Zoonotic Potential of *Escherichia coli* Isolates from Retail Chicken Meat Products and Eggs

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Chicken products are suspected as a source of extraintestinal pathogenic *Escherichia coli* (ExPEC), which causes diseases in humans. The zoonotic risk to humans from chicken-source *E. coli* is not fully elucidated. To clarify the zoonotic risk posed by ExPEC in chicken products and to fill existing knowledge gaps regarding ExPEC zoonosis, we evaluated the prevalence of ExPEC on shell eggs and compared virulence-associated phenotypes between ExPEC and non-ExPEC isolates from both chicken meat and eggs. The prevalence of ExPEC among egg-source isolates was low, i.e., 5/108 (4.7%). Based on combined genotypic and phenotypic screening results, multiple human and avian pathotypes were represented among the chicken-source ExPEC isolates, including avian-pathogenic *E. coli* (APEC), uropathogenic *E. coli* (UPEC), neonatal meningitis *E. coli* (NMEC), and sepsis-associated *E. coli* (SEPEC), as well as an undefined ExPEC group, which included isolates with fewer virulence factors than the APEC, UPEC, and NMEC isolates. These findings document a substantial prevalence of human-pathogenic ExPEC-associated genes and phenotypes among *E. coli* isolates from retail chicken products and identify key virulence traits that could be used for screening.

Extraintestinal pathogenic *Escherichia coli* (ExPEC), the etiologic agent of colibacillosis in chickens, also causes diverse infections in humans, including urinary tract infections (UTIs), neonatal meningitis, and sepsis (1). The ExPEC strains that cause these different syndromes are sometimes regarded as representing distinct pathotypes, which are designated avian-pathogenic *E. coli* (APEC), uropathogenic *E. coli* (UPEC), neonatal meningitis *E. coli* (NMEC), and sepsis-associated *E. coli* (SEPEC). Apart from their tremendous associated morbidity and mortality, ExPEC-associated human diseases impose a large economic burden due to both medical costs and lost productivity (2).

Pathogenic bacteria in poultry are a threat to both the poultry industry and human health, due to decreased production and/or transmission to consumers via contaminated poultry products. Both meat and eggs are known to be a source of human pathogens such as *Campylobacter*, *Listeria*, and *Salmonella*. These products, when inspected and found to be contaminated with these organisms, are sometimes recalled if the recommended limit for microbial load is exceeded (3). Recent studies have suggested that meats, particularly poultry, can also be a source of ExPEC strain transmission to humans (4).

Multiple virulence traits have been identified in ExPEC that allow these bacteria to invade, colonize, and cause infections in bodily sites outside the gastrointestinal tract (1, 5). Many human and animal-source ExPEC isolates exhibit similar virulence genes and clonal backgrounds, suggesting that they may represent zoonotic pathogens (6). ExPEC virulence genes are common among *E. coli* isolates from food products, particularly raw meats (5) and especially poultry meat (7). However, although poultry-source *E. coli* isolates have been shown to cause UTI, sepsis, and meningitis in rodent models that mimic human ExPEC infections (8), the human health risk posed by poultry products is still a matter of some debate, since direct transmission of ExPEC from poultry to humans is difficult to document (4).

An additional challenge in assessing the zoonotic risk of poultry products is that ExPEC isolates are genotypically heterogeneous. Not only do they share multiple genomic similarities with

commensal, nonpathogenic *E. coli* (9), but also the different putative subgroups of ExPEC are difficult to differentiate. Although recent evidence indicates that the evaluation of phylotypes and virulence genotypes permits discrimination of ExPEC from commensal *E. coli* (10–12), host-pathogen interactions can lead to differential expression of genes *in vivo* (13). Genotypic analysis alone is thus unlikely to definitively differentiate the various pathotypes.

A more complete understanding of the zoonotic risk of ExPEC is required to develop treatments and preventative measures against infections and food contamination. Accordingly, we sought to further define the zoonotic risk posed by ExPEC in poultry products and to fill existing knowledge gaps regarding ExPEC transmission. Specifically, we addressed two study questions. First, since we and others have shown that chicken meat from retail markets is contaminated with ExPEC isolates that resemble the strains that cause human infections, we sought to determine whether similar ExPEC-like bacteria are found on chicken eggs, another popular poultry-source food product. Second, since *E. coli* isolates from chicken products currently are identified as ExPEC based solely on their molecular characteristics, we assessed whether testing chicken-source *E. coli* for selected phenotypic traits could assist in distinguishing ExPEC from non-ExPEC and in the identification of different subpathotypes of ExPEC.

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FIG 1 Schematic diagram of experimental design described in this study. The study included four steps: (i) all chicken meat-source and egg-source *E. coli* isolates were genotypically screened for 5 ExPEC-defining genes and were phylotyped, (ii) a subset of ExPEC and non-ExPEC isolates were randomly selected for this study, (iii) all selected ExPEC study isolates were further screened for an additional 34 ExPEC-associated virulence genes, and (iv) all selected study isolates (ExPEC and non-ExPEC) underwent phenotypic testing. Boxes highlighted in dark gray represent *E. coli* isolates identified in previous studies, as described in Materials and Methods. Boxes highlighted in light gray represent *E. coli* isolates identified in this study. ExPEC, extraintestinal pathogenic *E. coli*.

MATERIALS AND METHODS

Bacterial strains and growth. In total, 282 chicken-source *E. coli* isolates were studied (Fig. 1). Of the 282 isolates, 174 were from raw chicken meat products, selected randomly from our collection of isolates previously recovered between 1999 and 2004 in several retail market surveys done at sites around the United States (7, 14–17) (Fig. 1). The remaining 108 *E. coli* study isolates represented all available shell-egg isolates recovered in 2003 at different stages of chicken egg processing in three commercial egg facilities in Georgia, USA (18), and were kindly provided by Michael Musgrove (Agricultural Research Service, U.S. Department of Agriculture, Athens, GA) (Fig. 1). Unless otherwise stated, bacteria were routinely grown at 37°C in Luria-Bertani (LB) broth, on LB agar, or on MacConkey agar. Stocks were maintained at –80°C in peptone-glycerol medium.

Genotypic and phylogenetic screening. Using boiled lysates as target DNA (19, 20), *E. coli* isolates were screened by multiplex PCR for ExPEC status, which was inferred based on detection of ≥ 2 of the following 5 ExPEC-defining markers: *papA* and/or *papC* (P fimbriae; counted as 1), *sfa* and/or *foc* (S and F1C fimbriae, respectively), *afa* and/or *dra* (Dr-binding adhesins), *kpsM II* (group 2 capsule), and *iutA* (aerobactin system) (21). This first screening was done either previously, for the 174 chicken meat isolates (7, 14–17), or in this study, for the 108 chicken egg isolates (Fig. 1). A total of 129 isolates (124 from meat and 5 from eggs) qualified molecularly as ExPEC; the remaining 99 (50 from meat and 49

from eggs) did not (Fig. 1). All isolates (Fig. 2) from both sources (chicken meat and eggs) underwent triplex PCR (22) to define the major *E. coli* phylogenetic groups (A, B1, B2, and D) (22) (Fig. 1). ExPEC-qualifying isolates were screened for 34 additional ExPEC-associated virulence genes by multiplex PCR (7, 10, 14) (Table 1).

Phenotypic screening. Study isolates were tested for ExPEC-associated phenotypic traits (Fig. 1) as described below.

Siderophore production was detected using chrome azurol S (CAS) agar to test for iron mobilization (23). After overnight (O/N) incubation at 37°C, bacterial colonies with orange halos on the blue agar were considered positive and halo diameters were recorded.

Resistance to complement was determined using a quantitative microtiter plate method (24). Guinea pig serum (Innovative Technologies) was serially 2-fold diluted from 50% to 3.125% in flat-bottom 96-well plates (Costar; Corning Inc., NY). A volume of 100 μ l of phosphate-buffered saline (PBS) containing $\sim 10^4$ CFU was mixed with fresh serum, heat-inactivated serum (control), or peptone-glucose broth (growth control). The absence of antibodies against *E. coli* in the serum, which would interfere with the complement-mediated killing assay, was confirmed in the heat-inactivated serum control that was tested against the positive- and negative-control *E. coli* strains. After a 4-h incubation at 37°C, plates were read by an absorbance spectrophotometer (SpectraMax M2; Molecular Devices, Sunnyvale, CA, USA) at 492 nm. Samples were considered com-

FIG 2 Schematic diagram of pathotype determination described in this study and its limitations. ExPEC, extraintestinal pathogenic *E. coli*; APEC, avian-pathogenic *E. coli*; NMEC, neonatal meningitis *E. coli*; UPEC, uropathogenic *E. coli*; SEPEC, sepsis-associated *E. coli*. Isolates were classified as ExPEC if positive for 2 of the following 5 ExPEC-defining markers: *papA* and/or *papC* (P fimbriae; counted as 1), *sfa* and/or *foc* (S and F1C fimbriae, respectively), *afa* and/or *dra* (Dr-binding adhesins), *kpsM* II (group 2 capsule), and *iutA* (aerobactin system). APEC genes include (i) *kii*; (ii) *iss*; (iii) *tsh*; (iv) one of the 5 genes *sfa*, *foc*, *papA*, *papC*, and *papEF*; and (v) one of the two genes *iutA* and *fyuA*.

plement resistant if the optical densities (ODs) of all diluted serum wells equaled or exceeded the optical density of the (no-serum) growth control well, normalized to the initial serum absorbance.

Total colicin production was tested using the double-agar diffusion method (25) on Trypticase soy agar. Clearance zone diameters of the *E. coli* K-12 indicator strain χ 6092 (26), after overnight incubation at 37°C, were recorded.

Biofilm production was evaluated in round-bottom 96-well microtiter plates (Microtest U-Bottom; Becton, Dickinson, Franklin Lakes, NJ). The optical density at 600 nm (OD₆₀₀) of an overnight (O/N) LB culture was normalized to 1.0, and 200 μ l of a 1:100 dilution was put into quadruplicate wells and incubated O/N at 37°C standing. Biofilms were quantified using crystal violet staining (27). The absorbance was measured at 570 nm in an absorbance spectrophotometer (SpectraMax M2; Molecular Devices). All tests were carried out at least three times, and the results were averaged. Isolates were considered positive for biofilm formation if the crystal violet-stained biofilm had an OD₆₀₀ equal to or greater than 3-fold the value obtained in the well containing bacterium-free medium.

Growth in human urine was assessed using filter-sterilized human urine. Urine was collected from adult volunteers. Each batch of urine used was collected from 4 individuals (2 men and 2 women) under a protocol approved by the local Institutional Review Board (IRB) (identifier [ID] 1012005820), with subjects' written informed consent. As is standard in

the field, urine from multiple donors was pooled to ensure that the composition of the tested urine samples represented more than just one person, to increase generalizability by avoiding artifacts arising from individual urine composition variations.

Urine samples from two male and two female donors were filter sterilized and then pooled. Dilute bacterial suspensions in urine were prepared by adding 1:100 volume of an overnight broth culture of the test strain to an aliquot of pooled sterile urine. After adjusting the OD₆₀₀ to $\sim$ 1.0, 200 μ l was placed into a growth curve machine (Bioscreen; Growth Curves USA, NJ, USA) 100-well honeycomb plate, in triplicate. OD measurements at 420 nm to 580 nm (wide band) were taken every 15 min for 8 h. The *E. coli* K-12 strain (χ 6092) and UPEC strain (CFT073) (28) were used as negative and positive controls, respectively, with urine blanks to ensure sterility. Strains that, based on mean values from triplicate experiments, had stationary-phase optical densities within or greater than the standard error of the positive control were considered positive for growth in urine.

Criteria for ExPEC pathotype groups. To define ExPEC pathotype groups, i.e., APEC, UPEC, and NMEC, we used either previously published or newly defined criteria. For APEC, the criterion was detection of $\geq$ 4 of 5 genes/groups, i.e., (i) *kii*; (ii) *iss*; (iii) *tsh*; (iv) one of the 5 genes *sfa*, *foc*, *papA*, *papC*, and *papEF*; and (v) one of the two genes *iutA* and *fyuA* (29). For UPEC and NMEC, the criteria represented genotypic and phenotypic traits that are characteristic of these pathotypes and would iden-

TABLE 1 Prevalence of ExPEC-associated genes among chicken-associated ExPEC isolates

Functional category	Specific gene	No. of isolates with gene ^a (%)		
		All ExPEC (n = 129)	Meat (n = 124)	Eggs (n = 5)
Adhesin	<i>afaE8</i>	7 (5)	7 (6)	0 (0)
	<i>bmaE</i>	7 (5)	7 (6)	0 (0)
	<i>fimH</i>	128 (99)	123 (99)	5 (100)
	<i>gafD</i>	7 (5)	7 (6)	0 (0)
	<i>hra</i>	68 (53)	64 (52)	4 (80)
	<i>papA</i>	19 (15)	19 (15)	0 (0)
	<i>papC</i>	70 (54)	68 (55)	2 (40)
	<i>papEF</i>	63 (49)	61 (49)	2 (40)
	<i>papG</i>	23 (18)	23 (19)	0 (0)
	<i>sfa</i> and/or <i>focDE</i>	3 (2)	3 (2)	0 (0)
	<i>sfaS</i>	4 (3)	3 (2)	1 (20)
Toxin	<i>cdtB</i>	1 (1)	0 (0)	1 (20)
	<i>cnf1</i>	1 (1)	1 (1)	0 (0)
	<i>astA</i>	59 (46)	57 (46)	2 (40)
	<i>hlyA</i>	3 (2)	3 (2)	0 (0)
	<i>hlyF</i>	85 (66)	83 (67)	2 (40)
	<i>pic</i>	6 (5)	4 (3)	2 (40)
	<i>tsh</i>	89 (69)	86 (69)	3 (60)
	<i>sat</i>	2 (2)	2 (2)	0 (0)
Siderophore	<i>fyuA</i>	67 (52)	66 (53)	1 (20)
	<i>ireA</i>	41 (32)	39 (32)	2 (40)
	<i>iroN</i>	68 (53)	67 (54)	1 (20)
	<i>iutA</i>	123 (95)	119 (96)	4 (80)
Protectin	<i>cvaC</i>	37 (29)	37 (30)	0 (0)
	<i>iss</i>	75 (58)	75 (61)	0 (0)
	<i>kpsM</i> K1	39 (30)	38 (31)	1 (20)
	<i>kpsM</i> K2 and K100	1 (1)	1 (1)	0 (0)
	<i>kfiC</i> K5	4 (3)	4 (3)	0 (0)
	<i>rfe</i>	4 (3)	3 (2)	1 (20)
	<i>traT</i>	48 (37)	48 (39)	0 (0)
Miscellaneous	H7 <i>fliC</i>	3 (2)	2 (2)	1 (20)
	<i>ibeA</i>	18 (14)	17 (14)	1 (20)
	<i>ompT</i>	71 (55)	67 (54)	4 (80)
	<i>malX</i>	23 (18)	22 (18)	1 (20)
	<i>usp</i>	24 (19)	24 (19)	0 (0)

^a All isolates were negative for certain adhesin (*afa* and *draBC*, *clpG*, *focG*, *f17*, and *iha*), toxin (*hlyD*), and capsule (*kpsM* K15) genes.

tify the majority of isolates of a specific pathotype. These included, for UPEC, growth in urine (13, 30), and for NMEC, positive results for two genes, *kpsMT* K1 and *ibeA* (31). APEC, UPEC, and NMEC isolates that were resistant to complement (32) were considered SEPEC (Fig. 2).

Statistical analysis. Comparisons involving continuous variables were tested using a two-tailed *t* test, and those involving categorical variables were tested using a chi-squared test with a *P* value of <0.05 considered significant. The area-proportional Venn diagram was created using the open-source eulerAPE program (<http://www.eulardiagrams.org/eulerAPE>) (33).

RESULTS

Identification of ExPEC among chicken isolates and distribution of virulence genes. In the previous surveys of retail meat products that provided the present study's chicken meat-source *E. coli* isolates, 130 (21%) of 606 chicken meat isolates qualified mo-

TABLE 2 Phylotype distribution among ExPEC and non-ExPEC *Escherichia coli* isolates from chicken meat and eggs

Sample type	ExPEC	No.	Phylotype, no. (row %)			
			A	B1	B2	D
Meat	No	50	21 (42)	12 (24)	2 (4)	15 (30)
	Yes	124	25 (20) ^a	9 (7) ^a	34 (27) ^a	56 (45)
Eggs	No	49	25 (51)	21 (43)	3 (6)	0 (0)
	Yes	5	4 (80)	0 (0)	1 (20)	0 (0)
Total	No	99	46 (47)	33 (33)	5 (5)	15 (15)
	Yes	129	29 (23) ^a	9 (7) ^a	35 (27) ^a	56 (43) ^a
	Yes or no	228	75 (33)	42 (18)	40 (18)	71 (31)

^a Difference between ExPEC and non-ExPEC isolates (same source and phylogroup) was statistically significant (*P* < 0.05).

lecularly as ExPEC (7, 14–17), of which 124 ExPEC and 50 non-ExPEC isolates were selected randomly for this study (Fig. 1). In the present study, the same PCR-based ExPEC screening method was applied to 108 *E. coli* isolates from chicken eggs. Five (4.7%) of 108 chicken egg isolates qualified as ExPEC (versus chicken meat, *P* < 0.001). Isolates that did not qualify molecularly as ExPEC were considered non-ExPEC (Fig. 1 and 2). The 5 ExPEC egg isolates and 49 randomly selected non-ExPEC egg isolates were analyzed further in this study.

Extended virulence genotyping of the 129 total ExPEC study isolates (124 from chicken meat and 5 from eggs) identified 34 of the 41 studied virulence genes in at least one isolate each. The 7 exceptions were genes for certain adhesins (*afa* and *draBC*, *clpG*, *focG*, *f17*, and *iha*), toxin *hlyA*, and a group 2 capsule variant (*kpsMT* K15). Among the 34 detected genes, prevalence values ranged from 1% to 100%. At a very low prevalence (1 to 5%) were certain adhesin (*afaE8*, *bmaE*, *gafD*, *sfa* and/or *focDE*, and *sfaS*), toxin (*cdtB*, *cnf1*, *hlyA*, *pic*, and *sat*), protectin (*kpsMT* K2/K100, *kfiC* K5, and *rfe*), and flagellin (H7 *fliC*) genes. At a low prevalence (14 to 37%) were other adhesin (*papA* and *papG*), siderophore (*ireA*), protectin (*cvaC*, *kpsM* K1, and *traT*), and miscellaneous (*ibeA*, *malX*, and *usp*) genes. At a medium prevalence (46 to 69%) were still other adhesin (*hra*, *papC*, and *papEF*), toxin (*hlyF* and *tsh*), siderophore (*fyuA* and *iroN*), protectin (*iss*), and outer membrane protein T (*ompT*) genes. Finally, at the highest prevalence (95 to 100%) were the adhesin *fimH* and siderophore *iutA* genes (Table 1).

Some virulence genes—for adhesins (*afaE*, *bmaE*, *gafD*, *papA*, *papG*, and *sfaS*), toxins (*cnf1*, *hlyD*, and *sat*), protectins (*cvaC*, *iss*, *kpsMT* K2, *kfiC* K5, and *traT*), and uropathogen-specific protein *usp*—were detected only in meat isolates (Table 1). In contrast, *cdtB* (cytolethal distending toxin) was specific to egg isolates.

Phylogenetic groups. Phylogenetic group distribution varied with both meat versus egg source and ExPEC status (Table 2). Considering all isolates combined, groups B2 and D were more prevalent among ExPEC isolates, whereas groups A and B1 were more prevalent among non-ExPEC isolates (Table 2). However, group D was not found in any egg isolates, regardless of ExPEC status. The prevalence of groups A, B1, and B2 among the egg isolates followed a trend similar to those of the meat isolates, with the only significant difference involving the B1 phylotype (Table 2).

Virulence-associated phenotypes. All 129 ExPEC isolates (124 from meat and 5 from eggs) and a subset of non-ExPEC

TABLE 3 Prevalence of virulence-associated phenotypes among ExPEC and non-ExPEC *Escherichia coli* isolates from chicken meat and eggs

Sample type	ExPEC	No.	Siderophore		Complement resistance (row %)	Colicin		Biofilm production (row %)	Growth in urine (row %)
			Production (row %)	CAS ^a zone diam, mean (mm)		Production (row %)	Zone diam, mean (mm)		
Meat	No	50	100	13	8	78	14.3	74	6
	Yes	124	100	15 ^b	48 ^b	93 ^b	19 ^b	86	24 ^b
Eggs	No	49	90	10	20	22	4	96	4
	Yes	5	100	17 ^b	80 ^b	80 ^b	18 ^b	100	0
Total	No	99	95	12	14	50	9	85	5
	Yes	129	100	16 ^b	64 ^b	86 ^b	19 ^b	93	12 ^b
	Yes or no	228	97	14	39	68	14	89	9

^a CAS, chrome azurol S.^b Difference between ExPEC and non-ExPEC isolates (same source and phenotype) was statistically significant ($P < 0.05$).

isolates from each source group ($n = 99$; 50 from meat and 49 from eggs) were compared for five virulence-associated phenotypes, including siderophore production, serum resistance, colicin production, biofilm formation, and growth in human urine (Fig. 1; Table 3).

Siderophore production (as indicated by an iron mobilization zone around the bacterial colony on CAS agar) was similarly prevalent among the 129 ExPEC isolates (100%) and the 99 tested non-ExPEC isolates (95%) (Table 3). However, zone diameters (overall range, 0 to 22 mm) were significantly larger among ExPEC than non-ExPEC isolates, both overall (mean, 16.0 mm, ExPEC, versus 11.6 mm, non-ExPEC; $P < 0.001$) and for each source group separately (Table 3). Zone diameters were correlated positively with the presence of siderophore genes *iutA* (aerobactin; $P < 0.001$) and *iroN* (salmochelin; $P = 0.003$), negatively with *ireA* (ferrisiderophore; $P = 0.047$), and indifferently with *fyuA* (yersiniabactin; $P = 0.78$).

Overall, 77 (33.8%) of the 228 tested isolates were complement resistant. ExPEC isolates had a significantly higher prevalence of complement resistance than did non-ExPEC isolates, for both specimen types (Table 3). Among the 129 ExPEC isolates, multiple virulence genes were more prevalent among the 63 complement-resistant than the 66 complement-sensitive isolates. These included adhesin *gafD* (64% versus 41%, $P = 0.01$); toxin genes (*hlyF*, 77% versus 56%, $P = 0.02$; *pic*, 13% versus 1.5%, $P = 0.01$); siderophore genes (*fyuA*, 64% versus 41%, $P = 0.01$; *iroN*, 65% versus 41%, $P = 0.006$); protectin *iss* (71% versus 45%, $P = 0.003$), and outer membrane protein *ompT* (65% versus 45%, $P = 0.03$). In contrast, the reverse was true for the toxin gene *astA* (19% versus 62%, $P < 0.001$).

Colicins were produced by 169 (74%) of the 228 tested isolates, with inhibition zone diameters ranging from 9 to 39 mm. Colicin production was significantly more prevalent among ExPEC isolates than non-ExPEC isolates for both egg and meat isolates (Table 3). Similarly, among colicin-producing isolates, inhibition zones were significantly larger among ExPEC isolates than non-ExPEC isolates for both sample types ($P < 0.001$). Colicin inhibition zone size was significantly correlated with the presence of *cvaC* ($P < 0.001$), which encodes microcin V.

Biofilm was produced by 195 (86%) of the 228 tested isolates (Table 3) and was similarly prevalent among ExPEC and non-ExPEC isolates (86% versus 85%). However, overall, non-ExPEC isolates produced optically denser biofilm than did ExPEC isolates ($P < 0.001$) (Fig. 3).

Growth in human urine was evident for only 35 (15%) of the 228 tested isolates overall. Among meat isolates, growth in human urine was significantly more common among ExPEC isolates (30/124; 24%) than non-ExPEC isolates (3/50; 6%) ($P < 0.001$). In contrast, the 2 egg isolates that grew in human urine were both non-ExPEC.

Pathotypes of ExPEC. Consensus is lacking regarding which traits define the putative ExPEC pathotypes. Here, to operationally define presumptive ExPEC pathotypes among the 129 ExPEC isolates, we used criteria as specified in Materials and Methods and Fig. 2, which allowed some isolates to be classified as APEC ($n = 76$; 59%), UPEC ($n = 30$; 23%), and NMEC ($n = 14$; 11%). With SEPEC considered a subpathotype of APEC, UPEC, and NMEC isolates, 42 (33%), 15 (12%), and 14 (11%) isolates of these groups, respectively, qualified as SEPEC (Table 4). Combined pathotype groups with overlapping traits (number of isolates; % of 129 ExPEC isolates) included NMEC/UPEC ($n = 5$; 3.9%), APEC/UPEC ($n = 21$; 16%), APEC/NMEC ($n = 4$; 3.1%), APEC/UPEC/NMEC ($n = 4$; 3.1%), and APEC/UPEC/NMEC/SEPEC ($n = 3$; 2%). The remaining 34 ExPEC isolates (26.3%) do not fit in any of the three major

FIG 3 Biofilm density in ExPEC and non-ExPEC chicken isolates. Values are shown as the mean $OD_{600} \pm$ standard deviation. Differences between the two groups were statistically significant ($P < 0.001$). ExPEC, extraintestinal pathogenic *E. coli*. ExPEC, $n = 129$; non-ExPEC, $n = 99$.

TABLE 4 Prevalence of specific pathotypes among 129 chicken-source ExPEC isolates

Pathotype	Selection-based criterion		No. (%)
	Phenotype	Genotype	
Main pathotypes			
UPEC	Growth in urine	NA ^a	30 (23)
NMEC	NA	<i>kpsMT</i> K1 + <i>ibeA</i>	14 (11)
APEC	NA	≥4 of 5 selected APEC genes ^b	76 (59)
With overlapping traits			
UPEC/NMEC	Growth in urine	<i>kpsMT</i> K1 + <i>ibeA</i>	5 (4)
NMEC/APEC	NA	<i>kpsMT</i> K1 + <i>ibeA</i> + ≥4 of 5 selected APEC genes ^b	12 (9)
APEC/UPEC/NMEC	Growth in urine	<i>kpsMT</i> K1 + <i>ibeA</i> + ≥4 of 5 selected APEC genes ^b	4 (3)
Septicemic			
UPEC/SEPEC	Growth in urine + complement resistance	NA	15 (12)
NMEC/SEPEC	Complement resistance	<i>kpsMT</i> K1 + <i>ibeA</i>	7 (5)
APEC/SEPEC	Complement resistance	≥4 of 5 selected APEC genes ^b	42 (33)
NMEC/UPEC/SEPEC	Growth in urine + complement resistance	<i>kpsMT</i> K1 + <i>ibeA</i>	4 (3)
NMEC/UPEC/APEC/SEPEC	Growth in urine + complement resistance	<i>kpsMT</i> K1 + <i>ibeA</i> + ≥4 of 5 selected APEC genes ^b	3 (2)

^a NA, not applicable.
^b APEC genes include (i) *kii*; (ii) *iss*; (iii) *tsh*; (iv) one of the 5 genes *sfa*, *foc*, *papA*, *papC*, and *papEF*; and (v) one of the two genes *iutA* and *fyuA*.

pathotype groups (NMEC, UPEC, or APEC). The meat-source ExPEC isolates included all 3 major pathotypes and the SEPEC subset, whereas the egg-source ExPEC isolates included only 2 pathotypes (2 APEC and 1 NMEC/SEPEC), plus 2 isolates of undefined pathotype (Fig. 4). Analysis of the prevalence by pathotype group of virulence genes other than those used to define the pathotypes (Table 5) showed that three adhesin genes (*afaE8*, *bmaE*, and *gafD*) were found in APEC isolates and the undefined-pathotype group but not in UPEC or NMEC isolates (Table 5). Similarly, an adhesin gene (*hra*) and a siderophore gene (*ireA*) were more prevalent

among APEC than NMEC isolates, whereas pathogenicity-associated island marker *malX* was more prevalent among NMEC than APEC isolates (Table 5). Genes uniquely present (or more prevalent) in UPEC isolates compared with NMEC isolates included two adhesin genes (*hra* and *sfa* [and/or *focDE*; the primer cannot distinguish *sfaDE* and *focDE*, so either or both may be present]) and siderophore gene *ireA*, whereas genes uniquely present (or more prevalent) in NMEC isolates compared with UPEC isolates included toxin gene *cdtB*, two siderophore genes (*fyuA* and *iroN*), and *malX* (Table 5). In the undefined-pathotype group, many virulence genes

FIG 4 Venn diagram illustrating the ExPEC pathotype groups. The size of each circle is proportional to the percentage of the samples in that pathotype. APEC, avian-pathogenic *E. coli* (purple circle); UPEC, uropathogenic *E. coli* (green circle); NMEC, neonatal meningitis *E. coli* (pink circle); those with overlapping traits are indicated within the Venn diagram. The undefined group is in a blue circle. The sum of the percentages equals 100% of isolates.

TABLE 5 Prevalence of ExPEC-associated genes and phenotypes among 129 ExPEC isolates in relation to pathotype

Functional category	Specific genes	Prevalence of gene ^a /phenotype, no. of isolates (column %)					P value ^b			
		Human and avian, all ExPEC, n = 129	Human UPEC, ^c n = 30	Human NMEC, ^c n = 14	Avian, APEC, ^c n = 76	Human and avian, undefined, n = 34	UPEC vs NMEC	APEC vs UPEC	APEC vs NMEC	Undefined vs other ExPEC
Adhesin	<i>afaE8</i>	7 (5)	0 (0)	0 (0)	6 (6)	1 (3)				
	<i>bmaE</i>	7 (5)	0 (0)	0 (0)	5 (7)	2 (6)				
	<i>fimH</i>	128 (99)	30 (100)	14 (100)	75 (99)	34 (100)				
	<i>gafD</i>	7 (5)	0 (0)	0 (0)	5 (7)	2 (6)				
	<i>hrrA</i>	68 (53)	12 (40)	0 (0)	37 (49)	23 (68)	0.005		<0.001	
	<i>papA</i>	19 (15)	6 (20)	0 (0)	14 (18)	4 (12)				
	<i>papC</i>	70 (54)	13 (43)	1 (7)	49 (65)	16 (47)	0.02		<0.001	
	<i>papEF</i>	63 (49)	10 (33)	0 (0)	43 (57)	14 (41)	0.01		<0.001	
	<i>papG</i>	23 (18)	3 (10)	0 (0)	10 (13)	8 (24)				
	<i>sfa</i> and/or <i>focDE</i>	3 (2)	1 (3)	0 (0)	2 (3)	1 (3)				
	<i>sfaS</i>	4 (3)	1 (3)	1 (7)	2 (3)	1 (3)				
Toxin	<i>cdtB</i>	1 (1)	0 (0)	1 (7)	0 (0)	0 (0)			0.02	
	<i>cnf1</i>	1 (1)	0 (0)	0 (0)	0 (0)	1 (3)				
	<i>astA</i>	59 (46)	9 (30)	3 (21)	28 (37)	21 (62)				
	<i>hlyF</i>	85 (66)	24 (80)	12 (86)	59 (78)	15 (44)				0.02
	<i>pic</i>	9 (7)	2 (7)	1 (7)	2 (3)	3 (9)				
	<i>tsh</i>	89 (67)	22 (73)	12 (86)	73 (96)	13 (38)		<0.001		<0.001
	<i>sat</i>	2 (2)	1 (3)	1 (7)	2 (3)	0 (0)				
Siderophore	<i>fyuA</i>	67 (52)	19 (63)	14 (100)	52 (68)	6 (18)	0.008		0.01	<0.001
	<i>ireA</i>	41 (32)	10 (33)	0 (0)	24 (32)	10 (29)	0.01		0.01	
	<i>iroN</i>	68 (53)	20 (67)	14 (100)	51 (67)	8 (24)	0.01	NA ^d	NA	0.002
	<i>iutA</i>	123 (95)	28 (93)	12 (86)	75 (99)	31 (91)		NA	NA	
Protectin	<i>cvaC</i>	37 (29)	12 (40)	7 (50)	27 (36)	5 (15)				
	<i>iss</i>	75 (58)	23 (77)	13 (93)	59 (78)	9 (27)		NA	NA	<0.001
	<i>kpsMT</i> K1	39 (30)	9 (30)	14 (100)	29 (38)	0 (0)	NA			
	<i>kpsM</i> K2 and K100	1 (1)	0 (0)	0 (0)	0 (0)	1 (6)				
	<i>kfiC</i> K5	4 (3)	0 (0)	0 (0)	2 (3)	2 (6)				
	<i>kii</i>	81 (63)	20 (67)	12 (86)	52 (68)	16 (47)				
	<i>rfe</i>	4 (3)	3 (10)	2 (14)	3 (4)	0 (0)				
Miscellaneous	<i>traT</i>	48 (37)	15 (50)	8 (57)	25 (33)	11 (32)				
	H7 <i>fliC</i>	3 (2)	2 (7)	1 (7)	2 (3)	0 (0)				
	<i>ibeA</i>	18 (14)	7 (23)	14 (100)	15 (20)	0 (0)	NA			0.02
	<i>ompT</i>	71 (55)	18 (60)	10 (71)	44 (58)	17 (50)		NA	NA	
	<i>malX</i>	23 (18)	10 (33)	10 (71)	18 (24)	0 (0)	0.02		<0.001	0.008
Complement resistant	<i>usp</i>	24 (19)	11 (79)	11 (37)	20 (26)	2 (6)	0.009		<0.001	
	Growth in urine	63 (49)	15 (50)	7 (50)	42 (55)	13 (38)				
Growth in urine		30 (23)	30 (100)	5 (36)	21 (28)	0 (0)	NA	NA	NA	0.002

^a All isolates were negative for certain adhesin (*afa* and/or *draBC*, *clpG*, *focG*, *fli7*, and *iha*), capsule (*kpsMT* K15), and toxin (*hlyD*) genes.

^b P values are shown only where they are <0.05.

^c Some isolates are overlapped between groups.

^d NA, not applicable; this characteristic is a defining criterion for one or both pathotypes.

either were not found, including those for toxins (*cnf1* and *sat*), protectins (*kpsMT* K1, *kfiC* K5, and *rfe*), H7 flagellin (*fliC*), invasion of brain endothelium (*ibeA*), and *malX*, or were significantly less prevalent than among other ExPEC isolates, including genes for toxins (*hlyF* and *tsh*), siderophores (*fyuA* and *iroN*), and serum resistance (*iss*). Although none of these un-

defined-pathotype isolates grew in urine, 13 (38%) were complement resistant (Table 5).

DISCUSSION

In this study, we genetically and phenotypically characterized a large collection of *E. coli* isolates from chicken-derived food prod-

ucts (meat and shell eggs), which are possible vehicles for transmission of chicken-source ExPEC to humans. Our goal was to identify and more fully characterize several proposed pathotypes (APEC, UPEC, and NMEC) and SEPEC subsets among ExPEC isolates from these products.

This study is, to our knowledge, the first to screen for ExPEC among *E. coli* isolates from chicken eggs, using the molecular criteria of Johnson et al. (16) for classifying *E. coli* isolates as ExPEC versus non-ExPEC. Although some egg-source *E. coli* isolates did qualify as ExPEC, the prevalence of ExPEC was much lower among egg-source than chicken meat-source *E. coli* isolates (4.7% versus 21%, $P < 0.001$). The higher prevalence of ExPEC among meat-source isolates might be explained by certain virulence-associated traits that could be required for (or promote) survival and persistence of chicken-source bacteria on meat. In contrast, the low prevalence of ExPEC on eggs could be due to the washing process, which might have eliminated most chicken-source *E. coli* isolates from the egg surface, leaving only those that were resistant to the washing process, thereby possibly favoring non-ExPEC over ExPEC; this could be tested experimentally. Alternatively, the egg-source *E. coli* isolates could have originated from environmental contamination during or after the washing process, making them less likely to be (host-adapted) ExPEC.

Prevalence of the main *E. coli* phylogenetic groups among animals varies in relation to host species, diet, body mass, and climate, reflecting between-group differences in preferred ecological niche (34, 35). Here, phylogroup distribution varied in relation not only to pathotype (ExPEC versus non-ExPEC) but also source (meat versus eggs). We determined that the major phylotypes of ExPEC chicken isolates are similar to those of ExPEC isolates that cause human disease, which belong mainly to the B2 and D phylogroups, while non-ExPEC isolates belong predominantly to groups A and B1, which are more associated with fecal isolates than disease isolates (36–38). Human isolates from phylogroups B2 and D typically contain more virulence factors than do those from groups A and B1 (39). This is consistent with our results with respect to chicken-source isolates. The finding that ExPEC isolates from different chicken-related sources belong predominantly to different phylogenetic groups, i.e., meat ($D > B2 > A > B1$) versus eggs ($A > B2$), suggests that these different populations could contain ExPEC isolates with differing abilities to cause specific diseases. Future work is needed to test this hypothesis using animal models for ExPEC infections (e.g., avian colibacillosis, UTI, meningitis, and sepsis) (40–42).

Previous studies devoted to the zoonotic potential of ExPEC focused on *in vivo* or genotypic evaluations; none provided large-scale evaluations of ExPEC-associated phenotypes. Here, to better distinguish different pathotypic groups (APEC, UPEC, NMEC, and SEPEC) among chicken-source ExPEC isolates and to improve ExPEC screening strategies, we compared ExPEC and non-ExPEC chicken-source isolates both qualitatively and quantitatively for selected virulence phenotypes, including survival or growth in body fluids (serum and urine), efficient iron uptake, persistence (biofilm formation), and competition with other bacteria (colicin production). Overall, virulence-associated phenotypes were more prevalent among ExPEC isolates, and significantly so for complement resistance and growth in urine, which could be used as discriminatory tests to distinguish between the two groups. Moreover, we identified links between genotypes and

TABLE 6 Prevalence of ExPEC-associated genes and phenotypes among complement-resistant and complement-sensitive ExPEC isolates

Functional category	Specific gene	Prevalence of gene, ^a no. of isolates (column %)		P value
		Complement resistant (n = 63)	Complement sensitive (n = 66)	
Adhesin	<i>afaE8</i>	6 (8)	8 (4)	
	<i>bmaE</i>	7 (9)	0 (0)	
	<i>fimH</i>	65 (84)	92 (43)	<0.001
	<i>gafD</i>	7 (9)	0 (0)	
	<i>hrrA</i>	31 (40)	39 (18)	<0.001
	<i>papA</i>	13 (17)	7 (3)	<0.001
	<i>papC</i>	39 (51)	32 (15)	<0.001
	<i>papEF</i>	33 (43)	30 (14)	<0.001
	<i>papG</i>	11 (14)	13 (6)	
	<i>sfa</i> and/or <i>focDE</i>	3 (4)	17 (8)	
Toxin	<i>sfaS</i>	4 (5)	0 (0)	
	<i>cdtB</i>	1 (2)	0 (0)	
	<i>cnf1</i>	1 (2)	0 (0)	
	<i>astA</i>	19 (25)	43 (20)	<0.001
	<i>hlyF</i>	49 (64)	40 (19)	<0.001
	<i>pic</i>	8 (10)	1 (1)	<0.001
	<i>tsh</i>	55 (71)	115 (54)	0.009
Siderophore	<i>sat</i>	2 (3)	0 (0)	
	<i>fyuA</i>	41 (53)	30 (14)	<0.001
	<i>ireA</i>	25 (33)	16 (8)	<0.001
	<i>iroN</i>	42 (55)	40 (19)	<0.0001
Protectin	<i>iutA</i>	61 (79)	80 (38)	<0.001
	<i>cvaC</i>	21 (27)	18 (9)	<0.001
	<i>iss</i>	46 (60)	42 (20)	<0.001
	<i>kpsMT K1</i>	16 (21)	23 (11)	
	<i>kii</i>	36 (47)	47 (22)	<0.001
	<i>rfe</i>	4 (5)	1 (1)	
Miscellaneous	<i>traT</i>	24 (31)	34 (16)	<0.001
	H7 <i>fliC</i>	2 (3)	16 (8)	
	<i>ibeA</i>	10 (13)	9 (4)	
	<i>ompT</i>	42 (55)	39 (18)	<0.001
	<i>malX</i>	9 (12)	14 (7)	
	<i>usp</i>	11 (14)	13 (6)	

^a All isolates were negative for certain adhesin (*afa* and/or *draBC*, *clpG*, *focG*, *f17*, and *iha*), toxin (*hlyD*), and capsule (*kpsMT K2*, *kfiC K5*, and *kpsMT K15*) genes.

phenotypes, as described below, which could be considered in future screening.

Virulence factors reported to protect ExPEC against complement-mediated killing include capsules, certain outer membrane proteins (Omps), *Iss*, *TraT*, and some O antigens (43–45). We found significant correlations with serum resistance not only for *ompT*, *iss*, and *traT* but also for multiple other genes, including those encoding adhesins (*fimH*, *hrrA*, *papA*, *papC*, and *papEF*), toxins (*hlyF*), siderophores (*fyuA*, *ireA*, *iroN*, and *iutA*), and protectins (*cvaC* and *kii*), which were more highly prevalent among complement-resistant isolates than complement-sensitive isolates (Table 6). This indicates that certain genotypes are more likely than others to be resistant to the host's complement system and, therefore, presumably to cause invasive disease.

The prevalence of siderophore production, as detected using CAS medium, did not differ between ExPEC and non-ExPEC isolates, since most isolates were positive. However, the amount of siderophore produced per isolate was significantly greater for ExPEC than non-ExPEC isolates. Iron is vital to many bacterial metabolic processes but is sequestered *in vivo* by host proteins (46). Multiple bacterial iron acquisition systems promote bacterial survival in different iron-limited environments (46). Nearly all *E. coli* isolates produce the catecholate siderophore enterobactin (enterochelin) (47), which probably explains why most study isolates exhibited some degree of siderophore production. However, the larger halos around some colonies, particularly the ExPEC isolates, are consistent with the known ability of ExPEC to produce other siderophores, such as aerobactin, salmochelin, and yersiniabactin (48, 49), and this is supported here by the positive correlation between CAS agar halo diameter and the siderophore receptor genes *iutA* and *iroN*. The role of siderophores in the virulence of ExPEC is well established, as exemplified by a recent study that showed the importance of aerobactin and salmochelin in the virulence of both APEC and UPEC in a chicken model (50). Future studies should assess whether these ExPEC-associated iron uptake systems also promote bacterial persistence on poultry products, especially meat.

No reported study has compared biofilm production with zoonotic potential among poultry-source *E. coli* isolates. Biofilm formation is a relevant pathogenetic mechanism for at least some subsets of ExPEC, such as UPEC (51). A biofilm-rich environment provides bacteria with resistance to host defense elements, antibiotics, and detergents (52) and allows them to be in proximity to other pathogens, which could allow them to acquire new genetic material and thereby become either more virulent or more antibiotic resistant. In the present study, *in vitro* biofilm formation on polyvinylchloride, a material used to make intravascular catheters (53, 54), was greater for ExPEC isolates than non-ExPEC isolates. It is conceivable that biofilms, which facilitate bacterial persistence in the genitourinary tract (55), could also allow bacteria to persist and survive on the surface of poultry products such as meat and eggs. It is unclear why the present non-ExPEC isolates produced denser biofilm than did the ExPEC isolates. Conceivably, this could help explain the high proportion of non-ExPEC *E. coli* isolates among egg isolates, since denser biofilm conceivably could allow for better persistence and survival on egg surfaces.

A previous study that used genotypic screening identified 15.6% of chicken meat *E. coli* isolates as presumptive ExPEC (7). Similarly, here, based on growth in human urine (which mimics loosely the *in vivo* urinary tract milieu), we determined that a small portion ($n = 35$, 16%) of study isolates presumably have the potential to cause human UTI, and these were mostly ExPEC ($n = 30$, 86% of 35) according to molecular criteria (Table 3).

We aimed to determine the presence and proportion of different putative ExPEC pathotypes among chicken-source ExPEC isolates. Using either published criteria, such as for APEC (29), or genotypes and/or phenotypes determined previously to be most associated with different pathotypes (Fig. 2), we detected all three major pathotypes (APEC, UPEC, and NMEC) and the SEPEC subset among the ExPEC isolates. Some isolates had overlapping traits of multiple groups, implying their potential to cause different diseases in different hosts. Additionally, a portion of ExPEC isolates (27%) did not correspond with any of the major pathotypes. These undefined-pathotype isolates had fewer virulence

genes than other ExPEC isolates, but since some were complement resistant, they still might be able to cause sepsis in certain individuals, especially if immunocompromised. Indeed, previous studies have shown that different ExPEC strains exhibit variable degrees of virulence when tested in animals (56–58).

Although our approach to categorizing ExPEC isolates into pathotypes was discriminatory, it also could underestimate the proportion of certain groups, such as NMEC (Fig. 2). We classified as NMEC only those isolates that were positive for both K1 and *ibeA*. However, in previous human isolate surveys, although around 80% of neonatal meningitis isolates were K1 positive (59–61), only ~30% of K1 isolates were also *ibeA* positive (59, 62, 63). It is therefore possible that some K1-positive, *ibeA*-negative isolates, which here were not considered NMEC, could indeed cause neonatal meningitis by utilizing non-*ibeA*-related mechanisms (64).

Conclusion. Diagnostic tools to distinguish ExPEC from non-ExPEC isolates have been proposed previously, based solely on genotypic screening (10, 16, 65, 66). Here, inclusion of both genotypic and phenotypic assessments helped to distinguish pathotype groups among the ExPEC isolates. We propose that genotypic testing could be combined with phenotypic testing to better select isolates to test in animal models of human diseases, since phenotype can indicate the host-pathogen interactions associated with differential expression of genes related to *in vivo* virulence. Our future studies will test the present putative ExPEC isolates in animal models of different ExPEC diseases.

This study provides new insights into the extent and possible human health implications of contamination of poultry products with ExPEC by including isolates from different chicken-related sources, i.e., meat and eggs, and by using a combination of genotypic, phenotypic, and phylogenetic analyses to identify isolates with presumed zoonotic potential. Our findings suggest that poultry meat could be a source of potentially virulent ExPEC that could cause multiple diseases, including UTI, neonatal meningitis, and sepsis. Significant correlations between phenotypic and genotypic traits that are highly associated with ExPEC were identified, which could be used for future ExPEC screening purposes.

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