Extraintestinal Pathogenic *Escherichia coli* Strains of Avian and Human Origin: Link between Phylogenetic Relationships and Common Virulence Patterns[∇]

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Extraintestinal pathogenic *Escherichia coli* (ExPEC) strains of human and avian origin show similarities that suggest that the avian strains potentially have zoonotic properties. However, the phylogenetic relationships between avian and human ExPEC strains are poorly documented, so this possibility is difficult to assess. We used PCR-based phylotyping and multilocus sequence typing (MLST) to determine the phylogenetic relationships between 39 avian pathogenic *E. coli* (APEC) strains of serogroups O1, O2, O18, and O78 and 51 human ExPEC strains. We also compared the virulence genotype and pathogenicity for chickens of APEC strains and human ExPEC strains. Twenty-eight of the 30 APEC strains of serogroups O1, O2, and O18 were classified by MLST into the same subcluster (B2-1) of phylogenetic group B2, whereas the 9 APEC strains of serogroup O78 were in phylogenetic groups D (3 strains) and B1 (6 strains). Human ExPEC strains were closely related to APEC strains in each of these three subclusters. The 28 avian and 25 human strains belonging to phylogenetic subcluster B2-1 all expressed the K1 antigen and presented no significant differences concerning the presence of other virulence factors. Moreover, human strains of this phylogenetic subcluster were highly virulent for chicks, so no host specificity was identified. Thus, APEC strains of serotypes O1:K1, O2:K1, and O18:K1 belong to the same highly pathogenic clonal group as human *E. coli* strains of the same serotypes isolated from cases of neonatal meningitis, urinary tract infections, and septicemia. These APEC strains constitute a potential zoonotic risk.

A wide range of extraintestinal infections in humans and vertebrate animals are caused by *Escherichia coli* strains that belong to the large group of extraintestinal pathogenic *E. coli* (ExPEC) as defined by Russo and Johnson (50). These strains are frequently categorized into pathotypes according to the host's clinical symptoms. The pathotypes include uropathogenic *E. coli* (UPEC), isolated from humans and animals with urinary tract infections; neonatal-meningitis *E. coli* (NMEC), isolated from human neonates; septicemic *E. coli*, isolated from humans and animals with septicemia cases of various origins; and avian pathogenic *E. coli* (APEC), responsible for colibacillosis in poultry. The most frequent form of avian colibacillosis is a systemic infection that starts in the respiratory tracts of chickens and turkey chicks. It is characterized by fibrinous lesions of internal organs (airsacculitis, pericarditis, perihepatitis) associated with septicemia (4, 15).

Phylogenetic studies based on the *E. coli* reference collection (ECOR), a set of 72 *E. coli* strains isolated from a variety of animal hosts and a variety of geographical origins (44), show that there are four main phylogenetic groups of *E. coli*, designated A, B1, B2, and D (25, 53), and that most ExPEC strains belong to group B2, although some belong to group D (31, 46). However, none of the strains of the ECOR collection is of

avian origin, and the place of APEC strains in the phylogenetic tree of *E. coli* has not been extensively studied.

Molecular epidemiology studies show that many APEC strains can be grouped into a limited number of clones (11, 12, 19, 33, 42). The clonal nature of APEC strains has also been demonstrated by phylogenetic analyses (11, 12, 64–66). Several studies also reveal the prevalence of various serogroups and of particular combinations of virulence-associated genes among APEC strains. These observations suggest that there may be only a limited number of virulence genotypes (5, 19, 41, 47, 48).

Some avian and human ExPEC strains share common characteristics, including various serogroups and various virulence-associated factors such as the iron acquisition systems aerobactin and the *iro* gene cluster, P and S fimbriae, the K1 capsule, IbeA invasin, the autotransporter Tsh, cytolethal distending toxin (CDT), and hemolysin F (7, 38, 47). Furthermore, some avian strains, especially those harboring the K1 capsule, have been shown to be closely related to the O18:K1 clone in phylogenetic group B2, originally identified by Achtman and colleagues (1, 39). However, the phylogenetic relationships between human and avian strains are not well documented. Despite similarities and genetic relationships between human and avian strains, the extent of the zoonotic risk associated with APEC strains remains unclear (2, 19, 47). A better knowledge of the phylogenetic relationships and virulence factor (VF) patterns of APEC strains would contribute to answering this question.

Here we document the phylogenetic location of APEC strains in the *E. coli* population and their phylogenetic rela-

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TABLE 1. Primers used for PCR amplifications[a]

Gene	Primer name	Primer sequence (5′ to 3′)	Size of PCR product (bp)	Annealing temp (°C)	Reference
fimA	fimA1[b]	CGGCTCTGTCCCTSAGT	500	52	39
	fimA2[b]	GTCGCATCCGCATTAGC			
fimA$_{MT78}$	fimA201	TCTGGCTGATACTACACC	266	52	37
	fimA215	ACTTTAGGATGAGTACTG			
fimH	fimH2	GATCTTTCGACGCAAATC	389	52	3
	fimH17	CGAGCAGAAACATCGCAG			
sfa/foc	sfa1	CTCCGGAGAACTGGGTGCATCTTAC	410	64	34
	sfa2	CGGAGGAGTAATTACAAACCTGGCA			
papC	pap1	GACGGCTGTACTGCAGGGTGTGGCG	328	61	34
	pap2	ATATCCTTTCTGCAGGGATGCAATA			
felA	fel1	GGTCAASCAGCTAAAAACGGTAAGG	239	61	39
	fel2	CCTTCAGAAACAGTACCGCCATTCG			
stgC	stgC-F	TCTGGTTCACATACACTACG	208	58	35
	stgC-R	CCAATCATAATCTGGCTTCT			
neuC	neu1	AGGTGAAAAGCCTGGTAGTGTG	676	61	39
	neu2	GGTGGTACATCCCGGGATGTC			
iutA	iutA1	ATGAGCATATCTCCGGACG	587	58	39
	iutA15	CAGGTCGAAGAACATCTGG			
iroD (D11)	20F	ATGCTGAACATGCAACAACA	1,230	50	51
	20R	TCAACCCTGTAGTAAACCAAT			
ibeA	ibeAF	TGAACGTTTCGGTTGTTTTG	814	55	21
	ibeAR	TGTTCAAATCCTGGCTGGAA			
tsh	tsh03	GGTGGTGCACTGGAGTGG	640	58	17
	tsh15	AGTCCAGCGTGATAGTGG			
cdt	cdt-s1	GAAAGTAAATGGAATATAAATGTCCG	466	55	59
	cdt-as1	AAATCACCAAGAATCATCCAGTTA			
	cdt-s2	GAAAATAAATGGAACACACATGTCCG	466	55	59
	cdt-as2	AAATCTCCTGCAATCATCCAGTTA			
hlyF	hlyF1	TCGTTTAGGGTGCTTACCTTCAAC	444	60	This study
	hlyF2	TTTGGCGGTTTAGGCATTCC			
A9	13F	TTTCGACTGCTGGATGAAC	934	50	51
	13R	AATCATGATTGACCGTGC			
A12	16F	ATGCACTCGATAAAAAAAGT	860	50	51
	16R	TTAAGAAGGTCGATATACGT			
D1	17F	ATGAATTCACAATTACTGGC	1,998	50	51
	17R	TTAGCTGTTCAGTAGCTCAC			
D7	cat31	TCAGTAAGAACGAAAGTGTG	565	50	This study
	cat32	ACAGGAACAATCCCGTGGAT			
D10	D10F2	ATCTTTACCGTCCTCACC	135	50	39
	D10R2	CGTACCGCCTTCATTATC			
chuA	chuA.1	GACGAACCAACGGTCAGGAT	279	59	8
	chuA.2	TGCCGCCAGTACCAAAGACA			
yjaA	yjaA.1	TGAAGTGTCAGGAGACGCTG	211	59	8
	yjaA.2	ATGGAGAATGCGTTCCTCAAC			
tspE4.C2	tspE4C2.1	GAGTAATGTCGGGGCATTCA	152	59	8
	tspE4C2.2	CGCGCCAACAAAGTATTACG			
adk	adk-F	CGGGCGCGGGGAAAGGGACTC	595	59	2
	adk-R	GCGCGAACTTCAGCAACCG			
gcl	gcl-F	GCGTTCTGGTCGTCCGGGTCC	758	58	2
	gcl-R	GCCGCAGCGATTTGTGACAGACC			
zwf	gdh-F	TCGGCGTAGGGCGTGCTGAC	796	59	2
	gdh-R	CTGCTCTTGTTCGCGCCCTCTTC			
mdh	mdh-F	CCCGGTGTGGCTGTCGATCTGA	706	59	2
	mdh-R	CGCCGTTTTTACCCAGCAGCAGC			
metA	metA-F	CGCAACACGCCCGCAGAGC	601	59	2
	metA-R	GCCAGCTCGCTCGCGGTGTATT			
ppk	ppk-F	TGCCGCGCTTTGTGAATTTACCG	758	58	2
	ppk-R	CCCCGGCGCAGAGAAGATAACGT			

[a] VFs searched for by PCR are as follows: *fimA* and *fimH*, type 1 fimbriae; *fimA*$_{MT78}$, type 1 variant of APEC strain MT78; *sfa/foc*, S and F1C fimbriae; *papC*, P fimbriae; *felA*, F11 variant of P fimbriae; *stgC*, Stg fimbriae; *neuC*, K1 antigen; *iutA*, aerobactin receptor; *iroD* (D11), siderophore; *ibeA*, invasin; *tsh*, autotransporter Tsh; *cdt*, cytolethal distending toxin; *hlyF*, putative hemolysin; A9, A12, D1, D7, and D10, genomic fragments putatively associated with ExPEC virulence; *chuA*, *yjaA*, and TspE4.C2, sequences used for classification of *E. coli* strains into phylogenetic groups; *adk*, *gcl*, *zwf*, *mdh*, *metA*, and *ppk*, housekeeping genes used for MLST.
[b] Primers fimA1 and fimA2 were designed from consensus sequences of various *fimA* genes.

tionships with human ExPEC strains. We focused on strains belonging to serogroups prevalent among APEC strains (O1, O2, O18, and O78) that are also common among human ExPEC strains. We used the rapid PCR-based phylotyping method of Clermont et al. (8), which allows one to assign *E. coli* strains to one of the four major phylogenetic groups. The multilocus sequence typing (MLST) method was then used to locate APEC strains in the phylogenetic tree of *E. coli* (36). MLST is currently one of the best phylogenetic grouping methods for investigating the genetic relationships between clinical pathogenic strains and reference strains. It has been found to be discriminating and reproducible (40) and has the advantage over pulsed-field gel electrophoresis of allowing pertinent phylogenetic analysis as well (60). For MLST analysis, we chose a set of six housekeeping genes previously used by Adiri et al. (2) in a comparison of O78 strains of avian and human origins. To estimate the zoonotic potential of avian *E. coli*, we compared the virulence genotypes and pathogenicities for chicks of APEC and human ExPEC strains.

MATERIALS AND METHODS

Bacterial strains. A total of 102 *Escherichia coli* isolates were used in this study: 51 ExPEC strains of human origin, 39 APEC strains, 4 strains from animals (other than poultry) with septicemia, 2 enterohemorrhagic *E. coli* (EHEC) strains, and 6 strains from the feces of healthy human (*n* = 4) or animal (*n* = 2) hosts.

The 39 APEC strains were recovered previously from the heart blood or livers of chickens or turkeys with clinical signs of colibacillosis and were lethal for 1-day-old chicks following subcutaneous inoculation. Strain 789 AC/1 was kindly provided by E. Ron (2), and the other 38 strains were selected from a collection of 1,601 avian colibacillosis isolates collected in Europe between 1995 and 2000 (57) as belonging to the most frequently isolated serogroups of APEC: O1, O2, O18, and O78.

Human strains used included isolates from the main clinical extraintestinal sources (newborn meningitis, septicemia, and urinary tract infections) as well as strains from the ECOR collection and archetypal ExPEC strains to facilitate comparison with other phylogenetic studies. Twenty of the strains used were isolated from the cerebrospinal fluid of neonates and belonged to serogroup O2, O18, or O78. They included six strains from France (61), six strains from The Netherlands (29), five strains from Hungary (10), two strains from Finland (32, 54), and the NMEC type strain RS218 (1). Sixteen strains were isolated from humans with septicemia: eight from the blood of newborns without meningitis (61), seven from adults with septicemia in France (this study), and one from a septicemic patient in Hungary (10). Fifteen strains were from urinary tract infections, including one strain from Hungary (10), five from Germany (68, 69), six from the ECOR collection (44) (http://foodsafe.msu.edu/whittam/ecor), and the UPEC type strains CFT073, 536, and J96 (22, 43, 62). Four animal ExPEC strains were isolated from cases of septicemia in a sheep (S5), a pig (P72), and calves (BM2-1 and Orne6) (9, 13, 45, 56). Five of the ECOR collection (ECOR5, ECOR17, ECOR26, ECOR55, and ECOR70) (44), isolated from feces from healthy humans or animals, and the nonpathogenic K-12 strain MG1655 (6) were also included. Two EHEC strains were also included in the study: EDL933 (ATTC 43895) and SAKAI (RIMD0509952) (24). *Escherichia fergusonii* (ATCC35469[T]) was used as an outgroup for phylogenetic analysis. The bacterial strains were routinely grown in Luria-Bertani broth (LB) at 37°C and stored at −70°C in 20% glycerol until use.

Serotyping. For strains that had not been serotyped previously, slide agglutination with specific antisera (Biovac, Angers, France) was used to test for O1, O2, and O78 antigens. We tested for the O18 antigen by agglutination using the O18 antiserum kindly provided by Jorge Blanco (Laboratorio de Referencia de *Escherichia coli*, Lugo, Spain). The presence of K1 capsular antigen was detected phenotypically using the Wellcogen *Neisseria meningitidis* B/*E. coli* kit (Oxoid), and the *neuC* gene was detected by PCR as described below.

PCR-based phylotyping. Strains were classified into the four main phylogenetic groups of the ECOR collection by PCR as described by Clermont et al. (8) using three primer pairs: chuA.1–chuA.2, yjaA.1–yjaA.2, and TspE4C2.1–TspE4C2.2 (Table 1). Strains were assigned to phylogenetic group A, B1, B2, or D according to the amplification of the *chuA* and *yjaA* genes and the TspE4C2.1 fragment. Strains MG1655, ECOR26, ECOR62, and ECOR50 were used as controls for phylogenetic groups A, B1, B2, and D, respectively.

Virulence for chicks. The virulence of each *E. coli* isolate was determined using a lethality test for 1-day-old chicks as previously described (14). Groups of five 1-day-old specific-pathogen-free (SPF) chicks were inoculated subcutaneously with 0.5 ml of a 24-h LB culture (in stationary phase, at about 10^8 CFU), and the mortality was recorded 4 days postinoculation. The 50% lethal dose (LD_{50}) was determined for some strains by inoculating chicks with 10-fold dilutions of the culture and was calculated by the method of Reed and Muench (14).

Experimental infection of SPF chickens via the air sacs. Thirteen groups of 12 3.5-week-old White Leghorn SPF chickens from the INRA Infectiology Platform were reared in separate cages with food and water available ad libitum. The experiments were conducted as previously described with some modifications (17). Each chicken was inoculated in the right thoracic air sac with 0.1 ml (1×10^7 CFU) of a bacterial inoculum consisting of a 24-h LB stationary-phase culture of *E. coli*. Blood samples of 50 μl were collected aseptically from each chicken 24 h and 48 h later and were incubated in 2 ml of brain heart infusion (BHI) for qualitative detection of *E. coli*. Positive growth of *E. coli* in BHI was confirmed by plating enriched cultures on Drigalski agar (Bio-Rad).

All birds were euthanized by injection of Nesdonal (Rhône-Mérieux, Lyon, France) 48 h postinoculation and underwent necropsy. Macroscopic fibrinous lesions were observed and scored (air sacs, 0 to 4; pericardium, 0 to 2; liver, 0 to 2). The colonization of organs by *E. coli* was detected as follows: swabs of the left thoracic air sac and of the pericardial fluid were incubated in BHI; 100 μl of heart blood and liver fragments was streaked onto Drigalski agar.

All experiments involving chickens were licensed by the "Préfecture d'Indre et Loire, France," agreement 006707.

Virulence genotyping. Virulence genes were detected by PCR amplification in a Perkin-Elmer 9700 temperature cycler (Applied Biosystems). Crude DNA extracts were prepared by a rapid boiling method. Four multiplex PCR assays were designed to detect simultaneously (i) *fimA* (with consensus primers), a *fimA* variant (*fimA*$_{MT78}$), and *fimH*, (ii) *neuC*, *felA*, and *papC*, (iii) *tsh* and *iutA*, and (iv) the *cdt* gene. Single PCR assays were used to detect four other genes (*sfa/foc*, *iroD*, *ibeA*, and *hlyF*) and five genomic fragments (A9, A12, D1, D7, and D10) previously identified in APEC strain BEN2908 and putatively associated with the virulence of ExPEC isolates (51). The corresponding primers are listed in Table 1. DNA fragments were amplified in a 25-μl PCR mixture including 5 μl of DNA crude extract, 1 U of *Taq* DNA polymerase (Promega), 12.5 pmol each of the forward and reverse primers, and 5 nmol of each deoxynucleoside triphosphate (Promega) in 1× buffer. PCR conditions were as follows: 94°C for 3 min; 30 cycles of 94°C for 1 min, the annealing temperature for 1 min, and 72°C for at least 30 s according to the size of the amplified fragment (1 min/kbp); and a final extension at 72°C for 10 min. The following *E. coli* strains were used as positive controls in PCR assays: strain BEN2908 (51) for the *fimA*, *fimA*$_{MT78}$, *fimH*, *neuC*, *iutA*, and *ibeA* genes; strain MT189 (16) for *felA* and *papC*; strain χ7122 (17) for *tsh*, *iroD*, and *stgC*; strain KH576 (67) for *iutA*; strain 536 (23) for *sfa/foc*; strain E6468/62 (52) for *cdt*; strain SP15 (29) for *hlyF*; and strain BEN2908 or CFT073 (51, 62) for genomic fragments A9, A12, D1, D7, and D10. The nega-

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FIG. 1. Phylogenetic relationships between 102 *E. coli* isolates originating from human clinical extraintestinal sources (*n* = 51), avian colibacillosis (*n* = 39), nonpoultry animal cases of septicemia (*n* = 4), healthy hosts (*n* = 6), and hemolytic-uremic syndrome (*n* = 2). The phylogenetic tree was constructed by neighbor-joining analysis based on the sequence analysis of six housekeeping genes. The phylogenetic groups and subclusters are given on the right. Bootstrap values are given above the nodes where values are higher than 60% (bootstrap values from the maximum-parsimony analysis are in parentheses). The serogroup of each strain (when known) is given in parentheses. The clinical origins of ExPEC strains are coded by colors and letters as follows: A (red), avian colibacillosis; M (purple), neonatal meningitis; S (blue), septicemia; U (green), urinary tract infection; no letter (black), strain from a healthy host. The animals from which the six nonpoultry isolates originated are given. *E. coli* EDL933 and SAKAI are EHEC strains.
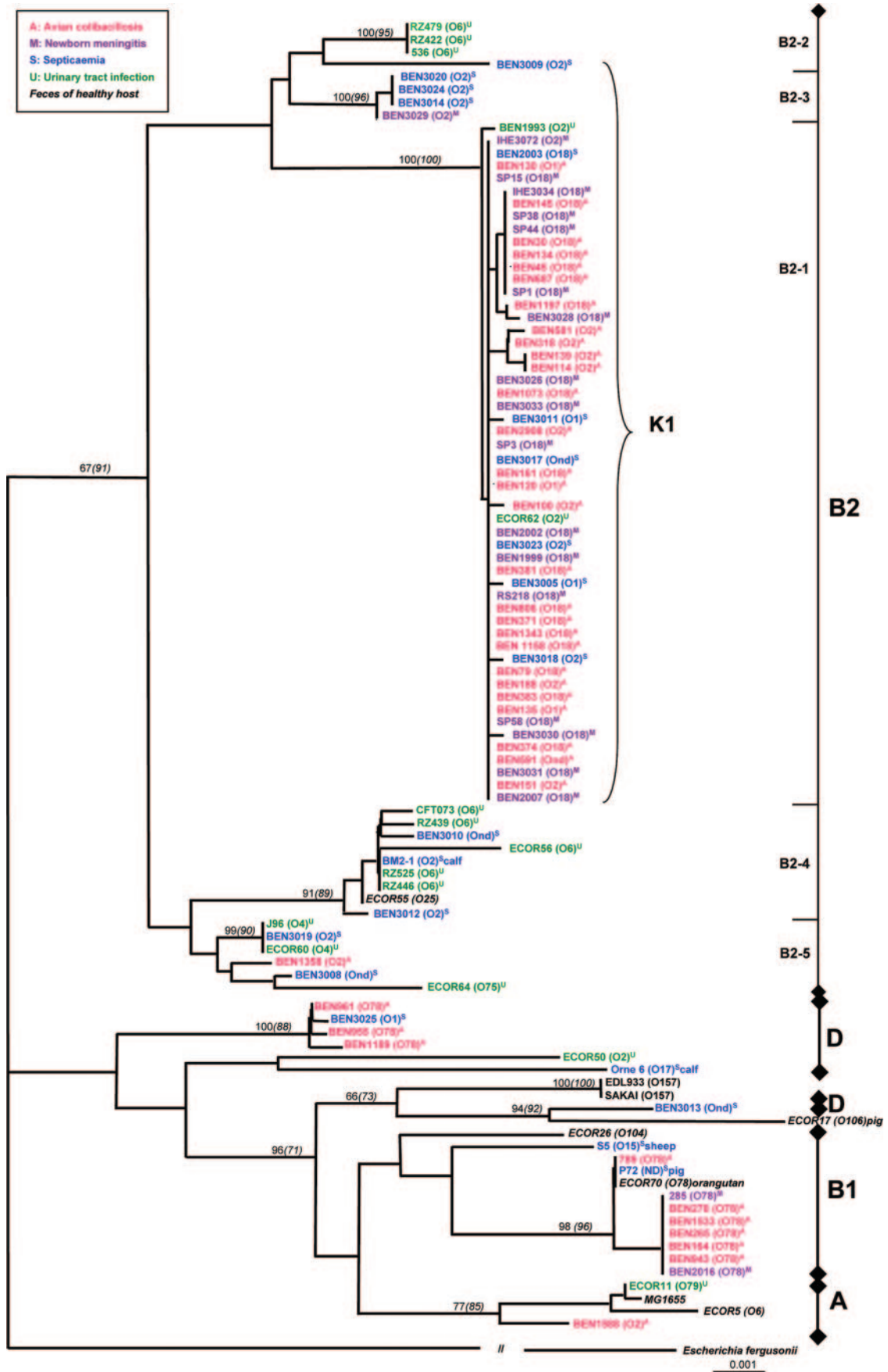
tive-control strains were *E. coli* MG1655 and the nonpathogenic *E. coli* avian strain EC79 (6, 14).

MLST. The phylogenetic relationships between strains were studied using the MLST method initially described by Maiden et al. (36). Six housekeeping genes were chosen according to the method of Adiri et al. (2): *adk* (adenylate kinase), *gcl* (glyoxylate carboligase), *zwf* (also commonly referred to as *gdh*) (glucose-6-phosphate dehydrogenase), *mdh* (malate dehydrogenase), *metA* (homoserine transsuccinylase), and *ppk* (polyphosphate kinase). The corresponding primers are listed in Table 1. DNA fragments (600 to 800 bp) were amplified in a Perkin-Elmer 9700 temperature cycler (Applied Biosystems) in a total volume of 50 µl containing 8 µl of DNA crude extract as a template, 0.5 U of *Taq* DNA polymerase (Promega), 25 pmol of the forward and reverse primers, and 5 nmol of each deoxynucleoside triphosphate (Promega) in 1× buffer. PCR conditions were as follows: 94°C for 5 min; 30 cycles of 94°C for 40 s, 58°C (or 59°C) for 45 s, and 72°C for 45 s; and a final extension at 72°C for 5 min. For strains CFT073, EDL933, and SAKAI, sequences were extracted from the GenBank database. The amplicons were sequenced on both strands by Genome Express (Meylan, France). No *gcl* fragment could be amplified by PCR from the *Escherichia fergusonii* strain, and recent sequencing of the whole genome of *E. fergusonii* (strain ATCC 35469[T]) confirmed the absence of *gcl* from this strain (E. Denamur, personal communication).

Sequences were then aligned using Clustal X (58), and a maximal common readable sequence was defined for each gene: *adk* (451 bp), *gcl* (534 bp), *zwf* (530 bp), *mdh* (468 bp), *metA* (472 bp), and *ppk* (545 bp). Sequences of each strain were then concatenated.

Phylogenetic analysis. The PILEUP program (Genetics Computer Group, Madison, WI) was used for multiple sequence alignments and the phylogenetic inference package PHYLIP for phylogenetic analyses (20). Phylogenetic relationships were inferred using (i) the DNAPARS program, based on the principle of maximum parsimony, and (ii) the DNADist program (for estimation of the distances between pairs of sequences) followed by neighbor-joining programs (building of the trees) based on the principle of phenetics. Bootstrap support percentages were calculated for each branch point of the tree by the Seqboot procedure (100 replicates), and the majority-rule consensus tree was determined by the CONSENSE program. Trees were plotted using DRAWGRAM.

Statistical analysis. Prevalences of VFs in the various phylogenetic groups were compared using a chi-square test. *P* values of ≤0.02 were considered significant.

A factorial analysis of correspondences (FCA) was undertaken to describe associations between the presence of virulence genes, the phylogenetic group, and the clinical and avian origin of the strains by using SPAD 6.0 software (Decisia, France). Only strains from chickens (39 strains) and humans (55 strains, excluding EHEC strains) were included in this analysis. The informative variables retained and used for calculations included the presence or absence of 16 genes or genomic fragments (*neuC, sfa/foc, papC, felA, fimA*MT78, *ibeA, iutA, tsh, cdt, iroD* [D11], *hlyF, stgC*, A9, D1, D7, and D10). Each strain was defined by its coordinates, these coordinates being the values obtained for each variable for this strain. A modality was defined as a possible value for each variable (e.g., *ibeA* = 0 or *ibeA* = 1), and the coordinates of each modality correspond to whether or not it was observed for each of the 94 strains. The coordinates obtained for each strain and each modality were transformed using matrices into new coordinates on several factorial axes. The variance of the distribution of the strains or modalities was calculated for each factorial axis. Factorial axes were then ordered by decreasing level of variance. Results are represented graphically in a plane defined by two axes: F1, which accounts for most of the variance, and F2, which accounts for the largest part of the variance not accounted for by F1. Data concerning the clinical and animal origins of the strains and the phylogenetic groups of the strains were considered supplementary characteristics and projected on the F1/F2 plane.

Nucleotide sequence accession numbers. The sequences of the housekeeping genes used in this study have been deposited in GenBank and are available under the following accession numbers: DQ999496 to DQ999598 (*adk*), DQ999291 to DQ999392 (*gcl*), DQ999188 to DQ999290 (*zwf* [*gdh*]), EF011985 to EF012087 (*mdh*), DQ999085 to DQ999187 (*metA*), and DQ999393 to DQ999495 (*ppk*).

RESULTS

Location of APEC strains in the *E. coli* population. We used PCR-based phylotyping to classify the 102 *E. coli* strains studied into the four main phylogenetic groups of *E. coli* (8). Results for strains of the ECOR collection tested corre-

sponded to their previously reported phylogenetic classifications, with the exception of strain ECOR70, which belongs to phylogenetic group B1 (25) but was identified as A by the PCR-based phylotyping method, as already described by Clermont et al. (8).

Most of the strains studied belonged to phylogenetic group B2 (76 strains), as expected for ExPEC strains (31, 46); some were in phylogenetic group D (7 strains: BEN955, BEN961, BEN1189, BEN3013, BEN3025, ECOR50, and Orne6). PCR-based phylotyping assigned only 2 strains to phylogenetic group B1 (ECOR26 and S5), and 15 strains, including strain ECOR70, showed a "group A profile": the absence of *chuA* and of TspE4.C2 and the presence of *yjaA*.

The sequences of six housekeeping genes (*adk, gcl, zwf, mdh, metA*, and *ppk*) were determined and used to generate phylogenetic trees by the neighbor-joining method (Fig. 1) and the maximum-parsimony method. Irrespective of the method used, similar main clusters of strains were consistently identified with high bootstrap values. The phylogenetic tree obtained by the neighbor-joining method presented a large cluster of 76 strains (bootstrap value, 67) comprising all strains of phylogenetic group B2 distributed into five subclusters. The main subcluster, referred to as B2-1 (bootstrap value, 100), included 28 avian and 25 human ExPEC strains belonging to serotypes O18:K1, O2:K1, and O1:K1. The human ExPEC strains were from cases of septicemia (6 strains), newborn meningitis (17 strains), or urinary tract infections (2 strains). They include the archetypal NMEC strains RS218 and IHE3034 and the reference strain ECOR62. The phylogenetic distance between these strains is close to zero.

Other subclusters in the B2 phylogenetic group contained only human strains, with the exception of one APEC strain in subcluster B2-5. Subcluster B2-2 contained three human strains (serogroup O6) from urinary tract infections, including the archetypal UPEC strain 536 (bootstrap value, 100). Another subcluster, named B2-3, included four human strains of the O2:K1 serotype, isolated mainly from patients with septicemia or newborn meningitis (bootstrap value, 100). Subclusters B2-4 and B2-5 included human strains isolated from patients with septicemia or urinary tract infections. UPEC archetypal strains CFT073 (serogroup O6) and J96 (serogroup O4) were in B2-4 and B2-5, respectively.

Another subcluster with a high bootstrap value (100) comprised strains belonging to phylogenetic group D; it included three O78 APEC strains (BEN955, BEN961, and BEN1189) and a strain from a human with septicemia of respiratory origin (BEN3025). Thus, the PCR-determined phylogenetic groups B2 and D and the locations of strains in the phylogenetic trees were in good agreement.

However, strains ECOR50 and Orne 6, identified as belonging to phylogenetic group D by the PCR-based phylotyping method, were not classified by any analysis into the same subcluster as other D strains. They may belong to a different subcluster of phylogenetic group D, but no sentinel strain was used that could allow allocating them to one of the D subgroups as recently defined by Johnson et al. (30). EHEC strains EDL933 and SAKAI showed a "phylogenetic group D pattern," as usually observed when the PCR-based phylotyping method of Clermont et al. (8) is used.

Reference strains of phylogenetic group A (ECOR5,

ECOR11, and MG1655) were clustered with the APEC strain BEN1588 (phylogenetic group A) with a bootstrap value of 77. Strain ECOR17, identified as belonging to phylogenetic group A both by multilocus enzyme electrophoresis (53) and by PCR-based phylotyping (this study), was not clustered with other group A reference strains by our MLST method.

An interesting cluster (bootstrap value, 98) contained 10 strains, including strain ECOR70 (serogroup O78), 8 O78 strains of human and avian origins, and a strain from a pig with septicemia (*E. coli* P72). All these strains were first identified as belonging to phylogenetic group A by PCR-based phylotyping. Misclassification into other phylogenetic groups of some B1 strains, in particular strain ECOR70, has been reported by this phylotyping method (8), so we investigated these strains in more detail. Lymberopoulos et al. (35) have demonstrated that the presence of the *stgC* gene is significantly associated with phylogenetic groups B1 and D and not with groups A and B2; we therefore tested for the presence of the *stgC* gene in the 102 *E. coli* strains of the study. The results confirmed the observations of Lymberopoulos et al. (35): none of the strains of phylogenetic group B2 possessed the *stgC* gene, whereas strains ECOR26 and ECOR70 (phylogenetic group B1) did. The 10 strains of the "ECOR70 cluster," initially assigned to phylogenetic group A, all possessed the *stgC* gene, whereas ECOR5, ECOR11, and MG1655, known to belong to phylogenetic group A, and strain BEN1588 did not. Thus, the 10 strains of the "ECOR70 cluster" were finally assigned to phylogenetic group B1 rather than A (Fig. 1).

APEC strains of serogroups O1, O2, and O18 were clearly differentiated from O78 strains: 28 of the 30 APEC strains of serogroups O1, O2, and O18 belonged to phylogenetic group B2 (subcluster B2-1; bootstrap value, 100), whereas the 9 APEC strains of serogroup O78 were grouped into two subclusters with high bootstrap values, in phylogenetic groups D (3 strains) and B1 (6 strains). Human ExPEC strains appeared to be closely related to APEC strains in each of these three subclusters.

The virulence genotype correlates with the phylogenetic classification of the strains. We tested for the presence of 13 VF genes commonly found in ExPEC strains and of 5 genomic fragments putatively associated with the virulence of ExPEC isolates (51). Differences in the incidence of each individual VF gene between the main phylogenetic group in our study (B2) and other phylogenetic groups (A, B1, and D) were tested using the chi-square test: the presence of *fimA*$_{MT78}$, *sfa/foc*, *neuC*, *ibeA*, *cdt*, A9, D1, D7, D10, and *iroD* (D11) was significantly more frequent ($P \leq 0.02$) in B2 strains than in strains belonging to phylogenetic groups A, B1, and D (Table 2). A theoretical virulence index (VI) was calculated for each group by scoring the total number of VF genes present (*fimA*, *sfa/foc*, *papC*, *neuC*, *iutA*, *iroD*, *ibeA*, *tsh*, *cdt*, *hlyF*, A9, A12, D1, D7, D10) and then dividing by the number of strains in the group. The VI was higher for phylogenetic group B2 (11.3/15) than for other groups (5.6/15).

FCA was also used to search for associations of multiple combinations of VF genes with the phylogenetic status of strains and with their origin (human or avian). FCA confirmed that strains of phylogenetic group B2 were clearly differentiated from strains of the other groups and were tightly associated with the presence of VF genes *sfa/foc*, *neuC*, A9, D1, D7,

TABLE 2. Distribution of VF genes in the phylogenetic groups for 100 *E. coli* isolates of different origins[a]

VF gene	No. (%) of strains positive for the indicated VF gene			
	Phylogenetic groups		B2 phylogenetic subclusters	
	B2	A, B1, or D	B2-1	Other B2 subclusters
fimA	76 (100)	23 (96)	53 (100)	23 (100)
fimA$_{MT78}$	46 (61)[b]	2 (8)	44 (83)[b]	2 (9)
fimH	76 (100)	24 (100)	53 (100)	23 (100)
sfa/foc	47 (62)[b]	3 (13)	30 (57)	17 (74)
papC	34 (45)	6 (25)	18 (34)[b]	16 (70)
felA	17 (22)	4 (17)	17 (32)	0
neuC	62 (82)[b]	0	53 (100)[b]	9 (39)
iutA	60 (79)	20 (83)	50 (94)[b]	10 (43)
iroD (D11)	72 (95)[b]	18 (75)	52 (98)	20 (87)
ibeA	40 (53)[b]	0	37 (70)[b]	3 (13)
tsh	24 (32)	8 (33)	22 (42)[b]	2 (9)
cdt	27 (36)[b]	2 (8)	24 (45)[b]	3 (13)
hlyF	52 (68)	16 (67)	50 (94)[b]	2 (9)
A9	64 (84)[b]	1 (4)	50 (94)	14 (61)
A12	76 (100)	20 (83)	53 (100)	23 (100)
D1	73 (96)[b]	5 (21)	50 (94)	23 (100)
D7	75 (99)[b]	6 (25)	52 (98)	23 (100)
D10	76 (100)[b]	7 (29)	53 (100)	23 (100)

[a] Isolates were from human clinical extraintestinal sources ($n = 51$), avian colibacillosis ($n = 39$), nonpoultry animal cases of septicemia ($n = 4$), and healthy hosts ($n = 6$). A VI was calculated for each phylogenetic subcluster by adding the number of strains positive for *fimA*, *sfa/foc*, *papC*, *neuC*, *iutA*, *iroD* (D11), *ibeA*, *tsh*, *cdt*, *hlyF*, A9, A12, D1, D7, and D10, and dividing by the total number of strains in the group. The VIs were 11.3 for phylogenetic group B2; 5.6 for A, B1, or D; 12.2 for the B2-1 subcluster; and 9.2 for other B2 subclusters.

[b] The incidence of the corresponding gene was significantly different ($P \leq 0.02$) between phylogenetic groups or subclusters as assessed by the chi-square test.

and D10 and the absence of *stgC* (Fig. 2). Among human and avian isolates of phylogenetic group B2, those associated with serogroup O18 were also associated with the same VF genes. By contrast, human isolates from urinary tract infections that were associated with serogroups O4 and O6 showed characteristics different from those of avian colibacillosis isolates (Fig. 2). FCA confirmed that APEC strains belonging to phylogenetic group B2 were associated with VF genes different from those of APEC strains of phylogenetic group B1 (Fig. 2).

Avian and human strains in subcluster B2-1 could not be differentiated by their virulence genotypes. The major subcluster in the phylogenetic tree (B2-1, with 53 strains) appeared to be a homogeneous group of human and avian strains with very low, sometimes even undetectable phylogenetic distances between strains (Fig. 1). Thus, we investigated if the B2-1 subcluster was associated with a particular genotype, compared with other B2 subclusters, and if avian and human strains could be differentiated by their virulence genotypes. The frequencies of various VF genes in the B2-1 subcluster—*fimA*$_{MT78}$ (83%), *neuC* (100%), *iutA* (94%), *ibeA* (70%), *tsh* (42%), *cdt* (45%), and *hlyF* (94%)—were higher than their frequencies in other B2 subclusters. Other VF genes were significantly less frequent in subcluster B2-1 than in other B2 subclusters (for example, *papC* [34%]) (Table 2). When present on strains of the B2-1 subcluster, P fimbriae were of the F11 variant type, as shown by the presence of the *felA* gene. The B2-1 subcluster included avian ($n = 28$) and human ($n = 25$) strains that did not differ
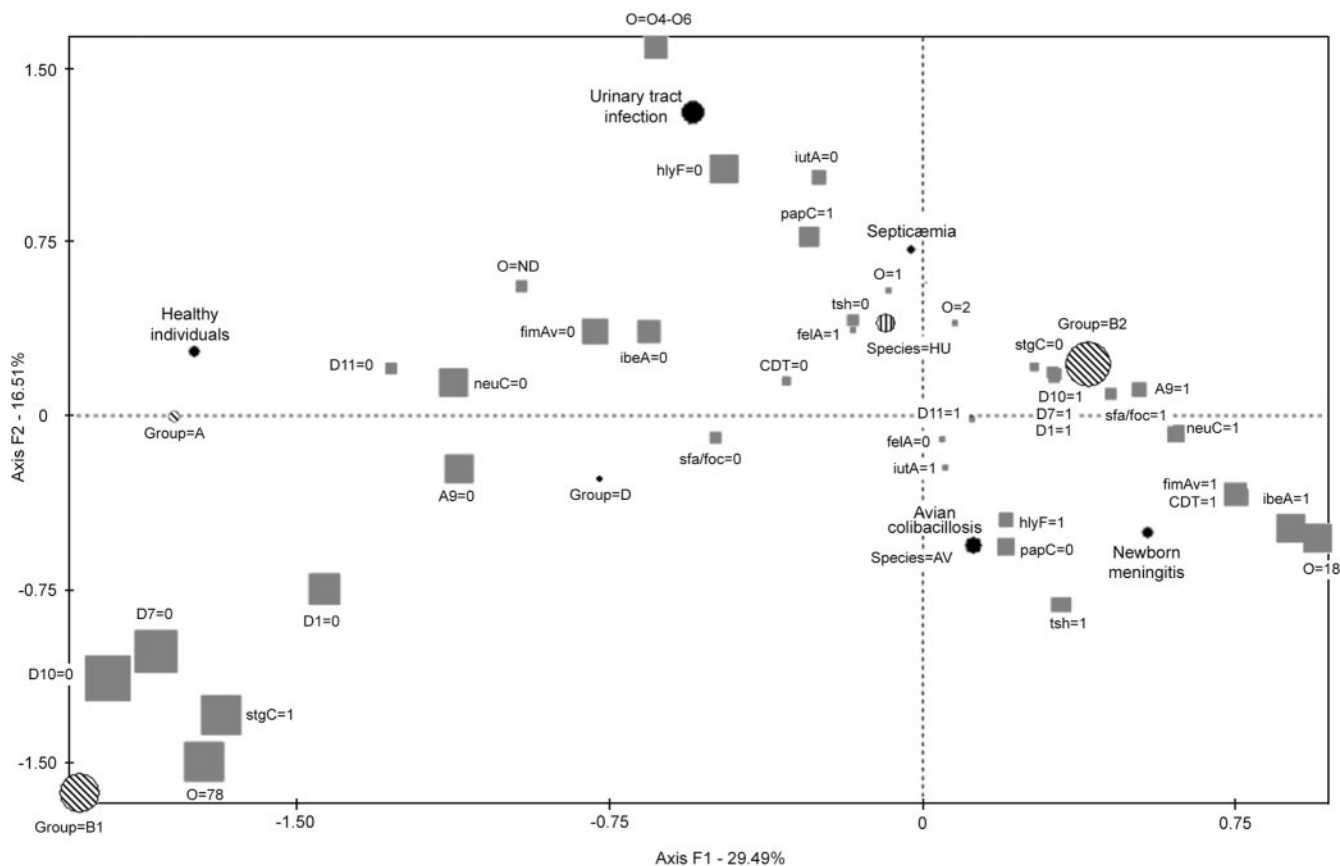
FIG. 2. Association of VFs with phylogenetic groups in *E. coli* strains originating from human clinical extraintestinal sources ($n = 51$), feces of healthy humans ($n = 4$), or avian colibacillosis ($n = 39$). In this schematic representation of the FCA, the distributions of VFs and major serotypes are represented on a factorial plane defined by the most discriminating axes: F1 and F2. Factorial axis F1 accounts for 29.49% of the variance, and factorial axis F2 accounts for 16.51% of the remaining variance. The strength of the associations is indicated by the distance between the symbols and the sizes of the symbols (the larger the size of the symbol, the stronger the association). The species of the host (avian or human), phylogenetic group (A, B1, B2, or D), clinical origin (avian colibacillosis, neonatal meningitis, septicemia, urinary tract infection, or healthy individual), O serogroup, and VFs are indicated. The presence or absence of a virulence factor is indicated by "1" or "0," respectively.

significantly in the incidence of any of the VF genes studied (data not shown). These results were confirmed by FCA, which demonstrated that among human and avian isolates of phylogenetic group B2, those associated with serogroup O18 (which belonged to the B2-1 subcluster) were also associated with the same VF genes. Conversely, human isolates from urinary tract infections that were associated with serogroups O4 and O6 (which belonged to subclusters B2-2, B2-4, and B2-5) showed characteristics different from those of avian colibacillosis isolates (Fig. 2).

Other VF genes commonly assessed in studies of human-source ExPEC, such as *hlyA* and *cnf*, are infrequently present on *E. coli* isolates from neonatal meningitis and avian colibacillosis cases (29, 39, 61) and thus were not systematically tested in the present study. However, in phylogenetic subcluster B2-1, all 28 avian isolates were *hlyA* negative and 27 were *cnf* negative while 15 of 17 human isolates tested were negative for both genes (data not shown).

Human ExPEC strains of subcluster B2-1 were highly virulent for chickens. We tested the hypothesis of host specificity of human B2 strains by determining their virulence for chickens in several models of infection.

All ExPEC strains of phylogenetic group B2 were lethal when used to inoculate 1-day-old chicks subcutaneously: 72 of the 76 B2 strains killed 5/5 chicks, 2 strains killed 4/5 chicks (ECOR55 and CFT073), and 2 strains killed 1 or 2 chicks (RZ525 and ECOR56). The LD_{50}s for 1-day-old chicks were also calculated for 25 strains of phylogenetic group B2: two groups of strains could be differentiated on the basis of the LD_{50} (Fig. 3). Strains of phylogenetic subcluster B2-1 were more virulent than strains of other B2 subclusters (Fig. 3). Of 17 strains belonging to phylogenetic subcluster B2-1, 14 were highly virulent (LD_{50}, $\leq 1.20E + 04$ CFU). All these strains belonged to serotype O18:K1 or O2:K1 and were isolated in cases of meningitis, septicemia, urinary tract infection, or avian colibacillosis. Three strains showed lower virulence levels: RS218, IHE3034, and BEN79, with LD_{50}s between $5.10E + 05$ and $2.10E + 06$ CFU. Of the eight strains of other B2 phylogenetic subgroups that had been isolated from humans with septicemia or urinary tract infections, all but one (BEN3012) were less virulent (LD_{50}, $>1.0E + 06$ CFU) than strains of phylogenetic subcluster B2-1. Most belonged to serogroup O6, which is infrequently isolated in cases of avian colibacillosis.
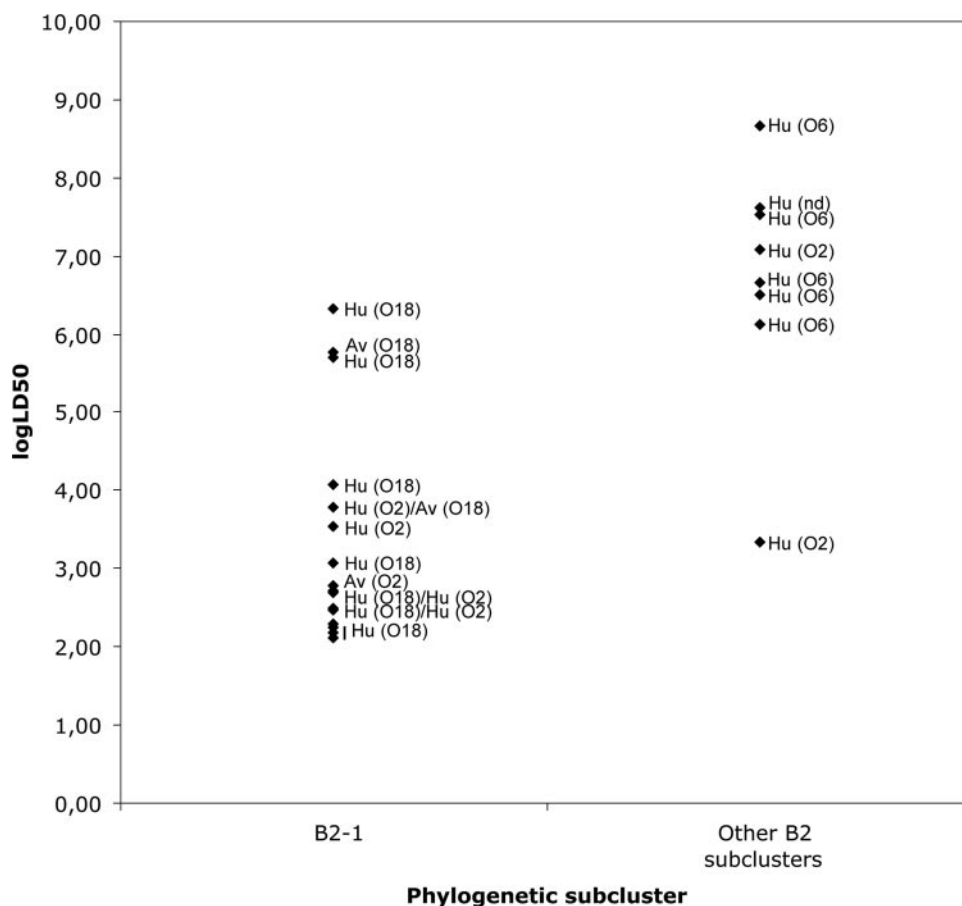
FIG. 3. LD_{50}s for chicks of human ($n = 22$) and avian ($n = 3$) ExPEC strains of phylogenetic group B2. Decimal dilutions of bacterial cultures of ExPEC strains of human and avian origins were inoculated into 1-day-old chicks, and the lethality was recorded. The virulence of the $E.$ $coli$ strains was expressed as the LD_{50} for chicks; the log LD_{50} is represented. The O serogroups and origins (Hu, human; Av, avian) of the strains are given.

Because the lethality test on 1-day-old chicks bypasses the first steps of natural infection in chickens (which usually starts in the respiratory tract), we tested the pathogenicities of nine human strains by using bacterial cultures to inoculate the thoracic air sacs of 3.5-week-old SPF chickens. Three APEC strains and one nonpathogenic strain of avian origin were also used for comparison. Human isolates of phylogenetic subcluster B2-1 caused a typical avian colibacillosis similar to that caused by avian strains, and the criteria used to assess avian colibacillosis (bacterial contamination of air sacs, blood, pericardial fluid, liver, and typical fibrinous lesions) were fulfilled (Table 3). In contrast, only a few chickens developed the disease when inoculated with the archetypal human strains RS218 and IHE3034, showing few lesions and no contamination of pericardial fluid or the liver. This result may be due to the fact that strains RS218 and IHE3034 have undergone numerous subcultures, possibly resulting in genetic modifications and partial loss of virulence properties, as is sometimes observed for intensively used reference strains.

Neither pathogenicity for chicks nor MLST and virulence genotyping data allowed us to distinguish APEC strains from human ExPEC strains belonging to the highly pathogenic sub-

cluster B2-1; thus, we could find no evidence of host specificity for strains belonging to this subcluster.

DISCUSSION

In this study we used two approaches, PCR-based phylotyping and MLST, to establish the phylogenetic positions of 39 APEC strains belonging to the prevalent serogroups O1, O2, O18, and O78 in relation to 63 $E.$ $coli$ strains of human and other mammalian origins. The positions of the 102 strains studied in the phylogenetic trees reconstructed from MLST analysis were consistent with results obtained by PCR-based phylotyping, with a few exceptions. Strain ECOR17, identified as belonging to phylogenetic group A both by multilocus enzyme electrophoresis (44) and by PCR-based phylotyping (this study), was not clustered with other A strains by use of our MLST method. As discussed by Johnson et al. (30), this could be a consequence of recombined genes or gene fragments at the loci we used for MLST. Strain ECOR70, originally identified as belonging to phylogenetic group B1 (44), and the other O78 strains located in the same cluster by MLST analysis were first identified as belonging to group A by the

TABLE 3. Pathogenicities of ExPEC strains of different clinical origins for SPF chickens following intra-air sac inoculation[a]

E. coli strain	Phylogenetic subcluster	Clinical origin[b]	Serotype	% of chickens with contamination in:				Lesion score/8 (SD)	Death (%)
				Air sacs	Blood	Pericardial fluid	Liver		
SP15	B2-1	Hu M	O18:K1	100	100	100	100	7.0 (0.6)	41.6
BEN3031	B2-1	Hu M	O18:K1	100	100	100	100	5.8 (1.2)	0
BEN3033	B2-1	Hu M	O18:K1	100	100	100	91.7	5.1 (0.8)	33.3
SP3	B2-1	Hu M	O18:K1	75	50	58.3	75	3.6 (3.1)	33.3
IHE3034	B2-1	Hu M	O18:K1	50	33.3	33.3	16.7	2.6 (1.8)	0
RS218	B2-1	Hu M	O18:K1	11.1	5.5	5.5	5.5	1.6 (1.2)	0
BEN79	B2-1	Av C	O18:K1	91.7	83.3	83.3	100	4.9 (0.8)	25
BEN374	B2-1	Av C	O18:K1	100	100	100	100	7.1 (0.9)	30
IHE3072	B2-1	Hu M	O2:K1	100	100	100	100	6.2 (1.0)	25
BEN2908	B2-1	Av C	O2:K1	100	100	100	100	5.0 (1.6)	37.5
BEN3009	B2-2	Hu S[c]	O2:K1	25	0	0	0	1.5 (1.2)	0
BEN3012	B2-4	Hu S[d]	O2:K1	100	58.3	100	100	4.8 (0.8)	0
EC79[e]	A	Av healthy	O2	6	0	0	0	0.5	0

[a] Groups of 12 chickens were inoculated in the air sacs with bacterial cultures of E. coli strains. Bacterial colonization of internal organs, fibrinous lesions, and deaths were recorded.
[b] Strains were from newborn meningitis (n = 7), human septicemia (n = 2), and avian colibacillosis (n = 3). Hu, human origin; Av, avian origin; M, newborn meningitis; S, septicemia; C, avian colibacillosis.
[c] Isolated from an adult with a urinary tract infection.
[d] Isolated from a newborn with septicemia.
[e] Nonpathogenic E. coli strain of avian origin used as a negative control (14).

PCR-based phylotyping method. As discussed by Clermont et al. (8), the PCR method may fail to identify some B1 strains, so we used the association between the presence of the *stgC* gene, serogroup O78, and phylogenetic group B1 (35) to demonstrate that this cluster of strains was more likely to belong to phylogenetic group B1. Moreover, this result is in agreement with the O78 clade described by Johnson et al. (30), which includes strain ECOR70 and an O78 *E. coli* strain from a case of human bacteremia. Thus, testing for the *stgC* gene could be a useful complement to the PCR-based phylotyping method of Clermont et al. (8) for the discrimination of strains belonging to phylogenetic groups B1 and A.

Comparison of the phylogenetic trees we obtained with those published in previous studies revealed similar subclusters of strains according to various sentinel strains, especially in the B2 phylogenetic group. The four important clades described by Johnson et al. (30) in group B2 were detected as B2-1 (strains RS218, IHE3034, and ECOR62), B2-2 (strain 536), B2-4 (strains CFT073 and ECOR56), and B2-5 (strains J96 and ECOR60). Similar clades in the B2 group were also differentiated by Escobar-Páramo et al. and Hommais et al. (18, 26).

Most of the APEC strains in this study (29/39) belong to phylogenetic group B2, and fewer belong to the B1, D, and A phylogenetic groups (6 strains, 3 strains, and 1 strain, respectively). This distribution does not necessarily reflect the incidence of the four main phylogenetic groups in the APEC population, because common serogroups (O1, O2, and O18) of avian and human ExPEC strains were overrepresented in our sample, and serogroup O78, which is prevalent among avian colibacillosis isolates in numerous countries (19, 42, 47), was underrepresented.

Only three major phylogenetic clusters could be identified among APEC strains of the predominant serogroups included in our study: B1, B2, and D. This finding is consistent with previous observations of a few clone complexes of APEC iso-

lates (39, 63, 65). We demonstrated an association between the phylogenetic clusters of APEC strains and their serogroups. Although the O1, O2, and O18 APEC strains originated from various geographical locations (France, Spain, and Belgium) and had been isolated over a 10-year period (and can thus be considered representative of APEC strains), they were very closely related: all but one were clustered in the same phylogenetic subcluster (B2-1). Also, APEC strains of serogroup O78 all fell into phylogenetic groups B1 and D. This agrees with the observation of two phylogenetic clusters of O78 APEC strains by Adiri et al., using MLST analysis (2); it also clearly demonstrates that O78 strains are phylogenetically different from O1, O2, and O18 APEC strains. A similar pattern of differences was also observed by virulence genotyping: the three clusters of APEC strains were associated with different sets of virulence genes. As already reported by Mokady et al. and by Ron, O2 and O78 APEC strains differ with respect to several VFs (38, 49).

Phylogenetic trees reconstructed from MLST analysis demonstrated close relationships between human and avian ExPEC strains belonging to the same phylogenetic group, whether B1, B2, or D. The few strains in groups B1 and D did not allow useful analysis, and further investigations are currently under way with a larger sample of O78 *E. coli* strains to analyze the relationships between human and avian strains in these groups.

However, phylogenetic group B2 included 28 APEC strains and 25 human ExPEC strains, all in a single, highly homogeneous subcluster designated B2-1: human and avian ExPEC strains belonging to this subcluster were highly virulent for chickens and possessed the same major VFs ($fimA_{MT78}$, *neuC*, *iutA*, *ibeA*, *tsh*, *cdt*, and *hlyF*). These results suggest that some particular subclusters of strains within the ExPEC population have little or no host specificity. As already suggested by Johnson et al. (28), this highly pathogenic clonal group including O1:K1, O2:K1, and O18:K1 *E. coli* strains isolated in cases of

neonatal meningitis, septicemia, or urinary tract infections, as well as avian colibacillosis, contradicts the classical definition of pathotypes as host specific in the case of ExPEC isolates.

Whereas we could demonstrate the virulence of human strains for chickens, it is difficult to assess the virulence of avian strains for humans. However, previous studies have shown that APEC isolates are lethal for mice when inoculated intraperitoneally (10). Moreover, experimental models of mammalian infections, such as those developed by Skyberg et al. and Johnson et al., indicate that APEC strains are able to express pathogenicity for mammals (27, 55).

According to our results, avian and human strains belonging to subcluster B2-1 can be indistinguishable in terms of phylogenetic location and the presence of VFs. Moreover, no host specificity could be shown: avian and human strains of this phylogenetic subcluster were highly and similarly virulent for chickens. These results are in favor of the possibility that *E. coli* strains of avian origin are virulent for humans and consequently constitute a zoonotic risk.

ACKNOWLEDGMENTS

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