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Virulence Factors of Escherichia coli from Cellulitis or Colisepticemia Lesions in Chickens

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SUMMARY. This study was designed to compare virulence factors of cellulitis-derived *Escherichia coli* to colisepticemic *E. coli* in order to clarify whether *E. coli* associated with cellulitis comprise a unique subset of pathogenic *E. coli.* Isolates were tested for serotype, capsule, aerobactin production, colicin production, the presence of the *iss* gene, and serum resistance. Untypable isolates made up the greatest percentage of each group. Serotypes O2 and O78 were the most commonly identified among both groups of isolates. No statistical differences in the distribution of aerobactin or colicin production, capsule, or *iss* gene were observed between groups. Cluster analysis showed that 90% of the *E. coli* isolates had greater than 42% livability in serum-resistance tests. No separation of colisepticemic *vs.* cellulitis *E. coli* isolates was observed on the basis of SR. Colicin production by *E. coli* was highly correlated with serum resistance ($P = 0.0029$). These data suggest that cellulitis *E. coli* have virulence traits similar to those of colisepticemic *E. coli.*

RESUMEN. Factores de virulencia de cepas aviares de Escherichia coli aisladas de lesiones de celulitis o colisepticemia.

Este estudio fue diseñado para comparar los factores de virulencia entre las cepas de *Escherichia coli* asociadas con celulitis y las cepas asociadas con colisepticemia, con el fin de comprobar si las cepas de *E. coli* relacionadas con celulitis conprenden un subgrupo único entre las cepas patógenas de *E. coli.* Los aislamientos se analizaron por serotipo, antígenos capsulares, producción de aerobactina y colicina, así como por la presencia del gen *iss* y la resistencia al suero. Los aislamientos no tipificables constituyeron el mayor porcentaje de cada grupo. Los serotipos O2 y O78 fueron los más comúnmente identificados entre ambos grupos de aislamientos. No se observaron diferencias estadísticas en la producción de aerobactina ó colicina, antígenos capsulares o el gen *iss* entre grupos. El análisis de los grupos mostró que el 90% de los aislamientos de *E. coli* tuvieron una supervivencia mayor al 42% en pruebas de resistencia al suero. Basados en la prueba de resistencia al suero, no se pudieron separar los aislamientos relacionados con colisepticemia de los asociados con celulitis. La producción de colicina por *E. coli* estuvo altamente correlacionada con la resistencia al suero ($P = 0.0029$). Estos datos sugieren que las cepas de *E. coli* asociadas con celulitis tienen mecanismos de virulencia similares a los observados en las cepas asociadas con colisepticemia.

Key words: cellulitis, colisepticemia, chicken, virulence, *Escherichia coli*

Abbreviations: % livability = average percentage of cells alive; s.c. = subcutaneous

Escherichia coli is the most common secondary bacterial infection of commercial poultry flocks and may also be a primary pathogen (6). Disease syndromes caused by *E. coli* include both disseminated systemic conditions like colisepticemia and focal infections such as cellulitis (1). Cellulitis is characterized by the presence of subcutaneous (s.c.) fibrinonecrotic

plaques and inflammation of the overlying skin but not by lesions of internal organs, depression, weight loss, or fever (1,13; pers. obs.). Conversely, chickens with colisepticemia generally have depression, fever, yellowish or greenish droppings, and multiple lesions of internal organs including fibrinous pericariditis, perihepatitis, and airsacculitis (6). Numerous virulence-related factors have been identified among the *E. coli* associated with disease in humans and other animals. Particular sets of virulence traits have been found among isolates causing specific disease syndromes, for example, urinary tract infections or diarrhea (6). Among avian isolates of *E. coli,* researchers have historically recognized O serogroups such as, O1, O2, O5, O35, and O78 as virulent or pathogenic for severe systemic infection (1,18). Aerobactin production, which facilitates iron scavenging by *E. coli,* has been linked to pathogenicity of avian *E. coli* (6). The ability of *E. coli* to cause embryo lethality has been used as an indicator of pathogenicity of avian *E. coli* and has been correlated with complement resistance, motility, and the presence of type 1 pili (24). Complement resistance and virulence of *E. coli* have also been associated with colicin V production (23). The *iss* gene was recently shown to occur much more frequently in *E. coli* isolates from birds with colibacillosis than in *E. coli* from healthy birds (15). We previously reported on the pathogenicity of 12 cellulitis-derived *E. coli* after s.c. inoculation of a standardized dose. These isolates caused disease conditions ranging from acute septicemia to mild s.c. lesions, indicating a range of virulence among isolates (9). The present study was designed to compare virulence factors of cellulitis-derived *E. coli* with those of colisepticemic *E. coli* in order to clarify whether *E. coli* associated with cellulitis comprise a unique subset of pathogenic *E. coli.*

MATERIALS AND METHODS

Bacteria isolates. Fifty *E. coli* isolates from cellulitis lesions in commercial broiler chickens were cultured onto triple sugar iron agar slants from frozen stocks in the Jeffrey laboratory and shipped to the Nolan laboratory for comparison with 50 *E. coli* isolates from colisepticemic lesions in chickens. All isolates were stored in Luria–Bertani broth (Difco Laboratories, Detroit, MI) with 20% glycerol at −70 C prior to use. In preparation for amplification procedures, isolates were grown on MacConkey agar (Difco) overnight at 37 C.

Serotyping. Isolates were serotyped by the Pennsylvania State University *Escherichia coli* Reference Center (University Park, PA).

Capsule staining. All isolates were examined for the presence of capsule by the method of Hiss (7). Stained smears were examined with oil immersion. Known encapsulated (*K. pneumoniae,* obtained from Meritcare Hospital, Fargo, ND) and unencapsulated (*E. coli* DH5∝) (2) strains were included in this study as controls.

Amplification studies. Isolates were examined for *iss* sequences with primers specific to *iss* by a modification of the protocol described by Pfaff-McDonough *et al.* (15). Briefly, primers were obtained from Sigma-Genosys (The Woodlands, TX). Template DNA was generated as follows. A single colony of an isolate to be tested was transferred into 40 μl of lysing buffer (10 mM Tris-Cl, pH 7.5, 1 mM ethylenediaminetetraacetic acid, with 50 μg/ml proteinase K added the day of use), incubated in a thermocycler (Perkin Elmer 2400, Branchburg, NJ) at 55 C for 10 min followed by 10 min at 80 C, and, after these incubations, 80 μl ddH_2O was added. Next, 45 μl of a polymerase chain reaction master mix (28 μl ddH_2O, 5.0 μl deoxynucleoside triphosphate mix [Life Technologies, Inc., Gaithersburg, MD], 0.25 μl of Taq DNA polymerase [5 U/μl; Life Technologies, Inc.], 0.1 mM *iss* upper primer [GTGGCGAAAACTAGTAAAACAGC], 0.1 mM *iss* lower primer [CGCCTCGGGGTGGATAA], and 4 μl of 25 mM $MgCl_2$) was added to tubes in a thermocycler (Perkin Elmer 2400), paused at 95 C. Five microliters of template DNA was added to the reaction tubes. Amplification was performed according to the following parameters: 5 min at 95 C; nine cycles of 1 min at 95 C, 30 sec at 51.6 C, and 30 sec at 72 C; 25 cycles of 30 sec at 51.6 C and 30 sec at 72 C; 7 min at 72 C; and held at 4 C in a thermocycler (Perkin Elmer 2400). Amplicons were subjected to horizontal gel electrophoresis. An isolate was considered to contain *iss* if it produced an amplicon of 760 bp (8).

Colicin bioassay. Colicinogeny was assessed by the principles of Fredericq (3). Briefly, chloroform-killed colonies of the test organisms were overlaid with a half-strength agar (brain–heart infusion agar; Difco) containing a colicin-sensitive strain of *E. coli* K12 (ATCC 23559). Colicin-producing (*E. coli* ATCC 23558 and *Salmonella typhimurium* ATCC 23854 and 23594) and non-colicin-producing (*E. coli* ATCC 23559) strains were included on each plate as controls. Plates were incubated at 37 C for 18 hr and observed for zones of growth inhibition in the overlay.

Aerobactin bioassay. The assay used to assess an isolate's ability to elaborate aerobactin is based on

the assays described by Viditto *et al.* (20) and Williams and Warner (22). Briefly, test and control organisms were stab inoculated into low iron agar (M9 containing 2,2 dipyridyl) containing *E. coli* LG1522 (obtained from Dr. Viditto, Departmento de Patologia Geral, Universidade Estadual de Londrina, Parana, Brazil), which cannot produce aerobactin but uses exogenous aerobactin. Plates were incubated at 37 C for 18 hr and observed for growth of the indicator organism around the test isolates. Positive (*E. coli* LG1315 and negative *E. coli* LG1522) controls were included on each plate.

Complement-resistance assay. The ability of isolates to resist the effects of serum complement was determined by a flow cytometric assay modified from Virta *et al.* (21). Human complement (Sigma, St. Louis, MO; 25% final concentration of complement) was mixed with washed bacteria from an exponentially growing culture. These mixtures were allowed to incubate in a 37 C water bath for 2 hr without shaking. Reactions were stopped in an ice bath, and bacterial cells were then collected by centrifugation, washed, and resuspended in ddH_2O. Cells were stained with the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Eugene, OR). The two fluorescent stains in the kit, SYT09 and propidium iodide, when used together in the proper proportion, allow for live and dead bacteria to be distinguished in a quantitative fashion by flow cytometry. Stained samples were examined with a Becton Dickinson FACSCalibur flow cytometer (San Jose, CA) to determine percentage of live bacteria. Three replications of this assay were run for all isolates.

Statistical methods. Cellulitis and colisepticemic *E. coli* were compared for significant differences in the presence of each virulence factor by using the chi-square test (17). A cluster analysis approach was used to assign isolates according to their complement-resistance status (19). The average percentage of cells alive (% livability) after exposure to complement was calculated for each isolate in two to four replicates. These data were then sorted on descending % livability and plotted with isolate on the x-axis and % livability on the y-axis. The plot revealed that isolates with either very high % livability or very low % livability tended to have low variability, whereas more intermediate % livability values were associated with generally higher variability. Complement-resistance categories were assigned with the % livability and range of % livability as input variables in a cluster analysis. Euclidean distances were calculated from these two input measures, and the isolates were then clustered by an average distance method. On the basis of the resulting dendrogram, isolates were assigned as either complement resistant, complement susceptible, or of intermediate or variable complement resistance. Contingency tables between complement-resistance status and other virulence attributes (*iss,* capsule, aerobactin, colicin, and disease condition, either cellulitis or septicemia) were generated. The null hypothesis of no association between complement-resistance status and each of these attributes was tested by Fisher exact tests (17).

Table 1. Serotype results for *E. coli* isolates from lesions of chickens with colisepticemia or cellulitis.

Serotype	Septicemic *E. coli* (n = 49)	(%)[A]	Cellulitis *E. coli* (n = 50)	(%)[A]
Untypeable	13	27	17	34
2	13	27	4	8
5	2	4	2	4
7	2	4	0	0
8	1	2	0	0
11	1	2	1	2
15	1	2	0	0
20	1	2	1	2
21	0	0	1	2
23	1	2	0	0
36	2	4	4	8
41	0	0	1	0
53	0	0	3	6
78	9	18	9	18
100	1	2	0	0
106	1	2	0	0
109	0	0	1	2
115	0	0	2	4
119	0	0	1	2
120	1	2	0	0
132	0	0	3	6

[A]Percentage of each serotype rounded to the nearest whole number.

RESULTS

Thirteen serotypes were identified within the colisepticemic and cellulitis *E. coli* groups (Table 1). Within the isolates from colisepticemic lesions, five pathogenic serotypes, O2, O5, O8, O15, and O78, were identified and made up 58% of the isolates; O2 was the most common serotype. Within the cellulitis *E. coli,* three pathogenic serotypes were identified, O2, O5, and O78, representing 34% of the isolates; O78 was the most common serotype. The serotype O36 was also common (10%) within cellulitis isolates. A large number of isolates in both the colisepticemic and cellulitis groups were untypeable, 26% and 34%, respectively.

The prevalence of virulence factors among isolates is shown in Table 2. A few isolates in the cellulitis group demonstrated a capsular an-

Table 2. Results of testing for virulence-related factors for *E. coli* isolated from lesions of chickens with colisepticemia or cellulitis.

Virulence factor	Colisepticemia *E. coli*	Cellulitis *E. coli*	Chi-square *P*-value
iss gene	40/50 (80%)	36/50 (72%)	$P = 0.88$
Aerobactin	19/22 (86%)	48/52 (92%)	$P = 0.64$
Colicin	19/22 (86%)	45/52 (86%)	$P = 0.00$
Capsule	0/22 (0%)	3/49 (6%)	$P = 1.41$

tigen, whereas none of the colisepticemia isolates had capsules. Aerobactin and colicin production were observed in >86% of the *E. coli* isolates from both groups. The *iss* gene was found in 72% and 80% of the cellulitis and colisepticemia isolates, respectively. The cluster analysis of % livability in serum-resistance tests divided the *E. coli* isolates into three major groups. Sensitive isolates had 7.5%–15% livability, intermediate isolates had 16%–42% livability, and resistant isolates had 64%–92% livability. No separation of colisepticemic *vs.* cellulitis *E. coli* isolates was observed on the basis of cluster analysis. The probabilities for a significant correlation between the presence of a specific virulence factor and serum resistance are shown in Table 3. Colicin production was highly correlated with serum resistance ($P = 0.0029$). Other virulence factors and the origin of the isolate from cellulitis or colisepticemia were not significant.

DISCUSSION

Cellulitis has been established as a distinct pathologic condition, supported by the presence of s.c. lesions in the absence of other lesions commonly associated with *E. coli* infection. Previous experimental studies have shown that *E. coli* cultured from cellulitis lesions are more efficient in reproducing cellulitis lesions than are fecal isolates or those from airsacculitis lesions (11,16). Also, some cellulitis-derived *E. coli* cause only localized lesions even when inoculated at high doses (7). The presence of unique virulence traits has been used to distinguish *E. coli* that are responsible for diseases of specific host systems or bodily compartments from other *E. coli* that are not associated with that disease (24). Clonal analysis based on electrophoretic typing of cellulitis *E. coli* bacteria has shown that many are similar to other pathogenic *E. coli* but that some (25%) of the isolates tested were clonally distinct and perhaps specific cellulitis pathogens (13). These studies suggested the hypothesis that cellulitis *E. coli* are different than other pathogenic *E. coli* and could be differentiated by standard virulence assays. In this study, virulence factors unique to cellulitis *E. coli* or colisepticemic *E. coli* were not identified. The predominant serotypes identified among cellulitis and colisepticemic *E. coli,* O78, O2, and untypeable, are similar to those found in previous studies (4,5,10,13). A high percentage of isolates were positive for colicin and aerobactin consistent with characteristics of pathogenic *E. coli* identified in other studies (5,23,24). The *iss* gene, recently suggested as a marker for *E. coli* associated with colibacillosis (8,15), was common in both sets of isolates. Serum resistance was variable among isolates, ranging from 7.5% to 92% livability after exposure to complement; however, this trait was not correlated with the origin of the isolate. The present study confirms the work of others (5) by concluding that the two groups of isolates are similar and suggests that the limitation of cellulitis lesions to the s.c. tissue after infection by *E. coli* is not due to reduced virulence on the part of the bacteria but may be moderated by portal of entry or host defenses.

Table 3. Correlation of serum resistance with other virulence related factors.[A]

Virulence factor	Probability (*P*)
iss gene	$P = 0.764$
Aerobactin	$P = 0.875$
Colicin	$P = 0.0029$
Capsule (K antigen)	$P = 1.0$
Disease syndrome	$P = 0.817$

[A]Fisher exact test.

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