Virulence Genes in Expanded-Spectrum-Cephalosporin-Resistant and -Susceptible *Escherichia coli* Isolates from Treated and Untreated Chickens

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This study investigated antimicrobial resistance, screened for the presence of virulence genes involved in intestinal infections, and determined phylogenetic groups of *Escherichia coli* isolates from untreated poultry and poultry treated with ceftiofur, an expanded-spectrum cephalosporin. Results show that none of the 76 isolates appeared to be Shiga toxin-producing *E. coli* or enteropathogenic *E. coli*. All isolates were negative for the major virulence factors/toxins tested (*ehxA*, *cdt*, heat-stable enterotoxin [ST], and heat-labile enterotoxin [LT]). The few virulence genes harbored in isolates generally did not correlate with isolate antimicrobial resistance or treatment status. However, some of the virulence genes were significantly associated with certain phylogenetic groups.

Resistance to expanded-spectrum cephalosporins (ESCs) is a major health threat. There are reports of ESC-resistant *Enterobacteriaceae* in the microbiota of broiler chickens and layer hens (1), and possible transmission to humans via the food chain or, in the case of farm personnel, via direct contact is cause for concern. ESC-resistant *Escherichia coli* strains are often detected in poultry during their first weeks of life, possibly because ESCs are injected *in ovo* in broilers and subcutaneously in future layers (as 1-day-old chicks). These hatchery practices have been shown to select for resistance to ESCs (2). In addition to selection, antimicrobial usage may also modify the *E. coli* population with regard to virulence, increasing the risks to human health. We thus compared virulence genes and phylogenetic groups of *E. coli* isolates from ESC-treated or nontreated broilers or future layers, to determine whether the *E. coli* present could be a reservoir of diarrheagenic strains for humans, namely, the Shiga toxin (Stx)-producing *E. coli* (STEC) strains. However, only a subset of STEC strains is responsible for severe human infections, such as hemorrhagic colitis or hemolytic-uremic syndrome (HUS): the so-called enterohemorrhagic *E. coli* (EHEC) strains. Typical EHEC strains carry Shiga toxins (*stx* genes) and intimin (*eae* gene), an adhesion factor that causes attaching and effacing lesions on enterocytes. Other gastrointestinal pathogenic *E. coli* types, such as enteropathogenic *E. coli* (EPEC), which is associated with watery diarrhea, lack Shiga toxins but share the *eae* gene typical of EHEC strains. These definitions of STEC, EHEC, and EPEC do not include the possible association with other virulence factors (i.e., type III effector proteins) that are carried by genetic mobile elements.

We examined 38 cefotaxime-susceptible (CTXS) and 38 cefotaxime-resistant (CTXR) *E. coli* isolates from a previous study (2) on fecal samples from flocks of broilers and future layers that had been treated with or without ceftiofur. Samples were collected during the first week after hatching from hatcheries A (26 broiler isolates, including 13 CTXR isolates), B (15 broiler isolates, including 6 CTXR isolates), C (12 future layer isolates, including 6 CTXR isolates), and D (23 future layer isolates, including 13 CTXR isolates). For each fecal sample, either one or two pairs of

CTXR and CTXS isolates were studied. The susceptibility of isolates to gentamicin (GEN), streptomycin (STR), tetracycline (TET), ciprofloxacin (CIP), kanamycin (KAN), ceftiofur (FOX), and trimethoprim-sulfamethoxazole (SXT) in a ratio of 1:19 was (2) based on EUCAST clinical breakpoints or epidemiological cut-offs. Phylogenetic groups were determined as described in reference 3. A high-throughput real-time PCR microarray (4) was used to screen for a panel of virulence-associated genes related to gastrointestinal pathogenic *E. coli* (STEC, EHEC, and EPEC). DNA was prepared from bacterial cultures with an InstaGene matrix (Bio-Rad). The *wecA* gene was included as a positive control. Statistical associations between resistance, presence of virulence genes, or phylogenetic groups were tested using Fisher exact tests. The relationship between the presence of virulence genes and hatchery or ceftiofur treatment was evaluated using logistic regression.

The most frequently observed virulence genes were *lpfA*_{O113}, a chromosomal gene coding for long polar fimbriae (36/76 isolates); *ehaA*, a chromosome-borne gene coding for a type V secretion system (35/76 isolates); and *paa*, coding for the porcine attaching-and-effacing-associated adhesion (28/76 isolates) (Table 1). Genes *irp2* (iron-repressible protein 2) and *fyuA* (pectin receptor), both known to be present on the high-pathogenicity island (HPI), were always found together. Virulence genes *stx*, *vxt*_{2a}, *eae*, *pagC*, *nleB*, *nleE*, *efa1*, *nleH1-2*, *nleA*, *ehxA*, *sfp*, *bfp*, *cdt-I*, *cdt-III*, *sta*, *lt*, *fasA* (F6 and P987), *fedA* (F18 and F107), *F41*, *orfA*, *orfB*,

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TABLE 1 Virulence genes and phylogenetic groups of isolates

Gene	Protein(s) encoded or phylogenetic group	Genomic location ^a	No. of isolates positive for virulence gene, belonging to phylogenetic group, or having resistance profile						
			Nontreated flocks		Treated flocks		CTXS (n = 38)	CTXR (n = 38)	Total (n = 76)
			Broilers (n = 22)	Layers (n = 8)	Broilers (n = 19)	Layers (n = 27)			
<i>iha</i>	Iron-regulated gene A homolog	C	4	5	4	3	7	9	16
ECs1763	Hypothetical protein putative marker for EHEC	C	0	0	0	1	1	0	1
<i>lpfA</i> _{O26}	Long polar fimbriae	C	3	1	2	2	4	4	8
<i>lpfA</i> _{O113}	Long polar fimbriae	C	10	4	12	16	21	21	42
<i>lpfA</i> _{O157}	Long polar fimbriae	C	4	0	2	5	6	5	11
<i>astA</i>	Enterotoxigenic <i>E. coli</i> heat-stable enterotoxin	C or P	7	6	5	6	14	10	24
<i>ecf3</i>	Enzyme that enhances bacterial membrane structure	P O157	0	0	0	1	1	0	1
<i>irp2</i>	Iron-repressible protein 2	HPI	8	6	4	6	10	14	24
<i>fyuA</i>	Pectin receptor	HPI	8	6	4	6	10	14	24
<i>ehaA</i>	Autotransporter, type V secretion system	C	10	4	10	15	20	19	39
<i>paa</i>	Porcine attaching and effacing associated	C or P	9	1	9	10	16	13	29
<i>terE</i>	Tellurite resistance gene	C	1	0	0	4	3	2	5
	Phylogenetic group A		5	3	8	8	17	7	24
	Phylogenetic group B1		8	1	7	9	11	14	25
	Phylogenetic group B2		3	1	0	0	1	3	4
	Phylogenetic group C		1	0	0	0	0	1	1
	Phylogenetic group D		0	0	0	2	0	2	2
	Phylogenetic group E		4	1	3	4	6	6	12
	Phylogenetic group F		1	2	1	4	3	5	8

^a Abbreviations: C, chromosome; P, plasmid; HPI, high-pathogenicity island.

ecf1, *ecf2*, *ecf4*, *stcE*, *eibG*, *epeA*, *saa*, *subAB*, *sab*, and *ureD* were not detected in any of the 76 tested isolates. The mean number of virulence genes per isolate was 2.9. None of the isolates appeared to be STEC or EPEC (i.e., all were negative for the *stx* and *eae* genes). Similarly, all isolates were negative for the major virulence factors/toxins tested (*ehxA*, *cdt*, heat-stable enterotoxin [ST], and heat-labile enterotoxin [LT]). However, all but 14 isolates harbored at least one of the tested adhesion factors allowing bacterial adhesion to the intestinal epithelial cells.

When two pairs of *E. coli* isolates were from the same sample, the four virulence profiles were either different (two samples from treated flocks) or identical for the two CTXR isolates (three samples from nontreated flocks) or the two CTXS isolates (two samples from treated flocks).

A total of 36 different virulence profiles were obtained, 23 for 46 isolates from treated flocks and 23 for 30 isolates from nontreated flocks and 25 in the 38 CTXS isolates and 18 in the 38 CTXR isolates. The most frequent profile was the absence of all screened virulence genes, being shared by seven isolates (six CTXR and one CTXS isolate) obtained from five treated and two nontreated flocks. The most frequent profile for isolates from nontreated flocks was *irp2*⁺-*fyuA*⁺, whereas treated flocks showed five isolates with no virulence genes and five isolates with *lpfA*_{O113} and *ehaA* genes. The absence of all virulence genes was not significantly associated with the treatment status of the flock, the strain, or its CTX, FOX, STR, KAN, CIP, or TET resistance (Fisher test, $P > 0.05$ for each). However, nonvirulent isolates were more frequently resistant to SXT ($P = 0.007$). There was no significant association between CTX, FOX, STR, CIP, KAN, SXT, TET, or GEN resistance and the presence of each virulence gene, except for the *irp2* and *fyuA* genes, which were more frequent in FOX-susceptible isolates ($P = 0.047$), and *lpfA*_{O26}, which was more fre-

quently found in TET-susceptible isolates ($P = 0.03$). The presence of virulence genes was not associated with hatchery or with treatment except for the *terE* gene, which was absent in 26 isolates from hatchery A and 12 from hatchery C but present in 4/23 (17%) isolates from hatchery D ($P = 0.04$), and *irp2* and *fyuA* genes, which were present in 10/46 (22%) and 14/30 (47%) isolates from treated and nontreated poultry, respectively.

The phylogenetic groups are given in Table 1. Most isolates belonged to phylogenetic groups A and B1, typically associated with commensal strains, in accordance with the absence of major virulence genes. Isolates from the same sample with the same virulence profile belonged to the same phylogenetic group. No association was observed between phylogenetic groups and resistance to the different antimicrobial families, hatchery, or treatment. However, isolates with virulence genes were unevenly distributed among phylogenetic groups for genes *iha* ($P = 0.001$), *lpfA*_{O26} ($P = 0.018$), *lpfA*_{O113} ($P < 0.001$), *lpfA*_{O157} ($P < 0.001$), *irp2* ($P < 0.001$), *fyuA* ($P < 0.001$), and *ehaA* ($P < 0.001$) (Fig. 1). Thus, most isolates harboring the *lpfA*_{O26}, *lpfA*_{O113}, or *ehaA* gene belonged to group B1, whereas isolates carrying *lpfA*_{O157} belonged to group E.

Virulence genes of extraintestinal pathogenic *E. coli* (ExPEC), such as *iss*, *iutA*, *iroN*, *hlyF*, and *ompT* (5), were not investigated in this study, but *E. coli* strains of avian origin are increasingly considered zoonotic agents (6). In contrast, various methods have been used to detect STEC in fecal samples from poultry. In most studies, STEC isolates are not (7–9) or only rarely (10, 11) detected, and their prevalence is usually estimated to be low in broilers compared with other animal species. However, one report suggests that laying hens may be a potential reservoir of *E. coli* O157:H7 (12). EPEC seemed more prevalent in chickens than in other animal species in a study in Burkina Faso (13). In contrast,

FIG 1 Distribution of the phylogenetic groups of *E. coli* isolates with and without the *iha*, *lpfA*_{O26}, *lpfA*_{O113}, *lpfA*_{O157}, *irp2*, *fyuA*, and *ehaA* virulence genes. The distribution of virulence genes was not even across all phylogenetic groups for *iha* ($P = 0.001$), *lpfA*_{O26} ($P = 0.018$), *lpfA*_{O113} ($P < 0.001$), *lpfA*_{O157} ($P < 0.001$), *irp2-fyuA* ($P < 0.001$), and *ehaA* ($P < 0.001$). The distribution of the *fyuA* gene is exactly the same as that of the *irp2* gene.

no potentially pathogenic strains (STEC, potential EPEC, and non-STEC *ehxA*-positive *E. coli*) were detected in a study of 144 chicken and 126 turkey *E. coli* isolates in the United States (14).

Overall, the results obtained in our study confirm that the fecal *E. coli* isolates from ceftiofur-treated or nontreated broilers or layers contain few genes involved in intestinal infections and are distributed unevenly over the phylogenetic groups. These virulence genes contributing to *E. coli* pathogenicity are present regardless of antimicrobial resistance and antimicrobial regimens, including the hatchery's questionable use of ceftiofur.

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