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Original article

Characterizing the APEC pathotype**

Kylie E. RODRIGUEZ-SIEK[a], Catherine W. GIDDINGS[b], Curt DOETKOTT[c], Timothy J. JOHNSON[a], Lisa K. NOLAN[a]*

[a] Department of Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA 50011, USA
[b] Department of Veterinary and Microbiological Sciences, North Dakota State University, Fargo, ND 58105, USA
[c] Information Technology Services, North Dakota State University, Fargo, ND 58105, USA

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Abstract – The purpose of this study was to compare avian pathogenic *Escherichia coli* (APEC) isolates to fecal isolates of apparently healthy poultry (avian fecal *E. coli* or AFEC) by their possession of various traits in order to ascertain whether APEC and AFEC are distinct and if the APEC strains constitute a distinct pathotype. Four hundred and fifty-one APEC and one hundred and four AFEC isolates were examined for possession of traits associated with the virulence of human extraintestinal pathogenic *E. coli* (ExPEC) as well as APEC. Several of the genes occurred in the majority of APEC and only infrequently in AFEC, including *cvaC*, *iroN*, *iss*, *iutA*, *sitA*, *tsh, fyuA*, *irp2*, and *ompT*. Of these genes, several have been found on large plasmids in APEC. Other genes occurred in significantly more APEC than AFEC but did not occur in the majority of APEC. Isolates were also evaluated by serogroup, lactose utilization, and hemolytic reaction. Twenty-nine and a half percent of the APEC and forty-two and three tenths percent of the AFEC were not serogrouped because they were not typeable with standard antisera, typed to multiple serogroups, were rough, autoagglutinated, or were not done. Around 65% of the typeable APEC (205 isolates) and AFEC (41 isolates) were classified into shared serogroups, and about a third of both fell into APEC- (113 isolates) or AFEC- (19 isolates) unique serogroups. Most were able to use lactose. No isolate was hemolytic. Overall, the majority of the APEC isolates surveyed shared a common set of putative virulence genes, many of which have been localized to an APEC plasmid known as pTJ100. This common set of genes may prove useful in defining an APEC pathotype.

avian pathogenic *Escherichia coli* (APEC) / pathotype / virulence plasmid / ExPEC / pTJ100

1. INTRODUCTION

Although *Escherichia coli* are likely widespread in the normal gastrointestinal flora and production environment of poultry, it is thought that only specific *E. coli* strains are endowed with virulence factors, enabling them to cause disease [8]. The disease they cause, avian colibacillosis, is extraintestinal in nature, manifesting in various ways, including as air sacculitis, cellulitis, and septicemia [2]. Colibacillosis in all its forms is one of the most significant infectious diseases in production birds, resulting

* Corresponding author: lknolan@iastate.edu
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in large annual losses for the poultry industry due to mortality, lost production, and condemnations [2, 14, 15, 31].

Although several virulence traits have been associated with the *E. coli* causing disease in birds [2], an avian pathogenic *E. coli* (APEC) pathotype remains ill-defined [8]. In the present study, a comparison of traits occurring among avian *E. coli* implicated in colibacillosis to those of *E. coli* from the feces of apparently healthy poultry (avian fecal *E. coli* or AFEC) was undertaken to determine if an APEC pathotype could be defined. The factors chosen for this study, included serogroup, since certain groups have been linked to the ability to cause disease [2, 42]; the ability to ferment lactose, given that some recent outbreaks have involved lactose-negative strains of *E. coli* [44]; and the ability to cause hemolysis, since this trait has been linked to virulence in other *E. coli* pathotypes [27]. Additionally, the occurrence of several genes among these avian *E. coli* was determined. Genes assayed included those previously associated with the disease-causing abilities of human extraintestinal pathogenic *E. coli* (ExPEC) and APEC. ExPEC genes were included since ExPEC cause extraintestinal diseases in human beings, much as APEC do in birds. All the APEC-related genes used in this study have been found on a large, conjugative R plasmid, known as pTJ100, from a virulent avian *E. coli* strain [25, 26].

Therefore, the purpose of this study was to compare APEC and AFEC isolates in order to ascertain whether APEC and AFEC are distinct and if APEC strains constitute a pathotype by their possession of a particular set of virulence-associated traits.

2. MATERIALS AND METHODS

2.1. Bacterial strains

Four hundred and fifty one *Escherichia coli* isolates incriminated in avian colibacillosis (APEC) and 104 fecal *E. coli* isolates from the feces of apparently healthy birds (AFEC) were used in this study. The APEC isolates were collected from various diagnostic laboratories throughout the USA and originated from different avian hosts, primarily chickens and turkeys, and sites within these hosts, including conjunctiva, sinus, trachea, air sacs, pericardium, spleen, liver, blood, brain, bone marrow, joint, skin, ovary, salpinx, uterus, and yolk stalk. Some were described previously by Pfaff-McDonough et al. [33]. All isolates were serogrouped through the *Escherichia coli* Reference Center (Pennsylvania State University, University Park, PA, USA) and screened for certain traits, associated with the virulence of ExPEC and APEC, using the techniques described below. Organisms were stored at –80 °C in Brain Heart Infusion broth (Difco Laboratories, Detroit, MI, USA) with 10% glycerol till use [38].

2.2. Hemolytic reaction

Test and control organisms were plated on 5% sheep blood agar plates (Midland Hospital, Fargo, North Dakota, USA) and incubated overnight at 37 °C. The 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 [12].

2.3. Fermentation of lactose

Test and control organisms were plated on MacConkey agar (Difco) and incubated overnight at 37 °C. Isolates were considered positive for lactose utilization if pink colonies were observed [12].

2.4. Virulence genotyping

The test and control organisms were examined for the presence of several genes, known for their association with ExPEC or APEC virulence using a number of multiplex PCR assays. Targeted genes and their descriptions are summarized in Table I, and the primer sequences used in the amplification studies are summarized in Table II. Genes from APEC that were used in genotyping

Table I. ExPEC/APEC genes used in virulence genotyping*.

Gene	Description	Source or reference
pTJ100-related genes		
cvaC†	Structural gene for the colicin V operon	[13]
iroN‡	Catecholate siderophore receptor gene	[36]
iss†	Increased serum survival gene	[3, 5]
iucC‡	Involved in aerobactin synthesis	[6, 7]
iutA‡	Ferric aerobactin receptor gene; iron transport	[7]
sitA‡	Putative iron transport gene	[35]
traT†	Outer membrane protein gene; surface exclusion; serum resistance	[1, 30]
tsh§	Temperature-sensitive hemagglutinin gene	[34]
Iron-Related		
feoB	Gene which mediates ferric iron uptake	[35]
ireA	Encodes an iron-responsive element; putative sideropohore receptor gene	[37]
irp-2	Iron repressible gene associated with yersiniabactin synthesis	[39]
Toxins		
hlyD	Transport gene of the hemolysin operon	[16]
Miscellaneous		
fliC (H7)	Produces flagellin protein associated with the H7 antigen group	[11]

* Descriptions of genes encoding components of certain adhesins (i.e., genes encoding parts of the P pilus, *papA*; *papC*; *papEF; papG*, including *papG* alleles I, II, and III; the S pilus, *sfa* and the gene encoding the S fimbrial tip, *sfa*S; the Type 1 fimbrial adhesin, *fim*H; the F1C fimbrial tip, *focG*; and other genes encoding portions of miscellaneous adhesins, *iha*; *afa*; *gafD*; and *bmaE*); toxins (*cnf*-1 and *cdtB*); protectins (*kpsMT* K1; *kpsMT* II; *kpsMT* III; and *rfc*); siderophores (*fyuA*); and other miscellaneous structures (*ibeA*; *ompT*; and $PAI_{(CFT073)}$, a fragment from archetypal UPEC strain CFT073) can be found in Johnson and co-workers [24]. Also, the description of *papG* allele I' can be found in Johnson and Stell [22].
† These genes are listed as pTJ100-related, but they could also be listed as protectins.
‡ These genes are listed as pTJ100-related, but they could also be listed with the iron-related genes.
§ These genes are listed as pTJ100-related, but they also could be listed in the miscellaneous group.

have been localized to a large, conjugative R plasmid, known as pTJ100 that encodes aerobactin and ColV production [25, 26]. All primers used in the amplification studies were obtained from Sigma-Genosys (The Woodlands, TX, USA) and Integrated DNA Technologies (Coralville, IA, USA).

Template DNA was generated as follows. Individual colonies from blood agar were inoculated into 1 mL of Luria Bertani broth (Difco) and grown overnight at 37 ºC with shaking. Boiled lysates were used as template DNA for the amplification procedure as previously described [21].

Six multiplex PCR panels targeting different gene combinations were used in the bulk of the genotyping studies. The first five panels have been described elsewhere [22]. The Group 1 panel targeted a pathogenicity island (PAI) marker, *papA*, *fimH*, *kpsMT III*, *papEF*, *ireA*, and *ibeA*. The Group 2 panel was designed to amplify *cnf1*, *fyuA*, *iroN*, *bmaE*, *sfa, iutA*, and *papG* allele III. The Group 3 primer panel amplified *hlyD*, *rfc, ompT*, *papG* allele I', *papG* allele I, *kpsMT II*, and *papC*. Group 4 targeted *gafD*, *cvaC*, *fliC* (H7), *cdtB*, *focG*, *traT*, and *papG* allele II, and the Group 5 panel included primers for

Table II. Primers used for the amplification of virulence genes.

Gene	Amplicon size (bp)	Primer seq. (5'–3')	Reference
pTJ100-related genes			
*cvaC**	679	F cacacacaaacgggagctgtt R cttccgcagcatagttccat	[22]
iroN†	667	F aagtcaaagcaggggttgcccg R gatcgccgacattaagacgcag	Personal communication with J.R. Johnson, 2003
*iss**	323	F cagcaacccgaaccacttgatg R agcattgccagagcggcagaa	Personal communication with J.R. Johnson, 2003
iucC†	541	F cgccgtggctggggtaag R cagccggttcaccaagtatcactg	[40]
iutA†	302	F ggctggacatcatgggaactgg R cgtcgggaacgggtagaatcg	[22]
sitA†	608	F agggggcacaactgattctcg R taccgggccgtttttctgtgc	[35]
*traT**	290	F ggtgtggtgcgatgagcacag R cacggttcagccatccctgag	[22]
tsh‡	420	F gggaaatgacctgaatgctgg R ccgctcatcagtcagtaccac	[29]
Adhesins			
afa	594	F ggcagagggccggcaacaggc R cccgtaacgcgccagcatctc	[22]
bmaE	507	F atggcgctaacttgccatgctg R agggggacatatagccccttc	[22]
fimH	508	F tcgagaacggataagccgtgg R gcagtcacctgccctccggta	[22]
focG	364	F cagcacaggcagtggatacga R gaatgtcgcctgcccattgct	[22]
gafD	952	F tgttggaccgtctcagggctc R tcccggaactcgctgttact	[22]
iha	829	F ctggcggaggctctgagatca R tccttaagctcccgcggctga	Personal communication with J.R. Johnson, 2003
papA	717	F atggcagtggtgttttggtg R cgtcccaccatacgtgctcttc	[22]
papC	205	F gtggcagtatgagtaatgaccgtta R atatccttttctgcagggatgcaata	[22]
papEF	326	F gcaacagcaacgctggttgcatcat R agagagagccactcttatacggaca	[22]
papG allele I§	461	F tcgtgctcaggtccggaattt R tggcatcccccaacattatcg	[22]
papG allele I'§	479	F ctactatagttcatgctcaggtc R cctgcatcctccaccattatcga	[22]
papG allele II§	190	F gggatgagcgggcctttgat R cgggcccccaagtaactcg	[22]
papG allele III§	258	F ggcctgcaatggatttacctgg R ccaccaaatgaccatgccagac	[22]
sfa	410	F ctccggagaactgggtgcatcttac R cggaggagtaattacaaacctggca	[22]
sfaS	244	F gtggatacgacgattactgtg R ccgccagcattccctgtattc	[22]

Table II. Continued.

Gene	Amplicon size (bp)	Primer seq. (5'–3')	Reference
Iron-Related			
feoB	470	F aattggcgtgcatgaagataactg R agctggcgacctgatagaacaatg	[35]
fyuA	787	F tgattaaccccgcgacgggaa R cgcagtaggcacgatgttgta	[22]
ireA	254	F gatgactcagccacgggtaa R ccaggactcacctcacgaat	Personal communication with J.R. Johnson, 2003
irp-2	287	F aaggattcgctgttaccggac R tcgtcgggcagcgtttcttct	[18]
Protectins			
kpsMT (K1)	153	F tagcaaacgttctattggtgc use with *kps*MT II-Reverse	[22]
kpsMT II	272	F gcgcatttgctgatactgttg R catccagacgataagcatgagca	[22]
kpsMT III	392	F tcctcttgctactattcccct R aggcgtatccatccctcctaac	[22]
rfc	788	F atccatcaggaggggactgga R aaccataccaaccaatgcgag	[22]
Toxins			
cdtB	430	F gaaaataaatggaacacacatgtccg F' gaaagtaaatggaatataaatgtccg R aaatctcctgcaatcatccagtta R' aaatcaccaagaatcatccagtta	[22]
cnf-1	1105	F atcttatactggatgggatcatcttgg R gcagaacgacgttcttcataagtatc	[22]
hlyD	904	F ctccggtacgtgaaaaggac R gccctgattactgaagcctg	Personal communication with J.R. Johnson, 2003
Miscellaneous			
fliC (H7)	547	F acgatgcaggcaacttgacg R gggttggtcgttgcagaacc	Personal communication with J.R. Johnson, 2003
ibeA	171	F aggcaggtgtgcgccgcgtac R tggtgctccggcaaaccatgc	[22]
ompT	559	F atctagccgaagaaggaggc R cccgggtcatagtgttcatc	Personal communication with J.R. Johnson, 2003
$PAI_{(CFT073)}$	925	F ggacatcctgttacagcgcgca R tcgccaccaatcacagccgaac	[22]

* These genes are listed as pTJ100-related, but they could also be listed with the protectins.
† These genes are listed as pTJ100-related, but they could also be listed with the iron-related genes.
‡ These genes are listed as pTJ100-related, but they also could be listed in the miscellaneous group.
§ *pap*G genotypes were assessed using an allele-specific assay developed by Johnson and Brown [21]. Protocols and control strains were obtained directly from Dr James R. Johnson.

papG allele 1, *papG* allele 2 and 3, *iha, afa, iss, sfaS*, and *kpsMT* (K1).

Amplification was performed in a 25 μL reaction mixture that included 2 μL of template DNA, 12.7 μL of double distilled water (ddH_20), 2.5 μL of 10× PCR buffer, 4.0 μL of 25mM $MgCl_2$, 0.25 μL of Amplitaq Gold *Taq* (5 U/μL) (Roche Molecular Biosystems, Branchburg, New Jersey, USA), 0.625 μL of each 10 mM dNTP (USB, Cleveland, Ohio, USA), and 0.075 μL of 0.1 mM upper and lower primers. The amount of ddH_20 varied according to the number of primers used in each panel. Reaction mixtures were subjected to the following conditions in a Mastercycler Gradient (Perkin-Elmer, Boston, Massachusetts, USA): 12 min at 95 ºC to activate the Amplitaq Gold *Taq*, 25 cycles of 30 s at 94 ºC, 30 s at 63 ºC, and 3 min at 68 ºC, with a final cycle of 10 min at 72 ºC, followed by a hold at 4 ºC.

Additionally, another multiplex amplification procedure was used to detect *sitA*, *feoB*, and *irp*2. Amplification of these genes was performed in a 25 μL reaction that included 2 μL of template DNA, 18.9 μL of double distilled water (ddH_2O), 1.5 μL of 10× PCR Buffer, 0.75 μL of 50 mM $MgCl_2$, 0.25 μL of DNA *Taq* Polymerase (Invitrogen, Carlsbad, CA, USA) 1.0 μL of a dNTP mixture with a concentration of 2.5 mM of each dNTP (USB, Cleveland, Ohio, USA), and 0.1 μL of 0.1 mM upper and lower primers. The reaction mixture was subjected to the following conditions: 5 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 59 °C, and 30 s at 72 °C, with a final 5 min cycle at 72 °C, followed by a 4 °C hold.

To amplify *tsh* and *iucC*, individual amplification runs were used. For *tsh*, a 25 μL reaction mixture was used that included 15.88 μL of ddH_2O, 2.5 μL of 10× PCR Buffer, 2.0 μL of 50 mM $MgCl_2$, 0.25 μL of DNA *Taq* Polymerase (Invitrogen), 2.0 μL of a dNTP mixture with a concentration of 2.5 mM of each dNTP (USB), and 0.25 μL of 0.1 mM upper and lower primers. The reaction mixture was subjected to the following conditions: 5 min at 95 °C, 1 min at 95 °C, 9 cycles of 30 s at 55 °C, 30 s at 72 °C, and 30 s at 94 °C, followed by 25 cycles of 30 s at 55 °C and 1 min at 72 °C, followed by 7 min at 72 °C with a final hold at 4 °C. The *iuc*C gene was amplified in a 25 μL reaction mixture, containing 15.15 μL of ddH_2O, 2.5 μL of 10× PCR Buffer, 3.0 μL of 50 mM $MgCl_2$, 0.15 μL of DNA *Taq* Polymerase (Invitrogen) 2.0 μL of a dNTP mixture with a concentration of 2.5 mM of each dNTP (USB), and 0.25 μL of 0.1 mM upper and lower primers. This mixture was subjected to the conditions described by Skyberg et al. [40].

Samples were subjected to horizontal gel electrophoresis in 1.5% agarose, and the size of the amplicons was determined by comparison to the Hi-Lo DNA marker (Minnesota Molecular Inc., Minneapolis, Minnesota, USA). Strains known to possess or lack the genes of interest were examined with each amplification procedure. An isolate was considered to contain the gene of interest if it produced an amplicon of the expected size (Tab. II). All amplifications were repeated three times to ensure reproducibility and reduce the chances of false negatives.

2.5. Biostatistics

The null hypothesis that the proportion of APEC exhibiting each factor was equal to the proportion of AFEC isolates with that factor was tested by a Z-test on the difference between the proportions [41]. Also, the Z-test of proportions was used to assess differences between the occurrence of these traits in APEC from chickens versus those from turkeys and in APEC isolated from systemic lesions (heart, liver, spleen, brain, and blood) versus those from cellulitis.

3. RESULTS

3.1. Serogroups

Of the 451 APEC isolates, only 70.5% (318 isolates) could be classified to a single serogroup using standard antisera; of the AFEC, only 57.7% (60 isolates) were classified into a single serogroup (Tab. III).

Table III. Serogroups among APEC and AFEC isolates*.

Serogroup	APEC $n = 451$ No.	AFEC $n = 104$ No.	Serogroup	APEC $n = 451$ No.	AFEC $n = 104$ No.
Shared serogroups			**Unshared serogroups continued**		
O1	7	1	O60	0	1
O2	53	9	O61	1	0
O9	5	4	O69	0	1
O15	1	1	O73	0	5
O20	5	3	O74	1	0
O25	7	1	O75	1	0
O51	3	1	O82	1	0
O77	2	1	O83	2	0
O78	103	7	O85	1	0
O86	1	1	O88	0	1
O106	2	4	O91	2	0
O108	2	3	O100	3	0
O119	6	1	O102	0	1
O120	3	1	O103	3	0
O153	3	1	O109	4	0
O158	2	2	O111	13	0
			O114	2	0
Subtotal	**205**	**41**	O115	2	0
Total shared	**205/318 = 64.5%**	**41/60 = 68.3%**	O117	1	0
			O123	1	0
Unshared serogroups			O127	1	0
O4	1	0	O132	4	0
O5	4	0	O139	0	1
O6	1	0	O143	1	0
O7	3	0	O144	0	1
O8	10	0	O149	0	1
O11	7	0	O159	1	0
O17	2	0	O167	2	0
O18	11	0	O171	3	0
O19	4	0			
O21	1	0	**Subtotal**	**113**	**19**
O23	1	0	**Total unshared**	**113/318 = 35.5%**	**19/60 = 31.7%**
O26	0	1			
O27	0	1	**Total typed**	**205 + 113 = 318 (70.5%)**	**41 + 19 = 60 (57.7%)**
O28	2	0			
O29	2	0	*** Isolates not serogrouped**		
O32	0	3	Non-typeable	110	33
O35	3	0	Multiple serogrups	10	2
O36	6	0	Rough	8	8
O37	0	1	autoagglutinated	2	0
O41	1	0	Not done	3	1
O45	0	1			
O53	3	0	**Subtotal**	**133**	**44**
O55	1	0	**Total untyped**	**133/451 = 29.5%**	**44/104 = 42.3%**

* Some isolates were not serogrouped because they failed to react with standard antisera, were rough, autoagglutinated, or were classified into more than one serogroup. In addition, serogrouping was not performed on three of the APEC isolates and one of the AFEC. Overall, only 70.5% of APEC isolates and 57.5% of the AFEC were typed to a single serogroup. Of the typeable isolates, only about a third of both APEC and AFEC isolates fell into unshared serogroups, i.e., most typeable isolates were assigned to shared serogroups.

A large number of both groups could not be assigned to a serogroup because they were not typeable with standard antisera, were rough, grouped to multiple serogroups, or autoagglutinated. In addition, three APEC isolates and one AFEC isolate were not tested. In the APEC that typed to a single serogroup, 55 serogroups were represented with 39 of these unique to this group. Among the AFEC isolates, there were 29 different serogroups with 13 of these unique to AFEC. Around 65% of the typeable APEC (205 isolates) and 68% (41 isolates) of the typeable AFEC were classified into shared serogroups, and about a third of both fell into APEC- or AFEC-unique serogroups. Serogroups that contained 5% or more of the typeable APEC isolates were O78 and O2; among the typeable AFEC, 5% or more were assigned to the O2, O78, O73, O9, O106, O20, O108, and O32 serogroups.

3.2. Lactose utilization and hemolytic activity

The abilities of the APEC and AFEC isolates to ferment lactose and hemolyze blood agar were determined by standard methods. Of the APEC, 92% of the isolates were positive for lactose fermentation; whereas 99% of the AFEC group was lactose positive. No isolate from either group was hemolytic.

3.3. Virulence genotypes

In addition to analyzing the isolates by serogroup, lactose utilization, and hemolytic activity, they were also examined for possession of ExPEC virulence genes as well as genes associated with a large avian *E. coli* plasmid called pTJ100. Several of the genes studied occurred in the majority of APEC (> 50%), while occurring only infrequently in AFEC. In fact, the difference in distribution of *cvaC*, *iroN*, *iss*, *iutA*, *sitA*, *tsh, fyuA*, *irp2*, and *ompT* across the two groups was highly significant with all these genes much more likely to occur in APEC (Tab. IV). Several other genes were found to occur in significantly more APEC than AFEC but did not occur in the majority of APEC tested (*afa*, *papA*, *papC*, *papEF*, *papG* allele II, *sfa*, *ireA*, *kpsMT* (K1), *kpsMT II*, and *ibeA* (Tab. IV). *iucC*, a gene of the aerobactin operon, occurred in about 75% of both APEC and AFEC, while the aerobactin receptor gene, *iutA*, was significantly more likely to occur in APEC.

Also, the prevalence of these same genes among the APEC isolated from chickens and turkeys was compared using the Z-test of proportions (Tab. V). Several statistically significant differences were detected in distribution of these genes by host species. Differences were found for *cvaC*, *iroN*, *iss*, *iucC*, *traT*, and *tsh*, all of which have been found to reside on the same APEC plasmid, pTJ100. However, significant differences in the distribution of two other pTJ100-associated genes, *iutA* and *sitA*, across host species were not detected. Differences in distribution were found for several adhesin genes, the *kpsMT II* capsular gene, and the *cdtB* gene. The isolates from turkeys were more likely than the chicken isolates to contain *cvaC*, *iucC*, *traT*, *tsh*, *papC*, *E*, *F*, and *G* allele II, *kpsMT II*, and *cdtB*. Only *iroN*, *iss*, and *afa* were significantly more likely to be found in the APEC isolates of chickens. Of special note, about 54% the APEC of turkeys contained *papC*, *E*, *F*, and *G* allele II; whereas, less than a third of the chicken APEC contained these same genes. No other significant differences were observed in gene prevalence by host species.

Differences in the distribution of these genes by disease syndrome (systemic versus cellulitis) were also examined for significance with the Z test (Tab. VI). Several significant differences were detected, including in the *cvaC*, *iucC*, *iutA*, *tsh*, *afa*, *iha*, *fyuA*, *irp2*, *kpsMT II*, *fliC* (H7), and *ibeA* genes. The cellulitis isolates were more likely than the systemic isolates to contain *iutA*, *tsh*, and *afa*. The systemic isolates were more likely than the cellulitis isolates to contain *iha*, *fyuA*, *irp2*, *kpsMT II*, *fliC* (H7), and *ibeA*.

Table IV. Prevalence of virulence genes in avian *E. coli.*

Gene	APEC $n = 451$ (%)	AFEC $n = 104$ (%)	Z	p-value
Plasmid-Related (pTJ100)				
cvaC	67.4	9.6	10.970	< 0.0001
iroN	88.2	13.5	13.750	< 0.0001
iss	82.7	18.3	11.850	< 0.0001
iucC	79.6	74.0	1.212	0.2256
iutA	81.2	25.9	10.170	< 0.0001
*sitA**	86.4	42.7	8.362	< 0.0001
traT	78.0	70.2	1.649	0.0991
tsh	62.5	41.3	3.897	< 0.0001
Adhesins				
afa	8.2	0.0	3.802	< 0.0001
bmaE	0.4	0.0	0.867	0.3861
fimH	98.0	99.0	–0.788	0.4309
focG	0.0	0.0	--------	--------
gafD	0.4	0.0	0.867	0.3861
iha	3.3	1.0	1.501	0.1334
papA	7.5	0.0	3.639	0.0003
papC	40.4	9.6	6.527	< 0.0001
papEF	39.0	8.7	6.552	< 0.0001
papG allele I	0.0	0.0	--------	--------
papG allele I'	0.0	0.0	--------	--------
papG allele II	40.6	11.5	6.082	< 0.0001
papG allele III	0.7	0.0	1.062	0.2882
sfa	4.4	0.0	2.769	0.0056
sfaS	4.0	1.0	1.792	0.0731
Iron-Related				
*feoB**	99.3	98.1	1.027	0.3043
fyuA	58.8	32.7	4.810	< 0.0001
ireA	48.1	9.6	7.811	< 0.0001
*irp2**	57.2	30.1	5.009	< 0.0001
Protectins				
kpsMT (K1)	16.0	8.7	2.046	0.0408
kpsMT II	25.3	16.3	2.022	0.0431
kpsMT III	1.8	0.0	1.739	0.0820
rfc	0.4	0.0	0.867	0.3861
Toxins				
cdtB	1.1	0.0	1.373	0.1698
cnf1	1.3	1.0	0.319	0.7500
hlyD	0.9	0.0	1.227	0.2198
Miscellaneous				
fliC (H7)	4.7	5.8	–0.460	0.6453
ibeA	14.4	7.7	1.970	0.0488
ompT	70.5	45.2	4.713	< 0.0001
$PAI_{(CFT073)}$	15.3	8.7	1.882	0.0599

* In this case, $n = 449$ in APEC and $n = 103$ in AFEC due to the loss of some isolates during testing.

Table V. Prevalence of virulence genes in APEC by host species.

Gene	Chicken $n = 292$ (%)	Turkey $n = 122$ (%)	Z	p-value
Plasmid-related (pTJ100)				
cvaC	63.4	73.8	2.081	0.0374
iroN	89.7	82.0	–2.065	0.0389
iss	84.9	76.2	–2.041	0.0413
iucC	74.3	88.5	3.389	0.0007
iutA	82.2	75.4	–1.539	0.1238
sitA	84.9	89.3	1.235	0.2167
traT	73.6	85.2	2.666	0.0077
tsh	56.9	70.5	2.631	0.0085
Adhesins				
afa	12.7	0.0	–4.825	< 0.0001
bmaE	0.7	0.0	–1.088	0.2768
fimH	98.3	99.2	0.741	0.4589
focG	0.0	0.0	--------	--------
gafD	0.3	0.0	–0.768	0.4423
iha	3.4	4.1	0.329	0.7426
papA	7.2	6.6	–0.233	0.8161
papC	32.9	54.1	3.971	0.0001
papEF	31.2	54.1	4.302	< 0.0001
papG allele I	0.0	0.0	--------	--------
papG allele I'	0.0	0.0	--------	--------
papG allele II	32.2	54.9	4.252	< 0.0001
papG allele III	0.0	0.0	--------	--------
sfa	5.1	1.6	–1.793	0.0729
sfaS	5.5	1.6	–1.923	0.0545
Iron-related				
feoB	99.0	100.0	1.335	0.1820
fyuA	61.0	53.3	–1.440	0.1500
ireA	44.4	54.9	1.929	0.0537
irp2	58.8	55.7	–0.567	0.5707
Protectins				
kpsMT (K1)	14.7	18.0	0.829	0.4072
kpsMT II	21.6	33.6	2.497	0.0125
kpsMT III	0.7	0.8	0.145	0.8850
rfc	0.0	0.8	1.190	0.2340
Toxins				
cdtB	0.0	3.3	2.400	0.0166
cnf1	0.0	1.6	1.687	0.0917
hlyD	0.3	0.0	–0.768	0.4423
Miscellaneous				
fliC (H7)	4.8	5.7	0.392	0.6953
ibeA	12.4	18.0	1.462	0.1437
ompT	70.6	67.2	–0.668	0.5040
$PAI_{(CFT073)}$	12.3	18.9	1.668	0.0953

Table VI. Prevalence of virulence genes in APEC by disease syndrome.

Gene	Systemic* $n = 292$ (%)	Cellulitis** $n = 122$ (%)	Z	p-value[a]
Plasmid-related (pTJ100)				
cvaC	703	51.5	2.501	0.0124
iroN	89.2	88.2	0.196	0.8448
iss	86.5	76.5	1.674	0.0941
iucC	82.0	97.1	–3.196	0.0014
iutA	79.3	92.7	–2.500	0.0125
sitA	89.1	94.1	–1.175	0.2400
traT	75.7	85.3	–1.576	0.1150
tsh	65.8	89.7	–3.737	0.0002
Adhesins				
afa	1.8	20.6	–3.869	0.0001
bmaE	0.0	0.0	--------	--------
fimH	97.3	97.1	0.094	0.9255
focG	0.0	0.0	--------	--------
gafD	0.0	0.0	--------	--------
iha	4.5	0.0	1.971	0.0487
papA	7.2	14.7	–1.559	0.1190
papC	44.1	33.8	1.374	0.1694
papEF	42.3	38.2	0.544	0.5866
papG allele I	0.0	0.0	--------	--------
papG allele I'	0.0	0.0	--------	--------
papG allele II	44.1	32.4	1.576	0.1151
papG allele III	0.9	1.5	–0.342	0.7325
sfa	4.5	4.4	0.029	0.9767
sfaS	3.6	2.9	0.242	0.8090
Iron-related				
feoB	100.0	100.0	--------	--------
fyuA	58.6	41.2	2.258	0.0240
ireA	47.8	58.8	–1.442	0.1494
irp2	60.0	35.3	3.207	0.0013
Protectins				
kpsMT (K1)	19.8	10.3	1.730	0.0837
kpsMT II	36.0	14.7	3.183	0.0015
kpsMT III	0.9	2.9	–0.965	0.3344
rfc	0.0	0.0	--------	--------
Toxins				
cdtB	0.9	0.0	0.870	0.3845
cnf1	2.7	1.5	0.560	0.5756
hlyD	0.9	1.5	–0.342	0.7325
Miscellaneous				
fliC (H7)	6.3	0.0	2.343	0.0191
ibeA	20.0	7.4	2.386	0.0170
ompT	68.5	70.6	–0.299	0.7649
$PAI_{(CFT073)}$	18.0	9.0	1.752	0.0799

* The systemic category was restricted to isolates originating from liver, heart, spleen, brain, or blood.
** Only APEC isolates identified as cellulitis were included in this category.

4. DISCUSSION

If the *E. coli* causing disease in birds use a common set of virulence factors to do so, they would constitute a group of strains known as a pathotype [27]. Since *E. coli* infections in poultry take many forms including colisepticemia, peritonitis, cellulitis, salpingitis, synovitis, omphalitis, air sacculitis, and coligranuloma [2], it seems reasonable that multiple pathotypes of disease-causing avian *E. coli* might exist. However, these different forms of the disease are similar in that they are extraintestinal in nature, leading to the hypothesis that virulence factors, contributing to survival in extraintestinal environments, may be used to define a single pathotype of *E. coli* capable of causing avian colibacillosis.

In this study, 451 *E. coli* isolates from different avian host species and forms of avian colibacillosis were compared to 104 AFEC isolates for their possession of various traits, including those associated with extraintestinal disease of human beings. Several genes were significantly more likely to be found in the disease-associated isolates. Many of these occurred in over half the APEC tested. Of these differences in gene prevalence, it was the occurrence of plasmid-related genes among the disease-associated strains that might prove most useful in defining an APEC pathotype. With few exceptions, possession of sequences previously localized to large APEC plasmids was strongly associated with the APEC, not the AFEC isolates. All 8 of the pTJ100-related genes studied were found in more than 60% of the APEC, while some of these genes were found in around 80% of the APEC. Such results indicate that these plasmids or these plasmid-associated genes are widely distributed among APEC. Of the pTJ100-associated genes studied, only *cvaC* and *tsh* occurred in less than 70% of the APEC. By contrast, AFEC were much less likely to contain *iroN, iutA, cvaC*, *sitA*, *tsh*, and *iss*, although *iucC* and *traT* were similarly distributed in APEC and AFEC. Therefore, it would seem that the presence of pTJ100-associated genes, and perhaps, pTJ100-like plasmids, may be an important factor in defining the APEC pathotype.

However, there are important caveats to this statement. First, these pTJ100-associated genes may occur elsewhere in the *E. coli* genome, not just on plasmids. For example, several of these pTJ100-related genes are known to occur in PAI of human ExPEC strains. The salmochelin operon, containing *iroN*, has been found in PAI III_{536} along with several other genes that might be linked to avian *E. coli* virulence, including *tsh* and certain components of the ColV operon [9, 10]. Therefore, future researchers will need to be aware that although these pTJ100-associated genes are widespread among APEC that they may not be plasmid-located in all APEC. Second, it should be noted that although several of these pTJ100-related genes, such as *iss* [3], have been experimentally proved to contribute to the virulence of certain *E. coli* strains, the role of these genes in avian *E. coli* virulence has not yet been confirmed. Therefore, any conclusions made about the inclusion of these genes in the definition of an APEC pathotype may need to be regarded as tentative till the role of these genes in the pathogenesis of avian colibacillosis has been confirmed.

In addition to the finding that these pTJ100-associated genes were widespread among the APEC of this study, the data also revealed that certain genes and operons involved in iron metabolism have wide distribution among these APEC. In fact, the APEC isolates were significantly more likely than the AFEC to contain several of the iron-related genes studied. This apparent redundancy in iron-acquisition genes among the APEC may be an indicator of iron's importance in the pathogenesis of avian colibacillosis. However, it should be noted that no effort was made in this study to determine if any of these iron acquisition operons were complete or functional. Certainly, a limitation of the approach using PCR assays for single genes, as reported

here, is that the assays fail to indicate whether a functional operon is present. For example, our data indicate that *iucC* [6] of the aerobactin operon is present at similar frequencies in APEC and AFEC (Tab. IV); whereas, *iutA* [7], another gene in the operon, is present at high frequency in APEC but low frequency in AFEC. Had only *iucC* been used to determine the presence of the aerobactin operon, the operon's association with APEC would have been overlooked. Regardless, it would appear that at least for the avian *E. coli* surveyed here that the presence of multiple iron acquisition systems may be characteristic of APEC isolates.

Other genes, not involved in iron metabolism or linked to pTJ100 that showed a significant association with the APEC of this study included those encoding adhesins and protectins. Also, a few genes with less well-known functions were more likely to occur in the APEC as well. All these "other" genes occurred in less than half the APEC tested except for *ompT*, which occurred in 70.5% of the APEC. *ompT* encodes OmpT protease, which has been shown to cleave colicins A, E1, E2, and E3 [4]. To our knowledge, its role, if any, in the pathogenesis of avian colibacillosis is unknown.

Although none of the *pap* genes or their various alleles were found in the majority of APEC studied, several were found in a substantial number of them and were much more likely to be found in APEC rather than AFEC. Although many of the *pap* operon's components were studied here, the prevalence of all the genes that comprise the *pap* operon was not determined [17, 28]. Interestingly, about 40% of the isolates surveyed contained several of the major genes of the *pap* operon, suggesting that about 40% of the disease-associated avian *E. coli* surveyed might produce P pili. However, few of these isolates contained *papA*. Since *papA* encodes the subunits of the pilus shaft [17], it seems unlikely that these *E. coli* could make intact P pili. Perhaps, the avian *E. coli* surveyed here make *pap* pili using a variant *papA* allele not detectable with the primers used in this study. Variants of *papA* have been described in human ExPEC [23]. In this regard, assays to assess the functionality of the *pap* operon among APEC might prove very revealing.

Also, of interest in regards to the *pap* operon among these APEC was that the allele of *papG* that occurred commonly among APEC was *papG* allele II, which characterizes the human ExPEC strains causing pyelonephritis [19, 20, 32]. Similarly, Vandemaele and coworkers [43] reported that *papG* allele II was the predominant *papG* allelic variant occurring among the APEC they studied. Further, they speculated that this similarity between APEC and ExPEC causing urinary tract infection might be indicative of a link between APEC and human disease.

In addition to virulence genotyping, the lactose and hemolytic reactions and serogroups of the avian *E. coli* of this study were determined in order to better define the APEC pathotype. Both APEC and AFEC groups were comprised primarily of *E. coli* that use lactose, and all isolates were found to be non-hemolytic. Therefore, these traits were not helpful in discriminating between the APEC and AFEC of this study. Additionally, there was a high degree of variability in serogroup with much overlap between APEC and AFEC. In fact, the most commonly occurring serogroups among the APEC and AFEC isolates were O2 and O78. Also, many isolates of both groups could not be typed to a single serogroup. Based on the failure of many of the isolates in this study to type to a single serogroup and the overlap in serogroup between the APEC and AFEC, it appears that serogroup might not be especially useful in defining an APEC pathotype.

Virulence genotyping using ExPEC and pTJ100-associated genes may be more useful than these other traits in defining an APEC pathotype, especially if certain criteria are used to interpret the data. For example, a trait could be excluded from the definition of an APEC pathotype if it failed to occur in 50% or more of APEC or if it were not

significantly more likely to occur in APEC as opposed to AFEC. Using these criteria, a typical member of an APEC pathotype, based on the results of this study, would likely contain *ompT*, several genes involved in iron metabolism (*iroN*, *iutA*, *sitA*, *fyuA*, and *irp2*), and several genes identified previously on a large APEC plasmid known as pTJ100 (*cvaC*, *iss*, *tsh*, *iroN*, *iutA*, and *sitA*). *ireA* also deserves mention here since it occurred in 48.1% of the APEC, just missing the "cut-off" value for inclusion in the definition of the APEC pathotype. *ireA* was, however, much more likely to occur in the APEC than in the AFEC. Its "near miss" in meeting these criteria emphasizes the arbitrary nature of these criteria. Although other genes, studied here, may play a role in virulence of APEC, they did not occur in the majority of APEC or were not significantly associated with APEC. Thus, they would not be typical of the APEC pathotype using these criteria. Perhaps, with further study, the "significant but minority traits" could be used to assign APEC isolates to subcategories within an APEC pathotype. Indeed, the significant differences in gene prevalence (in both majority and minority traits) seen within the APEC isolated from different host species (Tab. V) or from different syndromes (Tab. VI) suggest that sub-groupings of an APEC pathotype may be possible.

It will be interesting to see if our observations on the nature of an APEC pathotype hold true using larger and more varied samples of avian *E. coli*. Also, study of other traits among avian *E. coli* and their roles in the pathogenesis of colibacillosis may greatly refine the rudimentary definition of an APEC pathotype offered here. Further, more research will be needed to assess the utility, value, and validity of the criteria used here to define the APEC pathotype.

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