Escherichia coli*. Avian Pathol. 23:425–433. 1994.

15. Gyimah, J. E., B. Panigrahy, and J. D. Williams. Immunogenicity of an Escherichia coli multivalent pilus vaccine in chickens. Avian Dis. 30:687–689. 1986.

16. Horne, S. M., S. J. Pfaff-McDonough, C. W. Giddings, and L. K. Nolan. Cloning and sequencing of the iss gene from a virulent avian Escherichia coli. Avian Dis. 44:179–184. 2000.

17. Ike, K., K. Kawahara, H. Danbara, and K. Kume. Serum resistance and aerobactin iron uptake in avian Escherichia coli mediated by conjugative 100-megadalton plasmid. J. Vet. Med. Sci. 54:1091–1098. 1992.

18. Johnson, J. R., P. Delavari, A. L. Stell, T. S. Whittam, U. Carlino, and T. A. Russo. Molecular comparison of extraintestinal Escherichia coli isolates of the same electrophoretic lineages from humans and domestic animals. J. Infect. Dis. 183:154–159. 2001.

19. Johnson, T. J., S. J. Johnson, and L. K. Nolan. Complete DNA sequence of a ColBM plasmid from avian pathogenic Escherichia coli suggests that it evolved from closely related ColV virulence plasmids. J. Bacteriol. 188:5975–5983. 2006.

20. Johnson, T. J., S. Kariyawasam, Y. Wannemuehler, P. Mangiamele, S. J. Johnson, C. Doetkott, J. A. Skyberg, A. M. Lynne, J. R. Johnson, and L. K. Nolan. The genome sequence of avian pathogenic Escherichia coli strain o1:k1:h7 shares strong similarities with human extraintestinal pathogenic E. coli genomes. J. Bacteriol. 189:3228–3236. 2007.

21. Johnson, T. J., C. M. Logue, Y. Wannemuehler, S. Kariyawasam, C. Doetkott, C. DebRoy, D. G. White, and L. K. Nolan. Examination of the source and extended virulence genotypes of Escherichia coli contaminating retail poultry meat. Foodborne Pathog. Dis. 6:657–667. 2009.

22. Johnson, T. J., K. E. Siek, S. J. Johnson, and L. K. Nolan. DNA sequence of a ColV plasmid and prevalence of selected plasmid-encoded virulence genes among avian Escherichia coli strains. J. Bacteriol. 188: 745–758. 2006.

23. Johnson, T. J., Y. Wannemuehler, C. Doetkott, S. J. Johnson, S. C. Rosenberger, and L. K. Nolan. Identification of minimal predictors of avian pathogenic Escherichia coli virulence for use as a rapid diagnostic tool. J. Clin. Microbiol. 46:3987–3996. 2008.

24. Johnson, T. J., Y. Wannemuehler, S. J. Johnson, A. L. Stell, C. Doetkott, J. R. Johnson, K. S. Kim, L. Spanjaard, and L. K. Nolan. Comparison of extraintestinal pathogenic Escherichia coli strains from human and avian sources reveals a mixed subset representing potential zoonotic pathogens. Appl. Environ. Microbiol. 74:7043–7050. 2008.

25. Kariyawasam, S., B. N. Wilkie, and C. L. Gyles. Construction, characterization, and evaluation of the vaccine potential of three genetically defined mutants of avian pathogenic Escherichia coli. Avian Dis. 48:287–299. 2004.

26. Kariyawasam, S., B. N. Wilkie, and C. L. Gyles. Resistance of broiler chickens to Escherichia coli respiratory tract infection induced by passively transferred egg-yolk antibodies. Vet. Microbiol. 98:273–284. 2004.

27. Kariyawasam, S., B. N. Wilkie, D. B. Hunter, and C. L. Gyles. Systemic and mucosal antibody responses to selected cell surface antigens of avian pathogenic Escherichia coli in experimentally infected chickens. Avian Dis. 46:668–678. 2002.

28. Kuby, J. Immunology, 3rd ed. W. H. Freeman and Company, New York. 1998.

29. Kwaga, J. K., B. J. Allan, J. V. van der Hurk, H. Seida, and A. A. Potter. A carAB mutant of avian pathogenic Escherichia coli serogroup O2 is attenuated and effective as a live oral vaccine against colibacillosis in turkeys. Infect. Immun. 62:3766–3772. 1994.

30. Lynne, A. M., S. L. Foley, and L. K. Nolan. Immune response to recombinant Escherichia coli Iss protein in poultry. Avian Dis. 50:273–276. 2006.

31. Lynne, A. M., J. A. Skyberg, C. M. Logue, and L. K. Nolan. Detection of Iss and Bor on the surface of Escherichia coli. J. Appl. Microbiol. 102:660–666. 2007.

32. Melamed, D., G. Leitner, and E. D. Heller. A vaccine against avian colibacillosis based on ultrasonic inactivation of Escherichia coli. Avian Dis. 35:17–22. 1991.

33. Moulin-Schouleur, M., C. Schouler, P. Tailliez, M. R. Kao, A. Bree, P. Germon, E. Oswald, J. Mainil, M. Blanco, and J. Blanco. Common virulence factors and genetic relationships between O18:K1:H7 Escherichia coli isolates of human and avian origin. J. Clin. Microbiol. 44:3484–3492. 2006.

34. Nolan, L. K., S. M. Horne, C. W. Giddings, S. L. Foley, T. J. Johnson, A. M. Lynne, and J. Skyberg. Resistance to serum complement, iss, and virulence of avian Escherichia coli. Vet. Res. Commun. 27:101–110. 2003.

35. Nolan, L. K., R. E. Wooley, and R. K. Cooper. Transposon mutagenesis used to study the role of complement resistance in the virulence of an avian Escherichia coli isolate. Avian Dis. 36:398–402. 1992.

36. Peighambari, S. M., D. B. Hunter, P. E. Shewen, and C. L. Gyles. Safety, immunogenicity, and efficacy of two Escherichia coli cya crp mutants as vaccines for broilers. Avian Dis. 46:287–297. 2002.

37. Pfaff-McDonough, S. J., S. M. Horne, C. W. Giddings, J. O. Ebert, C. Doetkott, M. H. Smith, and L. K. Nolan. Complement resistance-related traits among Escherichia coli isolates from apparently healthy birds and birds with colibacillosis. Avian Dis. 44:23–33. 2000.

38. Provence, D. L., and R. Curtiss III. Role of crl in avian pathogenic Escherichia coli: a knockout mutation of crl does not affect hemagglutination activity, fibronectin binding, or curli production. Infect. Immun. 60:4460–4467. 1992.

39. Rodriguez-Siek, K. E., C. W. Giddings, C. Doetkott, T. J. Johnson, M. K. Fakhr, and L. K. Nolan. Comparison of Escherichia coli isolates implicated in human urinary tract infection and avian colibacillosis. Microbiology 151(Pt 6):2097–2110. 2005.

40. Rodriguez-Siek, K. E., C. W. Giddings, C. Doetkott, T. J. Johnson, and L. K. Nolan. Characterizing the APEC pathotype. Vet. Res. 36:241–256. 2005.

41. Roland, K., K. Karaca, and D. Sizemore. Expression of Escherichia coli antigens in Salmonella Typhimurium as a vaccine to prevent airsacculitis in chickens. Avian Dis. 48:595–605. 2004.

42. Skyberg, J. A., T. J. Johnson, J. R. Johnson, C. Clabots, C. M. Logue, and L. K. Nolan. Acquisition of avian pathogenic escherichia coli plasmids by a commensal E. coli isolate enhances its abilities to kill chick embryos, grow in human urine, and colonize the murine kidney. Infect. Immun. 74(11): 6287–6292. 2006.

43. Sojka, W. J., and R. B. A. Carnaghan. Escherichia coli infections in poultry. Res. Vet. Sci. 2:340–352. 1961.

44. Tivendale, K. A., C. M. Logue, S. Kariyawasam, D. Jordan, A. Hussein, G. Li, Y. Wannemuehler, and L. K. Nolan. Avian-pathogenic Escherichia coli strains are similar to neonatal meningitis E. coli strains and are able to cause meningitis in the rat model of human disease. Infect. Immun. 78:3412–3419. 2010.

45. Trampel, D. W., and R. W. Griffth. Efficacy of aluminum hydroxide-adjuvanted Escherichia coli bacterin in turkey poults. Avian Dis. 41:263–268. 1997.

46. Vandemaele, F., N. Bleyen, O. Abuaboud, E. vanderMeer, A. Jacobs, and B. M. Goddeeris. Immunization with the biologically active lectin domain of PapGII induces strong adhesion-inhibiting antibody responses but not protection against avian pathogenic Escherichia coli. Avian Pathol. 35:238–249. 2006.

47. Vandemaele, F., C. Ververke, N. Bleyen, J. Geys, C. D'Hulst, T. Addwebi, P. van Empel, and B. M. Goddeeris. Immunization with the binding domain of FimH, the adhesin of type 1 fimbriae, does not protect chickens against avian pathogenic Escherichia coli. Avian Pathol. 34:264–272. 2005.

48. Vidotto, M. C., E. E. Muller, J. C. de Freitas, A. A. Alfieri, I. G. Guimaraes, and D. S. Santos. Virulence factors of avian Escherichia coli. Avian Dis. 34:531–538. 1990.

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