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SUMMARY. A group of complement-resistant, virulent avian *Escherichia coli* isolates were compared with a group of complement-sensitive, avirulent avian isolates for the presence of K-1 capsule, smooth lipopolysaccharides (LPS), the *traT* gene, and Colicin V (ColV) production. These parameters were selected because of their reported association with complement resistance and virulence in *E. coli*. Lethality in chicken embryos has also been shown to be correlated with virulence of avian *E. coli* for chickens. The complement-resistant, virulent *E. coli* isolates did not possess a K-1 capsule. Production of ColV and the presence of smooth LPS were significantly correlated with embryo lethality. There was no correlation between the presence of *traT* and embryo lethality. These results suggest that complement resistance and virulence in avian *E. coli* are associated with ColV production and smooth LPS but not with K-1 antigen or *traT*.

RESUMEN. Asociación de producción de cápsula K-1, lipopolisacáridos lisos, gen traT y colicina V, con la resistencia del complemento y la virulencia de Escherichia coli aviar.

Se comparó un grupo de cepas virulentas de *Escherichia coli* aviar, resistentes al complemento, con un grupo de cepas avirulentas, sensibles al complemento, para la presencia de cápsula K-1, lipopolisacáridos lisos, el gen *traT* y la producción de Colicina V (ColV). Estos parámetros fueron seleccionados debido a que en *E. coli* se ha reportado asociación con la resistencia al complemento y la virulencia. La letalidad en embriones de pollo también se ha demostrado que se correlaciona con la virulencia de *E. coli* aviar en pollos. Las cepas de *E. coli* resistentes al complemento y virulentas no tenían la cápsula K-1. La producción de ColV y la presencia de lipopolisacárido suave fueron significantemente correlacionadas con la letalidad embrionarias. No hubo correlación entre la presencia de *traT* y letalidad embrionaria. Estos resultados sugieren que la resistencia al complemento y la virulencia en *E. coli* aviar están asociadas con la presencia de ColV y lipopolisacárido liso, pero no con el antígeno K-1 ó el *traT*.

Resistance of avian *Escherichia coli* to the effects of host complement has been shown to contribute to its virulence (23,31). Microbial complement resistance has been attributed to the presence of smooth lipopolysaccharide (LPS) layer (13), K-1 capsular antigen (1,27), and production of plasmid-encoded proteins such as TraT (4,27) and Colicin V (ColV) (3,30). ColV production is correlated with complement resistance because of the common plasmid location of the ColV biosynthesis genes and certain complement resistance genes (30). TraT, a surface-exposed lipoprotein, mediates serum resistance by interfering with the correct assembly or functioning of the complement membrane attack complex (28,29). Strains with a smooth LPS layer release the terminal comple-

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ment constituents from their surfaces, but in rough strains, the terminal complex is not released and inserts into the outer membrane to exert its killing effect (13,15). The K-1 capsular antigen might prevent binding of complement components to the cell wall by masking surface structures that activate complement (27).

The purpose of the present study was to determine whether complement resistance and virulence of avian *E. coli* were associated with the presence of K-1 capsule antigen, a smooth LPS phenotype, *traT* gene, or production of ColV.

MATERIALS AND METHODS

Test organisms. Fourteen *E. coli* isolates were studied; eight were complement-resistant isolates cultured from chickens with natural cases of colisepticemia, and six were complement-sensitive isolates from normal birds. Isolates from infected chickens were obtained from the liver, spleen, bone, or multiple organs. Isolates from normal, healthy birds came from the intestinal tracts of broiler chickens at the time of slaughter.

Bacterial resistance to chicken complement. The lytic activity of complement on the test isolates was determined with a quantitative microtiter test (17). Each isolate was classified as either sensitive, intermediate, or resistant to the action of chicken complement. Two-hour cultures grown in Luria-Bertani broth were diluted 1:50 in PG broth (1% peptone plus 1% glucose) (18), and 100-μl (10^4 colony-forming units [CFU]) samples were inoculated into 96-well flat-bottomed microtiter plates (Microtest III; Becton, Dickinson & Co., Rutherford, N.J.). Aliquots (100 μl) of chicken complement or heat-inactivated chicken complement were added in twofold dilutions from 50% to 0.8%. Media and culture controls were included. Plates were monitored for growth by an enzyme-linked immunosorbent assay reader (Titertek Multiskan; Flow Laboratories, McLean, Va.) at 492 nm, absorbance mode, with a range of 1.0. Plates were incubated at 35 C and read hourly from 0 to 6 hours (17).

Embryo lethality test. To assay for virulence, overnight cultures of the isolates were washed twice in phosphate-buffered saline (PBS), resuspended and diluted in PBS, and inoculated into the allantoic cavity of twenty 12-day-old chicken embryos. Twenty PBS-inoculated control embryos also were included. Eggs were candled daily, and deaths were recorded for 4 days. Twenty eggs were used for each of two inoculum dilutions, approximately 10^2 and 10^3 CFU in 0.1 ml of BPS (22).

ColV activity. The colicin test organisms consisted of *E. coli* K-12 (ATCC 23559 and ATCC 23561; American Type Culture Collection, Rockville, Md.), the colicin-sensitive controls, and the ColV producer, *E. coli* K-12 containing pHK11, a recombinant plasmid coding for ColV production (12). Presence of ColV production was tested by overlaying chloroform-killed colonies of the test organisms with a combination of *E. coli* K-12 ATCC 23559 and ATCC 23561. Plates were incubated at 37 C for 18 hours and observed for inhibition of growth of the colicin-sensitive organisms (10).

Isolation, purification, and identification of LPS. After LPS was extracted from the isolates by the method of Darveau and Hancock (8), samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained initially with Coomassie blue to detect gross protein contamination, followed by silver staining to detect the LPS (Bio-Rad Silver Stain Kit; Bio Rad Laboratories, Richmond, Calif.) (14).

DNA probe analysis. Probe DNA was obtained by digesting pKT107 with *Bst*EII (Promega Corp., Madison, Wisc.), separating the fragments electrophoretically, and extracting the chosen fragment with GENECLEAN (Bio101, La Jolla, Calif.). The 700-base-pair *traT*-containing fragment (20) was labeled with the Genius I kit (Boehringer Mannheim, Indianapolis, Ind.) for use as a probe. Bacteria to be tested were grown overnight at 37 C, and their plasmid DNA was separated by gel electrophoresis (5). Plasmid DNA was transferred to charge-modified nylon membranes (QIABRANE Nylon plus; QIAGEN, Inc., Chatsworth, Calif.) by the method of Southern (26) and hybridized with the probe under stringent conditions (25). Briefly, the filter was prehybridized in aqueous solution for 12 hours at 68 C and hybridized in the same solution with the denatured probe for 12 hours at 68 C. The filter was then washed twice in 2× SSC (5 Prime-3 Prime, Inc., Boulder, Colo.) with 0.1% sodium dodecyl sulfate (SDS) (Sigma Chemical Co., St. Louis, Missouri); in 1× SSC with 0.1% SDS; and in 0.1× SSC with 0.1% SDS. Each wash was carried out with mild agitation for 15 minutes at 68 C. Plasmid DNA recognized by the probe was detected using the protocol recommended by Boehringer Mannheim.

K-1 capsule. The presence of a K-1 capsular antigen was determined by a latex agglutination test (Wellcome Diagnostics, Dartford, England) (21).

Biostatistics. Presence of K-1 capsular antigen, smooth LPS phenotype, *traT* antigen, or the production of ColV are dichotomous features of avian *E. coli*. The relationship between these variables and percent embryo lethality were examined using the point-biserial correlation technique. Correlation coefficients ($r\,pbs$) and coefficients of determination ($r\,pbs^2 \times 100$) were also calculated. If two or more of these dichotomous variables are significantly related to embryo

Table 1. Characteristics of complement-resistant, virulent, and complement-sensitive, avirulent avian *Escherichia coli.*

Isolates[A]	% Embryo lethality	ColV production	Smooth LPS	traT gene
V1	85.0	+[B]	+	+ (35)[C]
V2	77.5	+	+	+ (35)
V3	85.0	+	+	−
V4	75.0	+	+	−
V5	60.0	−	+	+ (6.6)
V6	75.0	+	−	−
V7	90.0	−	+	+ (35)
V8	70.0	−	+	−
Av1	10.0	−	−	+ (35)
Av2	5.0	−	−	+ (35)
Av3	5.0	−	+	−
Av4	10.0	−	+	−
Av5	5.0	−	−	+ (8 & 35)
Av6	0	−	−	+ (35)

[A]V = virulent, Av = avirulent.
[B]+ = present, − = not present.
[C]Kilobase plasmid.

lethality, then their dichotomous (1 or 2) values would be added together and percent embryo lethality would be regressed against them using an analysis of variance for linear regression. Correlation coefficients (r) and coefficients of determination ($r^2 \times 100$) also were calculated (32).

RESULTS AND DISCUSSION

None of the 14 avian *E. coli* isolates studied had a K-1 capsule.

There was a significant correlation ($r\,pbs$) between percent embryo lethality and the production of ColV ($r\,pbs = 0.68$, $df = 12$, $t = 3.22$, $P < 0.01$). The correlation of determination ($r\,pbs^2 \times 100 = 0.68^2 \times 100$) is 46.3%. This means that 46.3% of the variation in embryo lethality can be explained by the production of ColV. The $r\,pbs$ between percent embryo lethality and smooth LPS phenotype is 0.57 ($df = 12$, $t = 2.41$, $P < 0.05$), which means that 32.6% of the variation in percent embryo lethality can be explained by the smooth LPS phenotype. The $r\,pbs$ between the presence of *traT* and embryo lethality is −0.16 ($df = 12$, $t = 0.6$, not significant), which means that 2.6% of the variation in percent embryo lethality can be explained by the presence or absence of *traT* gene (Table 1).

Because ColV and smooth LPS phenotype were significantly associated with percent embryo lethality, their scores were combined and percent embryo lethality was regressed against them. Percent embryo lethality regressed significantly ($F = 20.4$, $df = 1/12$, $P < 0.001$) against the combined scores. In this instance, $r = 0.79$ and $r^2 \times 100 = 63\%$, which means that 63% of the variation in percent embryo lethality can be explained by the combined presence of ColV and smooth LPS.

The two features that distinguish the virulent, complement-resistant isolates from the avirulent, complement-sensitive isolates were smooth LPS phenotype and ColV production. A smooth LPS phenotype is known to contribute to the complement resistance and virulence of some gram-negative bacteria (7,27). However, the role of ColV production in virulence and complement resistance is more controversial. Some workers have reported that ColV production is associated with pathogenicity of *E. coli* (1,2,30), whereas others have found that it does not contribute to *E. coli* pathogenicity (3). These discrepancies may be due to the heterogeneous nature of ColV plasmids (30). Although some ColV-encoding regions are chromosomally located (9), the large ColV plasmids (30) might vary in composition, with some possessing several virulence and complement-resistance genes. Therefore, ColV production by a virulent organism, as seems likely in this study, may indicate that an organism possesses a battery of virulence genes carried by a ColV plasmid.

The *traT* gene, which might be carried on ColV plasmids (27,30), is another putative virulence factor associated with complement resistance whose contribution to virulence is controversial. Although *traT* has been shown to contribute to the pathogenicity (27) and complement resistance (19,27,29) of some *E. coli,* the present study and other reports have failed to confirm this association (16,20). One explanation for these ambiguous observations is that *traT* proteins of different organisms might be structurally and functionally distinct (27).

In certain *E. coli,* K-1 capsules have also been associated with complement resistance and virulence (1,2,11,24,27,29). In fact, virulent bacteria, which are complement-resistant and have a rough or partially rough LPS phenotype, often require K-1 encapsulation for full virulence (6,7). Since all but one of the virulent, com-

plement-resistant isolates in the present study had a smooth LPS phenotype, their lack of K-1 antigen is not surprising. Furthermore, it was predictable that the intestinal isolates—which were complement-sensitive, had a rough LPS phenotype, and were avirulent—would not possess K-1 capsular antigen.

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