Examination of the Source and Extended Virulence Genotypes of *Escherichia coli* Contaminating Retail Poultry Meat

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Abstract

Extraintestinal pathogenic *Escherichia coli* (ExPEC) are major players in human urinary tract infections, neonatal bacterial meningitis, and sepsis. Recently, it has been suggested that there might be a zoonotic component to these infections. To determine whether the *E. coli* contaminating retail poultry are possible extraintestinal pathogens, and to ascertain the source of these contaminants, they were assessed for their genetic similarities to *E. coli* incriminated in colibacillosis (avian pathogenic *E. coli* [APEC]), *E. coli* isolated from multiple locations of apparently healthy birds at slaughter, and human ExPEC. It was anticipated that the retail poultry isolates would most closely resemble avian fecal *E. coli* since only apparently healthy birds are slaughtered, and fecal contamination of carcasses is the presumed source of meat contamination. Surprisingly, this supposition proved incorrect, as the retail poultry isolates exhibited gene profiles more similar to APEC than to fecal isolates. These isolates contained a number of ExPEC-associated genes, including those associated with ColV virulence plasmids, and many belonged to the B2 phylogenetic group, known to be virulent in human hosts. Additionally, *E. coli* isolated from the crops and gizzards of apparently healthy birds at slaughter also contained a higher proportion of ExPEC-associated genes than did the avian fecal isolates examined. Such similarities suggest that the widely held beliefs about the sources of poultry contamination may need to be reassessed. Also, the presence of ExPEC-like clones on retail poultry meat means that we cannot yet rule out poultry as a source of ExPEC human disease.

Introduction

COLIBACILLOSIS IS THE MOST COMMON bacterial disease of poultry (Barnes *et al.*, 2008). Although the economic impact of this disease is difficult to assess, it is widely assumed to be substantial based on losses due to mortalities, condemnations, and lost productivity (Barnes *et al.*, 2008). Avian pathogenic *Escherichia coli* (APEC), the etiologic agent of this disease, belong to the broad *E. coli* pathotype known as extraintestinal pathogenic *E. coli* (ExPEC) (Russo and Johnson, 2000; Johnson *et al.*, 2002; Smith *et al.*, 2007). ExPEC are characterized by their ability to cause disease outside of the intestinal tract and include pathogenic strains isolated from various animals and human beings (Russo and Johnson, 2000;

Johnson *et al.*, 2002; Kaper, 2005). Similar to APEC, human ExPEC are responsible for significant morbidity and mortality, resulting in thousands of deaths and millions of days of lost productivity annually (Russo and Johnson, 2000; Johnson *et al.*, 2002; Barnes *et al.*, 2008). Every year in the United States alone, it is estimated that ExPEC-caused diseases result in over \$4 billion in direct healthcare costs (Russo and Johnson, 2003).

Because of the diversity of ExPEC, researchers have employed many means to define the ExPEC pathotype, including genotyping, multilocus sequence typing, pulsed-field gel electrophoresis, and virulence testing in laboratory models (Johnson, 1991; Johnson and Stell, 2000; Johnson *et al.*, 2001, 2008b; Rodriguez-Siek *et al.*, 2005a; Ewers *et al.*, 2007;

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Moulin-Schouleur *et al.*, 2007). These studies have shown that subpathotypes exist within the ExPEC pathotype, sharing similarities in the aforementioned traits, causing related conditions, and originating from the same host species. For instance, APEC cause colibacillosis in chickens and turkeys raised for meat and/or egg consumption, and they are characterized by possession of a conserved cluster of plasmid-linked virulence genes (Barnes *et al.*, 2008). Although many serogroups and untypeable strains are found among APEC, those belonging to the O1, O2, and O78 serogroups are said to predominate (Barnes *et al.*, 2008). Uropathogenic *E. coli* (UPEC) of human beings cause urinary tract infections (UTIs). Typical UPEC have chromosomal virulence genes, such as the *pap* operon, belong primarily to such serogroups as O4 and O6, and are often assigned to the B2 phylogenetic group (Johnson, 1991; Oelschlaeger *et al.*, 2002; Johnson and Russo, 2005). ExPEC isolated from meningitis in newborns are called neonatal meningitis *E. coli* (NMEC) (Bonacorsi and Bingen, 2005). Like UPEC, NMEC are characterized by assignment to the B2 phylogenetic group, possession of certain serogroups, and possession of chromosomal virulence factors, such as *ibeA* (Bonacorsi and Bingen, 2005). However, unlike UPEC but similar to APEC, NMEC typically contain ColV plasmid-associated virulence genes (Smith and Huggins, 1980; Agüero *et al.*, 1989; Ewers *et al.*, 2007; Johnson *et al.*, 2008b). Although the majority of APEC, UPEC, and NMEC contain traits typical of their respective subpathotypes, exceptions occur. In fact, a recent study involving over 1000 ExPEC, including APEC, NMEC, and UPEC, showed that a substantial subset of these strains have overlapping virulence profiles (Johnson *et al.*, 2008b). The identification of ExPEC from avian and human hosts, harboring traits associated with multiple subpathotypes, suggests that certain ExPEC may have zoonotic potential. If so, this would suggest that APEC from poultry could cause disease in humans, or that UPEC or NMEC of humans could cause disease in avian hosts. Even if ExPEC are able to cross host species lines, it only presents a problem if transmission of ExPEC between avian and human hosts actually occurs.

Certainly, possibilities exist for transfer of ExPEC from birds to humans and vice versa. One plausible scenario would involve the transmission of APEC to humans from contaminated poultry. For such transmission to occur, retail poultry meat would need to routinely contain APEC capable of causing human extraintestinal disease. Such contamination of poultry meat seems unlikely. After all, the source of bacterial contamination of poultry carcasses is presumed to be the feces of slaughtered birds, which contain *E. coli* that have been shown to lack ExPEC-associated traits (Rodríguez-Siek *et al.*, 2005b). Nevertheless, some researchers have reported that ExPEC can be isolated from poultry products and that these contaminants may be epidemiologically linked to human disease (Johnson *et al.*, 2003, 2005a, 2005b; Schroeder *et al.*, 2003; Manges *et al.*, 2007). These disparate observations present an intriguing conundrum, leaving us to question the actual source of *E. coli* contaminating retail poultry. Perhaps, ExPEC contamination of poultry meat is due to the entrance of preclinically or subclinically infected birds into the food chain, or perhaps, the carcasses are contaminated in other ways. Regardless, addressing this question and determining the virulence capacity of *E. coli* isolated from poultry meat (subsequently referred to as retail poultry *E. coli* [RPEC]) are critical in assessing the validity of the hypothesis that some

ExPEC are transmitted in retail poultry to human hosts where they cause disease. In an effort to address this question, we compared RPEC, APEC, avian fecal *E. coli* (AFEC), and *E. coli* isolated from the crops and gizzards of healthy birds (subsequently referred to as crop and gizzard *E. coli* [CGEC]) using comprehensive genotyping, serogrouping, and phylogenetic typing.

Materials and Methods

Bacterial strains

A total of 1671 avian *E. coli* isolates were used in this study. These included 590 APEC, 179 AFEC, and 80 CGEC. *E. coli* were recovered from crops and gizzards using the following protocol. Plant sampling was carried out on at least three separate occasions. Briefly, fresh crops and gizzards were collected from a turkey slaughter line and individually placed in sterile sampling bags. All samples were transported to the lab in chilled containers and analysis carried out on the same day of collection, within 4 hours of slaughter. In the case of the crops, the sac was usually empty and aseptically opened using flame-sterilized scissors with an opening of 1–2 cm in diameter made in the wall of the crop. Using a sterile cotton swab moistened in sterile water, the inside of the crop was swabbed ensuring as much of the internal surface area as possible was sampled. The swab was then streaked out directly on MacConkey and Eosin Methylene Blue (EMB) agars. In the case of the gizzards, a similar approach was used. However, the gizzards were opened completely and the internal contents removed before swabbing of the internal wall in the same manner as the crops. All plates were incubated at 37°C for 18–24 hours and inspected for suspect colonies, which were transferred to tryptone soy agar (Difco, Sparks, MD) and identified using the Sensititre® AP80 panels (Trek Diagnostics, Cleveland, OH).

Two hundred strains were isolated from retail chicken breasts (RPEC) obtained from three FoodNet laboratories as previously described (Maryland, Georgia, and Oregon) (Schroeder *et al.*, 2003). Retail chicken samples were stored at 4°C and processed no later than 96 hours after purchase. Portions from each sample were placed in separate sterile plastic bags with 250 mL of buffered peptone water, and the bags were vigorously shaken. Fifty milliliters of double-strength MacConkey broth was added to flasks containing 50 mL of rinsate to be used for *E. coli* isolation. The contents were mixed thoroughly and incubated at 35°C for 24 hours. One loopful from each flask was transferred to an EMB agar plate and streaked for isolation. Agar plates were incubated at 35°C for 24 hours in ambient air and examined for typical *E. coli* colonies (colonies having a dark center and usually a green metallic sheen). If no typical growth was observed on an EMB agar plate, the sample was considered negative and the appropriate documentation was made on the log sheet accompanying the sample. When *E. coli*-like growth was present, one typical, well-isolated colony was streaked for isolation onto a BAP. The BAP(s) were incubated at 35°C for 24 hours in ambient air and examined for purity. Indole-positive and oxidase-negative isolates were presumptively identified as *E. coli*. Isolates were confirmed as *E. coli* using the Vitek 2 Compact microbial identification system (BioMérieux, Hazelwood, MO).

Isolation of the APEC and fecal isolates has also been previously described (Rodríguez-Siek *et al.*, 2005a, 2005b;

Johnson *et al.*, 2006a, 2006b). The genotyping of some APEC and AFEC were previously described, but the data are included here for comparative purposes. Additionally, 91 human NMEC and 531 human UPEC from a previous study (Johnson and Russo, 2002; Johnson *et al.*, 2008a, 2008b) were included in the cluster analysis to illustrate similarities between avian-source *E. coli* and human ExPEC. Organisms were stored at -80°C in Brain Heart Infusion broth (Difco Laboratories, Detroit, MI) with 10% glycerol until used.

Hemolytic reaction

Test and control organisms were plated on 5% sheep blood agar plates and incubated overnight at 37°C . Plates were then examined for "greening" or clearing of the agar around areas of bacterial growth as an indication of alpha or beta hemolytic activity.

Serogroup analysis

Avian *E. coli* isolates were analyzed for O serogroup by the *E. coli* Reference Center (The Pennsylvania State University, University Park, PA) using antisera produced against serogroups designated O1–O181 with the exceptions of O14, O31, O47, O72, O93, O94, and O122 since these serogroups have not been designated.

Virulence genotyping

Test and control organisms were examined for the presence of 43 PCR products representing genes known for their association with human ExPEC or APEC virulence using multiplex PCR primer sets previously described (Johnson and Stell, 2000; Rodriguez-Siek *et al.*, 2005a; Johnson *et al.*, 2006a, 2006b). All primers used in the amplification studies were obtained from Integrated DNA Technologies (Coralville, IA). Boiled lysates were used as template DNA for the amplification procedure as previously described (Johnson and Stell, 2000). Amplifications were performed in $25\mu\text{L}$ reactions as previously described (Rodriguez-Siek *et al.*, 2005a). Samples were subjected to horizontal gel electrophoresis in 2.0% agarose, and sizes of the amplicons were determined via comparison to the Hi-Lo DNA marker (Minnesota Molecular, Minneapolis, MN). Control strains known to possess or lack the genes of interest were examined with each amplification procedure (Johnson and Stell, 2000; Rodriguez-Siek *et al.*, 2005a; Johnson *et al.*, 2006a, 2006b). An isolate was considered to contain the gene of interest if it produced an amplicon of the expected size. All amplifications were repeated to ensure reproducibility and reduce the chances of false negatives.

Phylogenetic typing

Isolates were assigned to phylogenetic groups according to the method of Clermont *et al.* (2000). Isolates were 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 PCR. Amplifications were performed as previously described (Clermont *et al.*, 2000). Samples were subjected to horizontal gel electrophoresis in 1.5% agarose, and the sizes of the amplicons were determined by comparison to the Hi-Lo DNA marker obtained from Minnesota Molecular. Positive and negative bacterial controls for each phylogenetic group were included in the analysis.

Biostatistics

Fisher's exact test was used to make two-way comparisons between the prevalence of each of the genes examined, and among each *E. coli* population examined (Snedecor and Cochran, 1989). A two-sided *p*-value was determined for each comparison and subsequently categorized as nonsignificant (NS; $p \geq 0.05$) or significant (S; $p < 0.05$). Multivariate hierarchical clustering with Ward's minimum variance was performed based upon the overall presence or absence of the 43 PCR products examined. Two-way clustering was performed in JMP (SAS Institute, Cary, NC) to create dendrograms portraying relationships between genes and between isolates, irrespective of isolate source or phylogenetic type. Results were presented in the form of a heat map based upon the presence or absence of a particular gene (Eisen *et al.*, 1998).

Results

Serogroup and hemolytic activity

Isolates were identified as belonging to 91 distinct serogroups, though a number of isolates were assigned to multiple serogroups, atypical serogroups (other), or non-typeable (NT) serogroups. In an effort to illustrate the top occurring serogroups among each group studied and overall, Table 1 was constructed. Overall, 262 (25%) of the isolates were NT and 126 (12%) of the isolates were classified as "other." For

TABLE 1. MOST COMMON SEROGROUPS AMONG THE AVIAN *ESCHERICHIA COLI* GROUPS STUDIED

AFEC	APEC	CGEC	RPEC	All	Shared ^a
	O1			O1	
O2	O2		O2	O2	O2
O5	O5	O8	O8	O8	O8
					O9
		O15	O11		
	O18	O15	O15	O15	O15
		O18	O18	O18	
		O19			
O20	O20	O20		O20	O20
		O21			
		O29	O25	O25	
	O36				
O78	O78		O69		
O86			O78	O78	
O88					
		O89			
O106					
O108					
	O111				
		O149			
NT	NT	NT	NT	NT	
Other	Other	Other	Other	Other	

^aTop 10 occurring serogroups are listed for each group, except for the "shared" group because only five serogroups were shared among all populations. Shared serogroups are those occurring among all groups examined.

APEC, avian pathogenic *E. coli*; CGEC, crop and gizzard *E. coli*; RPEC, retail poultry *E. coli*; NT, non-typeable; other, atypical or multiple serogroup.

TABLE 2. HEMOLYTIC ABILITIES, GENOTYPING, AND PHYLOGENETIC TYPING FOR AVIAN-SOURCE *E. COLI* ISOLATES

Gene/ trait	RPEC (n = 200)		APEC (n = 590)		CGEC (n = 80)		AFEC (n = 179)		Overall (n = 1049)		RPEC vs. APEC		RPEC vs. CGEC		RPEC vs. AFEC		APEC vs. CGEC		APEC vs. AFEC		CGEC vs. AFEC		A		B1		B2		D	
	No.	%	No.	%	No.	%	No.	%	No.	%																				
<i>sitA</i>	171	85.5	509	86.3	40	50.0	68	38.0	788	75.1	NS	NS	S	S	S	S	S	S	S	S	NS	NS	62.4	80.7	80.7	85.8	84			
<i>ompT</i> epi	152	76.0	460	78.0	37	46.3	41	22.9	690	65.8	NS	NS	S	S	S	S	S	S	S	S	NS	NS	56.8	67.4	67.4	71	74.7			
<i>hlyF</i>	151	75.5	446	75.6	38	47.5	39	21.8	674	64.2	NS	NS	S	S	S	S	S	S	S	S	S	S	54.9	67.9	67.9	66	74.4			
<i>iutA</i>	138	69.0	466	79.0	31	38.8	34	19.0	669	63.8	NS	NS	S	S	S	S	S	S	S	S	NS	NS	50.6	58.8	58.8	81.5	76.2			
<i>etsA</i>	130	65.0	397	67.3	31	38.8	22	12.3	580	55.3	NS	NS	S	S	S	S	S	S	S	S	S	S	42.7	59.4	59.4	64.2	65.8			
<i>etsB</i>	129	64.5	396	67.1	31	38.8	22	12.3	578	55.1	NS	NS	NS	NS	S	S	S	S	S	S	S	S	41.7	59.4	59.4	66	65.5			
<i>iroN</i>	122	61.0	503	85.3	39	48.8	38	21.2	702	66.9	S	S	NS	NS	S	S	S	S	S	S	S	S	57.6	63.6	63.6	76.5	77.2			
<i>iss</i>	120	60.0	474	80.3	31	38.8	39	21.8	664	63.3	S	S	NS	NS	S	S	S	S	S	S	NS	NS	53.7	61.5	61.5	74.1	72.6			
<i>cvaB</i> 5'	111	55.5	445	75.2	33	41.3	37	20.7	626	59.7	S	S	NS	NS	S	S	S	S	S	S	NS	NS	51.6	57.2	57.2	71.6	66.5			
<i>cvaA</i>	109	54.5	437	74.1	33	41.3	38	21.2	617	58.8	S	S	NS	NS	S	S	S	S	S	S	NS	NS	50.4	58.8	58.8	69.8	65.1			
<i>cvaB</i> 3'	88	44.0	367	62.2	25	31.3	30	16.8	510	48.6	S	S	NS	NS	S	S	S	S	S	S	NS	NS	44.6	44.4	44.4	61.1	50.5			
<i>eitB</i>	88	44.0	221	37.5	33	41.3	18	10.1	360	34.3	NS	NS	NS	NS	S	S	NS	NS	S	S	S	S	28.1	43.3	43.3	24.1	31.7			
<i>eitA</i>	86	43.0	221	37.5	33	41.3	18	10.1	358	34.1	NS	NS	NS	NS	S	S	NS	NS	S	S	S	S	26.9	39	39	50	32.3			
<i>tsh</i>	84	42.0	349	59.2	25	31.3	62	34.6	520	49.6	S	S	NS	NS	NS	NS	S	S	S	S	NS	NS	48.7	55.6	55.6	56.2	43.1			
<i>cbi</i>	73	36.5	203	34.4	16	20.0	35	19.6	327	31.2	NS	NS	NS	NS	NS	NS	NS	NS	S	S	NS	NS	28.1	43.3	43.3	24.1	31.7			
<i>cvaC</i>	72	36.0	370	62.7	18	22.5	13	7.3	473	45.1	S	S	NS	NS	S	S	S	S	S	S	NS	NS	37.9	42.2	42.2	61.7	48.4			
<i>cma</i>	48	24.0	159	27.0	11	13.8	35	19.6	253	24.1	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	22.5	28.9	28.9	16.7	27.4			
<i>fyuA</i>	67	33.5	322	54.6	27	33.8	48	26.8	464	44.2	S	S	NS	NS	NS	NS	NS	NS	S	S	NS	NS	36	23	23	79	50.5			
<i>ireA</i>	42	21.0	274	46.4	13	16.3	15	8.4	344	32.8	S	S	NS	NS	NS	NS	S	S	S	S	NS	NS	20.4	20.9	20.9	35.2	57.7			
<i>vat</i>	41	20.5	188	31.9	18	22.5	12	6.7	259	19.4	NS	NS	NS	NS	S	S	NS	NS	S	S	NS	NS	3.8	5.9	5.9	58.6	48.8			
<i>kpsMTII</i>	29	14.5	140	23.7	15	18.8	19	10.6	203	19.4	NS	NS	NS	NS	NS	NS	NS	NS	S	S	NS	NS	7.4	8.6	8.6	64.8	17.8			
<i>malX</i>	22	11.0	95	16.1	3	3.8	12	6.7	132	12.6	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	1.9	3.2	3.2	61.1	6.8			
<i>ibeA</i>	20	10.0	77	13.1	6	7.5	8	4.5	111	10.6	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	2.2	0.5	0.5	51.2	6.4			
<i>papC</i>	14	7.0	223	37.8	7	8.8	9	10.6	263	25.1	S	S	NS	NS	NS	NS	S	S	S	S	NS	NS	18	23.5	23.5	26.5	35.6			
<i>kpsMTkI</i>	13	6.5	92	15.6	1	1.3	9	5.0	115	11.0	NS	NS	NS	NS	NS	NS	S	S	S	S	NS	NS	4.1	3.7	3.7	45.7	5.7			
<i>papEF</i>	11	5.5	209	35.4	7	8.8	18	10.1	245	23.4	S	S	NS	NS	NS	NS	S	S	S	S	NS	NS	16.5	21.9	21.9	25.3	33.1			

<i>papG_23</i>	11	5.5	199	33.7	6	7.5	18	10.1	234	22.3	S	NS	NS	NS	S	NS	16.3	16	26.5	32.7
<i>ihg</i>	9	4.5	17	2.9	0	0.0	1	0.6	27	2.6		NS	NS	NS	NS	NS	1.2	6.4	4.3	1.1
<i>gimB</i>	7	3.5	59	10.0	1	1.3	2	1.1	69	6.6		NS	NS	NS	S	NS	0.2	2.1	35.8	2.1
<i>flhCH7</i>	6	3.0	29	4.9	2	2.5	12	6.7	49	4.7		NS	NS	NS	NS	NS	3.4	5.3	13	1.4
<i>kpsMTIII</i>	6	3.0	9	1.5	0	0.0	0	0.0	15	1.4	NS	NS	NS	NS	NS	NS	1	0.5	2.5	2.1
<i>papA</i>	2	1.0	57	9.7	0	0.0	7	3.9	66	6.3	S	NS	NS	NS	NS	NS	4.3	8.6	13	3.9
<i>cdtB</i>	2	1.0	6	1.0	2	2.5	0	0.0	10	1.0	NS	NS	NS	NS	NS	NS	0.2	0.5	3.7	0.7
<i>rfc</i>	2	1.0	2	0.3	2	2.5	2	1.1	8	0.8	NS	NS	NS	NS	NS	NS	1	0	2.5	0
<i>afa</i>	1	0.5	38	6.4	0	0.0	0	0.0	39	3.0	NS	NS	NS	NS	S	NS	1.9	3.7	0	8.5
<i>bmaE</i>	1	0.5	2	0.3	0	0.0	0	0.0	3	0.0	NS	NS	NS	NS	NS	NS	0.5	0.5	0	0
<i>gafD</i>	1	0.5	2	0.3	0	0.0	0	0.0	3	0.3	NS	NS	NS	NS	NS	NS	0.5	0.5	0	0
<i>sfafoc</i>	0	0.0	27	4.6	0	0.0	1	0.6	28	2.7	NS	NS	NS	NS	NS	NS	0.7	1.1	13.6	0.4
<i>sfuS</i>	0	0.0	19	3.2	0	0.0	1	0.6	20	1.9	NS	NS	NS	NS	NS	NS	0.7	1.1	9.3	0
<i>papG_I</i>	0	0.0	7	1.2	0	0.0	0	0.0	7	0.7	NS	NS	NS	NS	NS	NS	0.5	0	0	1.8
<i>cnfI</i>	0	0.0	11	1.9	0	0.0	2	1.1	13	1.2	NS	NS	NS	NS	NS	NS	0.7	1.1	4.3	0.4
<i>hlyD</i>	0	0.0	5	0.9	0	0.0	1	0.6	6	0.6	NS	NS	NS	NS	NS	NS	0.2	0.5	1.9	0.4
<i>facG</i>	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	NS	NS	NS	NS	NS	NS	0	0	0	0
<i>Phylotype</i>		<i>RPEC</i> (n = 200)%	<i>APEC</i> (n = 590)%	<i>CGEC</i> (n = 80)%	<i>APEC</i> (n = 179)%	<i>Overall</i> (n = 1049)%	<i>Overall vs.</i> <i>RPEC</i>	<i>Overall vs.</i> <i>APEC</i>	<i>Overall vs.</i> <i>CGEC</i>	<i>Overall vs.</i> <i>APEC</i>	<i>Overall vs.</i> <i>RPEC</i>	<i>Overall vs.</i> <i>CGEC</i>	<i>Overall vs.</i> <i>APEC</i>	<i>Overall vs.</i> <i>RPEC</i>	<i>Overall vs.</i> <i>CGEC</i>	<i>Overall vs.</i> <i>APEC</i>				
A	59	29.5	229	38.8	24	30	105	58.7	417	39.8	NS	NS	NS	NS	NS	S				
B1	52	26	85	14.4	18	22.5	32	17.9	187	17.8	S	NS	NS	S	NS	NS				
B2	24	12	105	17.8	10	12.5	23	12.8	162	15.4	NS	NS	NS	NS	NS	NS				
D	63	31.5	171	29.0	28	35	19	10.6	281	26.8	NS	NS	NS	NS	NS	S				
<i>Hemolysis</i>		<i>RPEC</i> (n = 200)%	<i>APEC</i> (n = 590)%	<i>CGEC</i> (n = 80)%	<i>APEC</i> (n = 179)%	<i>Overall</i> (n = 1049)%														
α	0	0.0	0.0	0	0	0.0	0	0	0	0.0	0	0	0.0	0	0	0.0				
β	1	0.5	0.5	7	0	0.2	0	0	0	0.0	0	0	0.0	0	8	0.8				
γ	199	95.5	98.8	583	80	100.0	179	100	1041	99.2	179	1041	100	179	1041	99.2				

RPEC, the most common serogroups included O8, O15, O69, and O78. However, the occurrence of these serogroups among the 200 RPEC only ranged from 5% to 6%, and the remaining RPEC serogroups were widely distributed. Among the APEC examined, O2 and O78 were the most common serogroups, occurring at rates of 11% and 18%, respectively. Other prevalent APEC serogroups included O1 (2.5%), O8 (2.4%), O18 (1.9%), and O111 (2.2%). Overall, the 590 APEC examined belonged to 63 different distinct serogroups. Among AFEC, the most common serogroups were O2 (6.1%) and O78 (3.4%), but the AFEC also had a wide distribution involving a large number of serogroups. No serogroups stood out as highly prevalent among the CGEC examined, with 20 different serogroups each occurring in less than 5% of the isolates examined. Of the 1049 isolates examined, only 8 isolates (7 APEC and 1 RPEC) were β -hemolytic in sheep blood agar (Table 2).

Phylogenetic types

The majority of the isolates examined belonged to the A (39.8%) and D (26.8%) phylogenetic groups, with fewer isolates belonging to the B1 (17.8%) and B2 (15.4%) groups (Table 2). Among the source groups, this pattern was generally maintained with a few exceptions. Among the AFEC, significantly more isolates (58.7%) belonged to the A phylogenetic group, and significantly fewer isolates ($p < 0.05$; 10.6%) belonged to the D phylogenetic group, as compared to the overall distribution. Also, the RPEC had a significantly higher number of isolates belonging to the phylogenetic group B1, as compared to the overall distribution.

Virulence genotypes

All isolates were examined for the presence of 43 PCR products representing genes associated with ExPEC (Table 2). Based upon gene prevalence within each group and overall, a series of two-way comparisons were performed using Fisher's exact test to identify any significantly different ($p < 0.05$) distributions of genes among the different *E. coli* groups. Among the RPEC, *sitA* had the highest prevalence at 85.5%, and genes of the conserved portion of the ColV plasmid pathogenicity-associated island (PAI) (Johnson *et al.*, 2006a) occurred at rates ranging from 60.0% for *iss* to 76.0% for *ompT*. Genes of the variable portion of the ColV PAI ranged from 24% to 44% in the RPEC examined. Other genes occurring among the RPEC examined included *fyuA* of the Yersinia-bactin operon (33%), iron-regulated element gene *ireA* (21%), and the vacuolating autotransporter gene *vat* (21%). The APEC examined displayed similar gene prevalence patterns to the RPEC examined, except at higher rates. For example, the genes of the conserved portion of the ColV PAI occurred at rates of 74% to 78% among the APEC examined, while genes of the variable portion of the ColV PAI ranged from 27% to 62%. The occurrence of the conserved portion of the ColV PAI did not differ significantly between RPEC and APEC, but the presence of the ColV operon, *iss*, *iroN*, and *tsh* was significantly higher among APEC than RPEC. Also, *fyuA* and *ireA* occurred at a significantly higher rate ($p < 0.05$) among APEC as compared to RPEC. Among the CGEC examined, lower percentages were observed for the above-mentioned genes, as compared to the APEC and RPEC examined. The genes of the conserved portion of the ColV PAI ranged from 39% to 48% among the CGEC examined, which was significantly lower

than the RPEC and APEC but generally higher than the AFEC. The variable portion of the ColV PAI was present in 14% to 41% of the CGEC examined, which was significantly lower than its occurrence among APEC, similar to its occurrence among RPEC, and similar to its occurrence among AFEC except for a significantly higher occurrence of the *eitA* and *eitB* genes among CGEC. The occurrences of *ireA*, *vat*, and *fyuA* were similar between CGEC and RPEC, but these genes were found in significantly fewer CGEC isolates as compared to APEC. The AFEC examined contained significantly lower percentages of genes within the conserved and variable portions of the ColV PAI, as compared to APEC and RPEC. However, the occurrence of several genes within this PAI was statistically similar between CGEC and AFEC, including *ompT*, *iutA*, *iss*, *tsh*, and genes of the ColV and ColBM operons.

Two-way hierarchical clustering was performed based upon overall genotype involving the 43 products examined here (Fig. 1). Additionally, human NMEC and UPEC were included to search for any overlap between human and avian isolates. From this analysis, nine genotyping clusters were identified (Table 3). The largest cluster identified was cluster #1, which contained 365 isolates and generally did not contain any of the genes examined. The isolates within this cluster originated from all six source groups examined (APEC, AFEC, CGEC, RPEC, NMEC, and UPEC) and mostly belonged to the A phylogenetic group. Most of the AFEC were found in this cluster. Most of the UPEC examined fell within clusters #2–4, which generally lacked ColV plasmid-associated genes but contained some chromosomal PAI-associated genes, such as *fyuA*, *vat*, *kpsMTII*, *malX*, and genes of the *pap* operon. Most of the RPEC examined fell within clusters #5–6. Members of these clusters contained genes of the ColV PAI, with or without the ColBM operon, and they lacked most of the chromosomal PAI-associated traits examined, except *fyuA* and *ireA* on occasion. Cluster #7 appeared to contain isolates with ColV PAI variants, many lacking the conserved and/or variable components of this PAI. Included within this cluster were isolates from all sources, but the majority of the isolates were APEC and RPEC. Most of the NMEC examined fell within clusters #8–9, which also included APEC and RPEC. These isolates contained the ColV PAI genes and a mosaic of chromosomal PAI-associated genes.

When chromosome-encoded virulence factors were involved (clusters 2–4 and 8–9), the majority of the isolates belonged to the B2 or D phylogenetic groups. When these traits were not involved, most of the isolates belonged to the A, B1, or D phylogenetic groups. There was no observed relationship between phylogenetic group and presence of the ColV PAI. However, clustering was able to identify at least 12 ColV variants with differing combinations of PAI-associated traits. Clustering also separated out most of the UPEC and AFEC, based upon overall genotype. However, the APEC, RPEC, NMEC, and CGEC examined were often mixed together throughout the gel diagram.

Additionally, the distribution of certain genes across phylogenetic types was examined to determine if some types were more likely to contain a given gene than other types (Table 2). Several genes were more likely to be found in certain phylogenetic groups, including *sitA* (B1, B2, and D), *iutA* (B2 and D), genes of the ColV operon (B2 and D), *fyuA* (B2 and D), *ireA* (D), *kpsMTII* (B2), *malX* (B2), *ibeA* (B2), *kpsMTI* (B2), and *gimB* (B2). In general, conserved ColV plasmid PAI-associated

FIG. 1. Two-way cluster analysis of individual PCR genotyping results for every avian-source *E. coli* isolate examined in this study. Also included for comparison purposes were human NMEC ($n = 91$) and human UPEC ($n = 531$) from a previous study (Johnson *et al.*, 2008b). For each of the 1671 isolates (top to bottom), PCR results are depicted as positive (black) or negative (lime green) from left to right. On the left, a source bar was included to reference the sources of individual isolates. On the right, a Phylotype bar was included to illustrate *E. coli* phylogenetic group of individual isolates (Clermont *et al.*, 2000). Isolates are clustered (top to bottom) according to their overall similarity in gene/trait possession, not including Source or Phylotype. Also, genes/traits correlated with one another are clustered (left to right). The dashed line over the dendrogram on the right depicts the cut-off for the generation of the nine clusters described in the text. NMEC, neonatal meningitis *E. coli*; UPEC, uropathogenic *E. coli*.

FIG. 2. Two-way cluster analysis of average gene prevalence data for the four groups of avian-source *E. coli*. Also included for comparison purposes were human NMEC ($n = 91$) and human UPEC ($n = 531$) from a previous study (Johnson *et al.*, 2008b). From this analysis a heat map was constructed to illustrate the relationships among the groups (Y-axis) with regard to traits examined (X-axis). Colors indicate gene prevalence, ranging from dark blue (least prevalent) to gray (of intermediate prevalence) to dark red (most prevalent). *E. coli* groups are clustered (top to bottom) according to their overall similarity in average gene prevalence. Also, traits correlated with one another are clustered (left to right).

TABLE 3. CONTENTS OF GENOTYPING CLUSTERS

	N	APEC (%)	AFEC (%)	CGEC (%)	RPEC (%)	NMEC (%)	UPEC (%)	A (%)	B1 (%)	B2 (%)	D (%)	Characteristics
Overall	1670	35.3	10.7	4.8	12.0	5.4	31.8	28.9	12.8	33.8	24.5	
Cluster #												
1	365	18.4	31.5	10.1	14.0	2.5	23.6	50.1	19.2	9.3	21.4	No genes
2	164	6.7	4.3	1.2	0.6	8.5	78.7	7.9	3.0	70.1	18.9	<i>sitA</i> , <i>iutA</i> , <i>fyuA</i> , <i>vat</i> , <i>kpsMTII</i> , <i>malX</i> PAI
3	142	2.1	9.2	2.8	0.0	7.0	78.9	12.7	2.8	35.9	48.6	<i>sitA</i> , <i>iutA</i> , <i>ihcA</i> , <i>fyuA</i> , <i>kpsMTII</i> , <i>papACGEF</i>
4	177	0.6	0.6	0.0	0.0	1.7	97.2	3.4	0.0	95.5	1.1	<i>sitA</i> , <i>cnfI</i> , <i>hlyD</i> , <i>fyuA</i> , <i>vat</i> , <i>kpsMTII</i> , <i>malX</i> PAI, <i>papACGEF</i>
5	164	65.2	4.9	3.0	18.9	1.8	6.2	44.5	15.2	7.3	33.0	ColV PAI minus <i>eitAB</i> , <i>fyuA</i>
6	169	50.9	3.0	11.8	34.3	0.0	0.0	39.1	29.6	3.0	28.3	ColV PAI, some ColBM
7	178	58.4	14.6	3.4	21.9	0.6	1.1	46.1	18.0	7.9	28.0	Conserved ColV PAI minus variable ColV PAI, some ColBM
8	109	40.4	0.9	3.7	15.6	33.9	5.5	1.8	0.0	93.6	4.6	ColV PAI, <i>kpsMTI</i> , <i>ibeA</i> , <i>gimB</i> , <i>fyuA</i> , <i>vat</i> , <i>kpsMTII</i> , <i>malX</i> PAI
9	202	82.2	1.5	1.0	1.5	6.9	6.9	19.8	13.4	30.7	36.1	ColV PAI, some <i>fyuA</i> , some <i>vat</i> , some <i>kpsMTII</i> , some <i>malX</i> PAI, <i>papCEFG</i> , some <i>papA</i>

Percentages shown are proportions of isolates within each cluster.
NMEC, neonatal meningitis *E. coli*; UPEC, uropathogenic *E. coli*.

genes were evenly distributed among the four phylogenetic types, although they were slightly higher among the B2 and D phylotypes. Genes of the variable portion of the ColV PAI were evenly distributed. Genes of the *pap* operon were also evenly distributed, although they too were slightly higher in the B2 and D phylotypes.

Two-way clustering was also performed using average gene prevalence for each of the populations examined (Fig. 2). This clustering identified CGEC and RPEC as being most closely related, on average. This clustering also clearly distinguished the human NMEC and UPEC from the avian source *E. coli* isolates.

Discussion

Poultry meat is a relatively inexpensive source of high-quality protein for human consumption. Unfortunately, it also may serve as a vehicle of foodborne pathogens, such as *Campylobacter* and *Salmonella* (Dufrenne *et al.*, 2001; Zhao *et al.*, 2001; Dominguez *et al.*, 2002; Wilson 2002; Burgess *et al.*, 2005; Meldrum *et al.*, 2005, 2006; Rodrigo *et al.*, 2006; Meldrum and Wilson, 2007; Trajkovic-Pavlovic *et al.*, 2007; Vindigni *et al.*, 2007). Similarly, we and others have hypothesized that retail poultry may be a foodborne reservoir of ExPEC causing such conditions as UTIs, meningitis, and sepsis of human beings (Johnson *et al.*, 2003, 2005a, 2005b, 2007a; Rodriguez-Siek *et al.*, 2005a; Manges *et al.*, 2007). For this hypothesis to be viable, it requires that retail poultry actually be contaminated with ExPEC. The ExPEC that cause disease in production birds are known as APEC (Barnes *et al.*, 2008), which share many virulence attributes with human ExPEC (Rodriguez-Siek *et al.*, 2005a; Moulin-Schouleur *et al.*, 2006; Ron, 2006; Ewers *et al.*, 2007; Johnson *et al.*, 2007b, 2008b; Moulin-Schouleur *et al.*, 2007). Also, APEC have been shown to cause disease in mammalian models of human UTI, and APEC plasmids have been shown to contribute to the pathogenesis of murine UTI (Skyberg *et al.*, 2006). Follow-up comparisons of the genomes of an APEC and several human ExPEC confirmed their strong similarities and prevented our ruling out the possibility that APEC might be involved in human disease (Johnson *et al.*, 2007a). Despite these similarities, a foodborne link between APEC and human disease seemed improbable since AFEC, which show a dearth of ExPEC virulence traits, were thought to be the source of RPEC contamination and because *E. coli* contamination is reduced during processing (Altekruse *et al.*, 2002; de Brito *et al.*, 2003; Delicato *et al.*, 2003; Rodriguez-Siek *et al.*, 2005b). If true, the hypothesis that retail poultry serves as a vehicle for ExPEC transmission might not be viable. Consequently, we compared RPEC with APEC and AFEC for their possession of a variety of ExPEC virulence-associated traits in an effort to test these assumptions. Evaluation of our results was facilitated by a group of plasmid-associated genes whose presence can be used to distinguish the vast majority of APEC from AFEC (Rodriguez-Siek *et al.*, 2005b; Johnson *et al.*, 2006b, 2008a, 2008b). Surprisingly, RPEC contained many ExPEC-associated virulence traits (Tables 2 and 3) and were more similar to CGEC and APEC than to AFEC. More than 60% of the RPEC examined contained the genes of the ColV plasmid PAI that typify APEC. These counterintuitive findings mean we still cannot rule out the possibility that retail poultry meat, contaminated with APEC, is a foodborne source of ExPEC with the capacity to cause human disease.

Based on what is assumed about meat contamination, it is difficult to explain these results, since the feces of healthy birds at slaughter seem an unlikely source of APEC. However, reports of a low level occurrence of *E. coli* with ExPEC virulence genes in the fecal flora of poultry suggest that feces could be a source of contamination (Altekruse *et al.*, 2002; de Brito *et al.*, 2003; Delicato *et al.*, 2003; Rodriguez-Siek *et al.*, 2005b). This contamination scenario seems plausible, especially if the growth of these *E. coli* is favored along the food safety continuum resulting in these low level contaminants coming to predominate in the final product. Also, the possibility that birds with preclinical or subclinical APEC infections end up in the processing line should be considered as an additional source of *E. coli* contamination of retail product. We also must consider the possibility that meat may be contaminated by other sources along the food chain.

Since RPEC were very similar to APEC in their content of plasmid PAI genes (Johnson *et al.*, 2006a), an avian source of RPEC seemed reasonable. Also, recognizing that healthy birds could be raised in a contaminated environment, we reasoned that the crops and gizzards of slaughtered birds might contain APEC picked up by birds during pecking. Thus, a potential source of carcass contamination was crop and gizzard contents of slaughtered birds. It was previously shown that *E. coli* could be recovered at levels as high as 4.4 log₁₀ CFU from broiler crops (Berrang *et al.*, 2000). Lending further credence to crops and gizzards as a source of meat contamination, Hargis *et al.* (1995) reported that a quarter of the crops of broilers at a commercial processing plant were damaged during removal (Hargis *et al.*, 1995). Other studies have shown that the gizzard can also serve as a source of pathogenic microorganisms (Smith and Berrang, 2006). Thus, it is plausible that the crop could serve as a significant source of *E. coli* contamination. Here, we found that about 40% of the CGEC contained typical APEC traits, whereas less than 20% of the AFEC did. Indeed, CGEC showed more overall similarity to RPEC and APEC than AFEC (Fig. 2). Therefore, based upon the limited sites sampled in this study, crop and gizzard contents appear to be a much more likely source of RPEC than are feces. Although studies have found that rupturing of the crop and gizzard during poultry processing might not significantly increase the total bacterial counts on prechill carcasses (Smith and Berrang, 2006), it appears from this study that rupturing of the crop and/or gizzard might result in the contamination of carcasses with *E. coli* possessing enhanced fitness and virulence capabilities. Since such organisms might be favored for growth along the food chain, crops and gizzards deserve further attention as a potential source of carcass contamination, especially since the results of such research may have implications for a number of food safety issues.

Conclusions

Although the possibility that crop and gizzard contents contaminate carcasses with ExPEC is intriguing, other potential sources of contamination will need to be considered in future research. Indeed, the possibility that current food-processing practices favor growth of pathogen-like *E. coli* over commensal strains should be considered. Of particular interest in this regard is that a large number of RPEC, like APEC, harbor genes that are typically linked to colicin-encoding plasmids, which are known to encode a variety of fitness-

related traits. Perhaps, these plasmids help their *E. coli* host outcompete other members of the meat microflora. Finally, while we can say that APEC and RPEC harbor traits known to contribute to ExPEC-caused diseases of human beings, we still cannot know whether these strains actually cause human disease. Clearly, most of the human NMEC and UPEC examined are distinct from avian-source *E. coli* (Fig. 1). However, subsets of these groups show considerable overlap in their virulence-associated traits and may represent potential zoonotic pathogens. Indeed, most of the NMEC and some of the UPEC examined fall within this subset, as do many APEC and RPEC. Future work to examine the zoonotic potential of this important subset is warranted. Overall, these results show that the source of RPEC, their disease-causing capacity, and their possible role in human disease are incompletely understood. In summary, while these results demonstrate that the source of RPEC, the disease-causing capacity of these organisms, and their possible role in human disease are incompletely understood, the presence of ExPEC-like clones on retail poultry meat means that we still cannot rule out poultry as a source of ExPEC human disease.

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