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Characterization of *Escherichia coli* Isolates from Peritonitis Lesions in Commercial Laying Hens

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SUMMARY. Five clinically normal chickens from three farms (farm A, farm B, and farm C), for a total of 15 clinically normal chickens, were examined bacteriologically. In a similar manner, five dead chickens with lesions of peritonitis from each of the same three commercial egg-laying operations were selected for bacterial culturing. *Escherichia coli* were isolated from the cloaca in 14 of 15 healthy chickens and from all 15 chickens with peritonitis. Oviducts of normal chickens did not contain *E. coli* (0/15) whereas oviducts from 13 of 15 hens with peritonitis were positive for this pathogen. No lesions and no *E. coli* (0/15) were found in the peritoneal cavity of healthy hens, but peritonitis lesions from 13 of 15 dead chickens yielded *E. coli*. On farm A and farm B, a flock consisted of all chickens within a single house and all chickens in each flock were of the same age and same genetic strain. In flock 1 from farm A, all five *E. coli* isolates from the oviduct and all five isolates from the peritoneal cavity were serogrouped as O78; contained the virulence genes *iroN*, *sitA*, *iutA*, *tsh*, and *iss*; and belonged to phylogenetic group A. In flock 2 from farm B, all four *E. coli* isolates from the oviduct and all four isolates from the peritoneal cavity were serogrouped as O111; contained virulence genes *iroN*, *sitA*, *iutA*, *traT*, *iss*, and *ompT*; and belonged to phylogenetic group D. These data suggest that all chickens with peritonitis in a single flock on farms A and B were likely infected by the same *E. coli* strain. *Escherichia coli* isolates from the magnum and peritoneum had the same serogroup, virulence genotype, and phylogenetic group, which is consistent with an ascending infection from the oviduct to the peritoneal cavity.

RESUMEN. Caracterización de aislamientos de *Escherichia coli* provenientes de lesiones de peritonitis en ponedoras comerciales.

Se examinaron bacteriológicamente cinco aves clínicamente normales provenientes de tres granjas (granjas A, B y C) para un total de 15 aves clínicamente sanas. De una manera similar, de las mismas granjas de ponedoras comerciales se seleccionaron cinco aves muertas con lesiones de peritonitis para cultivo bacteriano. De la cloaca de 14 de las 15 aves sanas y de todas las 15 aves con peritonitis, se aisló *Escherichia coli*. Los oviductos de las aves normales no contenían *E. coli* (0/15) mientras los oviductos de 13 de las 15 aves con peritonitis resultaron positivos para este patógeno. No se observaron lesiones ni *E. coli* (0/15) en la cavidad peritoneal de las gallinas sanas, pero las lesiones peritoneales de 13 de las 15 aves muertas generaron *E. coli*. En las granjas A y B, una parvada estaba formada por todas las aves dentro de una caseta individual y todas las aves en cada parvada eran de la misma edad y la misma línea genética. En la parvada 1 de la granja A, todos los cinco aislamientos de *E. coli* provenientes del oviducto y todos los cinco aislamientos provenientes de la cavidad peritoneal fueron serotipificados como O78; contenían los genes de virulencia *iroN*, *sitA*, *iutA*, *tsh*, y *iss*; y pertenecían al grupo filogenético A. En la parvada 2 de la granja B, todos los cuatro aislamientos de *E. coli* del oviducto y todos los cuatro aislamientos de la cavidad abdominal fueron serotipificados como O111; contenían los genes de virulencia *iroN*, *sitA*, *iutA*, *traT*, *iss* y *ompT*; y pertenecían al grupo filogenético D. Estos datos sugieren que todas las aves con peritonitis en una parvada individual en las granjas A y B estaban probablemente infectadas con la misma cepa de *E. coli*. Los aislamientos de *E. coli* del magnum y del peritoneo pertenecían al mismo serogrupo, genotipo de virulencia y grupo filogenético, lo cual es consistente con una infección ascendente desde el oviducto a la cavidad peritoneal.

Key words: *E. coli*, peritonitis, virulence, genes, chickens

Abbreviations: APEC = avian pathogenic *Escherichia coli*; PCR = polymerase chain reaction

Peritonitis in commercial table-egg chickens is a frequent cause of morbidity, mortality, and, to the owners, significant economic loss (2,45). Lesions are characterized by fibrinoheterophilic exudate and are usually restricted to adult hens in egg production (17). Grossly, peritonitis appears as aggregations of yellow exudate on serosal surfaces that may be localized or widely disseminated throughout the body cavity. In laying hens, peritonitis is usually seen as one component of a constellation of lesions that frequently includes perihepatitis, septicemia, and salpingitis (2,32,45). Peritonitis has been associated with ascending bacterial infections of the oviduct. Oviduct infection in egg-laying chickens is caused by bacterial species that are normally present on cloacal mucus membranes (6), such as *Escherichia coli*. *Escherichia coli* can enter the oviduct of clinically normal hens in egg production. The heaviest contamina-

tion is in the vagina and the adjacent portion of the shell gland where coliforms are present in high numbers (18). *Escherichia coli* have been isolated from oviducts of chickens with salpingitis for 5 consecutive months (16). Infected oviducts continue to function, and most chickens with infected oviducts continue to produce eggs (1).

Pathogenic and nonpathogenic *E. coli* cannot readily be differentiated on the basis of antigenic or biochemical properties. Many potential virulence factors have been identified including complement resistance, iron acquisition, serum survival, colicin production, and type I fimbriae (2,21). Recently, it has been shown that genes encoding virulence factors are present on large, conjugative plasmids (22,23,26,27) that contribute to the lethality of *E. coli* to chick embryos (41) and that cotransfer with R plasmids in avian pathogenic *E. coli* (APEC) (25,29). Plasmid-linked virulence genes include *iss* (the increased serum survival gene), *tsh* (the temperature-sensitive

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hemagglutinin gene), *iucC* (a gene of the aerobactin operon), and *cvaC* (the structural gene of the ColV operon) (37,38). APEC containing these virulence plasmids are widespread. In general, these large plasmids appear to be a defining feature of the APEC pathotype (23,24,26,28,37,41). However, their occurrence among the APEC involved in peritonitis has not been examined.

In this study, *E. coli* were isolated from the cloaca, oviduct, and peritoneal surface of the ovary from apparently healthy chickens and from laying hens with peritonitis. These isolates were subsequently tested for the presence of plasmid-linked virulence genes in an effort to determine if the APEC causing peritonitis constitutes a unique subset of APEC and to gain insight into the pathogenesis of peritonitis.

MATERIALS AND METHODS

Isolate collection. Managers of three commercial in-line egg production facilities (farm A, farm B, and farm C) with multiple houses at each location were asked to submit 30 chickens from daily mortality in flocks experiencing elevated death loss because of peritonitis and five live, healthy hens from the same flock to the Iowa State University Veterinary Diagnostic Laboratory. On farm A and farm B, a flock consisted of all chickens within a single house and all chickens in each flock were of the same age and same genetic strain. Flock 1 consisted of 40-wk-old white egg-type chickens from one house on farm A. Flock 2 was composed of 28-wk-old hens from one house on farm B and chickens in flock 2 were of the same genetic strain as chickens in flock 1. The flock 3 submission contained 30 dead chickens from three houses on farm C that ranged in age from 23 to 76 wk and included birds from three different genetic strains in addition to five live, healthy hens from one of those three houses. Five dead chickens with yellow-white exudate on serosal surfaces within the body cavity were selected for bacteriological examination from flock 1, flock 2, and flock 3 for a total of 15 chickens with lesions typical of peritonitis. All five of the healthy chickens submitted from each of these three flocks were necropsied so that an additional 15 chickens without peritonitis lesions were cultured to serve as controls. For each of these 30 chickens, bacterial culture swabs were taken from the cloacal entrance to the vagina, the midpoint of the oviduct (magnum), and the peritoneal surface of the ovary.

Serogrouping. All isolates were O serogrouped at the *Escherichia coli* Reference Center (The Pennsylvania State University, University Park, PA).

Phylogenetic typing. Isolates were assigned to phylogenetic groups according to the method of Clermont *et al.* (11). Using this method, isolates are assigned to one of four groups (A, B1, B2, or D) based on their possession of two genes (*chuA* and *yjaA*) and a DNA fragment (TSPE4.C2), as determined by polymerase chain reaction (PCR). Boiled lysates of overnight cultures were used as a source of template DNA (20). Amplification was performed in a 25- μ l reaction mixture including 1 $\times$ PCR buffer (Invitrogen, Carlsbad, CA), 2.0 mM MgCl₂, 0.5 mM each of dATP, dCTP, dGTP, dTTP (USB, Cleveland, OH), 0.3 μ M of each primer (Integrated DNA Technologies, Coralville, IA) (Table 1), 1.25 units of *Taq* DNA polymerase (Invitrogen), and 2.0 μ l of template DNA. The reaction mixture was subjected to the following parameters in a Mastercycler Gradient thermocycler (Brinkmann Eppendorf, Westbury, NY): 4 min at 94 C, 30 cycles of 5 sec at 94 C and 10 sec at 59 C, and a final extension step of 5 min at 72 C, followed by a hold at 4 C.

Samples were subjected to horizontal gel electrophoresis in 2% (w/v) agarose, and the size of the amplicons was determined by comparison to the Hi-Lo DNA marker obtained from Minnesota Molecular Inc. (Minneapolis, MN). Strains known to possess or lack the genes of interest were examined with each amplification procedure. An isolate was considered to contain a gene or region of interest if it produced an amplicon of the expected size (Table 1). Isolates were assigned to phylogenetic group "A" if they exhibited the profile: *chuA* (–), *yjaA* (+/–), and TSPE4.C2 (–); "B1" by the profile of *chuA* (–), *yjaA* (+/–),

Table 1. Polymerase chain reaction primers used for the amplification studies.

Gene or DNA region	Amplicon size	Primer sequence (5'–3')	Source or reference
Virulence genes			
<i>iroN</i>	667	R gacgccgacattaagacgcag F aagtcaaagcaggggttgcgccg	26,38
<i>iss</i>	323	R agcttgccagagcggcagaa F cagcaacccgaaccacttgatg	26,38
<i>iutA</i>	302	F ggctggacatcatgggaactgg R cgtcggaacgggtagaatcg	22
<i>sitA</i>	608	F agggggcacaactgattctcg R taccgggcggttttctgtgc	39
<i>traT</i>	290	F ggtgtggtgcgatgagcacag R caggttcagccatccctgag	22
<i>tsh</i>	420	F gggaaatgacctgaatgctgg R ccgctcatcagtcagtaccac	32
<i>ompT</i>	559	F atctagccgaagaaggaggc R cccgggtcatagtgttcac	26,38
Phylogenetic typing			
<i>chuA</i>	279	F gacgaaccaacggctcaggat R tgccgcagtagtaccagagaca	12
<i>yjaA</i>	211	F tgaagtgtcaggagacgctg R atggagaatgcgttcctcaac	12
TSPE4.C2	152	F gtagtaatgtcggggcattca R cgcgccaacaaagtattacg	12

and TSPE4.C2 (+); "B2" by the genotype of *chuA* (+), *yjaA* (+), and TSPE4.C2 (+/–); or "D" if they were *chuA* (+), *yjaA* (–), and TSPE4.C2 (+/–).

Virulence genotyping. Test and control organisms (APEC O1 [24] and *E. coli* DH5 α [8]) were examined for the presence of several genes known for their association with APEC virulence, using multiplex PCR. The targeted genes occur on large APEC virulence plasmids, such as pAPEC-O1-ColBM (23) and pAPEC-O2-ColV (26). Both of these plasmids encode many traits associated with APEC virulence, including iron acquisition, serum resistance, and adhesion (22,23,26) and contain many genes and operons associated with the ability of APEC to cause disease in birds. In the present study, the following APEC plasmid-linked genes were targeted: *ompT*, which encodes a protease (10); *iroN*, which encodes the receptor of the salmochelin operon (14); *iutA*, the gene that encodes the aerobactin receptor (12); *sitA*, a gene of the *sit* operon (40); *tsh*, which encodes the temperature-sensitive hemagglutinin (36); and *traT* and *iss*, both of which encode outer membrane proteins important in increased serum survival (35,42). These genes were chosen for characterization of the APEC in this study, because these large plasmids are strongly associated with APEC and their genes occur widely and with some specificity among pathogenic but not commensal strains (37,38).

All primers used in amplification of the virulence genes were obtained from Sigma-Genosys (St. Louis, MO) and Integrated DNA Technologies (Table 1). Template DNA for all amplifications was generated as described elsewhere (36,37). Targeted genes were amplified in a single multiplex procedure. Amplification was performed in 25- μ l reaction mixtures that included 2.0 μ l of template DNA, 1 $\times$ PCR buffer (Invitrogen), 4.0 mM MgCl₂, 1.25 units of Amplitaq Gold *Taq* (Invitrogen), 0.125 mM dATP, dCTP, dGTP, dTTP (USB), and 0.3 μ M of each primer (Integrated DNA Technologies). These reaction mixtures were subjected to the following conditions in a Mastercycler Gradient thermocycler: 12 min at 95 C to activate the Amplitaq Gold *Taq*; 25 cycles of 30 sec at 94 C, 30 sec at 63 C, and 3 min at 68 C; with a final cycle of 10 min at 72 C, followed by a hold at 4 C.

All samples were subjected to horizontal gel electrophoresis in 2% agarose, and the size of the amplicons was determined by comparison to the Hi-Lo DNA marker (Minnesota Molecular Inc.). Positive and negative controls were examined with each amplification procedure, and all amplification procedures were repeated three times to reduce the

Table 2. *Escherichia coli* isolations from healthy chickens and chickens with peritonitis.

Flock	Cloaca		Magnum		Peritoneum	
	Healthy	Peritonitis	Healthy	Peritonitis	Healthy	Peritonitis
1	5/5	5/5	0/5	5/5	0/5	5/5
2	5/5	5/5	0/5	5/5	0/5	4/5
3	4/4	5/5	0/5	3/5	0/5	4/5
Total	14/15	15/15	0/15	13/15	0/15	13/15

possibility of false negatives. An isolate was considered to contain the gene of interest if it produced an amplicon of the expected size (Table 1).

RESULTS

In healthy chickens, *E. coli* were isolated only from the cloaca. In contrast, *E. coli* were isolated from the cloaca, oviduct, and peritoneal lesions of chickens with peritonitis. *Escherichia coli* were isolated from the cloaca in 14 of 15 healthy chickens and from all 15 chickens with peritonitis (Table 2). Oviducts of normal chickens did not contain *E. coli* (0/15), whereas oviducts from 13 of 15 hens with peritonitis were positive for this pathogen. No lesions and no *E. coli* (0/15) were found in the peritoneal cavity of healthy hens, but peritonitis lesions from 13 of 15 dead chickens yielded *E. coli*.

The serogroup, virulence genes, and phylogenetic group of *E. coli* isolates within a flock were the same when chickens originated from the same house but differed between houses on different farms. *Escherichia coli* were cultured from the peritoneal cavity of all five chickens with peritonitis from flock 1. In this flock, all five isolates from the peritoneal cavity, all five isolates from the oviduct, and three of five *E. coli* isolates from the cloaca were serogrouped as O78; had the same virulence genotype of *iroN*, *sitA*, *tsh*, *iss*, and *iutA*; and belonged to phylogenetic group A (Table 3). In laying hens from flock 2, *E. coli* were isolated from the peritoneal cavity of four out of five chickens with lesions of peritonitis. In these four chickens, all isolates from the peritoneal cavity, oviduct, and cloaca were serogrouped as O111; contained the virulence genes *iroN*, *sitA*, *ompT*, *iss*, *iutA*, and *traT*; and belonged to phylogenetic group D.

Characteristics of the *E. coli* isolated from chickens originating in different houses at the same egg production site were different. For *E. coli* isolated from flock 3, no consistent pattern was found for serogroup (O166, O3.34.73, O9w, and O15), virulence genotype, or phylogenetic type (A, B1, B2, and D).

DISCUSSION

Escherichia coli isolated from the magnum and peritoneum of chickens with peritonitis from the same house had the same O serogroup, phylogenetic group, and virulence genotype. The

exception was an *E. coli* isolated from one chicken in flock 1 that, unlike *E. coli* from the other four birds of this flock, carried the *traT* gene. In flock 1, 60% of cloacal isolates shared the same serogroup, phylogenetic group, and virulence genotype as those found in the reproductive tract and peritoneum. Also, 100% of cloacal isolates shared the same three traits as those found in the oviduct and peritoneum in flock 2. These findings are consistent with a pathogenesis of infection that begins with movement of cloacal *E. coli* into the oviduct followed by ascension of these bacteria up the oviduct, through the infundibulum, and into the peritoneal cavity (6,18,32). Also, based upon serogrouping, phylogenetic typing, and virulence genotyping, it appears as though peritonitis within a chicken house may be caused by a single *E. coli* strain that becomes dominant and is associated with most of the morbidity and mortality due to peritonitis in that house.

The location of *E. coli* in chickens producing eggs confirms that this bacterium is associated with disease and is not part of the normal microflora found in the oviduct or peritoneal cavity. Healthy laying hens had *E. coli* within the cloaca but not in the oviduct or peritoneal cavity. *E. coli* is a normal inhabitant of the chicken intestinal tract with up to 10^6 of these bacteria per gram of intestinal contents (2). Approximately 10% to 15% of intestinal *E. coli* are considered to be potential pathogens (19). It is believed that pathogenic *E. coli* from the normal intestinal microflora are the source of infection for the oviduct and, ultimately, the peritoneal cavity.

Virulence genes identified in O78 and O111 *E. coli* isolated from flock 1 and flock 2 confer certain physiological advantages to APEC. Iron is an essential nutrient required for *E. coli* growth. Not surprisingly, APEC's ability to obtain iron is well documented and is likely because of various iron-acquisition systems, such as those encoded by the aerobactin, *sit*, and *iro* operons (13,14,31,40,44). The *sit* operon encodes an ABC transport system, involved in the metabolism of iron and manganese and in resistance to hydrogen peroxide (40). In its plasmid location, *sit* is closely associated with the aerobactin siderophore operon and *iro* locus (23). The iron-related genes used in this study's virulence genotyping represented these three systems. That is, *iroN* was used to detect the salmochelin operon, *sitA* was used to detect the *sit* operon, and *iutA* was used to detect the aerobactin operon.

Table 3. Characteristics of *E. coli* isolated from laying hens with peritonitis.

Flock	Site ^A	Serogroup	Plasmid-mediated virulence genes							Phylogenetic type
			<i>iroN</i>	<i>sitA</i>	<i>iutA</i>	<i>tsh</i>	<i>traT</i>	<i>iss</i>	<i>ompT</i>	
1	O	O78	5/5	5/5	5/5	5/5	1/5	5/5	0/5	A
	P	O78	5/5	5/5	5/5	5/5	1/5	5/5	0/5	A
	C	O78	3/5	3/5	3/5	3/5	0/5	3/5	0/5	A
2	O	O111	4/4	4/4	4/4	0/4	4/4	4/4	4/4	D
	P	O111	4/4	4/4	4/4	0/4	4/4	4/4	4/4	D
	C	O111	4/4	4/4	4/4	0/4	4/4	4/4	4/4	D

^AO = oviduct; P = peritoneum; C = cloaca.

Additional *E. coli* virulence genes found in this study include *tsh* in O78 isolates from flock 1 and *traT*, *iss*, and *ompT* which were identified in O78 isolates from flock 1 and O111 isolates from flock 2. The *tsh* gene encodes Tsh, the first known serine protease autotransporter of the *Enterobacteriaceae* (15,30,36). Tsh is a bi-functional protein that acts as an adhesin and a protease (30,33) and mediates APEC's colonization of the host's respiratory tract during early infection (15). As do the other genes used in our genotyping scheme, *tsh* occurs on large APEC plasmids (22,23,26). Also, ability to resist complement is a common characteristic of APEC, regardless of the syndrome or avian host species of origin (35). Complement resistance of *E. coli* has been related to several structural factors including the outer membrane proteins TraT, Iss, and OmpA (5,9,10,34,43). The increased serum survival gene, *iss*, has been reported to increase the virulence of an *E. coli* 100-fold for day-old chicks (4) and its complement resistance over 20-fold (3,9,10). Also, *traT* is found in the virulence cluster of large APEC plasmids (3,26) and encodes an outer membrane protein thought to contribute to complement resistance (42). The *ompA* gene encodes for outer membrane protein A, a protease that has been shown to contribute to serum resistance and pathogenicity of *E. coli* in embryonated chicks and to cleave colicins (7,43).

Peritonitis in chickens from different houses in the same in-line egg-production complex was associated with different strains of *E. coli*. The dominant *E. coli* strain within one chicken house may be different from the dominant *E. coli* strain in adjacent houses at the same production site. If so, this observation has important implications for the use of bacterins to immunize laying hens at an egg production facility. Autogenous bacterins protect against homologous *E. coli* challenge but may offer little protection against infection by heterologous *E. coli* challenge (2). Using molecular typing to identify and match *E. coli* strains used in a bacterin with the dominant *E. coli* strain causing disease in a poultry house should enhance the probability of developing successful immunization programs for commercial flocks. Based upon results of this pilot study, it may be necessary to use a different bacterin in each house on the farm to protect against *E. coli*-associated peritonitis in laying hens. It would be desirable to have vaccines that offer protection against homologous and heterologous *E. coli* challenge. Vaccines targeting proteins encoded by large APEC virulence plasmids might provide widespread protection against heterologous *E. coli* challenge because these plasmids are common among APEC.

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