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Assessment of Virulence Factors Characteristic of Human *Escherichia coli* Pathotypes and Antimicrobial Resistance in O157:H7 and Non-O157:H7 Isolates from Livestock in Spain

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The distribution of virulence factors (VFs) typical of diarrheagenic *Escherichia coli* and the antimicrobial resistance (AMR) profiles were assessed in 780 isolates from healthy pigs, broilers, and cattle from Spain. VF distribution was broader than expected, although at low prevalence for most genes, with AMR being linked mainly to host species.

Pathogenic isolates of *Escherichia coli* are characterized by the presence of virulence factors (VFs) that can be combined, leading to different pathotypes (1). Six distinct intestinal pathotypes have been differentiated, including enteropathogenic (EPEC), enterotoxigenic (ETEC), enterohemorrhagic (EHEC), enteroaggregative (EAEC), enteroinvasive (EIEC), and diffusely adherent (DAEC) *E. coli* (2). The main reservoirs for EPEC, EAEC, and EIEC are humans (1, 3). In contrast, EHEC transmission from animals to humans may also occur through fecal contamination of food or water. In addition to the conventional *E. coli* pathotypes, novel hybrid pathotypes may emerge, as occurred with EAEC/EHEC O104:H4 of the German outbreak, which spread across several European countries, affecting almost 4,000 people and causing more than 50 deaths (4).

Limited information on the distribution of *E. coli* pathotypes in healthy livestock (especially non-Shiga toxin-producing *E. coli* [STEC]) and the possible association between the carriage of VF and their antimicrobial resistance (AMR) patterns is available. In this study, we evaluated the distribution of VFs and AMR profiles in *E. coli* isolates recovered from healthy livestock in Spain.

All these *E. coli* isolates were recovered in 2009 ($n = 780$) through the Spanish Surveillance Network of Antimicrobial Resistance in Bacteria of Veterinary Origin (VAV Network) (5). Isolate distribution was as follows: 50 O157:H7 *E. coli* strains from cattle and 730 *E. coli* strains (278 from pigs, 196 from broilers, and 256 from cattle) belonging to other serotypes (referred to here as non-O157:H7 *E. coli* regardless whether they were EHEC). These isolates were recovered from pooled samples collected at Spanish slaughterhouses selected according to their slaughter capacity and located in different regions within the country. Isolates were obtained by culturing pooled feces samples from pigs (2 animals per pool, 556 individual fecal samples analyzed), cattle (2 animals per pool, 512 individual fecal samples analyzed) and broilers (3 animals per pool, 588 individual fecal samples analyzed). Each pool represented one slaughter batch from one single farm.

Samples from pigs, broilers, and cattle were cultured on MacConkey agar plates (6). In addition, cattle feces were processed to obtain *E. coli* O157:H7 according to the ISO 16.654:2001 protocol.

Nine VFs (stx_1, stx_2, *eae*, *ehxA*, heat-stable enterotoxin [ST], heat-labile enterotoxin [LT], *bfpA*, p*Inv*, and *aggR*) and the somatic and flagellar antigens of O157:H7 and O104:H4 serotypes were assessed in all isolates using conventional PCR (see Table S1 in the supplemental material). The β-D-glucuronidase-encoding gene *uid*A was also included for *E. coli* confirmation (7). In addition, the stx_2 PCR product from two swine isolates was sequenced to assess their stx_2 type (8). All isolates were also tested against 14 antibiotics by broth microdilution (6).

All isolates were *E. coli*, as demonstrated by the positive *uidA* results, and all were negative for *bfpA*, LT, *aggR*, and $wzxo_{104}$/$fliC_{H4}$ (data not shown). Distribution of VFs varied depending on the host species, with pigs and cattle presenting more diversity of genotypes, while 57 (29.1%) of the isolates from broilers carried *eae* as the only detected VF, in agreement with previous reports on avian isolates (9).

In cattle *E. coli*, the occurrence of *eae* (3.9%) and Shiga toxins (7.7%) in this study was lower among our strains than in previous works (10, 11, 12).

In pigs prevalence of VF was also low, with one isolate being p*Inv* positive and two isolates being positive for both stx_2 and ST (Table 1). In fact, a significant association for the concurrent presence of stx_2 and ST was found in both cattle and swine isolates (Fisher's exact test, $P < 0.001$). Interestingly, this combination had not been previously described for cattle. The sequencing of the *stx* gene from the two ST^+ $stx_2{}^+$ swine strains identified them as *stx*2e. In previous studies, this pattern was detected in healthy pigs and piglets suffering from postweaning diarrhea (13, 14). Our *stx*2e/ST-positive strains were obtained from adult healthy pigs, highlighting their possible role as asymptomatic reservoirs of this intermediate strain.

Regarding *E. coli* O157:H7 strains, 100% of the isolates were

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TABLE 1 Positive samples in each animal species for detection of the selected VFs

E. coli serotype	Origin	No. of strains	No. (%) positive for:						
			stx_1	stx_2	*eae*	*ehxA*[a] (pO157)	p*Inv*	ST	$rbf_{O157}/fliC_{H7}$
Non-O157:H7	Cattle	256	1 (0.4)	1 (0.4)	10 (3.9)	ND[c]	0	3 (1.17)	3[b] (1.17)
	Swine	278	0	4 (1.4)	2 (0.72)	ND	1 (0.36)	2 (0.72)	0
	Broilers	196	0	0	57 (29.1)	ND	0	0	0
O157:H7	Cattle	50	33 (66)	50 (100)	50 (100)	50 (100)	0	0	50 (100)

[a] As enterohemolysin (*ehxA*) is present in EHEC strains, it was analyzed only in O157:H7 isolates.
[b] rbf_{O157}-positive $fliC_{H7}$-negative isolates.
[c] ND, not determined.

positive for at least three of the typical EHEC VFs (*ehxA*, *eae*, and stx_2), confirming the role of cattle as a relevant reservoir of this pathotype and a public health concern, as previously described (15).

Almost 20% of the isolates showed no resistance to the studied antimicrobials, and no resistance to colistin was detected (see Table S1 in the supplemental material). The highest resistance rates were found for tetracycline, streptomycin, and sulfonamides, especially in pig isolates. However, the largest proportion of isolates resistant to quinolones and beta-lactams, including third-generation cephalosporins, was observed in avian isolates. Bovine strains showed lower AMR percentages, although cattle *E. coli* O157:H7 isolates accounted for the highest levels of resistance to gentamicin, kanamycin, and chloramphenicol (see Table S1). Significant differences (Pearson chi-square test, $P < 0.05$) in the proportion of resistant isolates to the different antibiotics were observed depending on the host species. In cattle, there was a significantly ($P < 0.05$) higher proportion of resistant isolates among O157:H7 than in non-O157:H7 *E. coli* strains for all the antimicrobials except florfenicol ($P = 0.49$) and third-generation cephalosporins (no resistant cattle isolates).

Compared with previous data (16), resistance levels among our *E. coli* isolates from pigs and broilers were moderate to high for some antibiotics while they were low in the case of bovine *E. coli* isolates. A higher proportion of resistance to gentamicin, ciprofloxacin, tetracycline and nalidixic acid was observed in the O157:H7 *E. coli* cattle isolates analyzed in this study compared with previous reports.

The homogeneity of the limited VF distribution found in pigs and broilers made it impossible to detect resistance patterns related to VF combinations in these animal species. In contrast, differences in AMR between *E. coli* O157:H7 and non-O157:H7 isolates from cattle indicate that, although both types of strains were theoretically under the same selective pressure, there may be a specific mechanism that makes O157:H7 *E. coli* strains more resistant than non-O157:H7 *E. coli* strains. However, comparison of non-O157:H7 *E. coli* isolates revealed a strong association with the host species.

In summary, the selected VFs, commonly regarded in the literature as specific to a given *E. coli* pathotype, showed a broader distribution than expected among healthy livestock in Spain, although most of the VFs analyzed were present at low frequencies. The assessment of the presence of potentially pathogenic *E. coli* (not only EHEC) in healthy animals could be a useful tool to evaluate and predict the risk of the emergence of new pathogenic strains from animal reservoirs.

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