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Identification of Minimal Predictors of Avian Pathogenic *Escherichia coli* Virulence for Use as a Rapid Diagnostic Tool[▽]

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To identify traits that predict avian pathogenic *Escherichia coli* (APEC) virulence, 124 avian *E. coli* isolates of known pathogenicity and serogroup were subjected to virulence genotyping and phylogenetic typing. The results were analyzed by multiple-correspondence analysis. From this analysis, five genes carried by plasmids were identified as being the most significantly associated with highly pathogenic APEC strains: *iutA*, *hlyF*, *iss*, *iroN*, and *ompT*. A multiplex PCR panel targeting these five genes was used to screen a collection of 994 avian *E. coli* isolates. APEC isolates were clearly distinguished from the avian fecal *E. coli* isolates by their possession of these genes, suggesting that this pentaplex panel has diagnostic applications and underscoring the close association between avian *E. coli* virulence and the possession of ColV plasmids. Also, the sharp demarcation between APEC isolates and avian fecal *E. coli* isolates in their plasmid-associated virulence gene content suggests that APEC isolates are well equipped for a pathogenic lifestyle, which is contrary to the widely held belief that most APEC isolates are opportunistic pathogens. Regardless, APEC isolates remain an important problem for poultry producers and a potential concern for public health professionals, as growing evidence suggests a possible role for APEC in human disease. Thus, the pentaplex panel described here may be useful in detecting APEC-like strains occurring in poultry production, along the food chain, and in human disease. This panel may be helpful toward clarifying potential roles of APEC in human disease, ascertaining the source of APEC in animal outbreaks, and identifying effective targets of avian colibacillosis control.

Avian pathogenic *Escherichia coli* (APEC) isolates cause colibacillosis in birds raised for meat and eggs (2). This disease results in significant morbidity and mortality, which translates into multimillion-dollar annual losses for all facets of the world's poultry industry (2). It is estimated that at least 30% of the commercial flocks in the United States, at any point in time, are affected by colibacillosis. Recent reports have suggested a link between APEC and human disease (13, 36). Thus, the enhanced control of avian colibacillosis could prove beneficial to both animal and human health.

Management approaches based on the protection of poultry from predisposing conditions have proved largely ineffective in controlling avian colibacillosis (2). Also, evidence exists that APEC isolates are becoming more resistant to antimicrobial agents (17, 23, 29, 47), indicating that the control of colibacillosis is likely to become even more problematic in the future. Further complicating the control of this disease is the fact that antimicrobial usage in animal production is undergoing unprecedented scrutiny, with limitations placed on the use of certain agents in poultry production. Consequently, the vaccine-based control of avian colibacillosis, where appropriate, is likely to become increasingly desirable.

Unfortunately, vaccines designed to prevent avian colibacillosis have met with mixed results. Although vaccines against various APEC isolates have been produced (1, 3, 7, 14, 25, 26, 34, 38), some have proved effective only against homologous challenge (26, 34). This type of vaccine failure is a critical impediment to colibacillosis control, often because of the diversity of APEC populations (36, 37). Despite this diversity, recent efforts to define the APEC pathotype have shown that most APEC isolates contain a highly conserved cluster of plasmid-linked virulence genes that occurs in relatively few avian fecal commensal *E. coli* (AFEC) isolates (22, 37). Thus, the exploitation of these plasmid traits or other common APEC markers as the targets of future diagnostic tools and/or vaccines may yield colibacillosis control measures with widespread applicability.

Indeed, attempts to exploit this association between plasmid genes and APEC virulence to improve colibacillosis control already are under way. Lynne et al. (28) described the testing of a vaccine that targeted a plasmid-mediated trait, and others have described rapid diagnostic tools that identify APEC isolates based on the possession of certain genes, including plasmid-linked ones (11, 41). Unfortunately, these attempts were made prior to the recognition that these plasmid genes are ubiquitous among APEC isolates and before multiple APEC plasmid sequences had become available (21, 22). Also, these procedures were validated with relatively small samples of isolates. Here, we build on recent knowledge to more clearly define the APEC pathotype and to apply this definition to the

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TABLE 1. Bacterial strains used in this study

No. of strains	Geographical location/origin (reference)	Source(s)	Type of available results
APEC			
670	GA, MD, NC, ND, MN, Penn State University (22, 35, 36)	Chickens and turkeys clinically diagnosed with various forms of colibacillosis	Virulence genotyping, serogrouping, phylogenetic typing, and cluster analysis
124	Delmarva peninsula (6, 39, 40)	Commercially raised broilers clinically affected by colibacillosis	Virulence genotyping; serogrouping, phylogenetic typing, cluster analysis, pathotyping, and multiple correspondence analysis
AFEC			
200	ND, SD, MN (22, 35, 36)	Fecal/cloacal swabs from apparently healthy birds	Virulence genotyping, serogrouping, phylogenetic typing, and cluster analysis

TABLE 2. Primer sequences and gene descriptions

Gene	Amplicon size (bp)	Sequence	Description (reference)
iroN	553	AATCCGGCAAAGAGACGAACCGCCT GTTCGGGCAACCCCTGCTTTGACTTT	Salmochelin siderophore receptor gene (22)
ompT	496	TCATCCCGGAAGCCTCCCTCACTACTAT TAGCGTTTGCTGCACTGGCTTCTGATAC	Episomal outer membrane protease gene (22)
hlyF	450	GGCCACAGTCGTTTAGGGTGCTTACC GGCGGTTTAGGCATTCCGATACTCAG	Putative avian hemolysin (32)
iss	323	CAGCAACCCGAACCACTTGATG AGCATTGCCAGAGCGGCAGAA	Episomal increased serum survival gene (24)
iutA	302	GGCTGGACATCATGGGAACTGG CGTCGGGAACGGGTAGAATCG	Aerobactin siderophore receptor gene

TABLE 3. Relationship between APEC pathotype (high, intermediate, or low pathogenicity) and gene prevalence

Gene[a]	No. of isolates carrying (+) or lacking (−) the gene according to APEC pathotype						Probability[b]
	High + (n = 73)	High − (n = 73)	Intermediate + (n = 26)	Intermediate − (n = 26)	Low + (n = 25)	Low − (n = 25)	
etsA	69	4	22	4	17	8	0.0027
etsB	69	4	22	4	17	8	0.0027
hlyF	71	2	23	3	20	5	0.0133
iutA	71	2	23	3	20	5	0.0133
papC	30	43	10	16	3	22	0.0223
ireA	33	40	12	14	4	21	0.0232
kpsMT2	15	58	0	26	4	21	0.0232
Episomal *ompT*	69	4	23	3	19	6	0.031
papGII	27	46	10	16	3	22	0.0482
papEF	27	46	9	17	3	22	0.0549
cvaA	61	12	18	8	16	9	0.0716
cvaB5	61	12	18	8	16	9	0.0716
malPAI	10	63	1	25	0	25	0.0726
papG23	23	50	10	16	3	22	0.0841
Episomal *iss*	65	8	21	5	18	7	0.1077
kpsMT1	14	59	1	25	3	22	0.1722
fyuA	28	45	11	15	5	20	0.1913
iha	0	73	0	26	1	24	0.2016
papA	13	60	3	23	1	24	0.2297
vat	32	41	10	16	6	19	0.2405
ibeA	2	71	0	26	2	23	0.2727
cvaB3	49	24	14	12	14	11	0.3723
gimB	6	67	0	26	1	24	0.376
cvaC	41	32	12	14	11	14	0.4935
eitA	42	31	14	12	11	14	0.508
eitB	42	31	14	12	11	14	0.508
fliCH7	3	70	0	26	0	25	0.5678
cbi	27	46	10	16	12	13	0.6474
Chromosomal *ompT*	33	40	12	14	9	16	0.6996
cma	18	55	7	19	7	18	0.8833
bmaE	1	72	0	26	0	25	1
iroN	63	10	23	3	22	3	1

[a] Note that only 32 genes are listed, since *kpsMT3*, *cnf1*, *sfafoc*, *papGIII*, *hlyD*, *rfc*, *papG1*, *papGI*, *gafD*, *cdtB*, *focG*, *papGI′*, *afa*, and *sfaS* occurred in none of these isolates.
[b] The probability column shows the *P* values for Fisher's exact test of the homogeneity of prevalence rates across the pathogenicity groups for each gene.

development of a diagnostic test useful in predicting an avian *E. coli* strain's ability to cause disease. In addition to describing the use of multiple-correspondence analysis (MCA) in studying the traits of APEC isolates and their relationship to disease in birds, this study describes the use of MCA to predict the abilities of extraintestinal pathogenic *E. coli* (ExPEC) isolates to cause disease in their natural hosts rather than in a model system.

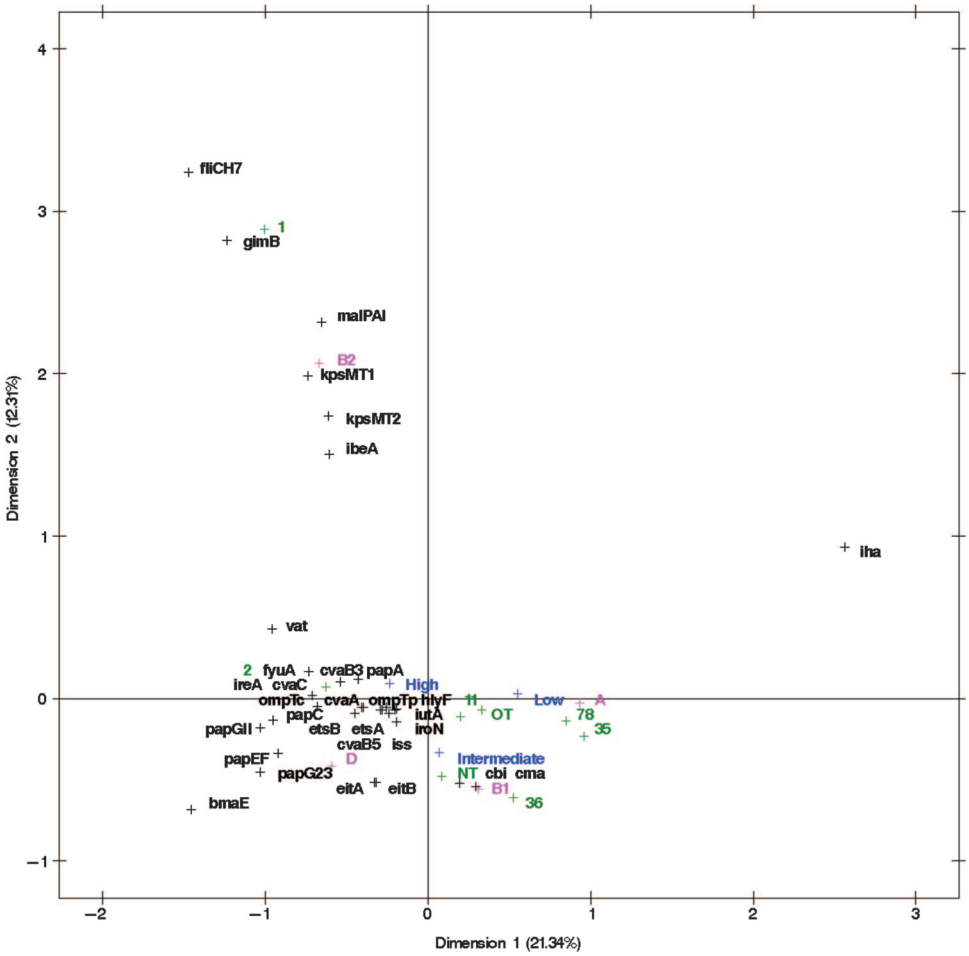

FIG. 1. MCA of 124 avian *E. coli* isolates of known pathogenicity, taking into account virulence genotypes, phylogenetic groups, and serogroups O1, O2, O11, O35, O36, O78, OT (other typeable serogroups are lumped together), and NT (nontypeable).

MATERIALS AND METHODS

Bacterial strains. For genotyping studies, a total of 994 isolates were used (Table 1). This population included 794 APEC isolates, which were defined as *E. coli* strains isolated from lesions of birds clinically diagnosed with colibacillosis, and 200 AFEC isolates, which were isolated from the feces of apparently healthy birds. These isolates originated from various farms and flocks throughout the United States. Of these, 556 isolates were previously characterized for the presence of a subset of the genes included in this study (37). Also included among the 794 APEC isolates were 124 strains that were previously assigned to low-, intermediate-, or high-pathogenicity groups based on lesions and mortalities observed in experimentally infected chicks (6, 39, 40). Isolates were serogrouped by the *E. coli* Reference Center at Pennsylvania State University, University Park.

Virulence genotyping. For multiplex PCR studies screening for virulence genes and phylogenetic typing, template DNA was prepared using boiled lysates, as previously described (20). DNA was stored at −20°C until used. This study used previously described results (37) in combination with novel data. Primers for this procedure have been previously described (37). Test and control organisms were amplified in several multiplex procedures.

Phylogenetic typing. Isolates were assigned to phylogenetic groups according to the method of Clermont et al. (5). Using this method, isolates are assigned to one of four groups (A, B1, B2, or D) based on their possession of two genes (*chuA* and *yjaA*) and a DNA fragment (TSPE4.C2) as determined by PCR. Boiled lysates of overnight cultures were used as a source of template DNA as described above.

Diagnostic pentaplex procedure. MCA was used to identify traits corresponding to APEC isolates assigned to different pathogenicity groups (6, 16). From this analysis, the best five genes in terms of correspondence with pathogenicity were targeted in a multiplex PCR procedure (Table 2). The amplification of the five gene targets for the diagnostic multiplex protocol was accomplished under the following reaction conditions: 4 mM magnesium chloride, 0.25 mM deoxynucleoside triphosphates (USB Corporation, Cleveland, OH), 0.3 μM each primer (Integrated DNA Technologies, Iowa City, IA), and 1 U HotMaster *Taq* DNA polymerase (Eppendorf, Westbury, NY). The reactions were performed using a Mastercycler EP machine (Eppendorf) using the following cycling parameters: 94°C for 2 min; 25 cycles of 94°C for 30 s, 63°C for 30 s, 68°C for 3 min; and a final cycle of 72°C for 10 min.

All samples were subjected to horizontal gel electrophoresis in 2% agarose, and amplicon sizes were determined by comparison to the Hi-Lo DNA marker obtained from Minnesota Molecular Inc. (MN). Strains known to possess or lack the genes of interest were examined with each amplification procedure. An isolate was considered to contain a gene of interest if it produced an amplicon of the expected size (Table 2). To verify the accuracy of the amplification procedure, amplicons from control organisms were excised from the gels and subjected to DNA sequencing. In all cases, amplicons of the sizes predicted had their identities confirmed by sequencing.

Statistical analyses. Fisher's exact test was used to test the null hypothesis of equal gene prevalence rates across the two populations studied. Due to the relatively large number of traits, stepdown permutation multiplicity adjustments were used to address the associated inflation of the type I error rate (44). In a further attempt to discern patterns among all isolates based on their content of virulence genes (*papGI′* was excluded, as it was absent in all isolates), multivariate statistics were used. MCA was used to look for associations among the presence of traits and pathotypes, phylogenetic groups, and serotypes simultaneously (16). A linear discriminant analysis (LDA) was used to determine if isolate type (APEC or AFEC) could be predicted based on the virulence genes present (15). Although the use of data from binary variables in an LDA, as done

TABLE 4. Extended virulence genotyping of APEC (n = 794) and AFEC (n = 200) isolates

Gene[a]	No. of APEC and AFEC isolates carrying (+) or lacking (−) the gene				*P* value
	APEC +	APEC −	AFEC +	AFEC −	
*iroN**	677	117	42	158	<0.0001
Episomal *ompT**	624	170	42	158	<0.0001
*hlyF**	621	173	48	152	<0.0001
Episomal *iss**	639	155	60	140	<0.0001
cvaB5′	594	200	44	156	<0.0001
cvaA	587	207	42	158	<0.0001
etsA	561	233	43	157	<0.0001
*iutA**	641	153	71	129	<0.0001
etsB	560	234	44	156	<0.0001
cvaC	485	309	24	176	<0.0001
cvaB3′	485	309	33	167	<0.0001
papGI	4	790	34	166	<0.0001
Chromosomal *ompT*	497	297	47	153	<0.0001
ireA	352	442	23	177	<0.0001
papEF	270	524	16	184	<0.0001
papC	289	505	21	179	<0.0001
papGII	286	508	23	177	<0.0001
cbi	278	516	23	177	<0.0001
vat	262	532	22	178	<0.0001
papG23	256	538	21	179	<0.0001
fyuA	413	381	60	140	<0.0001
cma	217	577	19	181	<0.0001
eitB	323	471	43	157	<0.0001
eitA	323	471	43	157	<0.0001
papA	79	715	2	198	<0.0001
gimB	75	719	3	197	0.0002
afa	43	751	0	200	0.0008
sfa/foc	29	765	0	200	0.0061
fliCH7	37	757	19	181	0.0080
cnf1	11	783	8	192	0.0158
sfaS	20	774	1	199	0.0760
kpsMT2	179	615	35	165	0.1209
kpsMT3	9	785	0	200	0.1304
papG1	7	787	0	200	0.1827
malPAI	125	669	24	176	0.1851
hlyD	5	789	0	200	0.2606
bmaE	4	790	0	200	0.3145
papGIII	3	791	0	200	0.3840
rfc	3	791	0	200	0.3840
kpsMT1	125	669	27	173	0.4308
gafD	2	792	0	200	0.4774
iha	18	776	3	197	0.5002
cdtB	7	787	1	199	0.5893
ibeA	94	700	21	179	0.5968
papG I′	0	794	0	200	1.0000
focG	0	794	0	200	1.0000

[a] Asterisks indicate genes selected for a pentaplex typing scheme based upon this analysis, LCA, and the MCA plot (Fig. 2).

here, violates the assumption of multivariate normality, LDA was used because parametric LDA can be very robust in spite of such violations (30). Additionally, a cluster analysis of the isolates was performed using the average linkage method based upon Jaccard's dissimilarity coefficient calculated from the presence of virulence genes (SAS 9.0). In order to better discern patterns among the isolates, the results of the cluster and discriminant analyses, along with the isolates' virulence genotypes, phylogenetic groups, and states of origin, were used to construct a single figure based on principles of Eisen et al. (10).

RESULTS

A group of 124 avian *E. coli* isolates were previously assigned to high-, intermediate-, and low-pathogenicity groups based on the lesions and mortality they caused in experimentally infected chickens (40). For the present study, these isolates were examined for serogroups, phylogenetic groups, and virulence genotypes. Several significant differences in gene distribution were found among the APEC isolates of the three pathotypes using Fisher's exact test ($P < 0.05$) and included *etsA*, *etsB*, *hlyF*, *iutA*, *papC*, *ireA*, *kpsMT2*, episomal *ompT*, and *papG2* (Table 3). In most cases, a higher proportion of APEC isolates of the high-pathogenicity group contained the genes of interest than did the APEC isolates assigned to the intermediate- or low-pathogenicity group.

Virulence genotypes, serogroups, and phylogenetic groups of these 124 APEC isolates also were subjected to MCA in order to determine which traits corresponded to the different pathogenicity groups (16, 40). Figure 1 graphs the results of this analysis. In this plot, corresponding factors associate with one another, but the distance between factors is not an indi-

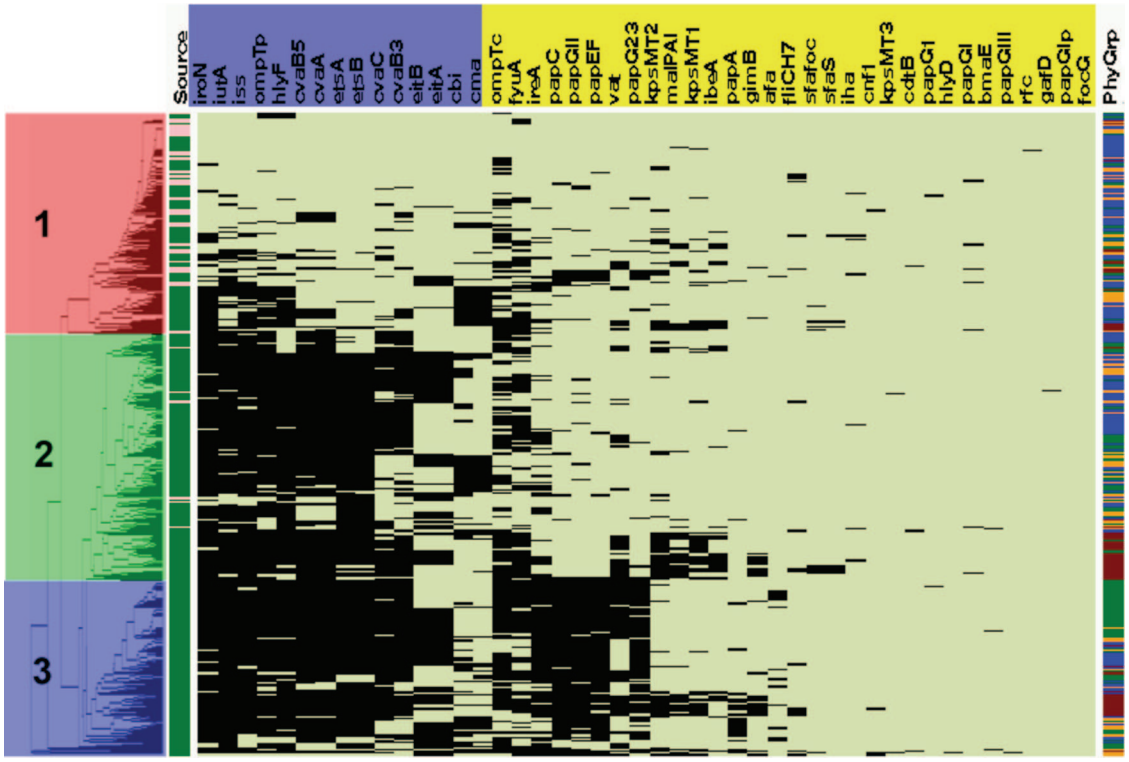

FIG. 2. Left-most portion of this figure is the dendrogram resulting from the cluster analysis. There appears to be three main clusters of isolates. Cluster 1 (highlighted in red) contains most of the AFEC isolates. The majority of isolates in cluster 2 (highlighted in green) are APEC isolates, and cluster 3 (highlighted in blue) consists entirely of APEC isolates. Just to the right of the dendrogram is column 1, which identifies an isolate as APEC (green) or AFEC (pink). Columns 2 to 47 show the virulence genotype of each isolate tested. Each column in this group shows the results for a single gene or trait. The identity of each gene tested is shown in the horizontal bar at the top of the diagram, with those in blue being plasmid mediated and those in yellow being chromosomally located. Black indicates that a gene is present, and pale green indicates that a gene is absent. Column 48 shows the phylogenetic group of each isolate: blue, group A; orange, B1; brown, B2; and green, D. ompTp, episomal *ompT*; ompTc, chromosomal *ompT*; iss, episomal *iss*.

cator of the degree of correspondence. Factors associated with the three pathogenicity groups clustered about the origin of the plot and included the phylogenetic groups A, B1, and D but not B2. Also, corresponding with these pathotypes were all of the plasmid genes studied (episomal *iss*, *iroN*, episomal *ompT*, *eitAB*, *cvaABC*, *cbi*, *cma*, *iutA*, *hlyF*, and *etsAB*) and some of the chromosomal genes (chromosomal *ompT*, *ireA*, *fyuA*, *papACEFG*, and *vat*). Certain serogroups also corresponded with these three pathotypes, including O2, O11, O35, O78, and others. The B2 phylogenetic group corresponded with *gimB*, *kpsMT1*, *ibeA*, *kpsMT2*, *malX*, the gene encoding the H7 flagellar antigen, and the O1 serogroup, but these did not correspond with any of the APEC pathogenicity groups (Fig. 1).

In addition, 794 APEC and 200 AFEC isolates were serogrouped, assigned to phylogenetic groups, and virulence genotyped, and the results were subjected to cluster analysis in an effort to identify patterns among the data (Table 4 and Fig. 2). Three major clusters could be discerned. Isolates in cluster 1 (red) generally were lacking in the traits examined, members of cluster 2 (green) were generally found to possess the plasmid-associated traits but lack the chromosome-associated traits, and isolates in cluster 3 (blue) possessed a combination of plasmid-associated and chromosome-associated traits. All but 40 AFEC isolates fell into cluster 1, with the remaining 40 AFEC isolates falling into cluster 2; no AFEC isolates fell into cluster 3. Notably, the 40 AFEC isolates falling into cluster 2 each had some, but not all, of the plasmid-associated genes. Thus, the assignment of an isolate to cluster 2 was due to the presence of only some of these genes. By contrast, most APEC

TABLE 5. Most commonly occurring serogroups among APEC isolates (n = 794)

Serogroup	Frequency	% Positive	Cumulative frequency	Cumulative %
NT[a]	175	22.3	175	22.3
78	145	18.5	320	40.8
2	103	13.1	423	53.9
Y	29	3.7	452	57.6
1	19	2.4	471	60
8	16	2	487	62
35	15	1.9	502	64
111	14	1.8	516	65.7
18	12	1.5	528	67.3
36	12	1.5	540	68.8
R	10	1.3	550	70.1
11	9	1.2	559	71.2
20	9	1.2	568	72.4
19	8	1	576	73.4
9	8	1	584	74.4
25	7	0.9	591	75.3

[a] NT, nontypeable.

TABLE 6. Relationship between an APEC isolate's (n = 794) phylogenetic group and gene prevalence

Gene (n = 44)	No. of isolates in a phylogenetic group carrying (+) or lacking (−) the gene								Probability
	A +	A −	B1 +	B1 −	B2 +	B2 −	D +	D −	
afa	9	274	9	126	1	143	24	208	<.0001
cbi	94	189	86	49	36	108	62	170	<.0001
cma	73	210	60	75	27	117	57	175	<.0001
cvaB3	164	119	66	69	108	36	147	85	<.0001
cvaC	151	132	74	61	106	38	154	78	<.0001
etsA	171	112	101	34	111	33	178	54	<.0001
etsB	167	116	101	34	114	30	178	54	<.0001
fliCH7	4	279	6	129	25	119	2	230	<.0001
fyuA	130	153	30	105	113	31	140	92	<.0001
gimB	2	281	3	132	65	79	5	227	<.0001
ibeA	9	274	1	134	68	76	16	216	<.0001
ireA	75	208	44	91	68	76	165	67	<.0001
Episomal *iss*	206	77	107	28	130	14	196	36	<.0001
iutA	203	80	107	28	127	17	204	28	<.0001
kpsMT1	13	270	6	129	91	53	15	217	<.0001
kpsMT2	22	261	14	121	108	36	35	197	<.0001
malX	9	274	7	128	97	47	12	220	<.0001
Chromosomal *ompT*	140	143	56	79	111	33	190	42	<.0001
papA	14	269	26	109	28	116	11	221	<.0001
papC	68	215	54	81	47	97	120	112	<.0001
papEF	62	221	54	81	41	103	113	119	<.0001
papG23	61	222	39	96	43	101	113	119	<.0001
papGII	69	214	49	86	55	89	113	119	<.0001
sfaS	2	281	2	133	16	128	0	232	<.0001
sfafoc	3	280	2	133	23	121	1	231	<.0001
vat	12	271	8	127	107	37	135	97	<.0001
iroN	221	62	116	19	129	15	212	20	0.0001
cvaA	190	93	98	37	123	21	176	56	0.0005
cvaB5	192	91	99	36	123	21	180	52	0.0006
Episomal *ompT*	201	82	106	29	122	22	195	37	0.0008
papG1	0	283	0	135	4	140	0	232	0.0019
hlyF	202	81	113	22	116	28	191	41	0.0058
iha	3	280	7	128	6	138	2	230	0.009
cnf1	2	281	2	133	6	138	1	231	0.0228
eitA	104	179	67	68	66	78	86	146	0.0282
eitB	104	179	67	68	66	78	86	146	0.0282
cdtB	1	282	1	134	4	140	1	231	0.0829
papGI	2	281	0	135	0	144	5	227	0.1241
rfc	1	282	0	135	2	142	0	232	0.1876
bmaE	1	282	2	133	0	144	1	231	0.4303
kpsMT3	4	279	1	134	3	141	1	231	0.4483
papGIII	1	282	1	134	1	143	0	232	0.5078
gafD	1	282	1	134	0	144	0	232	0.5353
hlyD	1	282	1	134	2	142	1	231	0.5906

isolates contained the plasmid pathogenicity-associated island (PAI) genes that previously were identified as being highly conserved among APEC isolates, including *sitA*, *iutA*, *hlyF*, episomal *ompT*, *etsAB*, episomal *iss*, *iroN*, and *cvaABC* (12, 13, 22, 27, 36, 46, 48). In addition to these plasmid genes, members of cluster 3, which were exclusively APEC isolates, also were characterized by the possession of certain chromosomal genes, including *fyuA*, *ireA*, the *pap* operon genes, *vat*, capsular biosynthesis genes (K1 and K2 capsule types), and other PAI markers (*malX*, *ibeA*, and *gimB*). The most commonly occurring serogroups among the APEC isolates examined were O78 (18.5%) and O2 (13.1%) (Table 5). However, 22.3% of the isolates examined were nontypeable, and a high degree of diversity was found among the remaining isolates. Because 68.4% of the APEC isolates examined did not belong to the O2 or O78 serogroup and no other serogroup was prominent among APEC isolates, no discernible patterns could be identified with regard to serogroup and virulence potential.

An analysis of the distribution of virulence genes among APEC isolates of the four phylogenetic groups revealed that most of the genes were differentially distributed (Table 6). In fact, the only genes without significantly different distributions across phylogenetic groups ($P < 0.05$) were those that had very low prevalence among all populations, such as *cdtB*, *rfc*, *papG1*, *papG3*, *bmaE*, *gafD*, *kpsMT3*, and *hlyD*. Among the AFEC isolates examined, only nine genes displayed significant differences across phylogenetic types. These included plasmid-associated genes, such as episomal *iss* and genes of the ColV operon, and PAI-associated genes, such as *fyuA*, *ibeA*, and the *mal* PAI marker (Table 7).

In an attempt to exploit these data to identify a minimum number of traits that could be used to distinguish an APEC

TABLE 7. Relationship between an AFEC isolate's (n = 200) phylogenetic group and gene prevalence

Gene[a]	No. of isolates in a phylogenetic group carrying (+) or lacking (−) the gene								Probability[b]
	A +	A −	B1 +	B1 −	B2 +	B2 −	D +	D −	
fyuA	13	64	19	41	22	16	6	19	0.0001
ibeA	5	72	5	55	11	27	0	25	0.0012
malPAI	7	70	4	56	12	26	1	24	0.002
Chromosomal *ompT*	10	67	13	47	17	21	7	18	0.0026
cvaC	4	73	7	53	11	27	2	23	0.0048
ireA	11	66	1	59	5	33	6	19	0.0061
iroN	10	67	13	47	15	23	4	21	0.0148
cvaB5	12	65	12	48	16	22	4	21	0.0159
Episomal *iss*	20	57	16	44	19	19	5	20	0.033
Episomal *ompT*	9	68	15	45	11	27	7	18	0.0592
papG23	5	72	4	56	7	31	5	20	0.0649
cvaA	12	65	12	48	14	24	4	21	0.0733
etsB	11	66	14	46	13	25	6	19	0.1047
kpsMT2	10	67	9	51	12	26	4	21	0.1078
kpsMT1	8	69	6	54	10	28	3	22	0.1173
gimB	0	77	1	59	2	36	0	25	0.1197
papG1	19	58	6	54	6	32	3	22	0.1354
etsA	12	65	13	47	13	25	5	20	0.1615
papEF	6	71	2	58	6	32	2	23	0.1804
papC	8	69	3	57	7	31	3	22	0.1996
eitA	14	63	12	48	13	25	4	21	0.2257
eitB	14	63	12	48	13	25	4	21	0.2257
fliCH7	8	69	4	56	2	36	5	20	0.2281
papGII	6	71	7	53	7	31	3	22	0.3843
iha	2	75	0	60	0	38	1	24	0.4563
hlyF	16	61	13	47	11	27	8	17	0.5609
cdtB	0	77	1	59	0	38	0	25	0.615
cma	6	71	6	54	3	35	4	21	0.6291
cbi	7	70	9	51	5	33	2	23	0.6773
cvaB3	11	66	11	49	8	30	3	22	0.7566
papA	2	75	0	60	0	38	0	25	0.7678
cnf1	4	73	3	57	1	37	0	25	0.8742
sfaS	1	76	0	60	0	38	0	25	1
iutA	20	57	16	44	29	9	6	19	<.0001
vat	0	77	1	59	16	22	5	20	<.0001

[a] The probability column represents the P value for Fisher's Exact test of the homogeneity of prevalence rates for each gene across the 4 phylogenetic groups. Note that there are only 35 genes in the table, even though testing has been done for 46 genes; no occurrences of *kpsMT3*, *bmaE*, *sfa*/*foc*, *papGIII*, *hlyD*, *rfc*, *papG* I′, *gafD*, *focG*, *papG1*, and *afa* were found among these isolates.

[b] The probability column represents the P value for Fisher's Exact test of the homogeneity of prevalence rates for each gene across the four phylogenetic groups.

from an AFEC isolate, further LCA was done (Table 8). This analysis identified a subset of genes, *iutA*, *hlyF*, episomal *iss*, *iroN*, and episomal *ompT*, which showed correspondence to APEC pathotypes and appeared to be capable of discriminating APEC from AFEC isolates to nearly the same degree as virulence genotyping for 46 genes. Using this subset of genes, a pentaplex PCR procedure targeting these genes was designed and validated using *E. coli* strains known to lack or possess these genes. In all cases, amplicons occurred as predicted, were of the size predicted (Table 2), and were confirmed as to their identities by DNA sequencing. Using this multiplex procedure, the 794 APEC and 200 AFEC isolates described above (Fig. 3) were analyzed, and the data generated were plotted in another cluster diagram. As seen previously in the cluster diagram using 46 genes, this cluster analysis showed a sharp demarcation between AFEC and APEC isolates, with the APEC isolates on average possessing 4.0 of the 5 genes and the AFEC averaging only 1.3 genes. These results suggest that screening for these genes is a useful tool in APEC diagnostics. Also, when the pentaplex results for the 200 AFEC and 124 APEC isolates assigned to pathotypes were plotted against one another (Fig. 4), the average number of genes possessed decreased from high (4.6) to medium (4.3) to low (3.9) for AFEC groups. However, despite strong differences in the distribution of these genes between APEC and AFEC, every gene studied could be found in both APEC and AFEC populations.

TABLE 8. Genes useful in predicting APEC (n = 794) or AFEC (n = 200) membership

Model	No. (%) correctly identified as APEC	No. (%) correctly identified as AFEC
All 46 genes	685 (86.3)	168 (84.0)
Stepwise (17-plex)	686 (86.4)	169 (84.5)
Best five[a]	678 (85.4)	158 (79.0)

[a] The genes in the best five model were *iutA*, *hlyF*, episomal *iss*, *iroN*, and episomal *ompT*.

DISCUSSION

This study validates a refined multiplex PCR scheme to be used for the prediction of virulence of avian *E. coli*. This

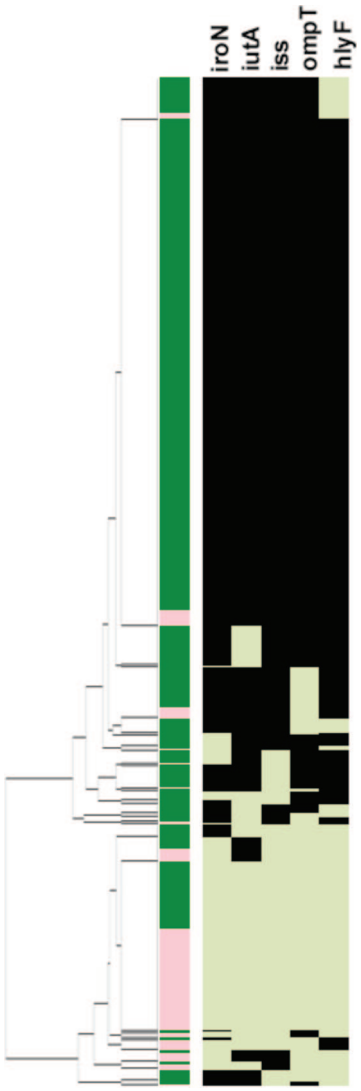

FIG. 3. Results using the pentaplex panel for the 994 avian *E. coli* isolates. The left-most portion of this figure is the dendrogram resulting from the cluster analysis. Just to the right of the dendrogram is column 1, which identifies an isolate as APEC (green) or AFEC (pink). Columns 2 to 6 show the virulence genotype of each isolate tested for *iroN*, *iutA*, *iss*, *ompTp*, and *hlyF*. Each column in this group shows the results for a single gene. Black indicates that the gene is present, and light green indicates that the gene is absent.

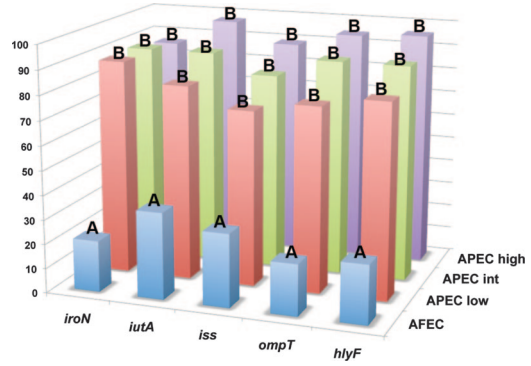

FIG. 4. Histogram comparing the prevalence of the genes targeted in the pentaplex procedure among APEC isolates of known pathogenicity (APEC high, high pathogenicity [$n = 73$]; APEC int, intermediate pathogenicity [$n = 26$]; APEC low, low pathogenicity [$n = 25$]). Also shown is the gene prevalence among 200 AFEC isolates. Letters above bars indicate levels of statistical significance according to Fisher's exact test ($P < 0.05$ is considered statistically significant).

scheme is based on extensive virulence genotyping on a large number of isolates from a variety of sources; takes advantage of recent advances in plasmid genomics; and correlates the presence of five genes with the ability of an APEC isolate to cause disease in 1-day-old chicks. A cluster analysis of multiplex PCR results of nearly 1,000 isolates, screened for the presence of more than 40 ExPEC-associated traits, showed that the majority of APEC isolates fall into two distinct clusters: those with plasmid-associated virulence genes but lacking chromosome-associated virulence genes and those possessing both plasmid- and chromosome-associated virulence genes. Although some APEC isolates lacked the plasmid genes studied here, the vast majority of APEC isolates were distinguished from AFEC isolates by their possession of plasmid-linked PAI genes. In fact, the sharp demarcation between most APEC isolates and most AFEC isolates due to their plasmid gene content suggests that APEC isolates are well equipped for a pathogenic lifestyle, which is contrary to the widely held belief that they are opportunistic pathogens. Perhaps APEC isolates assigned to cluster 1, which are characterized by a dearth of the genes tested, are opportunistic pathogens, while those of clusters 2 and 3 are frank pathogens. Indeed, an APEC isolate in this study was defined as an *E. coli* strain isolated from the lesions of birds with colibacillosis with no regard to any host factors that might have predisposed the birds to infection, including infection with commensal strains of *E. coli*. Thus, we speculate that APEC isolates falling into cluster 1 actually are commensal *E. coli* strains taking advantage of an immunocompromised host. A further examination of the APEC isolate of this cluster in a range of immunocompetent and immunocompromised hosts would be helpful in resolving this issue. Regardless, it is evident from this study that a distinction can be made between the majority of APEC and AFEC isolates examined here by their possession of ColV virulence plasmids.

Besides confirming that virulence plasmids are a defining trait of the APEC pathotype, these results help explain the assignment of APEC isolates to phylogenetic types that are not typically associated with ExPEC isolates of human beings. While the majority of human ExPEC isolates belong to phylogenetic type B2 and, to a lesser degree, D, the majority of APEC isolates belong to the A, B1, and D phylogenetic types (5, 36). Phylogenetic grouping, which relies on identifying certain chromosomal markers, does not account for virulence due to plasmid-mediated PAIs and other extrachromosomal and mobile elements. Since such extrachromosomally located PAIs are a defining trait of the APEC pathotype and appear to be critical to APEC virulence (8, 9, 42, 43, 45), PCR-based phylogenetic typing is not a clear predictor of avian *E. coli* virulence. However, the literature provides evidence that no absolute definition of an APEC or a human ExPEC isolate is possible (4, 13, 18, 19, 29, 31, 33). Overlap among all ExPEC subtypes in terms of serogroups, phylogenetic types, and viru-

lence genotypes exists to some degree (13, 33, 36). However, because most APEC isolates fall into phylogenetic groups other than the B2 group and possess ColV or ColBM virulence plasmid, one can conclude that these plasmids and/or some other genetic elements common to avian *E. coli* of the non-B2 types provide these strains with an enhanced ability to cause avian colibacillosis.

In summary, plasmid-linked PAIs are common among APEC isolates and provide a useful target for identifying these organisms. By exploiting this characteristic trait of APEC, we have developed and validated a pentaplex PCR panel that can distinguish most APEC isolates from AFEC isolates. Since APEC isolates remain an important concern for poultry producers and a potential one for public health professionals, such a diagnostic tool may be used to detect APEC-like strains occurring in poultry production, along the food chain, and in human disease, helping to clarify the role of APEC in human disease and identify targets for improved colibacillosis control.

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