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Comparison of Extraintestinal Pathogenic *Escherichia coli* Strains from Human and Avian Sources Reveals a Mixed Subset Representing Potential Zoonotic Pathogens[▽]

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Since extraintestinal pathogenic *Escherichia coli* (ExPEC) strains from human and avian hosts encounter similar challenges in establishing infection in extraintestinal locations, they may share similar contents of virulence genes and capacities to cause disease. In the present study, 1,074 ExPEC isolates were classified by phylogenetic group and possession of 67 other traits, including virulence-associated genes and plasmid replicon types. These ExPEC isolates included 452 avian pathogenic *E. coli* strains from avian colibacillosis, 91 neonatal meningitis *E. coli* (NMEC) strains causing human neonatal meningitis, and 531 uropathogenic *E. coli* strains from human urinary tract infections. Cluster analysis of the data revealed that most members of each subpathotype represent a genetically distinct group and have distinguishing characteristics. However, a genotyping cluster containing 108 ExPEC isolates was identified, heavily mixed with regard to subpathotype, in which there was substantial trait overlap. Many of the isolates within this cluster belonged to the O1, O2, or O18 serogroup. Also, 58% belonged to the ST95 multilocus sequence typing group, and over 90% of them were assigned to the B2 phylogenetic group typical of human ExPEC strains. This cluster contained strains with a high number of both chromosome- and plasmid-associated ExPEC genes. Further characterization of this ExPEC subset with zoonotic potential urges future studies exploring the potential for the transmission of certain ExPEC strains between humans and animals. Also, the widespread occurrence of plasmids among NMEC strains and members of the mixed cluster suggests that plasmid-mediated virulence in these pathotypes warrants further attention.

Speculation has long existed regarding a food-borne origin for extraintestinal pathogenic *Escherichia coli* (ExPEC) strains (28, 33, 42) and has spawned recent work investigating *E. coli* contaminants of food and the ExPEC strains of food-producing animals (15, 18, 24, 40). Of particular interest in this regard are avian pathogenic *E. coli* (APEC) strains that cause colibacillosis in poultry (3, 9, 35, 36, 38). Although it has been widely assumed that most APEC strains do not possess zoonotic potential, recent reports have suggested otherwise for certain groups of strains (2, 9, 29, 30, 35, 36), and some researchers have demonstrated that APEC strains and their plasmids may be transmitted to human hosts (27, 38). Recently, APEC isolates have been compared to ExPEC isolates from human urinary tract infections (UTIs) and neonatal meningitis, revealing that these "subpathotypes" have some overlap in serogroups, phylogenetic groups, virulence genotypes, and abilities to cause disease in certain animal models (9, 30, 31, 35, 36). The validity of these observations was sustained by comparison

of the first APEC genome sequence with sequenced ExPEC isolates of humans (25), which revealed that few differences existed between the sequenced APEC strain (APEC O1) and human strains. In fact, results of an in silico multilocus sequence typing (MLST) comparison of APEC O1 and all other sequenced *E. coli* genomes showed that APEC O1 belonged to the same sequence type (ST), ST95 (also referred to as ST29), as several well-characterized human ExPEC strains, including uropathogenic *E. coli* (UPEC) strains UTI89 and NU14 and neonatal meningitis *E. coli* (NMEC) strain RS218 (25).

While such data provide compelling evidence that APEC may be linked to human ExPEC, the results should not be overinterpreted to mean that all human ExPEC strains, or even most, are derived from APEC. APEC O1 was chosen for sequencing because it appeared to contain both UPEC- and APEC-like traits, not because it was representative of mainstream APEC (25). Regardless, other reports lend support to the idea that APEC and human ExPEC share chromosomal similarities. For instance, the *ibeA* gene, recognized for its contributions to the invasion of brain microvascular endothelial cells by human NMEC infection, was found significantly more often in APEC strains than in avian commensal strains (9, 10, 31, 34), and when *ibeA* was inactivated in the APEC
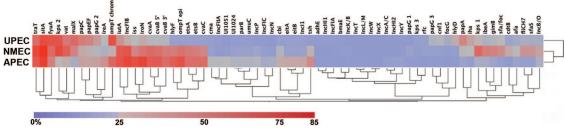
FIG. 1. Two-way clustering of gene prevalence results among the ExPEC subpathotypes. A blue-gray-red heat map was constructed based upon the percentage of each gene examined among each of the subpathotypes. Clustering was performed to illustrate similarities between the prevalence of the genes examined and between the subpathotypes with regard to gene prevalence.

strain BEN 2908, the mutant's ability to invade human brain microvascular endothelial cells and cause avian colibacillosis was significantly reduced compared to the wild type (10). *ibeA* occurs in 14% to 26% of APEC strains (9, 10, 34), and in APEC O1, *ibeA* is found in a chromosomal pathogenicity island (PAI) (25). Such examples of chromosomal virulence attributes occurring in both human and avian ExPEC strains are numerous (25).

In addition to these similarities in chromosomal attributes, similarities may occur between avian and human ExPEC strains in the plasmid-linked genes they possess. Two recent studies provided evidence that the *iss* gene, a marker of ColV virulence plasmids, was present in the majority of both APEC and NMEC populations (9, 20). However, these studies were limited in terms of sample sizes and the number of ColV-associated genes sought. This limitation and a lack of solid phylogenetic linkage between APEC and human ExPEC strains, leaves this a topic of much debate and little proof. Epidemiological studies involving poultry production facilities, their employees, and the consumer would be ideal but are complex and difficult to perform. Rather, we have utilized a genome-based approach to identify similarities and differences between these groups in an effort to provide more substantial evidence that highly related strains coexist in humans and poultry, causing a variety of extraintestinal illnesses. In this study, we performed comprehensive genotyping with large samples of NMEC, UPEC, and APEC strains in an effort to better understand the relationships between the ExPEC subpathotypes.

MATERIALS AND METHODS

Bacterial strains. A total of 1,074 isolates were used in this study, including 531 isolates from cases of human UTIs, 452 *E. coli* isolates implicated in avian colibacillosis, and 91 isolates from cases of human neonatal meningitis. Some of these isolates have been previously described, albeit to a lesser extent (21, 22, 32, 35). APEC isolates were taken from lesion sites of chickens and turkeys raised for meat consumption and laying hens. Lesion sites included the air sacs, liver, pericardium, spleen, reproductive tract, joints, and blood. These birds displayed the typical signs of colibacillosis, including respiratory distress, depression, reduced appetite, reduced mobility, ruffled feathers, and even recent death. APEC isolates came from commercial farms throughout the United States (21, 22, 32, 34). Seventy of the NMEC isolates came from the cerebrospinal fluid of newborns in The Netherlands, isolated from 1989 through 1997 (16). The remaining NMEC isolates were isolated in a similar fashion and over the same time period from patients in the United States. Two hundred of the UPEC isolates came from MeritCare Medical Center in Fargo, North Dakota (36). These isolates were taken from the urine of patients of various ages and sexs affected with uncomplicated UTI. Sixty-seven UPEC isolates came from four hospitals in Seattle, Washington, from the blood cultures of patients with bacteremia arising

from a urinary tract source during the 1990s (12, 13, 17, 19). Eleven UPEC isolates are members of the ECOR reference group and were implicated in human cystitis or pyelonephritis (11). One hundred seventy of the UPEC isolates were recovered at multiple locales in the United States during the 1990s, from the urine of pretherapy female patients with uncomplicated acute pyelonephritis (14, 39). Eighty-three UPEC isolates were collected during the 1990s at the University of Minnesota Student Health Center, from the urine of female patients with acute uncomplicated cystitis (14, 39). All organisms were stored at $-80°C$ in brain heart infusion broth (Difco Laboratories, Detroit, MI) with 10% (vol/vol) glycerol, until use.

Phylogenetic typing. Isolates were assigned to phylogenetic groups according to the method described by Clermont et al. (6). Using this method, we assigned isolates 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 for this study (12). Amplification was performed in a 25-μl reaction mixture as previously described (35).

Multiplex PCR genotyping. Multiplex PCR was performed for the presence or absence of 67 genes/traits. Some of these multiplex panels have been previously described (21–24, 35). Reaction mixtures included positive and negative control organisms. These panels included 50 ExPEC virulence-associated genes and 17 plasmid replicon types. All primers were obtained from Integrated DNA Technologies (Coralville, IA). In all, multiplex panels targeting 67 products were used. PCR was performed as previously described (35). Strains known to possess or lack the genes of interest were examined with each amplification procedure. Reactions were performed twice. An isolate was considered to contain a gene of interest if it produced an amplicon of the expected size.

Amplification of the *svg* gene for identification of the ST95/B2₁ strains. A previous study identified the *svg* gene as a distinguishing trait of strains belonging to the ST95/ST29/B2₁ subgroups (4). One hundred eight isolates falling into a mixed genotyping cluster (Fig. 1) were assessed for the presence of this gene, as previously described (4). APEC O1 and *E. coli* DH5α were used as positive and negative controls, respectively.

Biostatistics. For APEC, UPEC, and NMEC populations, Fisher's exact test was used to test the null hypothesis of equal gene prevalence rates across the three populations studied. Due to the relatively large number of traits, step-down permutation multiplicity adjustments were used to address the associated inflation of the type I error rate (43). An average linkage cluster analysis was performed based upon the Jaccarddissimilarity coefficient calculated from the presence or absence of all traits examined. This value was used to examine groupings among the isolates from the three populations. The dendrogram resulting from this cluster analysis was combined with a modified heat map (8) to allow visualization of all of the characters used in the analysis in the context of the groups obtained from the cluster analysis. Similarly, average gene prevalence values for each subpathotype were used to construct a two-way clustering diagram. Clustering images and dendrograms were constructed using SAS 9 and JMP 7 (SAS Institute) software.

RESULTS AND DISCUSSION

APEC and NMEC isolates share similarities in plasmid-associated genes but have different chromosomal backgrounds. When examined for the presence of plasmid-carried, ExPEC-associated genes, APEC and NMEC isolates were sim-

TABLE 1. Results of genotyping studies[a]

Gene, strain, or replicon	% of prevalence relative to the total no. of isolates (n)			Statistical significance of prevalence			
	UPEC (531)	NMEC (91)	APEC (452)	APEC vs human ExPEC	APEC vs UPEC	APEC vs NMEC	UPEC vs NMEC
traT	67.8	85.6	78.1	+	−	−	−
sitA	83.4	95.6	89.6	−	−	−	−
iutA	48.4	77.8	80.8	++	++	−	+
hlyF	5.6	58.9	75.4	++	++	−	++
etsA	6.0	61.1	67.0	++	++	−	++
etsB	6.0	58.9	66.8	++	++	−	++
ompT epi	5.6	64.4	81.6	++	++	−	++
iss epi	26.6	55.6	82.7	++	++	++	++
iroN	34.8	63.3	87.4	++	++	++	++
cvaA	23.4	68.9	77.4	++	++	−	++
cvaB5'	24.1	65.6	77.4	++	++	−	++
cvaB3'	22.0	61.1	68.1	++	++	−	++
cvaC	5.6	54.4	67.5	++	++	−	++
cmi	3.8	4.4	24.6	++	++	++	−
cba	4.0	21.1	34.3	++	++	−	++
tsh	2.6	31.1	52.7	++	++	+	++
eitA	4.3	5.6	37.2	++	++	++	−
eitB	4.5	5.6	37.2	++	++	++	−
UI1051	0.4	2.2	26.5	++	++	++	−
UI1024	2.4	5.6	19.7	++	++	−	−
parB	2.4	5.6	19.7	++	++	−	−
umuC	3.2	5.6	19.7	++	++	−	−
adhE	2.1	0.0	0.2	−	−	−	−
papA	54.8	28.9	7.5	++	++	++	++
papC	59.7	35.6	40.5	++	++	−	+
papEF	55.4	32.2	39.2	++	++	−	+
papG1	0.6	6.7	1.5	−	−	+	++
papG2	42.9	22.2	40.7	++	−	+	++
papG3	20.2	4.4	0.7	−	++	−	++
kps1	29.2	70.0	15.7	++	++	++	++
kps2	78.5	85.6	25.0	++	++	++	−
kps3	4.0	2.2	1.8	−	−	−	−
malX	68.2	56.7	15.0	++	++	++	−
ireA	26.0	17.8	48.0	++	++	++	−
ibeA	19.2	58.9	14.2	++	−	++	++
gimB	22.6	56.7	8.8	++	++	++	++
vat	62.3	74.4	33.4	++	++	++	−
cnf1	23.4	4.4	1.3	++	++	−	++
fyuA	80.6	68.9	58.2	++	++	−	−
cdtB	8.7	35.6	1.1	++	++	++	++
bmaE	1.3	2.2	0.4	−	−	−	−
sfa/foc	26.4	51.1	4.4	++	++	++	++
hlyD	34.1	3.3	0.9	++	++	−	++
rfc	5.3	4.4	0.4	++	−	−	−
ompT chrom	81.5	31.1	70.4	−	+	++	++
fliC$_{H7}$	16.0	47.8	4.6	++	++	++	++
focG	14.3	2.2	0.0	++	++	−	++
iha	39.2	26.7	3.5	++	++	++	−
afa	12.6	25.6	8.2	+	−	++	+
sfaS	14.1	46.7	4.0	++	++	++	++
IncB/O replicon	14.5	38.9	17.9	−	−	++	++
IncFIC replicon	1.1	3.3	12.4	++	++	−	−
IncA/C replicon	0.6	0.0	3.3	++	+	−	−
IncP replicon	0.8	8.9	21.7	++	++	−	+
IncT replicon	0.0	0.0	0.9	−	−	−	−
IncK/B replicon	0.0	2.2	1.5	−	−	−	−
IncW replicon	0.2	0.0	0.0	−	−	−	−
IncFIIA replicon	3.0	1.1	24.3	++	++	++	−
IncFIA replicon	2.6	1.1	1.5	−	−	−	−
IncFIB replicon	33.5	80.0	86.9	++	++	−	++
IncY replicon	1.7	1.1	4.2	−	−	−	−
IncI1 replicon	4.5	6.7	38.3	++	++	++	−
IncX replicon	0.0	0.0	0.0	−	−	−	−
IncHI1 replicon	1.9	0.0	1.1	−	−	−	−
IncN replicon	0.2	2.2	15.0	++	++	++	−
IncHI2 replicon	0.2	0.0	4.0	++	++	−	−
IncL/M replicon	0.0	0.0	0.7	−	−	−	−
Phylo A	10.5	11.1	36.9	++	++	++	−
Phylo B1	4.5	2.2	15.9	++	++	+	−
Phylo B2	62.7	76.7	17.3	++	++	++	−
Phylo D	22.2	11.1	29.9	++	−	+	−

[a] Values shown for results of genotyping are given in percentages. Two-way comparisons were performed for each gene, strain, or replicon studied between the different groups examined, using Fisher's exact test. For each comparison, a P value of <0.05 (+) was considered statistically significant, and a P value of <0.01 (++) was also considered statistically significant, while a P value of >0.05 (−) was not considered statistically significant. epi, episomal; chrom, chromosomal; Phylo, phylotype.
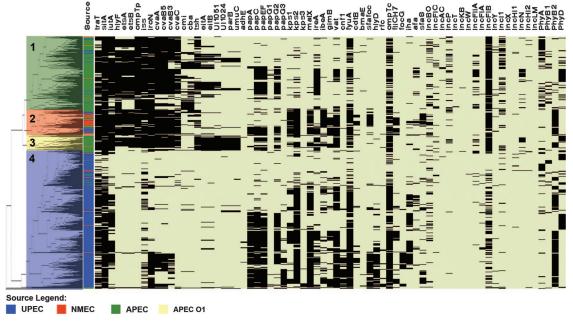
FIG. 2. Results of cluster and discriminant analyses based on the traits examined. From left to right, the dendrogram was constructed based upon the cluster analysis of common traits, and cluster numbers (1 to 4) were discerned using a cutoff based upon overall virulence genotype; the source column indicates the origin of an isolate; the following columns depict individual PCR results for the presence (black) or absence (light green) of plasmid-carried genes, chromosomal genes, plasmid replicons, and phylogenetic type. *ompT*p, episomal *ompT*; *iss*, episomal *iss*; *ompT*c, chromosomal *ompT*.

ilar in their possession of RepFIB and ColV virulence plasmids (Table 1). In particular, APEC and NMEC isolates did not differ significantly ($P > 0.05$) in their possession of most of the genes of the conserved PAI of ColV plasmids, including *sitA*, *iutA*, *hlyF*, *etsAB*, and *ompT* and genes of the ColV operon (21). With regard to plasmid replicon type, both APEC and NMEC isolates had a similarly high prevalence of the IncFIB plasmid replicon, with generally lower occurrences of other replicon types. The FIC, P, and I1 plasmid replicons occurred in a significantly higher proportion of APEC isolates than NMEC isolates. Chromosomal genes possessed by both groups ($P > 0.05$) included some genes of the *pap* operon (26) and *fyuA* of the yersiniabactin operon (41). However, these two groups did exhibit considerable differences in the prevalence of most other chromosomal genes, with NMEC isolates generally possessing them and APEC isolates generally not possessing them. These chromosomal differences were supported by the finding that APEC and NMEC isolates belonged to different phylogenetic groups, with most APEC isolates belonging to groups A (37%) and D (30%) and most NMEC isolates belonging to group B2 (77%). While the phylogenetic typing scheme originally described by Clermont et al. and used here is not the most discriminatory phylogenetic classification method, it has proven effective at rapidly distinguishing between pathogenic and nonpathogenic ExPEC organisms (6, 46). However, caution should be taken when interpreting such results, as more sensitive methods are available for classifying ExPEC isolates by phylogeny, such as MLST. Nevertheless, the rapid phylogenetic typing scheme was useful for the purposes of this study, when combined with virulence genotype.

UPEC isolates have different virulence genotypes than those of both APEC and NMEC. The 531 UPEC isolates examined

were significantly different from those of APEC and UPEC in many of the genes studied (Table 1). UPEC isolates possessed the ColV plasmid PAI genes at a significantly lower rate than those of APEC and NMEC, ranging from 5 to 27%. These rates excluded *iutA*, *sitA*, and *iroN*, because these genes can also occur on the UPEC chromosome (37, 44, 45). Chromosomal genes occurring at significantly different rates among the UPEC isolates examined included genes of the *pap* operon, *kps* type 1, *cnf1*, *focG*, *sfa/foc*, and IncFIB (compared to APEC and NMEC isolates); *fyuA*, *malX*, *ireA*, *kps* type 2, *vat*, IncFIC, IncP, IncFIIA, IncI1, and IncN (compared to APEC isolates); and *ireA*, *ibeA*, *gimB*, *cdtB*, *fliC*$_{H7}$, *afa*, chromosomal *ompT*, *sfaS*, and IncB/O (compared to NMEC isolates). Most of the UPEC isolates examined belonged to the B2 and D phylogenetic groups.

APEC strains are different from human ExPEC strains, as a whole. Compared to human ExPEC (UPEC and NMEC) strains, the APEC strains examined were significantly different ($P < 0.01$) in nearly all of the traits examined, with the exception of genes occurring at a high rate among all groups, such as *sitA*, *traT*, chromosomal *ompT*, and those occurring at low rates among all groups, including *adhE*, the *papG* allele 3, the *kps* type 3 capsular synthesis gene, *bmaE*, and several plasmid replicons.

What traits characterize each of the ExPEC subpathotypes? Using two-way clustering, we attempted to characterize the ExPEC subpathotypes examined based upon their possession of genes/traits (Fig. 1). Again, the APEC and NMEC strains appeared to be characterized by the presence of the plasmid-carried PAI of ColV plasmids (21). The UPEC strains examined generally did not contain any of these genes. All three subpathotypes were characterized by the presence of

sitA and *traT*, while only APEC strains were characterized as containing *tsh*.

With regard to chromosome-associated traits, the APEC strains were distinguished from the UPEC and NMEC strains because they lacked most of these genes. The UPEC and NMEC strains were characterized by their possession of genes of the *pap* operon, the *kps* capsular synthesis genes (type 2 for all human ExPEC and type 1 for NMEC), the *malX* PAI marker, *vat*, and their assignment to the B2 phylogenetic group. The NMEC strains also were further characterized by their possession of *ibeA*, *gimB*, and *sfa/foc*. All three groups were characterized by their possession of *fyuA*.

Cluster analysis for gene correlations showed close relationships overall between genes of the *pap* operon, *ireA*, and chromosomal *ompT*; between genes of the conserved portion of the ColV PAI; and between several chromosomal PAI-associated genes, including the *kps* type 1 capsular synthesis gene, *ibeA*, *gimB*, *sfa/foc*, *cdtB*, and *afa*. Clustering of the subpathotypes UPEC, NMEC, and APEC based upon gene prevalence illustrates that APEC and NMEC strains shared the highest similarities to one another (Fig. 1).

Cluster analysis further defines ExPEC subpathotypes. An additional cluster analysis was performed, grouping isolates together based upon their overall possession or the absence of traits examined. Such an analysis is an excellent supplement to gene prevalence because it allows for a visualization of genetic associations among individual isolates. Four major clusters could be discerned from this analysis (Fig. 2). Clusters 1 and 3 in Fig. 2 contained mostly APEC isolates. Most of the isolates from cluster 1 belonged to the phylogenetic group A, and nearly all of the isolates in cluster 1 contained the genes of the conserved ColV PAI. Some of the isolates within cluster 1 also appeared to contain the *pap* operon, *ireA*, *vat*, chromosomal *ompT*, and *fyuA*. Isolates in this cluster contained the ColB/M operon, the ColV operon, or both. This characteristic could reflect different variants of colicin virulence plasmids that have arisen over time. Isolates from cluster 3 belonged to either the phylogenetic group B2 or D. Isolates in this cluster generally contained the genes of the conserved portion of the ColV PAI, as well as other ColV-associated genes, such as *tsh* and *eitAB*. Cluster 3 isolates generally lacked chromosomal traits.

Cluster 4 (Fig. 2) contained mostly UPEC and some NMEC isolates. Most of the isolates in cluster 4 belonged to the B2 and D phylogenetic groups. These isolates generally lacked genes of the ColV plasmid PAI but contained *traT*, *sitA*, and *iutA*. These isolates also contained the *kps* type 2 capsular synthesis gene, *malX*, *vat*, *fyuA*, and chromosomal *ompT*. Some of the cluster 4 isolates possessed the IncFIB plasmid replicon, but these isolates lacked other known plasmid replicon types. Some of the cluster 4 isolates contained *iroN* and portions of the ColV operon but not other ColV-associated genes. This characteristic could reflect the presence of a chromosomal PAI similar to that of PAI III$_{536}$ of UPEC strain 536 in these isolates (7).

Cluster analysis defines a mixed subset representing B2 strains that also contain a virulence plasmid. Cluster 2 (Fig. 2) contained a mixture of all three ExPEC subpathotypes examined. This cluster contained 108 isolates, including 39 APEC, 50 NMEC, and 19 UPEC isolates (Table 2 and Fig. 3). Nearly all of these isolates appeared to contain the ColV PAI, with the

TABLE 2. Prevalence of genes and/or traits in a mixed genotyping cluster[a]

Gene, strain, or replicon	% of prevalence			
	UPEC	NMEC	APEC	Overall
% of total	8.6	3.6	54.9	10.1
traT	100.0	98.0	98.1	98.1
sitA	100.0	100.0	98.1	98.1
iutA	78.9	98.0	93.5	93.5
hlyF	94.7	94.0	88.9	88.9
etsA	100.0	94.0	90.7	90.7
etsB	94.7	92.0	89.8	89.8
ompT chrom	89.5	96.0	92.6	92.6
iss epi	89.5	80.0	88.9	88.9
iroN	100.0	86.0	90.7	90.7
cvaA	100.0	96.0	98.1	98.1
cvaB5	100.0	96.0	98.1	98.1
cvaB3	94.7	96.0	91.7	91.7
cvaC	89.5	88.0	88.0	88.0
cmi	15.8	2.0	12.0	12.0
cba	21.1	30.0	29.6	29.6
tsh	21.1	44.0	42.6	42.6
eitA	10.5	0.0	11.1	11.1
eitB	10.5	0.0	11.1	11.1
UI1051	5.3	0.0	4.6	4.6
UI1024	0.0	0.0	0.9	0.9
parB	0.0	0.0	0.9	0.9
umuC	0.0	0.0	0.9	0.9
adhE	0.0	0.0	0.0	0.0
papA	73.7	26.0	34.3	34.3
papC	73.7	30.0	40.7	40.7
papEF	84.2	26.0	39.8	39.8
papG1	0.0	0.0	0.0	0.0
papG2	73.7	18.0	37.0	37.0
papG3	5.3	0.0	0.9	0.9
kps1	94.7	90.0	88.9	88.9
kps2	100.0	100.0	98.1	98.1
kps3	0.0	2.0	0.9	0.9
malX	100.0	72.0	78.7	78.7
ireA	63.2	24.0	38.0	38.0
ibeA	31.6	80.0	71.3	71.3
gimB	73.7	78.0	67.6	67.6
vat	73.7	100.0	88.9	88.9
cnf1	0.0	0.0	1.9	1.9
fyuA	100.0	82.0	89.8	89.8
cdtB	5.3	56.0	28.7	28.7
bmaE	0.0	0.0	0.0	0.0
sfafoc	21.1	76.0	50.0	50.0
hlyD	0.0	0.0	0.0	0.0
rfc	0.0	4.0	1.9	1.9
ompT chrom	100.0	40.0	72.2	72.2
fliC$_{H7}$	68.4	72.0	58.3	58.3
focG	0.0	0.0	0.0	0.0
iha	5.3	34.0	16.7	16.7
afa	0.0	40.0	18.5	18.5
sfaS	15.8	56.0	39.8	39.8
IncB/O replicon	10.5	48.0	25.9	25.9
IncFIC replicon	0.0	2.0	2.8	2.8
IncA/C replicon	0.0	0.0	0.0	0.0
IncP replicon	5.3	10.0	13.0	13.0
IncT replicon	0.0	0.0	0.0	0.0
IncK/B replicon	0.0	2.0	0.9	0.9
IncW replicon	0.0	0.0	0.0	0.0
IncFIIA replicon	0.0	0.0	2.8	2.8
IncFIA replicon	0.0	2.0	0.9	0.9
IncFIB replicon	68.4	92.0	84.3	84.3
IncY replicon	5.3	2.0	1.9	1.9
IncI1 replicon	15.8	2.0	20.4	20.4
IncX replicon	0.0	0.0	0.0	0.0
IncHI1 replicon	5.3	0.0	1.9	1.9
IncN replicon	0.0	0.0	0.9	0.9
IncHI2 replicon	0.0	0.0	0.9	0.9
IncL/M replicon	0.0	0.0	1.9	1.9
Phylo A	0.0	2.0	5.6	5.6
Phylo B1	0.0	0.0	1.9	1.9
Phylo B2	100.0	96.0	89.8	89.8
Phylo D	0.0	2.0	2.8	2.8

[a] The cluster shown is that of cluster 2 from Fig. 1. epi, episomal; chrom, chromosomal; Phylo, phylotype.

prevalence of these genes within this cluster ranging from 88 to 99% (Table 2). About 25% of these isolates appeared to contain a plasmid variant involving genes of the ColB/M operons and *eitABC*, a putative ABC transporter system (22). Approximately one-third of the isolates from this cluster appeared to possess an intact *pap* operon, and most possessed the *kps*
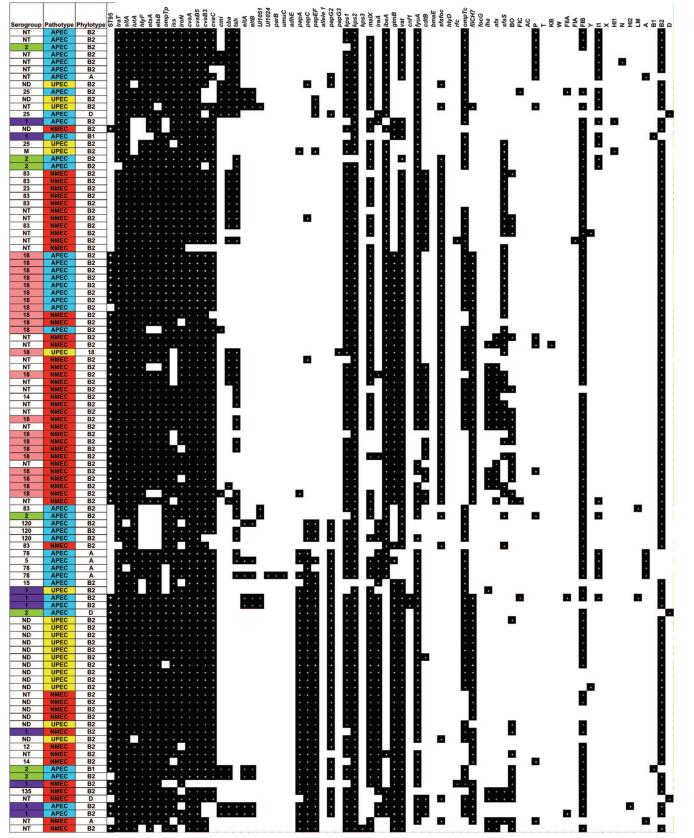
FIG. 3. Overview of isolates belonging to the mixed genotyping cluster. The 108 isolates from the mixed genotyping cluster shown in Fig. 1 are listed in order. For each isolate, the serogroup, pathotype (source), phylotype, and overall genotype are provided. For serogroup O1 (purple), O2 (olive), and O18 (salmon), isolates are colored. Isolates are also colored by source (APEC, sky blue; UPEC, yellow; NMEC, red). For genotype, black squares containing "+" represent a positive PCR, whereas open squares represent a negative PCR.

capsule biosynthesis type 1 or 2. Many of these isolates also contained a wide variety of chromosome-carried ExPEC traits, including *malX*, *ireA*, *ibeA*, *gimB*, *vat*, *fyuA*, *sfa/foc*, *ompT*, *fliC*$_{H7}$, and *sfaS*. Most of these isolates possessed the IncFIB plasmid replicon.

The 108 isolates within this mixed cluster were almost exclusively members of the B2 phylogenetic group (89.8%). Within this genotyping cluster is APEC O1, a strain which has been previously sequenced and analyzed in multiple models of ExPEC infection (25). Like other isolates in this cluster, APEC O1 possesses a ColV-type virulence plasmid with its highly conserved PAI (21). This strain has been shown to cause disease in the 1-day-old chick model of avian colibacillosis and the mouse model of human UTI (T. Johnson, unpublished data) (25). APEC O1 belongs to ST95, a potentially zoonotic sequence type strain, as determined through MLST analysis of housekeeping genes (30, 31). In fact, several recently sequenced or archetypal strains belong to this ST, including UPEC strains UTI89 (5) and NU14, and NMEC strain RS218 (47). These strains all contain a variety of chromosome-carried virulence factors such as those mentioned above. It was recently determined that the *svg* gene appears to be a distinguishing trait of *E. coli* strains belonging to ST95 and the B2$_1$ ribotype (4). When the 108 isolates from the mixed genotyping cluster in this study were analyzed for the presence of *svg*, it was found that 58% of the isolates contained this gene, suggesting their membership within the ST95 group (Fig. 3). Many of the *svg*$^+$ isolates belonged to the O1, O2, or O18 serogroup, all of which have been implicated with multiple forms of ExPEC disease. This is in agreement with the work of Achtman and Pluschke (1), who identified the K1 capsule-bearing O1:K1:H7, O2:K1:H7, and O18:K1:H7 strains shown to be closely related by multilocus enzyme electrophoresis. However, the implications and occurrence of ColV plasmids among the ST95/B2$_1$ subgroups have not been previously explored. The results of this study suggest that the acquisition of ColV virulence plasmids by hosts with B2 phylogeny has resulted in strains such as those within the mixed genotyping cluster, with an enhanced ability to cause disease and survive in multiple environments and in the face of multiple pressures. Future work should take unbiased approaches toward determining the prevalence of ColV virulence plasmids among ST95/B2$_1$-positive populations.

Conclusions. This study builds upon previous work involving extensive virulence genotyping of ExPEC populations and provides some insights into the evolution of ExPEC virulence. It is apparent from this study that most APEC, UPEC, and NMEC strains are genetically distinct from one another, and thus, their classification into subpathotypes appears to be justified. Expectedly, APEC strains are characterized by the presence of ColV-like virulence plasmids in strains belonging to the A and D phylogenetic groups. UPEC and NMEC strains are characterized by their possession of chromosome-carried virulence genes, presumably on PAIs, and they belong mostly to the B2 phylogenetic group. Many NMEC strains appear to contain ColV plasmids in addition to this chromosomal background, and cluster analyses suggest that APEC and NMEC strains share many genetic similarities, and, irrespective of host source, nearly 10% of the isolates in this study belong to a genotype cluster representing the most likely zoonotic pathogens. Nearly 50% of the NMEC strains examined belonged to this group, but it also included APEC and UPEC strains. It is evident from this study that the distribution of ColV plasmids is not limited to any particular phylogenetic type, as they are evenly distributed among all four phylotypes. Perhaps, the acquisition of ColV virulence plasmids by B2 strains has provided them with an enhanced ability to cause disease and survive under adverse conditions. If so, such strains thus present a threat to both human and animal health, and further work is required to determine the true zoonotic potential of these strains.

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