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An evaluation of the virulence and adherence properties of avian pathogenic *Escherichia coli*

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ABSTRACT

Avian pathogenic *E. coli* (APEC) cause disease primarily in poultry; however, the link between APEC and infections in humans is questionable. In this current study, a total of 100 APEC strains isolated from chickens in Delmarva were evaluated for the presence of virulence genes to investigate their zoonotic potential in humans. A total of 28 isolates possessed one Enterohaemorrhagic *E. coli* (EHEC) virulence factor each and 87 isolates possessed up to 5 extraintestinal pathogenic *E. coli* (ExPEC) virulence factors. Five APEC isolates exhibited stronger attachment to chicken breast than both human *E. coli* outbreak strains tested. Ten APEC isolates exhibited stronger attachment to human epithelial cells (HCT-8) than both *E. coli* outbreak strains. While the APEC isolates in this study were not found to possess all the virulence genes necessary to cause clinical illness in humans, their potential to acquire these genes in the environment as well as their ability to attach to food surfaces and human cells warrants further attention.

1. Introduction

Avian pathogenic *Escherichia coli* (APEC) cause severe colibacillosis and respiratory illness in poultry resulting in large economic losses [1]. APEC strains are classified into the extraintestinal pathogenic *E. coli* subgroup (ExPEC), which is phylogenetically distinct from commensal and intestinal pathogenic *E. coli* groups [2]. APEC are considered atypical *E. coli* because they are designated as single, heterogeneous population within the ExPEC group, whereas human ExPEC are further categorized into different subpathypes based on their ability to cause different diseases [2]. Members of the human ExPEC subgroup include uropathogenic *E. coli* (UPEC), the leading cause of urinary tract infections in humans. Establishment of extraintestinal disease in humans by ExPEC is complex. Similarities in virulence profiles continue to be studied given the importance and relative ease of transmission in our global society and the ability of these bacteria to cross host species barriers [3].

Because poultry serve as a reservoir for APEC, certain food commodities may serve as vehicles for human *E.coli* infections [4–6]. APEC has been linked to extraintestinal diseases in humans due to the fact that APEC share common virulence factors with UPEC [3,7]. The acronym FUTI (foodborne urinary tract infection) describes urinary tract infections associated with contaminated food [8]. In cross-species studies, APEC caused disease in rats [9] and human ExPEC strains were virulent to chicks [10]. The specific *E. coli* pathotypes responsible for FUTIs are not well defined [4]. Similarly to other foodborne organisms, evaluation of transmission and attribution is complex due to the variety of ExPEC sources in the environment and food supply, including the human gastrointestinal tract, food animals, retail meat products, companion animals, manure, and sewage [3,11]. APEC have been isolated from retail foods including chicken, turkey, pork, and produce [12]. Produce may become contaminated in the pre-harvest environment by fecal dissemination from wild birds or through the presence of contaminated poultry litter as a soil amendment [13]. While APEC are known to be subset of ExPEC, little is known if these APEC strains carry any virulence factors of enterohemorrhagic *E. coli* (EHEC). *E. coli* O157:H7, an EHEC, has been associated with multiple outbreaks associated with ground beef, leafy greens, and apple cider [14]. EHEC virulence factors can be located in genome, within pathogenicity islands, or on plasmids.

In the work presented here, APEC strains isolated from poultry flocks on the Delmarva Penninsula were assayed for ExPEC and EHEC virulence genes APEC isolates containing these VFs were also assayed for their attachment to foods and human epithelial cells to determine their ability to persist and potentially cause human illness.

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2. Materials and methods

2.1. Isolation of APEC isolates from commercial broilers

APEC isolates (n = 100) were collected from lesions (liver, hock joint, pericardium, yolk, crop, air sac, ceca, intestine, and cellulitis infection) of diseased chickens from commercial broiler houses in Delmarva [15] and serotyped (O-typed) by University of Pennsylvania New Bolton Center (Kennett Square, PA). Several non-APEC strains were used as positive controls and reference strains: *E. coli* O157:H7 strain 4407 (clinical isolate from 2006 spinach outbreak) and *E. coli* O157:H12 [16] served as positive and negative EHEC controls, respectively. Three *E. coli* strains originally isolated from cabbage in California (MW 416, MW 423, and MW 425) were also evaluated [17] to serve as reference strains. Two clinical strains of uropathogenic *E. coli* provided by Don Lehman of the Department of Medical Laboratory Science at the University of Delaware (Newark, DE), and a typical uropathogenic *E. coli* (ATCC 700928) served as ExPEC controls. A clinical isolate of *E. coli* O104:H4 (ATCC # BAA-2326) from the German sprout outbreak served as an enteroaggregative *E. coli* (EAEC) strain. Pure bacterial isolates were grown on MacConkey Agar (MAC) with or without Sorbitol (Fisher Scientific, Fair Lawn, NJ). A suspension of a single colony from each culture was placed in 50 µL of nuclease water, which was then used for subsequent PCR assays.

2.2. Multiplex PCR screening for E. coli genes of interest

Two multiplex PCR assays were developed in order to determine the presence of EHEC virulence factors in APEC isolates (Table 1). Multiplex PCR # 1 was developed to screen isolates for virulence factors *stx1, stx2, eae,* and *espA* from various mobile genetic elements including the LEE pathogenicity island and phage-based genes [18]. Multiplex PCR #2 assay was designed to screen APEC isolates for EHEC virulence factors located on the plasmid pO157 [19]. *E. coli* O157:H7 strain 4407 contained all eight virulence factors tested in Multiplex PCR #1 and #2 and was used as a positive control, while *E. coli* O157:H12 did not contain of these eight genes and was used a negative control. The enteroaggregative *E. coli* (EAEC) strain O104:H4 from the 2011 outbreak in Germany was shown to possess *stx2* and *espP* genes in order to verify the accuracy of multiplex PCR #1 and #2 assays [20].

Multiplex PCR # 3 was designed in order to characterize the APEC isolates based on extraintestinal pathogenic *E. coli* virulence genes [21]. PCR assays for all three multiplex PCR trials in 25 m were set up as follows: 12.5 µl of GoTaq Green Master Mix (Fisher Scientific, Nazareth, PA), 2 ×, 0.6 µl of 10 µM Forward primer (Sigma-Genosys, Woodlands, TX), 0.6 µl of 10uM Reverse primer, 1 µl of the bacterial suspenstion in nuclease free water, and an addition 6.7 µl of nuclease-free water. The samples were dispensed into 0.2 ml PCR tube strips and loaded into an Eppendorf thermocycler. A 5-minute initial denaturation step at 95 °C was initiated, followed by 35 cycles of: 95 °C for 45 s; 58 °C for 45 s; and 72 °C for 1 min. A final extension step at 72 °C for 5 min was executed. Amplified products from PCR reactions were identified by gel electrophoresis on a 2% agarose gel stained with ethidium bromide using AlphaImager software for observation and data collection.

Table 1
List of primers used in multiplex PCR assays to characterize APEC isolates.

Amplicon size	Gene category
384 bp	LEE pathogenicity island
180 bp	Shiga toxin
255 bp	Shiga toxin
100 bp	LEE pathogenicity island
914 bp	Virulence plasmid (pO157)
774 bp	Virulence plasmid (pO157)
399 bp	Virulence plasmid (pO157)
262 bp	Virulence plasmid (pO157)
714 bp	ExPEC
981 bp	ExPEC
116 bp	ExPEC
309 bp	ExPEC
501 bp	ExPEC
824 bp	ExPEC
1181 bp	ExPEC

2.3. Evaluation of APEC attachment to retail chicken breast

Whole chicken breast tenderloins were purchased from a local grocery (Newark, DE) and aseptically cut into 2.5 cm × 2.5 cm squares in a biosafety cabinet. APEC isolates (n = 28) were assayed for their attachment to the chicken breast.

Bacterial cultures were grown overnight in Luria-Bertani (LB) broth (Fisher Scientific, Fair Lawn, NJ) at 37 °C. Cultures were centrifuged at 5000 rpm for 10 min and resuspended in 1 ml LB broth for an inoculum of 10^8 cfu/ml. Each chicken piece was spot inoculated with 10 µl of a culture suspension and allowed to dry for 30 min in the biosafety cabinet. Samples were then placed in 25 ml of Buffered peptone water (BPW) (Fisher Scientific, Fair Lawn, NJ) in a sterile 50 ml conical tube, and the tube was inverted 25 times. Serial dilutions were prepared and 0.1 ml, in duplicate, were distributed on to sorbitol MacConkey agar (SMAC) to enumerate loosely attached bacteria (CFU/ml). Chicken pieces were then aseptically transferred to Whirlpack® bags with 25 ml of BPW and hand-massaged for 1 min. Serial dilutions were plated and *E. coli* recovered on SMAC media from this step were referred to as strongly attached bacteria (CFU/ml). Based on a previously published formula [22], the percentage of the total bacterial population that was strongly attached (S_R) was calculated as (strongly attached bacteria) / (loosely attached bacteria + strongly attached bacteria). All experiments were performed in duplicate.

2.4. HCT-8 cell attachment assay

HCT-8 human ileocecal colorectal adenocarcinoma cells (ATCC # CCL-244) were grown in Roswell Park Memorial Institute medium (RPMI 1640) (Mediatech Inc., Manassas, VA) with L-gluatamine and 25 mM HEPES plus 10% fetal bovine serum (FBS) and grown to confluency at 37 °C with 5% CO_2. The APEC cell association assay was based on previously published procedures [23]. Confluent cells in 75 cm^2 flasks in 10% media were trypsonized, washed with Hanks Balanced Salt Solution (HBSS), and 6-well plates seeded at ~10^6 cells/well.

Cell monolayers were challenged with a multiplicity of infection of ~100:1 (*E. coli*: HCT-8) with each of the 28 APEC isolates. Overnight cultures of APEC were centrifuged at 581 × *g* for 10 min and resuspended in RPMI. Cell monolayers were washed with HBSS and challenged with 500 µl (~10^8 CFU/ml) bacterial cells in RPMI or RPMI alone (control) and incubated at 37 °C with 5% CO_2 for 1 h. After incubation, media was removed and cells were washed twice with HBSS. Fresh media was added monlayers were incubated for an additional 30 min at 37 °C and 5% CO_2. Media was again removed and the monolayers lysed by addition of 1 ml of 1% Triton-X-100 prepared in HBSS. Serial dilutions were prepared in BPW to determine the population of attached *E. coli* to HTC-8 cells by enumeration to SMAC agar. Samples were analyzed in duplicate.

2.5. Statistical analysis

For the chicken breast attachment work, data was recorded, and S_R values were reported as mean ± the standard deviation. A student's *t*-test was used to determine the significant differences between S_R means of selected APEC isolates (CI = 95%; *p*-value < 0.05). For the HCT-8 attachment study, mean CFU/ml values were log transformed and reported as log CFU/ml ± standard deviation. Significant differences in

attachment to HCT-8 cells were determined by the student's *t*-test (p-value < 0.05). Statistical analysis was performed with JMP 10 software (SAS Cary, NC).

3. Results and discussion

3.1. Multiplex PCR for EHEC genes

The *stx1*, *stx2*, and *espA* genes were not found to be present in any of the 100 APEC isolates that were tested. One isolate out of 100 possessed the *eaeA* gene, an essential virulence gene in EHEC. This was an O13 serogroup APEC isolate originally obtained from a diseased broiler chicken.

The identification of *stx1* and *stx2* genes in APEC is important as they encode for the production of shiga toxin, an important virulence factor that can lead to the development of haemolytic uremic syndrome (HUS) in humans [24]. The *eae* gene encodes for the intimin outer membrane protein, which is a key factor for attachment to epithelial cells [25]. The *espA* gene is part of the type III secretion system (T3SS) and is partially responsible for the formation of attaching and effacing lesions [26].

A second multiplex PCR assay screened APEC for accessory virulence genes on the pO157 virulence plasmid (Table 1). Out of the 100 APEC isolates that were screened for EHEC virulence plasmid genes, 15 were positive for *katP*, 8 for *espP*, 2 for *ehxA*, and 2 for *stcE*. These accessory genes are often not considered hallmarks of disease like those primary EHEC virulence genes discussed above, but they contribute to pathogen survival in the host [19]. The presence of virulence genes from pO157 in the APEC isolates surveilled in thist study indicate that some APEC isolates may serve as reservoirs for these EHEC accessory virulence genes, but not for primary EHEC virulence genes. It may also indicate plasmid transfer between EHEC strains and APEC strains in the environment. The *stcE* and *espP* VF's were found in two of the three clinical isolates of UPEC. Three non-pathogenic environmental isolates of *E. coli*[17] did not contain any EHEC or ExPEC virulence genes.

Previous studies have identified the presence of cytotoxins in APEC: researchers reported 18 of 82 (22%) chicken isolates produced a verotoxin (now termed shiga-toxin) that was toxic toward Vero cells [27]. Verotoxin production was recognized from 11% of *E. coli* isolates derived from septic chickens [28]. In an examination of 97 APEC isolates from lesions of chickens showing symptoms of septicemia, cellulitis, and swollen head syndrome in chickens, PCR and colony hybridization assays found that 53% of the isolates contained *stx* gene sequences, with the majority containing the *stx1* allele [29]. However, no *stx*-positive isolates contained the *eae* and *E-hlyA* genes [29]. They determined that *stx1* genes were widespread among APEC however; cytotoxicity on Vero cells was uncommon. These results are vastly different from the results of our current study where no *stx* genes were found in 100 APEC isolated from chickens in Delmarva, and differences may be due to collection from different types of lesions from birds, different PCR methodology and different criteria for inclusion of the APEC isolates in that study *versus* the current one. Specifically, their PCR amplification strategy permitted for selection of the appropriate primers for amplification of the entire *stx1* gene. The DNA sequence they identified for *stx1* was identical to that for *S. dysenteriae*, except for one nucleotide 300 bp from the start of the *stx1* gene which is identical to the region of a variant stx1 in an O111 strain [29,30].

3.2. Multiplex PCR for ExPEC genes

Multiplex PCR #3 identified ExPEC genes in APEC isolates which could potentially cause extraintestinal disease in humans (Table 1). Isolates were evaluated for seven ExPEC genes: *papC* (P-fimbriae, an adhesion factor); *tsh* (temperature-sensitive hemagglutinin); *iucD* (iron-acquisition system); *iss* (surface exposed lipoprotein involved in increased serum survival); *cva/cvi* (colicin V plasmid), *astA*, enteroaggregative heat-stable toxin; and *vat* (vacuolating autotransporter toxin) [21]. In total, 18% of the APEC isolates contained only one ExPEC virulence factor, 39% contained two, 26% contained three, 3% contained four, and 1% had five. Similar to the results found in our current study, Ewers et al. [21] found a higher prevalence of ExPEC virulence genes within APEC and UPEC strains than in other pathotypes or non-pathogenic strains of *E. coli*. As expected the outbreak isolates of *E. coli* O157:H7 strain 4407 and *E. coli* O104:H4 did not possess any of these ExPEC genes. Two of the three clinical UPEC reference strains contained ExPEC virulence genes, with one containing both the *iss* and *iucD* genes and the other containing the *papC* gene. Finally, the *cva* gene was found to be present in one of the APEC (APEC 9) environmental isolates that was tested.

3.3. Attachment of APEC to retail chicken breast

Twenty-eight APEC isolates were chosen for attachment assays based on the presence of EHEC genes detected in multiplex PCR #1 and #2. An attachment assay was used to explore the ability of APEC to attach and subsequently be transmitted to consumers during the handling or consumption of raw or undercooked poultry, a potential route of infection [3,6,31].

Attachment strength (S_R) to chicken breast varied among the APEC isolates (Table 2). Significant differences in attachment were determined by a p value < 0.05. Nineteen APEC isolates had a significantly stronger S_R value than *E. coli* O104:H4, ten APEC isolates had a significantly stronger S_R value than *E. coli* O157:H7 strain 4407, and eight APEC isolates had a significantly stronger S_R value than the strongest attaching UPEC isolate (UPEC 3). These results illustrate the strong affinity of APEC for chicken breast and potential for human disease through handling and consumption of raw or undercooked poultry.

3.4. HCT-8 cell attachment assay

To gain a further understanding of the ability of APEC to cause intestinal disease in humans, the 28 APEC isolates and references strains were assessed for their ability to attach to human ileocecal colorectal adenocarcinoma (HCT-8) cells *in vitro*. According to previous studies, adherence of EHEC to HCT-8 cells is achieved by the localized adherence mechanism referred to as log jam and represents a basal means by which *E. coli* bacteria attach to the human intestine [32]. A later study by Fleckenstein et al., [33] demonstrated that the *E. coli* invasion protein A (Tia) interacts with the cell surface heparan sulfite proteogylcans on epithelial cells, including HCT-8, in order to initiate infection. Attachment strength to the HCT-8 cells varied among the isolates. All 28 APEC showed significantly stronger attachment strength than the reference isolate *E. coli* O157:H7 strain 4407, 12 APEC isolates attached significantly stronger than the most strongly attaching UPEC isolate, and 10 APEC isolates attached significantly more strongly than the *E. coli* O104:H4 isolate (Table 2). Interestingly isolate APEC 22 attached at a significantly higher level than all of the other isolates.

Effective cellular attachment is a key factor in pathogenicity, reaffirming the potential for APEC to be a zoonotic concern. In EHEC, this intimate attachment is aided by virulence factors such as intimin encoded by the *eae* gene and a host cell-bacteria bridge encoded by *espA*, *espB*, and *espD*[34]. The physiological attachment of *E. coli* cells was shown by Bouckaert et al. [31] to be different between fecal and uropathogenic strains with FimH was shown to have importance in mannose-binding during cell attachment. For APEC, a number of other attachment factors may be involved such as the *E. coli* pilus, curli fibers, and bundle-forming pili [32]. The ability of APEC isolates to strongly attach to chicken breasts and to HCT-8 cells shown here, combined with their reported presence on retail chicken breast [31], provides preliminary data to demonstrate a potential method of foodborne transmission of APEC to humans.

Table 2
Mean attachment values (S_R or log cfu/ml) of 38 APEC isolates and reference strains to chicken breast and HCT-8 cells.

Isolate	O-type	EHEC genes	ExPEC genes	Attachments	
				Chicken breast	HCT-8
				(S_R ± SD)[a]	(log CFU/ml ± SD)
APEC 1	O20	*katP*	*iss, iucD*	62.3 ± 9.1	6.93 ± 0.32
APEC 2	Untypable	*katP*	*papC, iucD*	28.9 ± 1.9	6.4 ± 0.11
APEC 3	O157	*espP*	*iss, iucD,vat*	35.2 ± 1	6.27 ± 0.1
APEC 4	Untypable	*katP*	*iss, iucD, tsh*	70.5 ± 6	6.79 ± 0.05
APEC 5	O35	*katP*	*iss, iucD*	12.4 ± 2.3	6.78 ± 0.12
APEC 6	O6	*katP*	*iss, iucD*	16.9 ± 1.7	6.83 ± 0.13
APEC 7	O157	*espP*	*none*	44.4 ± 12.7	6.46 ± 0.24
APEC 8	O157	*katP*	*iss*	27.2 ± 6.5	6.51 ± 0.03
APEC 9	O157	*espP*	*iss, vat, cva*	50 ± 1	2.98 ± 0.28
APEC 10	Untypable	*katP*	*iss, iucD, tsh*	12.3 ± 0.3	3.52 ± 0.06
APEC 11	O157	*espP*	*iss, iucD, vat*	16.6 ± 7	6.04 ± 0.03
APEC 12	O1	*stcE*	*iss, tsh*	8.8 ± 1.8	2.7 ± 0
APEC 13	O102	*katP*	*astA, iss, iucD*	21 ± 0.2	6.92 ± 0.02
APEC 14	O8	*espP*	*iss, iucD*	17 ± 1.9	5.54 ± 0.1
APEC 15	O157	*ehxA*	*astA, iss, iucD*	36.1 ± 12.8	3.78 ± 0.2
APEC 16	O5	*katP*	*astA, iucD*	13.2 ± 5	7 ± 0.13
APEC 17	O8	*ehxA*	*iss, iucD, vat*	24.8 ± 12	5.97 ± 0.07
APEC 18	O78	*espP*	*iss, iucD*	12.7 ± 0	5.44 ± 0.03
APEC 19	O12	*espP*	*iss, iucD*	34.8 ± 3.1	6.12 ± 0.13
APEC 20	O78	*katP*	*iss, iucD, tsh*	6 ± 0.5	6.95 ± 0
APEC 21	O78	*katP*	*iss, iucD, vat*	15.8 ± 12.6	6.62 ± 0.09
APEC 22	O9	*katP*	*iss, iucD*	0 ± 0	8.39 ± 0.12**
APEC 23	O157	*espP*	*none*	13.9 ± 0.6	6.88 ± 0.06
APEC 24	O13	*eaeA*	*iss, iucD*	31.6 ± 0.7	6.08 ± 0.09
APEC 25	O157	*katP*	*iucD, vat*	7.2 ± 3.8	6.79 ± 0
APEC 26	Untypable	*katP*	*astA, iss, iucD*	5.3 ± 0.3	6.83 ± 0.17
APEC 27	O5	*katP*	*astA*	44.4 ± 24	7.16 ± 0.01
APEC 28	O2	*stcE*	*iss, iucD, vat*	14.2 ± 2.3	6.19 ± 0
UPEC 1	nd	*stcE*	*none*	31.8 ± 5.8	6.43 ± 0.32
UPEC 2	nd	*espP*	*iss, iucD*	23.8 ± 5.2	6.13 ± 0.03
UPEC 3	nd	*none*	*papC*	32.1 ± 6	0 ± 0
4407	O157	*eae, ehx, espA, espP, katP, stcE, stx1, stx2*	*none*	27.7 ± 2.4	0 ± 0
O104:H4	O104	*stx2, espP*	*none*	13.4 ± 1.8	6.53 ± 0.14

[a] SR value = (strongly attached bacteria) / (loosely + strongly attached bacteria).

4. Conclusions

This study shows that APEC can potentially serve as a reservoir for EHEC virulence genes present on the pO157 plasmid. Results from this study demonstrate that APEC are unlikely to be reservoirs of primary EHEC virulence genes (*eae, espA, stx1,stx2*) however other studies have identified stx genes in APEC from other geographical areas [29]. This work also shows that APEC cells can attach to chicken breasts and human epithelial cells, indicating a potential path of disease transmission to humans. Our work supports previous hypotheses suggesting a role for ExPEC and related organisms in foodborne infections and food reservoirs for urinary tract infections [4,8]. Our preliminary findings highlight the need to characterize more APEC isolates to aid in identification of virulence profiles that could lead to FUTIs.

Conflict of interest

The authors declare no conflict of interest.

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