

Book Chapter

Characterization of Shiga-toxin producing *Escherichia coli* (STEC) Strains Isolated from Bovine Feces from Three Cattle-Growing Regions of Mexico

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Abstract

Intestinal infections represent an important public health concern worldwide. *Escherichia coli* is one of the main bacterial agents involved in the pathogenesis of different diseases. In 2011, an outbreak of hemorrhagic colitis (HC) and hemolytic uremic

syndrome (HUS) in Germany was related to a non-O157 STEC strain of O104:H4 serotype. The difficulty in identifying the origin of the bacteria related to the outbreak showed the importance of having epidemiological information from different parts of the world. The aim of this study was to perform a retrospective analysis to determine if *E. coli* strains isolated from cattle from different locations in Mexico have similar characteristics to those isolated in other countries. Samples obtained in different years from 252 cows belonging to 5 herds were analyzed. A total of 1,260 colonies were selected from the 252 samples, 841 (67%) of which corresponded to *E. coli* and 419 (33%) to other enterobacteria. In total, 78% (656) of the *E. coli* strains could be serotyped, of which 393 (59.9%) belonged to 5 diarrheagenic (DEC) pathotypes. Serotyping showed STEC (40.7%) and ETEC (26.7%) strains were more common. PCR assays were used to determine the presence of STEC (*eae*, *stx1*, *stx2*, *ehxA*) and EAEC (*aata*, *aggR*, *aapA*) genes, and phylogenetic groups. The results showed that 70 strains belonging to 23 serogroups were *stx1* and *stx2* positive, while 13 strains from the O9 serogroup were *ehxA*, *aggR* and *eae* positive. Phylogenetic analysis showed 58 (82.9%) strains belonged to A and B1 commensal phylogroups and 12 (17.1%) to B2, D and E virulent phylogroups. An assay to evaluate cross-antigenic reactivity in the serum of cattle between K9 capsular antigen and O104 LPS by ELISA showed similar responses against both antigens ($p > 0.05$). The antimicrobial sensitivity assay of the strains showed resistance to AM, CEP, CXM, TE, SXT, cephalosporins and fluoroquinolones. The results show that cattle are carriers and potential transmitters of STEC and ETEC strains containing virulence genes. Epidemiological retrospective studies in different countries are of great help for identifying virulent bacterial strains with the potential to cause outbreaks that may have epidemiological impact in susceptible countries.

Keywords

Escherichia coli; Serogroups, Serotypes; DEC; non-O157 STEC; phylogenetic groups

Introduction

Escherichia coli (*E. coli*) strains continue to be a frequent cause of human infection. These infections can be restricted to the gastrointestinal track or the cause of urinary, systemic or meningial infections [1]. In the case of gastrointestinal infections, *E. coli* strains associated with disease have been classified by their virulent characteristics into at least six different pathogenic clusters that constitute the so-called Diarrheagenic *E. coli* group (DEC) [2]. Human feces constitute the most frequent source of DEC strains, either through direct hand-to-mouth transmission or by the contamination of water and food [3-5]. Animal feces are a secondary source of human infection for some DEC strains, mainly those capable of producing Shiga-like toxin. These group of DEC strains have been called Shiga toxin-producing *E. coli* (STEC) and represent the most frequent cause of severe hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS), since their discovery in the early 80s [6,7]. While most of the large outbreaks of HC and HUS have been associated with specific STEC belonging to the O157:H7 serotype, STEC strains belonging to other O:H serotypes have also been linked to HC and HUS [4,8-10]. This latter group includes the recently isolated O104:H4 strains, which were associated with an important outbreak of HC and HUS in Germany [11]. These outbreak strains represent a hybrid with virulence traits from both STEC and Enteroaggregative *E. coli* (EAggEC) strains, which is something that had not been reported previously [12]. Given our interest in the frequency of DEC strains as a cause of diarrheal disease in Mexican children over the past 30 years and the capacity of our laboratory to conduct full serotyping of O and H antigens, and the determination of the virulence characteristics of putative DEC strains isolated from both humans and animals, we describe here the frequency and characteristics of *E. coli* strains isolated from cattle raised in three different regions of Mexico and discuss the capacity of these strains to cause human disease outbreaks.

Material and Methods

Biological Material

The protocol was approved by the Research Committee (IN225705) of the Faculty of Medicine at the National Autonomous University of Mexico. Sampling of dairy cows was conducted in accordance with specific techniques for the production, care and use of laboratory animals, as described in the Mexican Official Norm 062-Zoo-1999 (NOM-062-Zoo-1999).

Fecal, rectal and blood samples were collected from 252 cows belonging to five herds of lactating cows raised in Jalisco (Jal), Sinaloa (Sin) and Sonora (Son), Mexico. Sampling of the dairy cows was carried out in three different years (2004, 2005 and 2007). Fecal samples (10g) were collected from the rectum of the cattle. Following collection, cotton swabs were dipped into Cary-Blair transport medium and the samples were kept at 4 °C until they reached the laboratory for processing within 3 hours (3h) of arrival.

Bovine Serum Samples

Serum samples were obtained from total blood taken from the coccygeal vein of the cows, after cleaning and disinfecting the area with soap, water and alcohol (70%). Each blood sample was obtained using a 5 mL syringe with a 22G needle. Approximately 3 mL of blood was taken and placed in a Vacutainer tube without anticoagulant (BD). As with the fecal samples, the blood samples were kept under refrigeration at 4 °C until they reached the laboratory for processing. Serum separation was performed in the laboratory and kept until use at -20 °C in 100 µL aliquots.

Isolation and Identification of Strains

For the isolation of *E. coli* and other *Enterobacteriaceae*, the fecal samples were streaked onto MacConkey agar with lactose and sorbitol and the plates were incubated overnight at 37 °C. For the isolation of antigenic representative varieties of *E. coli*

and other enterobacteria, five or more isolated colonies were selected from the agar plates. The cultures were identified separately using the GNI card (Gram-negative identification) of the automated VITEK2 system (compact model, bioMérieux).

Serotyping

Strains identified as *E. coli* were serotyped by agglutination assays using 96-well micro titer plates and rabbit antisera against O1 to O187 somatic (O) antigens and 53 flagellar (H) antigens prepared in rabbits (SERUNAM, registered trademark in Mexico, with number 323158/2015) using the method described by Orskov and Orskov [13] with minor modifications.

Virulence Genes

Virulence genes for Shiga-toxin producing *E. coli* (STEC) and enteroaggregative *E. coli* (EAEC) were detected by PCR using the primers described in Table 1 under conditions previously described [14-19].

Phylogenetic Grouping

Phylogenetic groups were determined by PCR multiplex using the primers described in Table 1 and under conditions detailed by Clermont, et al. [20]. The designation of the phylogenetic groups (A, B1, B2, C, D, E, F and *Escherichia* cryptic clade I) was carried out by the presence or absence of *chuA*, *yjaA*, TspE4.C2 and the *arpA* gene using a flow scheme [20].

Table 1: Primers used to identify virulence genes and phylogenetic groups.

Genes	Code	Sequence (5' a 3')	Product size (pb)	Reference
<i>eae</i> (Universal)	Sk1-F	CCCGAATTCGGCACAAGCATAAGC	864	[15]
	Sk2-R	CCCGGATCCGTCTCGCCAGTATTC		
<i>stx1</i>	vtx1-F	GTACGGGGATGCAGATAAAATCGC	209	[16]
	vtx1-R	AGCAGTCATTACATAAGAACGYCCACT		
<i>stx2</i>	vtx2-F	GGCACTGTCTGAAACTGCTCCTGT	627	
	vtx2-F	ATTAAACTGCAC TTCAGCAAATCC		
<i>ehxA</i>	ehxA-F	GGTGCAGCAGAAAAAGTTGTAG	1551	[14]
	ehxA-F	TCTCGCTGATAGTGT TTGGTA		
<i>arpA</i>	arpA-F	AACGCTATTCGCCAGCTTGC	400	[20]
	arpA-R	TCTCCCCATACCGTACGCTA		
<i>chuA</i>	chuA-F	ATGGTACCGGACGAACCAAC	288	
	chuA-R	TGCCGCCAGTACCAAAGACA		
<i>YjaA</i>	yjaA-F	CAAACGTGAAGTGTCAGGAG	211	
	yjaA-R	AATGCGTTCCTCAACCTGTG		
<i>TspE4.C2</i>	TspE4-F	CACTATTCGTAAGGTCATCC	152	
	TspE4-R	AGTTTATCGCTGCGGGTCGC		
<i>aatA</i>	aatA-F	ATGTTACCAGATATAAATATAG	1064	[17]
	<i>aat-R</i>	CATTCCCCTGTATTGGAAATG		
<i>aggR</i>	aggR-F	CTAATTGTACAATCGATGTA	308	[18]
	aggR-R	ATGAAGTAATTCTTGAAT		
<i>aapA</i>	aapA-F	CTTGGGTATCAGCCTGAATG	310	[19]
	aapA-R	AACCCATTCGGTTAGAGCAC		

Antimicrobial Resistance

Antimicrobial susceptibility testing was performed according to the manufacturer's instructions (bioMérieux). In brief, antimicrobial resistance testing was performed by the VITEK2 automated system (compact model, bioMérieux). Each strain was inoculated into nutrient agar (pH 7.4) and incubated overnight at 37°C. The growth of each culture was diluted in 2.0 mL of 4.5% sterile saline solution and then adjusted to tube number 1 of McFarland nephelometer (3×10^8 bacteria/mL). The bacterial suspension was then inoculated by the automated system onto an AST-GN23 card to test its reaction against antimicrobial agents for gram negative organisms.

The concentration of antimicrobials in the AST-GN23 card was as follows: amoxicillin/clavulanic acid (AMC) 4/2, 16/8, 32/16 µg/mL; ampicillin (AM) 4, 8, 32 µg/mL; cephalothin (CEP) 2, 8, 32 µg/mL; cefazolin (CZ) 4, 16, 64 µg/mL; cefepime (FEP) 2, 8, 16, 32 µg/mL; ceftiofloxacin (FOX) 8, 16, 32 µg/mL; cefpodoxime (CPD) 0.5, 1, 4 µg/mL; ceftiazidime (CAZ) 1, 2, 8, 32 µg/mL; ceftriaxone (CRO) 1, 2, 8, 32 µg/mL; cefuroxime (CXM) 2, 8, 32 µg/mL; ciprofloxacin (CIP) 0.5, 2, 4 µg/mL; gentamicin (GM) 4, 16, 32 µg/mL; levofloxacin (LEV) 0.25, 0.5, 2, 8 µg/mL; nitrofurantoin (FT) 16, 32, 64 µg/mL; norfloxacin (NOR) 1, 8, 32 µg/mL; piperacilline/tazobactam (TZP) 4/4, 16/4, 32/4, 64/4 µg/mL; tetracycline (TE) 2, 4, 8 µg/mL; tobramycin (TM) 8, 16, 64 µg/mL; and trimethoprim/sulfamethoxazole (SXT) 1/19, 4/76 µg/mL. *E. coli* strains ATCC 25922 and ATCC 35218 from the American Type Culture Collection (ATCC) were used as controls.

Purification of O104 LPS and K9 Capsular Antigen

Extraction and purification of O104 LPS was carried-out by the phenol-water method described by Westphal and Jann [21] using *E. coli* strain H519 (O104:H12). K9 capsular antigen was obtained from *E. coli* strain B1316/42 (O9:K9:H12) using a modified method of that described by Jann [22].

ELISA

IgG antibodies from each bovine serum were determined against O104 LPS and K9 capsular antigen by an ELISA test, as described previously [23]. The assays were placed into separate 96-well plates (Maxisorp F96) to which, either 10 µg/mL of the O104 LPS or the K9 capsular antigen were added to each well dissolved in carbonate buffer pH 9.6. The plates were first incubated for 2 h at 37 °C and then for 18-24 h at 4 °C. The plates were then blocked with 200 µL of a 1.0% PBS/BSA solution and incubated for 2 h at 37 °C. The plates were washed 3 times with 0.05% PBS/Tween and each well was inoculated with 100 µL of 1:1000 dilution of bovine serum sample to be tested. The plates were incubated for 2 h at 37 °C and then washed 3 times with PBS/Tween20. To each well, 100 µL of rabbit anti-IgG conjugated with alkaline phosphatase (Invitrogen) diluted 1:1000 in PBS pH 7.4 was added. Plates were then incubated for 2 h at 37 °C before adding 200 µL p-nitrophenyl phosphate (1 mg/mL, Sigma) in diethanolamine buffer (pH 9.8; Sigma) to visualize the reaction, which was stopped with 25 µL of 3M NaOH. Optical density (OD) was determined in an ELISA reader (MR580 Dynatech) at 405 nm.

Statistical Analysis

The assays were carried out in duplicate and the arithmetic means of two assays were compared by a one-tailed analysis of variance with a significance level of < 0.05. A cut-off point of OD 0.7 and 1:1000 serum dilution was considered to be a positive ELISA result, as described previously [20].

Results

Identification

Of the 252 fecal samples studied, 150 corresponded to Jalisco herds, and of these 58 samples corresponded to 2004, and 92 to 2005 and 2007 (46 in each year); in addition, 56 samples were from Sinaloa herds and 46 from Sonora. Of each fecal sample, five colonies were selected obtaining 1,260 cultures. In the identification of the cultures, 841 (66.7%) were identified as *E.*

coli and 419 (33.3%) as other *Enterobacteriaceae* (*E. hermannii*, *Enterobacter spp.*, *Klebsiella spp.*, *Citrobacter freundii*, and *Proteus spp.*).

Diarrheagenic Serogroups

Serotyping of the 841 cultures identified as *E. coli* strains indicated that 656 (78%) belonged to different, recognized *E. coli* serogroups (Table 2) and 185 (22%) were non-typable (O?). Of the strains with a recognized O group, 202 (30.8%) belonged to 21 non-pathogenic serogroups (Table 3), 61 (9.3%) to 6 ExPEC serogroups, and 393 (59.2%) to 80 DEC serogroups (Tables 2). Of the DEC strains, 105 (26.7%) belonged to ETEC group, 68 (17.3%) to EPEC group, 45 (11.5%) to EIEC group, 15 (3.8%) to EAEC group (Table 2 and 4) and 160 (40.7%) to non-O157 STEC group.

Table 2: Pathotypes analysis of *E. coli* strains isolated from dairy cows from 5 herds of three states of Mexico.

Pathogenic group	Pathotypes	Strains N (%)	Serogroups number
Non DEC	ExPEC	61 (9.3)	6
	Others (Non-pathogenic)	202 (30.8)	21
DEC	ETEC	105 (26.7)	19
	EPEC	68 (17.3)	7
	EIEC	45 (11.5)	7
	EAEC	15 (3.8)	5
	STEC	160 (40.7)	42
Total		656	107

The analysis by serogroups showed that 393 of 656 (59.9%) strains belonged to 80 diarrheagenic *E. coli* (DEC) serogroups and 263 (40.1%) were non-DEC. Likewise, of the 841 *E. coli* strains 185 were nontypeable (O?).

Table 3: Non-pathogenic serogroup.

Biochemical identification	Serogroups	Number of serogroups	Number of strains
<i>E. coli</i>	O10	1	20
	O102	1	12
	O108	1	9
	O134	1	16
	O140	1	12
	O155	1	9
	O16	1	3
	O165	1	4
	O170	1	3
	O19	1	4
	O32	1	7
	O33	1	14
	O42	1	5
	O53	1	7
	O58	1	6
	O59	1	24
	O61	1	4
	O62	1	6
O66	1	8	
O80	1	6	
O93	1	23	
Total		21	202

Table 4: DEC strains of different pathotype.

Biochemical identification	Serogroups	Pathotypes	Number of strains	Number of serogroups
<i>E. coli</i>	O7	ETEC	4	
	O8	ETEC	29	
	O11	ETEC	1	
	O25	ETEC	1	
	O27	ETEC	6	
	O29	ETEC/EIEC	3	
	O48	ETEC	6	
	O71	ETEC	5	
	O73	ETEC	1	
	O77	ETEC	6	

	O85	ETEC	7	
	O105ab	ETEC	15	
	O128	ETEC	2	
	O133	ETEC	1	
	O134	ETEC	1	
	O139	ETEC	2	
	O141	ETEC	10	
	O149	ETEC	4	
	O166	ETEC	1	
				19
<i>E. coli</i>	O18ac	EPEC/UPEC/MN	7	
	O26	EPEC	7	
	O88	EPEC	26	
	O103	EPEC	13	
	O111	EPEC	2	
	O142	EPEC	11	
	O145	EPEC	2	
				7
<i>E. coli</i>	O112ab	EIEC	19	
	O115	EIEC	2	
	O121	EIEC	1	
	O124	EIEC	3	
	O135	EIEC	4	
	O136	EIEC	8	
	O152	EIEC	8	
				7
<i>E. coli</i>	O3	EAEC	3	
	O77	EAEC	4	
	O85	EAEC	1	
	O86	EAEC	1	
	O125ab	EAEC	6	
				5
<i>E. coli</i>	O1	UPEC	14	
	O2	UPEC/MN	10	
	O6	UPEC	18	
	O7	UPEC/MN	10	
	O16	UPEC	8	
	O75	UPEC	1	
				6
Total			294	44

Serotyping and Virulence Genes

Table 5 shows the results of the serotyping and pathogenic characteristics of the STEC strains detected in all the herds studied. The presence of *stx1* and *stx2* genes was determined in

160 STEC strains and of these, 70 (43.8%) could be grouped into 23 serogroups amplified for one or both genes, while the remaining 90 (56.3%) were negative for the presence of both genes (Tables 5 and 6). When considering the serotypes, as well as the place and year of isolation, three Jal2004 strains were O157:H7, three O179:H8, two O146:H21 serotypes and seven belonged to different serotypes. The presence of the *stx2* gene was detected in 8 (11.4%) of these STEC strains, *stx1+stx2* in 7 (10%), *eae* in 3 (4.3%) and *ehxA* in 8 (11.4%) strains. For the Jal2005 samples, three STEC strains belonged to serotype O116:H21, two were O88:H25 and three strains were of different serotypes. Gene detection for these strains showed *stx1* in 1 (1.4%) strain, *sxt2* in 5 (7.1%) and *ehxA* in 3 (4.3%) strains. Finally, in the Jal2007 samples, four were O116:H16, three O121:H11, three O48:NM (non-motile), two O154:H1, two O154:H4, two O180:NM and two strains were of different serotypes. The genes detected in these STEC strains were *stx2* in 18 (25.7%) strains and *ehxA* in 9 (12.9%) strains.

The serotypes of the Sinaloa strains isolated during 2005 included seven O88:H25 strains, four O146:H21, three O175:H2, two O non-typeable:H2, two O18ac:H7 and four strains of different serotypes. The genes detected were *stx1* in 4 (5.7) strains, *stx2* in 10 (14.3%), *stx1+stx2* in 9 (12.9%) and *ehxA* in 5 (7.1%) strains. In the Son2005 strains, two were O104:H12 strains and four were of different serotypes. The genes detected were *stx1* in 1 (14.4%) strain, *sxt2* in 5 (7.1%) and *ehxA* in 3 (4.3%) strains. Of the 90 STEC strains that did not amplify *stx1* or *stx2*, 13 were of serogroup O9 with different flagellar antigens (NM, H12, H21, H25 and H54) isolated from the Jal2005, Jal2007 and Sin2005 herds. These strains had genes *ehxA*, *aggR* and *eae* when amplified (Table 7)

Phylogenetic Groups

Most of the non-O157 STEC strains were included in the commensal B1 (61.4%) and A (21.4%) groups, with only 17% belonging to virulent groups B2, D and E (Table 5). The analysis of the *stx1* and *stx2* negative O9 strains showed that they belonged only to commensal group A (23.1%), group B1 (30.8%) and group C (46.1%) (Table 7).

Table 5: Association between phylogenetic groups and virulence genes of STEC strains isolated from dairy cows.

Location and year of sampling	Serotypes	Strains	Phylogenetic group ³	Virulence genes				
				<i>stx1</i> only	<i>stx2</i> only	<i>stx1+stx2</i>	<i>eae</i>	<i>ehxA</i>
Jal2004	O8:H8	1	B1 (15.7)	-	1	-	-	-
	O48:H8	1		-	-	1	-	-
	O58:H40	1		-	1	-	-	1
	O77:H18	1		-	1	-	-	-
	O80:H39	1		-	1	-	-	-
	O112ac:H7	1		-	1	-	-	1
	O146:H21	2		-	2	-	-	-
	O179:H8	3		-	-	3	-	2
	O157:H7 ¹	3	D (4.3)	-	-	3	3	3
O?:H48	1	E (1.4)	-	1	-	-	1	
Jal2005	O3:NM	1	A (1.4)	-	-	1	-	-
	O8:H8	1	B1 (10)	-	1	-	-	1
	O88:H25	2		-	2	-	-	2
	O116:H21	3		1	1	1	-	-
	O178:NM	1		-	1	-	-	-
Jal2007	O116:H16	4	A (15.7)	-	4	-	-	4
	O154:H1	2		-	2	-	-	1
	O154:H4	2		-	2	-	-	-
	O154:H32	1		-	1	-	-	1
	O180:NM	2		-	2	-	-	-
	O121:H11	3	B1 (4.3)	-	3	-	-	3
	O48:H54	1	B2 (1.4)	-	1	-	-	-
	O48:NM	3	D (4.3)	-	3	-	-	-
Sin2005	O18ac:H7	2	B1 (27.1)	-	2	-	-	-
	O39:H21	1		-	1	-	-	1
	O88:H25	7		-	2	5	-	3
	O103:H25	1		1	-	-	-	1
	O146:H21	4		1	2	1	-	-
	O174:H28	1		1	-	-	-	-
	O175:H21	3		-	-	3	-	-
	O?:H2	3		D (5.7)	1	2		
	O?:H20	1	-		1	-	-	-
Son2005	O104:12 ²	2	A (4.3)	-	2	-	-	2

	O176:H54	1		-	1	-	-	-
	O176:H11	1	B1 (4.3)	1	-	-	-	-
	O88:H25	1		-	1	-	-	1
	O146:H21	1		-	1	-	-	-
Total, N (%)		70		6 (8.6)	46 (65.7)	18 (25.7)	3 (4.3)	28 (40)

¹All the O157:H7 strains were *eae*, *stx1*, *stx2* and *ehxA* gene positives

²Positive strains for the *aggR* and *aapA* genes. The remaining of the strains were negative for *aggR*, *aapA*, and *aatA* genes.

³Group A, *arpA*+, *chuA*-, *yjaA*+, TspE4.C2-; Group B1, *arpA*+, *chuA*-, *yjaA*-, TspE4.C2+; B2, *arpA*-, *chuA*+, *yjaA*+, TspE4.C2+; group D, *arpA*+, *chuA*+, *yjaA*-, TspE4.C2+; group E, *arpA*+, *chuA*+, *yjaA*+, TspE4.C2-. Jalisco: JalSinaloa: Sin Sonora: Son Non-motile: NM

Table 6: STEC strains, *stx* negative

Identification biochemical	Serogroups	Strains	Virulence genes					
			Pathotypes	<i>stx1</i>	<i>stx2</i>	<i>eae</i>	<i>ehxA</i>	<i>aggR</i>
<i>E. coli</i>	O9	13	STEC	-	-	11	13	13
	O18ac	5	EPEC	-	-		-	
	O26	7	EHEC	-	-	-	7	-
	O48	5	EHEC	-	-	-	5	-
	O77	3	STEC	-	-		1	
	O81	2	STEC	-	-	-	-	
	O88	7	STEC	-	-		3	
	O91	1	EHEC	-	-	-	-	-
	O104	3	STEC	-	-	-		-
	O111	2	EHEC	-	-	-	-	-
	O116	2	STEC	-	-	-	-	-
	O117	1	STEC	-	-	-	-	-
	O135	2	EIEC	-	-		-	
	O145	2	EHEC	-	-	-	2	-
	O146	9	STEC	-	-	-	1	-
	O150	1	UTI	-	-		-	
	O176	5	EHEC	-	-	-	-	-
O178	1	EHEC	-	-	-	-	-	
O179	19	EHEC	-	-	-	-	-	
Total		90				11	32	13

Bovine Serum Response to O104 LPS and K9 Capsular Antigen

A cut-off point of 0.7 OD was considered as a positive value for the ELISA test to determine a response at a 1:1000 serum dilution. This value corresponded to the mean value, plus two standard deviations when the assays were read at 405 nm, as previously reported [23]. The analysis of these assays indicated that 89 serum samples (35%) reacted with the O104 LPS and 82 (33%) with the K9 capsular antigen. The results did not show significant differences in an X^2 analysis (Table 8). The serum response to these same antigens was the same for samples obtained from cows raised in the different herds ($p>0.05$). Similar observations were obtained when a cut-off point of 0.4 DO was applied to the ELISA test. In this case, the results showed that 183 (72.6%) serum samples reacted with the O104 LPS and 200 (79.4%) with the K9 capsular antigen ($p= 0.08$).

Antimicrobials Sensitivity

Antimicrobial susceptibility testing indicated that 52 out of 70 (74.3%) STEC strains were sensitive to all antimicrobials tested, while 18 (25.7%) were resistant to AMP, CF, CXM, TE and SXT in different combinations (Table 9). In difference, 92% of the non-STEC strains belonging to serotype O9, were resistant to most of the antimicrobials tested (Table 10).

Table 7: Phylogenetic group and virulence genes of *E. coli* serogroup O9* isolated from dairy cows.

Location and year	Serotype	Strains	Phylogenetic group	<i>eae</i>	<i>ehxA</i>	<i>aggR</i>
Jal2005	O9:H12	1	B1 (7.7)	1	1	1
Sin2005	O9:H21	1	A (7.7)	1	1	1
Jal2007	O9:NM	1	A (15.4)	1	1	1
	O9:H25	1		1	1	1
	O9:NM	1	B1 (23.1)	1	1	1
	O9:H21	1		1	1	1
	O9:H54	1		1	1	1
	O9:NM	4	C (46.2)	4	4	4
	O9:H9	1		-	1	1
O9:H12	1	-		1	1	
Total, N (%)		13		11 (85)	13 (100)	13 (100)

* All strains of *E. coli* O9 were negative for *stx1*, *stx2* and *aap* genes.

Non-motile:NM

Table 8: Analysis of the reactivity of serum samples from five herds of dairy cows from three states of Mexico against O104 LPS and the K9 capsular antigen.

Location and year of sampling	Samples	Antigens		<i>p</i> value*
		O104 LPS	K9 capsular antigen	
Jal2004	58	27 (47)	23 (40)	0.45
Jal2005	46	16 (35)	13 (28)	0.5
Jal2007	46	13 (28)	12 (26)	0.81
Sin2005	56	23 (41)	24 (43)	0.85
Son2005	46	10 (22)	10 (22)	1.0
Total	252	89 (35)	82 (33)	0.63

The results are shown as the number of positive samples with the percentage in parentheses.

The ELISA used a cut-off point of .0.7 at A₄₀₅ and a serum dilution of 1:1000.

*Statistical difference between the two antigens using a X² test.

Table 9: Resistance patterns of STEC strains isolated from dairy cows.

Herd and year	Serotype	Strains	Resistance to Antimicrobials				
			Penicillins	Cephalosporins		Tetracycline	Trimethoprim/Sulfa methoxazole
			AM	CEP	CXM	TE	SXT
Jal2004	O8:H8	1				R	
	O58:H40	1				R	
	O146:H21	1				R	
	O146:H21	1	R	R	R	R	
Jal2005	O88:H25	2				R	
	O178:NM	1		R			
Jal2007	O116:H16	3	R				
	O116:H16	1	R			R	
	O121:H11	2	R	R		R	R
Sin2005	O174:H28	1	R	R			
	O88:H25	1	R		R		
Son2005	O146:H21	1			R		
	O104:H12	1				R	
	O104:H12	1			R		R
Total N (%)		18	9 (50)	5 (27.8)	4 (22.2)	10 (55.6)	3 (16.7)

AM, ampicillin; CEP, cephalothin; CXM, cefuroxime; TE, tetracycline; SXT, trimethoprim/sulfamethoxazole. Non-motile:NM

Table 10: Patterns of antimicrobial resistance of *E. coli* O9 strains.

Herd and year	Serotypes	Strains	Resistance in common to β -lactams and cephalosporins	Other antimicrobials	Total antimicrobials
Jal2005	O9:H12	1		CIP, LEV TET y SXT	4
Jal2007	O9:H21	1		AMC, TZP, CEP, CPD	4
	O9:NM	1	AM, AMC, CEP, CZ, CXM, CPD, CAZ, CRO, FEP		9
	O9:NM	1		TZP, TET	11
	O9:NM	1		GM, CIP	11
	O9:NM	2		GM, FOX, TET, SXT	13
	O9:H12, O9:NM, O9:H54, O9:H25	4		TZP, GM, TM, FOX, SXT	14
	O9:H9	1		TZP, GM, TM, FOX, CIP, TET, SXT	16
	Total (N=13)	12 (92%)			

AM, ampicillin; AMC: amoxicillin/clavulanic acid; CEP, cephalothin; CZ, cefazolin; CXM, cefuroxime; CPD, cefpodoxime; CAZ, ceftazidime; CRO; ceftriaxona; FEP, cefepime; CIP, ciprofloxacin; LEV, levofloxacin; TE, tetracycline; SXT, trimethoprim/sulfamethoxazole; TZP, piperacilline/tazobactam; GM, gentamicin; FOX, ceftiofur; TM, tobramycin. Non-motile:NM

Discussion

Although diarrheal disease mortality has decreased significantly over the past 15 years, morbidity due to this problem continues to be high [24]. Although currently available vaccines against some of the most frequent viral pathogens have shown their capacity to reduce mortality, the search for the source of these infections is essential for their final control. Water and food sampling constitute the most accessible way to look for these sources. That is the reason why we conducted a sequential sampling of feces and sera of cows belonging to herds in three of the largest cattle-rearing regions in Mexico. Over a number of years, the search for putative pathogenic bacteria in these samples has shown the presence of putative pathogenic strains able to cause disease in humans. Around 40% of the putative pathogenic *E. coli* found in the sample animals belonged to non-O157 STEC strains. This has also been found in previous studies conducted in other parts of the world [25,26]. The isolation of O157:H7 strains is interesting since, to date, there have been no reported outbreaks of HC and HUS in Mexico associated with these type of isolates. Their presence in these herds is a wake-up call to continue this surveillance since it is clear that cows from these herds harbor both O157:H7 and non-O157 strains that could be the source of a human outbreak.

ETEC strains were the second most frequent DEC group of strains found in the present study. ETEC strains have been associated worldwide with cases of diarrhea in pigs, calves and rabbits and are an important cause of diarrhea in children in low and middle income countries [5,25,27,28] and in travelers to these areas of the world [10,29]. In the present study, we were able to identify ETEC strains belonging to 19 different serotypes, all of them previously reported from other parts of the world [28,30].

The lack of clinical symptoms in the cows harboring strains belonging to pathogenic STEC types O157:H7 and O104:H12 confirm previous findings and indicate the risk for humans coming in contact with these animals or their feces [31,32]. However, there is no clear explanation as to why these fully

pathogenic strains found in cows have not been able to cause human disease. Strains belonging to serotype O104:H7 have been previously found in healthy Mexican lambs [33]. No human cases associated with these strains have been reported to date, either. In the present study, strains of serotype O104:H12 were also isolated during 2005 from the herd raised in Sonora. These strains carried *stx2*, *aggR* and *aatA* genes, similar to the O104:H4 strains that caused the HC and HUS outbreak in Germany [12]. In addition to the O104 isolates, cows from herds in the three States also carried strains belong to the O88:H25 serotype that had been previously reported as belonging to STEC and EPEC groups [34,35]. Gene analysis of these O88:H25 strains indicated that they carried the *stx2* gene, making them potentially virulent non-O157 STEC strains.

The higher percentage of strains belonging to phylogenetic B1 groups found in the present study is similar to previous findings from other parts of the world where these B1 strains have been isolated from herbivorous animals, such as goats, lambs and cows [36-38].

Observations in our laboratory over the past 20 years have shown that *E. coli* of serotype O104 and STEC O9:K9 is not an infrequent finding in human fecal samples (unpublished results). This has led to our continued interest in studying the cross reactions that these two groups of strains have with each other and, as has been previously reported, to both the O104 LPS and the K9 capsular antigen [39,40]. Serum from the cows raised in the five herds tested showed a similar level of response, which signaled a common type of cross-reaction in the serum samples of all the animals tested. The development of antibodies (IgG) to both the O104 LPS and K9 capsular antigen in the same animal indicates the possibility that these animals have a gradual and low intensity repeated exposure to *E. coli* O104 strains. Another factor that may give rise to the presence of anti-O104 LPS and anti-K9 capsular antigen antibodies in the serum of these animals could be colonization by specific *E. coli* O9:K9 strains. These findings could be helpful to develop better oriented vaccines that might protect humans by inhibiting their capacity to colonize

animal reservoirs. The implication for animal vaccines against O157:H7 is obvious [41].

In terms of our findings concerning the frequency of antimicrobial resistance, the STEC Mexican strains, including the isolated O157:H7, showed a much lower presence of this characteristic than the one described in other countries [42]. On the other hand, the results of these assays in the O104:H12 strains indicated a similar frequency of antimicrobial resistance to one (CEP) or two (TE and SXT) antimicrobials, as previously reported by Schroeder *et al.* [41] but with different patterns to the O104:H4 strains isolated during the epidemic outbreak in Germany [11]. Interestingly, multiple antimicrobial resistance was found in O9 strains we studied, which is in line with reports from previous studies for *E. coli* strains isolated from extraintestinal sources [43]. All these results continue to point to the use of antimicrobials as part of the food given to farm animals, which needs to be addressed as one of the main causes for the increase in multiple antimicrobial resistance in human infections [44,45].

The presence of *E. coli* strains belonging to two of the most important human DEC pathotypes isolated from the feces of the animals studied indicates their importance as a potential source of virulence strains able to cause human infections. The participation of food, water and other environmental sources, as well as direct human contact with animals has been well documented in terms of infections by *E. coli* strains belonging to the O157:H7 serotype. [32,46]. In addition, the spread of pathogenic bacteria could be enhanced by the use of fresh manure to fertilize soil for the purpose of growing vegetable crops [47]. The results of the present study clearly support these findings and point to the need for continued surveillance of these putative sources of human disease.

Finally, the presence of multiresistant strains as part of the intestinal microbiota of cattle could be the source of difficult-to-treat extraintestinal infections caused by *E. coli* strains that have received these genes through horizontal transfer in the intestinal environment [48]. Bearing this in mind, our study reveals the

importance of epidemiologic surveillance of the natural reservoirs of DEC strains in groups of animals used for human consumption.

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