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Pathotype and Antibiotic Resistance Gene Distributions of *Escherichia coli* Isolates from Broiler Chickens Raised on Antimicrobial-Supplemented Diets▽†

Claudie Bonnet,[1] Fatoumata Diarrassouba,[2] Roland Brousseau,[1,3] Luke Masson,[1,3] Edward Topp,[4] and Moussa S. Diarra[2]*

National Research Council of Canada, Biotechnology Research Institute, Montreal, Quebec, Canada H4P 2R2[1]; Pacific Agri-Food Research Centre, Agriculture and Agri-Food Canada, Agassiz, British Columbia, Canada V0M 1A0[2]; Département de Microbiologie et Immunologie, Université de Montréal, Montréal, Québec, Canada H3T 1J42[3]; and Southern Crop Protection and Food Research Centre, Agriculture and Agri-Food Canada, London, Ontario, Canada N5V 4T3[4]

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The impact of feed supplementation with bambermycin, monensin, narasin, virginiamycin, chlortetracycline, penicillin, salinomycin, and bacitracin on the distribution of *Escherichia coli* pathotypes in broiler chickens was investigated using an *E. coli* virulence DNA microarray. Among 256 *E. coli* isolates examined, 59 (23%) were classified as potentially extraintestinal pathogenic *E. coli* (ExPEC), while 197 (77%) were considered commensal. Except for chlortetracycline treatment, the pathotype distribution was not significantly different among treatments ($P > 0.05$). Within the 59 ExPEC isolates, 44 (75%) were determined to be potentially avian pathogenic *E. coli* (APEC), with the remaining 15 (25%) considered potentially "other" ExPEC isolates. The distribution within phylogenetic groups showed that 52 (88%) of the ExPEC isolates belonged to groups B2 and D, with the majority of APEC isolates classified as group D and most commensal isolates (170, 86%) as group A or B1. Indirect assessment of the presence of the virulence plasmid pAPEC-O2-ColV showed a strong association of the plasmid with APEC isolates. Among the 256 isolates, 224 (88%) possessed at least one antimicrobial resistance gene, with nearly half (107, 42%) showing multiple resistance genes. The majority of resistance genes were distributed among commensal isolates. Considering that the simultaneous detection of antimicrobial resistance *tet*(A), *sulI*, and bla_{TEM} genes and the integron class I indicated a potential presence of the resistance pAPEC-O2-R plasmid, the results revealed that 35 (14%) of the isolates, all commensals, possessed this multigene resistance plasmid. The virulence plasmid was never found in combination with the antimicrobial resistance plasmid. The presence of the ColV plasmid or the combination of *iss* and *tsh* genes in the majority of APEC isolates supports the notion that when found together, the plasmid, *iss*, and *tsh* serve as good markers for APEC. These data indicate that different resistant *E. coli* pathotypes can be found in broiler chickens and that the distribution of such pathotypes and certain virulence determinants could be modulated by antimicrobial agent feed supplementation.

Several classes of antimicrobial agents, such as glycolipids (bambermycin), cyclic peptides (bacitracin), ionophores (monensin and salinomycin), streptogramins (virginiamycin), and β-lactams (penicillin), are widely used as food additives in modern animal husbandry to prevent infections and promote growth (6). Increasing antimicrobial resistance in animals and its potential threat to human health led to the ban of bacitracin, spiramycin, tylosin, and virginiamycin as feeding additives by the European Union in 1999 (7, 46). Although this precautionary measure is still controversial because of being seen as having a negligible impact on human health, negative consequences for animal health and welfare, including economic losses for farmers, were subsequently observed in Europe (7). In stark contrast, however, the ban has been beneficial in reducing the total quantity of antibiotics administered to food animals (7, 47). Under good production conditions and correct use of antibiotics, poultry production is reported to be competitive (14, 47, 48) and even beneficial in reducing antimicrobial resistance in important food animal reservoirs and thus the potential threat to public health (48).

Escherichia coli is generally considered a commensal member of the normal gastrointestinal microflora in humans and animals, yet some strains are known to cause serious morbidity and mortality. The expression of various virulence factors, which affect cellular processes, can result in different clinical diseases, such as cystitis, pyelonephritis, sepsis/meningitis, and gastroenteritis. The possession of different virulence gene subsets can further define the *E. coli* pathotype (31). The extraintestinal pathogenic *E. coli* (ExPEC) strains are epidemiologically and phylogenetically distinct from both intestinal pathogenic and commensal strains (43). In North America, annually, several million cases of urinary tract infections, abdominal infections, pelvic infections, pneumonia, meningitis, and sepsis are caused by ExPEC (42). In poultry production, avian pathogenic *E. coli* (APEC) is responsible for significant economic losses. APEC strains induce extraintestinal diseases such as air sacculitis, colibacillosis, polysorositis, and sep-

* Corresponding author. Mailing address: P.O. Box 1000, 6947 Highway 7, Agassiz, BC, Canada V0M 1A0. Phone: (604) 796-2221. Fax: (604) 796-0359. E-mail: Moussa.Diarra@agr.gc.ca.

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ticemia in birds (9, 21, 22, 31, 35, 45). Although no specific set of virulence factors has been clearly linked to APEC strains, most identified virulence factors are similar to those frequently associated with ExPEC (36).

Bearing in mind that the avian intestinal environment has been considered a reservoir of *E. coli* having zoonotic potential (15) and the possible contamination of poultry products with such bacteria during slaughter, the impact of antimicrobial feeding additives on the distribution and dissemination of bacterial pathotypes and antibiotic resistance needs to be explored to address human, animal, and environmental health concerns. To this end, an *E. coli* DNA virulence microarray previously employed to assess the genotypes (virulence and antibiotic resistance genes) of *E. coli* strains isolated from different environmental ecosystems and from the chicken intestinal tract (1, 10, 19, 20, 33) was used. The aim of the present trial was to investigate the distributions of pathotypes and of virulence and antibiotic resistance genes in *E. coli* isolates from broilers fed with antimicrobial supplementation diets including bambermycin, penicillin, salinomycin, bacitracin, chlortetracycline, virginiamycin, monensin, and narasin.

MATERIALS AND METHODS

Broiler chickens and treatments. The study was performed in two different trials with 1,800 1-day-old male broiler chicks and was conducted under feed, trial design, and raising conditions similar to those previously described (10). The birds were placed in 36 pens (18/study; 50 birds per pen) that were assigned at random to 11 treatments, including one control group fed without antibiotics and 10 groups fed rations containing the following per kg of feed: trial I, 2 mg bambermycin, 2.2 mg procaine penicillin, 60 mg salinomycin, 55 mg bacitracin, and a combination of 55 bacitracin plus 60 mg salinomycin; and trial II, 110 mg chlortetracycline, 11 mg virginiamycin, 22 mg virginiamycin, 99 mg monensin, and 70 mg narasin. Biosecurity and hygienic practices were applied to both trials as previously described (10). All experimental procedures were approved by the Animal Care Committee of the Pacific Agri-Food Research Center and followed principles described by the Canadian Council on Animal Care.

Sample collection and bacteriological analysis. A total of 256 *E. coli* isolates (109 and 147 from trials I and II, respectively) were analyzed for pathotypes and antimicrobial resistance genes. *E. coli* isolates were obtained from the ceca, cloacae, and litter of broiler chickens from day 7 to day 28 to 36. The isolation method for these *E. coli* isolates has been previously reported (10). Briefly, samples were aseptically collected from 18 separate pens for each trial at each sampling time. Samples were then transferred to peptone buffer in sterile containers, placed on ice, and transported to the microbiology laboratory for bacteriological analysis carried out the same day. The total *E. coli* population was estimated using *E. coli* and coliform Petrifilms (3 M, St. Paul, MN) at each sampling time as previously described (11). Presumptive *E. coli* isolates from each treatment group (two colonies per pen) were purified on blood agar and confirmed as *E. coli* using API20E strips (BioMérieux, St-Laurent, QC, Canada) according to the manufacturer's specifications.

DNA extraction. *E. coli* isolates were grown overnight in 3 ml of brain heart infusion broth (Becton Dickinson, Sparks, MD) at 37°C, after which 200 μl of culture was transferred to 1.5-ml Eppendorf centrifuge tubes and centrifuged at 13,000 × *g* for 2 min. Supernatants were removed, and the bacterial pellets were resuspended in 200 μl of sterile water with vortexing. The suspension was boiled for 10 min and centrifuged for 5 min, after which 150 μl of the supernatant containing DNA was removed for testing.

***E. coli* DNA labeling.** Bacterial DNA was labeled using a Bioprime DNA labeling system (Invitrogen Life Technologies, Burlington, ON, Canada). Fifteen microliters of the supernatant containing DNA was added to a final volume of 32.5 μl containing 10 μl of a random primer solution, 0.5 μl of high-concentration DNA polymerase (Klenow fragment, 40 U/μl), 5 μl of a deoxyribonucleoside triphosphate mixture (1.2 mM dATP, 1.2 mM dGTP, 1.2 mM dTTP, and 0.6 mM dCTP in 10 mM Tris [pH 8.0] and 1 mM EDTA), and 2 μl of 1 mM Cy5-dCTP. Labeling reactions were performed in the dark at 37°C for 3.5 h and stopped by the addition of 5 μl of 0.5 M Na_2EDTA (pH 8.0). The labeled samples were then purified with a PureLink PCR purification kit (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. The amount of incorporated fluorescent Cy5 dye was then quantified by scanning the DNA sample with a NanoDrop ND-1000 spectrophotometer from 200 to 700 nm. Data were analyzed using a Web-based percent incorporation calculator (http://www.pangloss.com/seidel/Protocols/percent_inc.html).

DNA microarrays. The microarray used in this study was based on earlier published work (5) and carries 302 oligonucleotides of 70 bases in length targeting 264 virulence or virulence-related genes and 38 antimicrobial resistance or antimicrobial resistance-related genes found in gram-negative bacteria. The microarray, designed to detect a complete set of virulence genes representative of all *E. coli* pathotypes, includes genes for virulence factors such as adhesins, the locus of enterocyte effacement, colicins and microcins, toxins, iron acquisition and transport systems, capsular and somatic antigens, hemolysins, and hemagglutinins, as well as newly recognized or putative *E. coli* virulence genes. Antimicrobial resistance genes included in the microarray represent different antimicrobial families, such as β-lactams, aminoglycosides, tetracycline, phenicols, trimethoprim, sulfonamide, and class I integron. The microarray also carries five positive oligonucleotide controls for *E. coli* derived from the sequences of genes encoding tryptophanase (*tnaA*), beta-glucuronidase (*uidA*), lactose permease (*lacY*), beta-galactosidase (*lacZ*), and glutamate decarboxylase (*gad*). Negative controls added to this microarray consist of oligonucleotides derived from the gene sequences for the green fluorescent protein of *Aequorea victoria*, the lactose permease of *Citrobacter freundii*, and the chlorophyll synthase of *Arabidopsis thaliana*.

Hybridization of labeled DNA. Prehybridization and hybridizations were performed as previously described (19), with the following modifications: hybridizations were performed using a Slide Booster hybridization work station (model SB800; Advalytix, Germany), and scanning was performed using a ScanArray Lite fluorescent microarray analysis system (Perkin-Elmer, Mississauga, ON, Canada) with images scanned at 5-μm resolution and laser power set to 95%. Acquisition of fluorescent spots and quantification of fluorescent spot intensities were performed as described by Hamelin et al. (19).

Phylogenetic analysis and virulence genotyping. Following the method developed by Clermont et al. (8), based on the presence or absence of the *chuA* (outer membrane receptor protein for heme transport and utilization), *yjaA* and *tspE4.C2* genes, isolates were assigned to one of four *E. coli* phylogenetic groups (A, B1, B2, or D). Isolates were designated intestinal *E. coli* pathotypes based on the presence of specific virulence genes, such as locus of enterocyte effacement genes and heat-stable, heat-labile, or Shiga-like toxin-encoding genes (5). The ExPEC pathotype is not yet clearly defined, as different groups have assigned different genes to it. The criteria elaborated by Johnson et al. (25) were used to classify *E. coli* isolates as ExPEC. Isolates must possess two or more of the following virulence genes: *pap* (P fimbriae), *sfa* or *foc* (S/F1C fimbriae), *afa* or *dra* (Dr binding adhesins), *iutA* (aerobactin receptor), and *kpsM* II (group II capsule synthesis).

The precise set of virulence factors possessed by APEC is also not clearly established. For the purpose of this work and based on the literature on APEC-related virulence factors (9, 13, 17, 22, 34, 39), we chose to classify isolates as potentially APEC based on the presence of at least four of the five following functional genes or gene groups: *iss* (increased serum survival protein); *tsh* (temperature-sensitive hemagglutinin); P, F, or S fimbriae; iron acquisition systems (aerobactin receptor or yersiniabactin), and *kpsM* II (group II capsule synthesis). It should be considered, however, that no true genotype of APEC is generally agreed upon and that isolates cannot be stated as truly APEC unless they have been proven to be infectious in vivo. Isolates that were not classified within an intestinal pathotype or within an extraintestinal pathotype (e.g., ExPEC or APEC) were considered commensals.

Although our virulence array does not directly detect the AEC-02-ColV or the APEC-02-R plasmid, the simultaneous detection of the genes *tsh*, *iss*, *cvaC* (structural gene for microcin V), *ompT* (outer membrane protein 3b, also called protease VII), *iroN* (siderophore receptor), and the aerobactin iron acquisition system, or of the complement resistance protein gene *traT* and the antimicrobial resistance *tet*(A), *sulI*, and bla_{TEM} genes as well as integron class I, was considered potentially representative of the presence of the virulence plasmid pAPEC-O2-ColV (29) or the antimicrobial resistance plasmid pAPEC-O2-R (28), respectively.

Statistical analysis. Data were analyzed according to a randomized complete block design using SAS software (2000) with the individual pens as experimental units (three pens per treatment group). The Cochran-Mantel-Haenszel association test was used to determine the relationship between feed supplementation and genotype using the FREQ procedures. Associations between pathotype and genotype were determined using the Pearson's chi-square and Fischer exact tests. A P value of 0.05 was used to declare significance.

TABLE 1. Pathotype distribution among the 256 *E. coli* isolates from chickens fed with antimicrobial agents in the two studies

Study and treatment (*n*)	No. (%) of isolates		
	"Other" ExPEC	APEC	Commensal
I			
Control (20)	3 (15.0)	6 (3.3)	11 (55.0)
Bambermycin (14)	2 (14.3)	3 (21.4)	9 (64.3)
Penicillin (17)	2 (11.8)	7 (41.2)	8 (47.0)
Salinomycin (21)	4 (19.0)	4 (19.0)	13 (62.0)
Bacitracin (19)	3 (15.8)	1 (5.3)	15 (78.9)
Salinomycin + bacitracin (18)	1 (5.6)	6 (3.3)	11 (61.1)
Total (109)	15 (13.8)	27 (24.7)	67 (61.5)
P[a]	0.50	0.17	0.50
II			
Control (29)	0 (0.0)	3 (10.3)	26 (89.7)
Chlortetracycline (18)	0 (0.0)	0 (0.0)	18 (100)
Virginiamycin, 11 ppm (23)	0 (0.0)	7 (30.4)	16 (69.6)
Virginiamycin, 22 ppm (31)	0 (0.0)	2 (6.5)	29 (93.5)
Monensin (21)	0 (0.0)	1 (4.8)	20 (95.2)
Narasin (25)	0 (0.0)	4 (16.0)	21 (84.0)
Total (147)	0 (0.0)	17 (11.6)	130 (88.4)
P	ND[b]	0.02*	0.02*
Total (256)	15 (5.8)	44 (17.2)	197 (77.0)

[a] *P* value obtained by Cochran-Mantel-Haenszel test. *, statistically significant difference between treatment groups ($P \leq 0.05$).
[b] ND, not determined (or estimated).

RESULTS

Pathotype distribution and prevalence according to supplementation diets. The hybridization array data from all 256 isolates examined in this study revealed the complete absence of any potential intestinal *E. coli* pathotypes. More specifically, no enterocyte effacement genes or heat-stable, heat-labile, or Shiga-like toxin-encoding genes were detected among the strains tested. However, of all 256 *E. coli* isolates examined with our DNA virulence microarray, 59 (23.0%) were classified as potentially ExPEC. The remaining 197 isolates (77.0%) were considered commensal strains. The 59 ExPEC isolates were further subdivided into two groups based on the criteria described in Materials and Methods. Among these 59 ExPEC isolates, 44 (74.6%) and 15 (25.4%) were classified as potentially APEC and "other" ExPEC, respectively. Although the majority of isolates were classified as commensal, all isolates did carry virulence-encoding genes to some extent. The categorical separation of ExPEC and commensal strains is empirical and is based on the absence of specific sets of virulence-encoding genes rather than on in vivo bioassays.

Within the different antibiotic supplementation groups, the distributions of all ExPEC and commensal strains do not seem to be influenced by antibiotic treatment, except for chlortetracycline in study II. Although ExPEC isolates appeared to be randomly distributed among the treatment groups, including the untreated controls, all 18 (100%) isolates from chickens fed with chlortetracycline were classified as commensal *E. coli* (Table 1).

Prevalence of virulence genes. Analysis of the virulence gene content revealed the presence of several virulence and virulence-related genes distributed among the isolates regardless of treatment. Isolates possessed an average of 28 ± 5 virulence or virulence-related genes, ranging from 16 to 38 genes per isolate. The number of virulence-encoding genes detected in APEC isolates was significantly higher than that in the "other" ExPEC or the commensal isolates ($P < 0.05$). The "other" ExPEC isolates possessed an average of 30 ± 1 virulence genes, while the APEC isolates harbored an average of 35 ± 2 virulence genes. As expected, commensal strains possessed an overall lower average, with approximately 26 ± 5 genes detected per isolate. No effect of treatment was noted for the number of virulence genes detected ($P > 0.05$).

Among the 256 isolates in this study, 99 different genotypes were observed, including antimicrobial resistance and virulence genes, underscoring the great genetic diversity among the isolates as a whole. Moreover, the rest of the isolates were not a homogeneous group per se but represented a distribution of genetic patterns showing similarity to at least one other isolate.

The prevalence of *tsh* was not influenced by treatments; however, this gene was significantly associated with "other" ExPEC isolates ($P < 0.05$). Of the 15 potentially "other" ExPEC isolates, 14 (93.3%) carried the *tsh* gene, compared to 22 (11.2%) of the 197 commensal isolates. As previously stated, the classification of strains as APEC was, in part, based on the presence of the *tsh* and the *iss* genes. Consequently, 43 (98%) of the 44 strains classified as APEC were *tsh* and *iss* gene positive. In contrast, all 15 "other" ExPEC isolates were negative for *iss*. The majority, i.e., 179 (91%), of the 197 commensal isolates harbored the *iss* gene.

The *hra1* (heat-resistant agglutinin 1) gene, coding for an integral membrane hemagglutinin, was detected in 101 (39.4%) of the 256 studied isolates. Apart from being found in all four isolates of phylogenetic group B2, it was present primarily in isolates from the commensal phylogenetic group A (34/42, 81.0%) and was significantly associated with isolates from bacitracin (12/19, 63.2%) and salinomycin (11/21, 52.4%) treatments ($P < 0.05$) in study I. In study II, the distribution of adhesion [*lpfA*(EHEC) and *lpfA*(O113)] genes, colicin and microcin (*cba*, *cei*, *cma*, *cvaC*, and *mccB*) genes, iron acquisition or transport system (*chuA*, *fepC*, and yersiniabactin biosynthesis and uptake) genes, capsular and somatic antigen [*kpsM II*, *wzy*(O103), and *wzy*(O7)] genes, and genes with various functions (*yjaA*, *iss*, *tia*, *traT*, and *tspE4.C2*) was found to be significantly different among the treatment groups (Table 2). For example, the *irp* and *fyuA* genes, coding for the yersiniabactin biosynthesis and yersiniabactin receptor protein, respectively, were found in 19/25 (76.0%), 22/54 (40.7%), and, 4/21 (19.0%) isolates from the narasin, virginiamycin (11 and 22 ppm), and monensin treatment groups, respectively.

The following virulence-encoding genes, not included in the criteria elaborated for pathotype classification, were detected at a higher frequency within the "other" ExPEC and APEC groups than within commensal isolates: *chuA* (heme transport protein), *fepC* (ferric enterobactin transport protein), and, in the case of ExPEC strains, *tsh* (Table 2). The *ccdB* (cytotoxic protein), *malX* (phosphotransferase system, pathogenicity island [PAI] associated), *ompT*, and the toxin-encoding *astA* and *astA(2)* genes were detected at a higher frequency in the APEC group than in commensal isolates or the "other" ExPEC group (Table 2). The enterotoxigenic *E. coli* invasion protein-encod-

TABLE 2. Distribution of virulence genes among pathotypes of 256 *E. coli* isolates from chicken

Function of gene product	Gene(s)	No. (%) of isolates				P^a
		"Other" ExPEC (n = 15)	APEC (n = 44)	Commensal (n = 197)	Total (n = 256)	
Adhesin	*agn43*	0 (0.0)	0 (0.0)	118 (59.9)	118 (46.1)	<0.01
	bfpA	13 (86.7)	39 (88.4)	182 (92.4)	234 (91.4)	0.96
	f165(1)A	0 (0.0)	7 (15.9)	0 (0)	7 (2.7)	<0.01
	hra1[b]	0 (0.0)	6 (13.6)	95 (48.2)	101 (39.5)	<0.01
	iha	0 (0.0)	0 (0)	1 (0.5)	1 (0.4)	0.73
	lpfA	0 (0.0)	0 (0)	48 (24.4)	48 (18.8)	<0.01
	lpfA(EHEC)[c]	0 (0.0)	39 (88.6)	172 (87.3)	77 (30.1)	0.56
	lpfA(O113)[c]	0 (0.0)	37 (84.1)	172 (87.3)	75 (29.3)	0.03
	lpfA(O157)	0 (0.0)	0 (0)	10 (5.1)	10 (3.9)	0.60
	papA(11)	0 (0.0)	7 (15.9)	0 (0.0)	7 (2.7)	<0.01
	papC	0 (0.0)	6 (13.6)	0 (0.0)	6 (2.3)	<0.01
	papGII	0 (0.0)	6 (13.6)	0 (0.0)	6 (2.3)	<0.01
Toxin	*astA*, *astA(2)*	0 (0.0)	5 (11.4)	5 (2.5)	10 (3.9)	<0.01
	cdtB-1	0 (0.0)	4 (9.1)	0 (0.0)	4 (1.6)	<0.01
Hemagglutinin	*tsh*	14 (93.3)	43 (97.7)	22 (11.2)	79.0 (30.9)	<0.01
Colicin or microcin	*cba*[c]	15 (100.0)	11 (25.0)	148 (75.1)	174 (70.0)	<0.01
	ce1a	0 (0.0)	1 (2.3)	51 (25.9)	52 (20.3)	0.27
	cei[c]	15 (100.0)	2 (4.5)	47 (23.9)	64 (25.0)	<0.01
	cia	0 (0.0)	11 (25.0)	13 (6.6)	24 (9.4)	<0.01
	cib	0 (0.0)	0 (0)	69 (35.0)	69 (27)	<0.01
	cma[c]	15 (100.0)	10 (22.7)	148 (75.1)	173 (67.6)	<0.01
	cvaC[c]	15 (100.0)	33 (75.0)	106 (53.8)	154 (60.2)	<0.01
	mcbA	0 (0.0)	4 (9.1)	2 (1.0)	6 (2.3)	<0.01
	mccB[c]	0 (0.0)	0 (0.0)	8 (4.1)	8 (3.1)	0.32
	mchB	0 (0.0)	0 (0.0)	1 (1.0)	1 (0.4)	0.73
	mcjA	0 (0.0)	0 (0.0)	4 (2.0)	4 (1.6)	0.27
	mtfS	0 (0.0)	0 (0.0)	2 (1.0)	2 (0.8)	0.53
Iron acquisition or transport system	Aerobactin gene	15 (100.0)	43 (96.3)	24 (12.2)	82 (32.0)	<0.01
	chuA[c]	15 (100.0)	37 (84.1)	27 (13.7)	79 (30.9)	<0.01
	fepC[c]	15 (100.0)	33 (75.0)	26 (13.2)	74 (28.9)	<0.01
	iroN	0 (0.0)	39 (88.6)	123 (62.4)	162 (63.3)	0.76
	Yersiniabactin gene[c]	0 (0.0)	42 (95.5)	63 (32.0)	105 (41)	<0.01
Capsular or somatic antigen	*kpsM II*[c]	15 (100.0)	38 (86.4)	14 (7.1)	67 (26.2)	<0.01
	mtfA	0 (0.0)	0 (0.0)	1 (0.5)	1 (0.4)	0.73
	neuA, *neuC*	0 (0.0)	1 (2.3)	0 (0.0)	1 (0.4)	0.22
	wzy(O103)[c]	0 (0.0)	0 (0.0)	14 (7.1)	14 (5.5)	0.15
	wzy(O7)[c]	15 (100.0)	3 (6.8)	10 (5.1)	28 (10.9)	<0.01
Newly recognized or putative *E. coli* virulence gene product	b1121	15 (100.0)	40 (90.9)	187 (94.9)	227 (94.5)	0.06
	b1432	0 (0.0)	4 (9.1)	0 (0.0)	4 (1.6)	<0.01
	ECs1282	0 (0.0)	0 (0)	9 (4.6)	9 (3.5)	0.09
	rtx	15 (100.0)	5 (11.4)	4 (2.0)	24 (9.4)	<0.01
	yjaA[c]	0 (0.0)	4 (9.1)	41 (20.8)	45 (17.6)	0.58
	virK	0 (0.0)	0 (0.0)	1 (0.5)	1 (0.4)	0.73
Various	*capU*	0 (0.0)	0 (0.0)	1 (0.5)	1 (0.4)	
	ccdB	0 (0.0)	36 (81.8)	59 (29.9)	95 (37.1)	<0.01
	eaf	15 (100.0)	1 (2.3)	4 (2.0)	20 (7.8)	<0.01
	fliC	15 (100.0)	38 (86.4)	152 (77.2)	205 (80.1)	0.58
	fliC(H7)	0 (0.0)	0 (0.0)	3 (1.5)	3 (1.2)	0.38
	flmA54	0 (0.0)	4 (9.1)	46 (23.4)	50 (19.5)	0.17
	iss, *iss(2)*[c]	0 (0.0)	44 (100)	155 (78.7)	199 (77.7)	<0.01
	malX	0 (0.0)	22 (50.0)	3 (1.5)	47 (18.4)	<0.01
	ompT	0 (0.0)	33 (75.0)	92 (46.7)	125 (48.8)	0.75
	pic	0 (0.0)	0 (0.0)	4 (2.3)	4 (1.6)	0.53
	tia[c]	0 (0.0)	31 (70.5)	0 (0.0)	31 (12.1)	<0.01
	tir-3	0 (0.0)	1 (2.3)	6 (3.1)	7 (2.7)	0.23
	tlrA	13 (86.7)	2 (7.4)	3 (1.5)	18 (7.0)	0.38
	traT[c]	15 (100.0)	43 (96.3)	170 (86.3)	228 (89.1)	0.5
	tspE4.C2[c]	15 (100.0)	10 (22.7)	153 (77.7)	178 (69.3)	<0.01
	shf	0 (0.0)	0 (0.0)	1 (0.5)	1 (0.4)	0.73

[a] P value obtained by Cochran-Mantel-Haenszel test. A P value of ≤0.05 was considered to indicate a statistically significant difference between pathotypes.
[b] Statistically significant difference ($P \leq 0.05$) between treatment groups in study I.
[c] Statistically significant difference ($P \leq 0.05$) between treatment groups in study II.

TABLE 3. Distribution of pathotypes among *E. coli* phylogenetic groups

Phylogenetic group	No. (%) of isolates			
	"Other" ExPEC ($n = 15$)	APEC ($n = 44$)	Commensal ($n = 197$)	Total ($n = 256$)
A	0 (0.0)	1 (2.3)	41 (20.8)	42 (16.4)
B1	0 (0.0)	6 (13.6)	129 (65.5)	135 (52.7)
B2	0 (0.0)	4 (9.1)	0 (0.0)	4 (1.6)
D	15 (100.0)	33 (75.0)	27 (13.7)	75 (29.3)

ing gene *tia* was detected only in 31/44 (70.5%) isolates of the APEC group.

Lateral transfer of mobile genetic elements of different origin such as plasmids and PAIs leads to the development of new and unexpected strains (2). Usually, these contiguous virulence genes and PAIs are absent from commensal strains (26). The *Yersinia* PAI was indeed detected in 42/44 (95.5%) of potentially APEC strains but also in 63/197 (32.0%) of the commensal strains.

Although the presence of the plasmid was not directly tested, the combined detection of *tsh*, *iss*, *iroN*, *cvaC*, and *ompT* genes as well as the aerobactin-iron acquisition system was considered an indicator of pAPEC-O2-ColV presence. This gene combination was detected in 36 (14.1%) of the 256 isolates, which were distributed between the two pathogenic phylogenetic groups B2 and D. A significant association ($P < 0.05$) between APEC and this potential pAPEC-O2-ColV plasmid was observed. Thirty-two (72.7%) of the 44 APEC isolates possessed the gene combination, while none of the 15 "other" ExPEC isolates and only three (1.5%) of the 197 commensal isolates possessed it. No significant association was observed between the presence of this gene combination and the treatments ($P > 0.05$).

Phylogenetic distribution of *E. coli* isolates. Isolates were classified into four different phylogenetic groups (A, B1, B2, or D) based on the presence or absence of the *chuA*, *yjaA*, and *tspE4.C2* genes (8). Most of the 197 commensal *E. coli* isolates (170, 86.3%), belonged to the normally avirulent A and B1 phylogenetic groups, with the rest falling into group D (Table 3). Of all 256 isolates, only four were classified as B2, and all four of these isolates belonged to the APEC group. All 15 "other" ExPEC isolates and 33 (75.0%) of the 44 APEC isolates were associated with phylogenetic group D ($P < 0.01$).

Among the 109 isolates in study I, no B2 group was found, and group A was found in only six isolates; among them three were from the bambermycin treatment group (Table 4). Pathotypes B1 and D were each found in 53 isolates, similarly distributed between the treatment groups ($P > 0.05$). In study II, only four isolates of B2 group were found, and none of the 36 isolates of group A was found in birds treated with 11 ppm of virginiamycin (Table 4). The frequency of this pathotype was significantly lower in isolates from the monencin treatment group than those from the other groups ($P < 0.05$). The distribution of the 83 isolates of group B1 was significantly influenced by the treatment ($P < 0.05$). The highest B1 frequencies were in bird treated with monencin, virginiamycin at 11 ppm, and chlortetracycline. The lowest frequency was found for naracin treatment. None of the 23 isolates of group D were found in the group treated with virginiamycin at 22 ppm. The lowest frequencies of group D were found in the control group and birds treated with chlortetracycline and monensin, whereas the isolates from birds treated with naracin and with virginiamycin at 11 ppm showed the highest frequencies of isolates of group D ($P < 0.05$).

TABLE 4. Phylogenetic distribution of the 256 *E. coli* isolates from chickens fed with antimicrobial agents

Study and treatment (n)	No. (%) of isolates in phylogenetic group:			
	A	B1	B2	D
I				
Control (20)	1 (5.0)	9 (45.0)	0 (0.0)	10 (50.0)
Bambermycin (14)	3 (21.4)	7 (50.0)	0 (0.0)	4 (28.6)
Penicillin (17)	2 (11.8)	5 (29.4)	0 (0.0)	10 (58.8)
Salinomycin (21)	0 (0.0)	9 (42.9)	0 (0.0)	12 (57.1)
Bacitracin (19)	0 (0.0)	13 (68.4)	0 (0.0)	6 (31.6)
Salinomycin + bacitracin ($n = 18$)	0 (0.0)	7 (38.9)	0 (0.0)	11 (61.1)
Total (109)	6 (5.5)	50 (45.9)	0 (0.0)	53 (48.6)
P[a]	0.04*	0.28	ND[b]	0.23
II				
Control (29)	10 (34.5)	16 (55.2)	0 (0.0)	3 (10.3)
Chlortetracycline (18)	5 (27.8)	12 (66.7)	0 (0.0)	1 (5.5)
Virginiamycin, 11 ppm (23)	0 (0.0)	16 (69.6)	0 (0.0)	7 (30.4)
Virginiamycin, 22 ppm (31)	11 (35.5)	18 (58.0)	2 (6.5)	0 (0.0)
Monensin (21)	1 (4.8)	17 (80.9)	1 (4.8)	2 (9.5)
Narasin (25)	9 (36.0)	6 (24.0)	1 (4.0)	9 (36.0)
Total (147)	36 (24.5)	85 (57.8)	4 (2.7)	22 (15.0)
P	<0.01*	0.01*	0.11	<0.01*
Total (256)	42 (16.4)	135 (52.7)	4 (1.6)	75 (29.3)

[a] P value obtained by Cochran-Mantel-Haenszel test. *, statistically difference between treatment groups ($P \leq 0.05$).
[b] ND, not determined (or estimated).

Prevalence of antimicrobial resistance genes. The impact of feed supplementation on the distribution of antimicrobial resistance determinants was previously analyzed for strains isolated from birds fed with either bambermycin, procaine penicillin, salinomycin, bacitracin, or a combination of bacitracin plus salinomycin (10). No significant association could be found between the prevalence of antimicrobial resistance genes and diet in *E. coli* strains isolated from chickens fed with chlortetracycline, virginiamycin, monensin, or narasin.

At least one antimicrobial resistance gene was found in 224 (87.5%) of all 256 *E. coli* isolates analyzed. As shown in Table 5, the combination of tetracycline [*tet*(A)], sulfonamide (*sulII*), and phenicol (*floR*) resistance genes was detected in all 15 "other" ExPEC isolates. Of the 44 APEC isolates, 37 (84.1%) possessed tetracycline resistance genes *tet*(B) and/or *tet*(A), whereas, eight (18.2%) were positive for the *sulII* sulfonamide resistance gene. None of the strains classified within the "other" ExPEC or APEC group possessed more than four antimicrobial resistance genes, with 27 (61.4%) of the 44 APEC isolates having only one and all 15 "other" ExPEC isolates possessing three to four each.

Only in the 197 commensal *E. coli* isolates could both sulfonamide resistance genes (45 isolates, 22.8%), both β-lactam

TABLE 5. Distribution of antibiotic resistance genes among the 256 *E. coli* isolates

Antibacterial	Gene(s)	No. (%) of isolates			
		"Other" ExPEC (n = 15)	APEC (n = 44)	Commensal (n = 197)	Total (n = 256)
Aminoglycoside (streptomycin)	*ant(3)-Ia (aadA1)*	0 (0.0)	6 (13.6)	50 (25.4)	56 (21.9)
Trimethoprim	*dhfrI*	0 (0.0)	6 (13.6)	1 (0.5)	7 (2.7)
Phenicol	*floR*	15 (100.0)	2 (4.5)	46 (23.4)	63 (24.6)
Class I integron	*int1(1) + int1(2) + int1(3)*	0 (0.0)	0 (0.0)	50 (25.4)	50 (19.3)
Sulfonamide	*sulI*	0 (0.0)	0 (0.0)	4 (2.0)	4 (1.7)
	sulII	15 (100.0)	8 (18.2)	5 (2.5)	28 (10.9)
	sulI + sulII	0 (0.0)	0 (0.0)	45 (22.8)	45 (17.6)
β-Lactam (ampicillin)	bla_{TEM}	0 (0.0)	3 (6.8)	47 (23.9)	50 (19.3)
	bla_{SHV}	0 (0.0)	0 (0.0)	13 (6.6)	13 (5.0)
	$bla_{TEM} + bla_{SHV}$	0 (0.0)	0 (0.0)	2 (1.0)	2 (0.8)
Tetracycline[a]	*tet*(A)	13 (86.7)	2 (4.5)	85 (43.1)	100 (39.0)
	tet(B)	0 (0.0)	35 (79.5)	43 (21.8)	78 (30.5)
	tet(A) + *tet*(B)	0 (0.0)	1 (2.3)	26 (13.2)	27 (10.5)
	tet(A) + *tet*(C)	2 (13.3)	0 (0.0)	6 (3.0)	8 (3.1)
	tet(A) + *tet*(B) + *tet*(C)	0 (0.0)	0 (0.0)	8 (4.1)	8 (3.1)

[a] None of the isolates were positive for *tet*(C) alone or for the *tet*(B)-*tet*(C) combination.

resistance genes (2, 1.0%), all tetracycline resistance genes (8, 4.0%), and all integron class I resistance genes (50, 25.4%) be found. Thirty-three (16.8%) of the 197 commensal isolates possessed 10 or more antimicrobial resistance genes, and 17 (8.6%) possessed between 5 and 9 antimicrobial resistance genes. The pAPEC-O2-R plasmid, originally isolated from an APEC strain, carries *traT* (complement resistance protein); antibiotic resistance genes *tet*(A), *sulI*, *and* $bla_{TEM;}$ and integron class I (28). The combination of these genes, which collectively implies plasmid presence, was detected in 35 (17.8%) of the 197 commensal strains but was not detected in either APEC or ExPEC strains.

DISCUSSION

Increasing resistance to first-line antimicrobial agents among *E. coli* isolates represents a potential threat to animal and human health (10, 28, 42). Therefore, it is important to identify factors that can negatively affect the balance of gut microflora and consequently the health status and production performance of chickens.

Previously we showed that multidrug-resistant *E. coli* strains can be found in chicken guts and that feed supplementation with some antimicrobial agents, such as salinomycin or bacitracin, could influence the phenotype and distribution of resistance determinants in *E. coli* (10). In the present study we examined the effect of supplementation on virulence gene distribution. With the exception of chlortetracycline, no correlation between the different antibiotic supplementation diets and the pathotype of *E. coli* isolates could be found, as isolates classified as ExPEC were similarly distributed among all other supplemented groups, including the control. The lack of ExPEC isolates in the chlortetracycline treatment group cannot be explained by clonal isolation of a non-ExPEC strain, since our microarray data revealed that one of every two to three isolates possessed a unique genotype. However, since the overall number of isolates in this group was relatively low, further exploration is needed before drawing any reliable conclusions on possible negative selection pressure of chlortetracycline against potentially virulent strains.

Even though commensal *E. coli* strains are classified on the basis of their weak virulence potential related to the possession of fewer virulence factors, they are known to share a considerable amount of virulence-associated genes with the ExPEC pathotype (12, 26). Unequivocal distinction between ExPEC and commensal strains is, as a result, problematic. Indeed, discrimination between commensal and potentially pathogenic *E. coli* strains in our study is artificial, since a true virulence phenotype can be assessed only when infectiousness is demonstrated in vivo (which was beyond the scope of this study). The virulence potential of ExPEC is determined largely by the presence of genes enabling the strains to colonize host mucosal surfaces, avoid or subvert local and systemic host defense mechanisms, scavenge essential nutrients such as iron, injure or invade the host, and stimulate a noxious inflammatory response (26). Classified by criteria elaborated by Johnson et al. (25) and on the assumption that strains carrying similar virulence determinants have the same pathotype, 23% of all 256 *E. coli* strains presented here could be grouped as ExPEC strains. Two other studies, based on the same ExPEC classification and performed with bacteria isolated from retail chicken products, demonstrated similar or higher levels of potentially pathogenic strains, i.e., 21% in the first case and 46% in the second (25, 28).

One of the difficulties in identifying *E. coli* pathotypes is that no single virulence gene can turn a commensal *E. coli* strain into a strain with a known pathotype. For example, APEC strains are known to possess several virulence genes encoding adhesins, hemolysins, resistance to the bactericidal effect of serum and phagocytosis, iron acquisition systems, toxins, and cytotoxins (9, 13, 17, 22, 34, 39), but no single factor can be linked unequivocally to APEC pathogenicity (9, 16, 32). Our virulence microarray with its capacity to scan individual iso-

lates for the full complement of known *E. coli* virulence genes represents a powerful tool with which to look for specific gene combinations, thus facilitating the search for other potential APEC markers as well as the study of gene distribution. Our study showed that the antimicrobial used in the diet could change the composition of *E. coli* pathotypes and the distribution of virulence determinants. More studies are needed to understand and explain these changes, which might be related to gut physiology and microflora.

The *tsh* gene has also been suggested as a potential APEC-specific marker (9, 34, 37, 49). In contrast with data of Maurer et al. (34), the *tsh* gene was observed in approximately 31% of our isolates from healthy chickens, with more than 8% of the commensal strains being positive for this gene. The *iss* gene has also been suggested as a specific marker for APEC identification (18, 21, 38, 39). Although 100% of our classified APEC strains possessed *iss*, the data clearly showed no correlation between the sole presence of the *iss* gene and specific APEC identification, as this gene was highly prevalent in commensal strains as well. Plasmids, such as ColV, are commonly found among APEC strains and are thought to be linked to the virulence of these organisms (29, 30). Rodriguez-Siek et al., (41) demonstrated a correlation between the APEC pathotype and the presence of the plasmid pAPEC-O2-ColV, carrying *tsh*, *iss*, and other virulence-related genes such as the aerobactin iron acquisition system and the siderophore receptor genes. Our data showed that among the 256 isolates examined, 14 isolates were *tsh* positive and *iss* negative, while of the 199 *iss*-positive isolates, only 65 carried both *iss* and *tsh*. This number is further reduced to 60 isolates when looking for an iron acquisition system (e.g., *iroN* or aerobactin) combined with *iss* and *tsh.* PAIs are also considered to be good virulence markers (40, 44) and have been shown to be absent from commensal *E. coli* genomes (31, 42). Although the *Yersinia* PAI was more frequently detected within APEC strains, it was also observed in more than 30% of the commensal strains. This result clearly highlights the strong genetic transfer potential between strains and the great variability of the *E. coli* genome. Interestingly, the enterotoxigenic *E. coli* invasion protein-encoding gene *tia* was found only associated with potential APEC isolates (31/44). Its absence in "other" ExPEC isolates and commensals suggests a role for this protein in avian virulence.

The results from the present study confirm the observations that ExPEC strains belong predominantly to the phylogenetic group B2 and to a lesser extent to group D, while commensal strains are typically classified within phylogenetic group A or B1 (3, 4, 26, 27, 40). The association of ExPEC with B2 was suggested to be more a result of the abundance of virulence factors in the B2 lineage (23). In agreement with this notion, we found that the highest number of virulence-encoding genes were in the B2 phylogenetic group. However, some strains classified within the A, B1, or D group, exhibiting a large number of important virulence factors, were also classified as potentially virulent regardless of their phylogenetic classification. The abundance of virulence genes, regardless of their functions, was significantly higher in our APEC isolates than in the "other" ExPEC isolates and commensal groups, suggesting, as previously mentioned (23), that the relative abundance of virulence factors is an important parameter in assessing the virulence potential of extraintestinal *E. coli* isolates.

A significant percentage of the *E. coli* isolates studied possessed one or more antimicrobial resistance genes. Interestingly, a series of antimicrobial resistance genes, i.e., *tet*(A), *sulI*, bla_{TEM}, and integron I, related to plasmid pAPEC-O2-R, were detected together within the same strains but were detected only among commensal strains. As suggested earlier for the ColV plasmid, the combined presence of those resistance genes with *traT* may indirectly reflect the presence of pAPEC-O2-R. Such horizontally transmissible plasmids may serve as a reservoir of antimicrobial resistance genes for other bacterial species of potentially great threat to human health (24). Interestingly, pAPEC-O2-R and pAPEC-O2-ColV were never detected together in the *E. coli* isolates analyzed, possibly implying that potentially pathogenic strains have less antimicrobial resistance potential than isolates classified as commensal. This observation cannot be explained by incompatibility, since both pAPEC-O2-R and pAPEC-O2-ColV have been cointroduced into an avirulent *E. coli* strain. The resultant transconjugant acquired both increased resistance and virulence (29).

In summary, no correlation between the pathogenic potential of *E. coli* strains isolated from broiler chickens and their antimicrobial supplementation diets could be determined, with the possible exception of chlortetracycline. Further work is needed to confirm and explain the possible association between virulence genes and antimicrobial agents used in diet. Consistent with results from other groups, the presence of pAPEC-O2-ColV was highly correlated with the APEC pathotype. Furthermore, despite the high numbers of both antibiotic resistance and virulence genes found among the isolates in this study, gene combinations reflecting the presence of the antimicrobial resistance plasmid pAPEC-O2-R and the virulence plasmid pAPEC-O2-ColV suggested that these plasmids were never detected together. Although potentially virulent *E. coli* isolates tended to carry few antibiotic resistance genes, our study clearly shows that broiler chickens act as a reservoir for commensal *E. coli* strains carrying large numbers of antibiotic resistance genes. These bacteria have the potential to spread through fecal waste, potentially contaminating both farm workers and processing plants, food, or the natural environment.

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