Genetic characterization of Shiga toxin-producing *Escherichia coli* O26:H11 strains isolated from animal, food, and clinical samples

Alejandra Krüger[1]*, Paula M. A. Lucchesi[1], A. Mariel Sanso[1], Analía I. Etcheverría[1], Ana V. Bustamante[1], Julia Burgán[1], Luciana Fernández[1], Daniel Fernández[1], Gerardo Leotta[2], Alexander W. Friedrich[3], Nora L. Padola[1]* and John W. A. Rossen[3]

[1] Laboratorio de Inmunoquímica y Biotecnología, Facultad de Ciencias Veterinarias, Centro de Investigación Veterinaria de Tandil, Consejo Nacional de Investigaciones Científicas y Técnicas, Comisión de Investigaciones Científicas, Universidad Nacional del Centro de la Provincia de Buenos Aires, Tandil, Argentina, [2] Línea Seguridad Alimentaria, Instituto de Genética Veterinaria Ing. F.N. Dulout, Consejo Nacional de Investigaciones Científicas y Técnicas, La Plata, Argentina, [3] Department of Medical Microbiology, University Medical Center Groningen, University of Groningen, Groningen, Netherlands

The Shiga-toxin producing *Escherichia coli* (STEC) may cause serious illness in human. Here we analyze O26:H11 strains known to be among the most reported STEC strains causing human infections. Genetic characterization of strains isolated from animal, food, and clinical specimens in Argentina showed that most carried either stx_{1a} or stx_{2a} subtypes. Interestingly, stx_{2a}-positive O26:H11 rarely isolated from cattle in other countries showed to be an important proportion of O26:H11 strains circulating in cattle and food in our region. Seventeen percent of the isolates harbored more than one gene associated with antimicrobial resistance. In addition to *stx*, all strains contained the virulence genes *eae*-β, *tir*, *efa*, *iha*, *esp*B, *cif*, *esp*A, *esp*F, *esp*J, *nle*A, *nle*B, *nle*C, and *iss*; and all except one contained *ehxA*, *esp*P, and *cba* genes. On the other hand, *tox*B and *esp*I genes were exclusively observed in stx_2-positive isolates, whereas *kat*P was only found in stx_{1a}-positive isolates. Our results show that O26:H11 STEC strains circulating in Argentina, including those isolated from humans, cattle, and meat products, present a high pathogenic potential, and evidence that cattle can be a reservoir of O26:H11 strains harboring stx_{2a}.

Keywords: Shiga toxin-producing *Escherichia coli*, O26:H11, genetic characterization, microarray, MLVA, hemolytic uremic syndrome, cattle

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) are *E. coli* strains that can cause human diseases, like gastrointestinal illnesses and hemolytic uremic syndrome (HUS). *E. coli* O157 has been the most commonly reported STEC serogroup since it was identified in the 1980s. However, the number of non-O157 STEC infections has increased substantially in the last years. The O26 is one of the few serogroups frequently reported in non-O157 cases (Rivas et al., 2010; EFSA, 2013; Gould et al., 2013; ISPCH, 2014). In particular, strains of serotype O26:H11/- have been associated with severe human diseases (Gerber et al., 2002; Zimmerhackl et al., 2010; Käppeli et al., 2011).

All STEC strains are characterized by the ability to produce Shiga toxins (Stx). The Stx family consists of two major types: Stx1 and Stx2, which can be further divided into several subtypes. A single STEC strain may carry one or more Shiga toxin-encoding genes (*stx*) which are generally carried by prophages. Epidemiological studies indicate that different subtypes of *stx* are related to different clinical manifestations after STEC infection (Krüger and Lucchesi, 2015). Particularly, the stx_{2a} subtype is associated with highly virulent strains and HUS. Characterization of *stx* genotypes showed that STEC O26 strains isolated from patients can harbor stx_{1a}, stx_{2a} or both, however, strains harboring only stx_{2a} were significantly associated with HUS (Bielaszewska et al., 2013). Furthermore, Bielaszewska et al. (2013) identified a new highly virulent stx_{2a}-positive O26 clone as an emerging cause of HUS in Europe.

The production of Stx seems to be essential but not solely responsible for STEC pathogenicity. Other known and putative virulence factors are usually present in pathogenic STEC strains. Some of them, like the adhesin intimin encoded on a bacterial chromosomal pathogenicity island and the enterohemolysin EhxA encoded on a plasmid have been found in association with severe clinical disease in humans (Boerlin et al., 1999; Aldick et al., 2007).

It is a well-known fact that ruminants are the main reservoir of STEC strains (Naylor et al., 2005). Some studies suggest that domestic animals serve as reservoir for human pathogenic O26 STEC strains (Leomil et al., 2005). The O26 STEC strains also seem to be widely distributed in cattle, since those strains have been isolated from bovines belonging to different animal categories and production systems (Monaghan et al., 2011; Fernández et al., 2012; Paddock et al., 2014; Bonardi et al., 2015; Ison et al., 2015). Moreover, O26:H11 strains have been isolated from beef and dairy products (Bosilevac and Koohmaraie, 2011; Madic et al., 2011; Mohammed et al., 2014). Several studies from Europe and the United States report that O26 strains isolated from food and cattle generally carry stx_1 or both stx_1 and stx_2 genes; moreover, O26 strains harboring only stx_{2a} have been rarely isolated from cattle and food (Pearce et al., 2006; Geue et al., 2009; Bonanno et al., 2015; Ison et al., 2015).

Our aim was to characterize O26:H11 STEC strains isolated from cattle, food and humans to contribute to the global knowledge of virulence profiles and epidemiology of O26 strains circulating in Argentina.

MATERIAL AND METHODS

Bacterial Strains and Growth Conditions

E. coli O26:H11 strains were selected from STEC collections in Argentina. The strains had been isolated between 1995 and 2013, from cattle, meat, human, and farm environment (**Table 1**). Most of the strains had been previously characterized by PCR regarding the presence of stx_1, stx_2, *eae, ehxA,* and *saa* genes. Strains were stored at $-70°C$ with 20% (v/v) glycerol and when necessary grown in Luria Bertani broth at $37°C$ overnight.

TABLE 1 | Characteristics of O26:H11 STEC isolates tested in this study.

Strain	Period of isolation	Source	References
1	2009	Milk-fed calf, Farm E	Fernández et al., 2012
2	2009–2010	Ground, Farm E	Polifroni et al., 2012
3	2009	Milk-fed calf, Farm E	Fernández et al., 2012
4	1995–1996	Calf	Blanco et al., 2004
5	1995–1996	Calf	Blanco et al., 2004
6	1995–1996	Calf	Blanco et al., 2004
7	1995–1996	Calf	Blanco et al., 2004
8	1995–1996	Calf	Blanco et al., 2004
9	1995–1996	Calf	Blanco et al., 2004
10	2002–2009	Human with diarrhea	Rivero et al., 2010
11	2002–2009	Human with diarrhea	Rivero et al., 2010
12	2009	Milk-fed calf, Farm D	Fernández et al., 2012
13	2009	Milk-fed calf, Farm E	Fernández et al., 2012
14	2010	Newborn calf, Farm A	Fernández et al., 2012
15	2010	Newborn calf, Farm A	Fernández et al., 2012
16	2010	Newborn calf, Farm D	Fernández et al., 2012
17	2009–2010	Cow, Farm A	Fernández et al., 2012
18	2009	Milk-fed calf, Farm E	Fernández et al., 2012
19	2010	Newborn calf, Farm A	Fernández et al., 2012
20	2010	Newborn calf, Farm B	Fernández et al., 2012
21	2010	Newborn calf, Farm B	Fernández et al., 2012
22	2009	Growing calf, Farm D	Fernández et al., 2012
23	2010	Human with diarrhea	This study
24	2009	Human with diarrhea	This study
25	2009	Cow	This study
26	2013	Beef	This study
27	2013	Beef	This study
28	2013	Ground meat	This study
29	2009	Calf feed, Farm E	This study

Microarray-based Characterization of Virulence Factors

Bacterial genomic DNA was extracted with the UltraClean Microbial DNA Isolation Kit (Mo Bio) or Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. Virulence and antimicrobial resistance genes were evaluated with a commercial oligonucleotide microarray for *E. coli* according to the manufacturer's protocol (CLONDIAG *Escherichia coli* combined Assay, Alere Technologies GmbH; Geue et al., 2010). The array contained 87 probes targeting virulence genes and 102 probes targeting antimicrobial resistance associated genes. Visualization of hybridization was achieved using the ArrayMate instrument (CLONDIAG GmbH) and signals were analyzed automatically. The results were converted into a binary numerical format (1– present, 0– absent) and further analyzed using BioNumerics (Version 6.6; Applied Maths).

stx Subtyping

Specific PCR reactions were performed to identify stx_{1a}, stx_{1b}, and stx_{1c} subtypes (Scheutz et al., 2012).

The presence of stx_{2b}, stx_{2e}, stx_{2f}, and stx_{2g} subtypes was evaluated with the oligonucleotide microarray. This assay also

detects stx_{2a}, stx_{2c}, and stx_{2d} subtypes but does not discriminate among them. Therefore, strains positive with the probe that detects $stx_{2a,c,d}$ subtypes where further subtyped with specific PCR reactions (Scheutz et al., 2012).

Detection of *eae*-β Gene

Strains were tested for the presence of the *eae*-β subtype by specific PCR using the primer set SK1/LP4 (Oswald et al., 2000).

Antibiotic Susceptibility Testing

Isolates carrying resistance genes were tested for resistance to several antimicrobials by the disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2014). The following antimicrobial disks were used: ampicillin (10 μg), cephalothin (30 μg), cefotaxime (30 μg), cefoxitin (30 μg), amoxicillin/clavulanate (20 μg-10 μg), gentamicin (10 μg), amikacin (30 μg), streptomycin (300 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), trimethoprim-sulfamethoxazole (1.25–23.75 μg), nitrofurantoin (300 μg), tetracycline (30 μg), colistin (10 μg), florfenicol (30 μg).

Multiple-locus Variable Number Tandem Repeat Analysis (MLVA)

For typing, an MLVA protocol that comprises nine variable number of tandem repeats (VNTR) loci, seven loci proposed by Lindstedt et al. (2007), i.e., CVN001, 002, 003, 004, 007, 014, 015, and two additional loci proposed by Løbersli et al. (2012), i.e., CVN016 and 017 was used. The seven loci proposed by Lindstedt et al. (2007) were amplified as described by Bustamante et al. (2010) and the two additional ones according to González et al. (2014). PCR products were analyzed in 6% denaturing polyacrylamide gels, and detected by silver staining (GenePrint STR Systems—Silver Stain Detection, Promega).

Allelic variants identified for each VNTR were sequenced with the same primers used to amplify those regions (Macrogen, Inc.). The sequences obtained were analyzed using Chromas 2.32 software (Technelysium Pty. Ltd.) and allele sequences of each VNTR were aligned with the software Clustal W (Larkin et al., 2007) in order to identify the number of tandem repeat units (TR). Alleles were named according to the number of TR. The absence of an amplification product was considered a null allele (-2).

The diversity index (D_N), based on Nei's marker diversity, was calculated for each locus using the formula $D_N = 1 - \Sigma(\text{fra})^2$, where fra is the allelic frequency (Noller et al., 2003). The discriminatory power of the method was assessed using the Simpson diversity index (D_S) (Hunter and Gaston, 1988).

RESULTS

Genetic Characterization of Virulence Factors

Figure 1 shows the genes encoding virulence factors detected in the O26:H11 strains, clustered according to the Bionumerics analysis. Genes that were not found in any isolate are summarized in a footnote in the figure.

The *stx* types identified with the array were in agreement with previous PCR results. Forty five percent of the isolates were stx_1-positve, 52% stx_2-positive, and one isolate (3%) was stx_1 and stx_2-positive. By using the PCR subtyping protocol, all stx_1 genes corresponded to the stx_{1a} subtype and all stx_2 corresponded to the stx_{2a} subtype.

All tested isolates harbored the genes encoding for the adhesin Intimin (*eae* subtype β), and its receptor Tir (*tir*). Other adhesion related genes were also identified. The presence of *efa*1, *esp*B, and *iha* genes was demonstrated in all isolates, whereas *esp*P was found in all except the stx_1 and stx_2-positive isolate. The *tox*B gene was only observed in stx_2-positive isolates. The *fas*A, *fed*A, *fed*F, *fim*41a, *nfa*E, and *saa* genes were not found.

Among toxin-encoding genes, *ehxA* (encoding for a hemolysin) was present in 97% of the isolates and *ast*A (encoding for EAST1, the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1) was detected in 72% of the isolates. The *cba* and *cel*B genes associated with colicin activity were identified in 97 and 17% of the isolates, respectively. Also, one bovine isolate was positive for the gene encoding colicin M (*cma*) and one isolate obtained from human with diarrhea was positive for *mch*B, *mch*C, *mch*F, and *mcm*A microcin genes.

Several type III secretion system components (translocators and effectors) were identified. All the strains were positive for *cif*, *esp*A, *esp*fO103, *esp*J, *nle*A, *nle*B, and *nle*C genes, and 76% of the isolates harbored *tcc*P. The *esp*I gene was only identified in stx_2-positive strains. The *etp*D, a gene encoding for a type II secretion pathway-related protein, was not found.
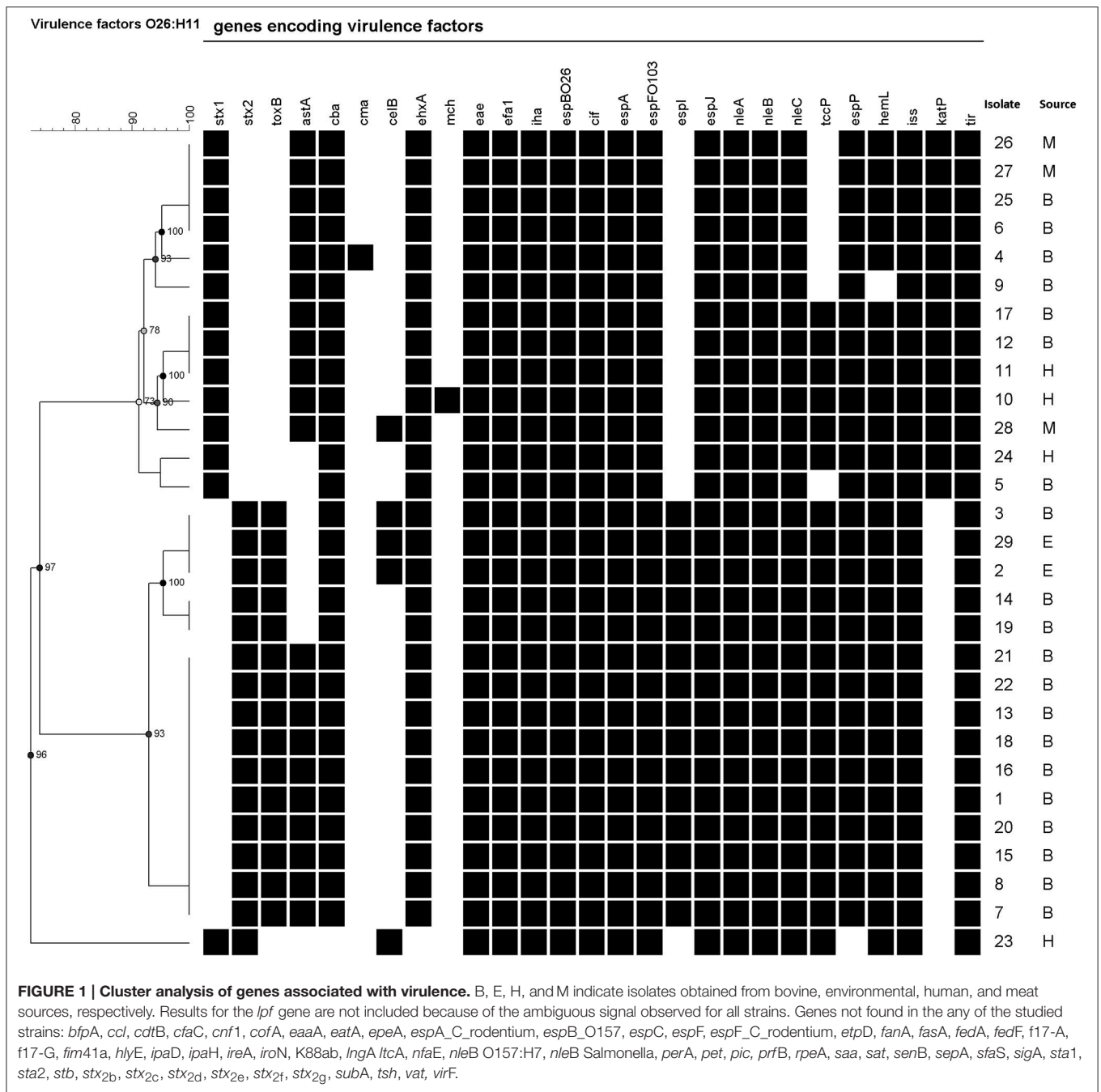
The *iss* and *hem*L genes were identified in 100 and 97% of the isolates, respectively. The *kat*P gene, encoding a catalase peroxidase, was detected in stx_1-positive isolates only.

Antimicrobial Resistance

Five O26:H11 isolates (17%) carried more than two antimicrobial resistance genes, being bla_{TEM}, *str*A-*str*B, and *sul*2 the most frequently detected (**Table 2**). Genes that were not found in any of the isolates are summarized in a footnote in the table. The integron integrase encoding gene (*int*I1) was detected in isolate 21. The antimicrobial susceptibilities of the five isolates carrying resistance genes were determined using disk diffusion method. Four isolates were resistant to multiple antimicrobial agents (**Table 2**), and most genetic resistances were phenotypically confirmed. The exception was isolate 23 (human isolate) that carried *str*A, *str*B, and *sul*2 genes but was susceptible for the tested antibiotics.

MLVA Typing

The isolates could be principally discriminated by alleles of loci CVN014 ($D_N = 0.83$) and CVN016 ($D_N = 0.70$), followed by those of CVN017 ($D_N = 0.63$). On the other hand, loci CVN002 and CVN003 showed a null allele in all the isolates. MLVA typing divided the total number of strains into 19 distinct genotypes, 13 of them being unique (**Figure 2**). The

FIGURE 1 | Cluster analysis of genes associated with virulence. B, E, H, and M indicate isolates obtained from bovine, environmental, human, and meat sources, respectively. Results for the *lpf* gene are not included because of the ambiguous signal observed for all strains. Genes not found in the any of the studied strains: *bfp*A, *ccl*, *cdt*B, *cfa*C, *cnf*1, *cof*A, *eaa*A, *eat*A, *epe*A, *esp*A_C_rodentium, *esp*B_O157, *esp*C, *esp*F, *esp*F_C_rodentium, *etp*D, *fan*A, *fas*A, *fed*A, *fed*F, f17-A, f17-G, *fim*41a, *hly*E, *ipa*D, *ipa*H, *ire*A, *iro*N, K88ab, *lng*A *ltc*A, *nfa*E, *nle*B O157:H7, *nle*B Salmonella, *per*A, *pet*, *pic*, *prf*B, *rpe*A, *saa*, *sat*, *sen*B, *sep*A, *sfa*S, *sig*A, *sta*1, *sta*2, *stb*, stx_{2b}, stx_{2c}, stx_{2d}, stx_{2e}, stx_{2f}, stx_{2g}, *sub*A, *tsh*, *vat*, *vir*F.

Simpson's index of diversity, calculated for the combined typing set, showed a value of $D_S = 0.96$. Three main clusters were obtained: one group included the stx_2-positive isolates and one stx_1-positive isolate (isolate 17); a second group, most of the stx_1-positive strains and the stx_1 and stx_2-positive isolate (isolate 23) and a third group included three stx_1-positive strains.

Isolates with the same MLVA type may indicate they are clonal. For some isolates this was supported by revealing a time-spatial relationship and by their identical virulence profile (isolates 14 and 19 from dairy farm A; 26 and 27 from a same

meat processing plant; 20 and 21 from dairy farm B; 16 and 22 from dairy farm D; 1 and 18 from dairy farm E; and 2 and 29 also from dairy farm E).

DISCUSSION

STEC O26:H11 strains are considered among the most reported STEC strains involved in human infections worldwide. To our knowledge, there is no specific study of O26:H11 STEC strains in Latin America. Our aim was to determine the characteristics of *E. coli* O26:H11 strains circulating in Argentina in the period

TABLE 2 | Antibiotic resistance profiles present in O26:H11 STEC isolates.

Isolate	Source[a]	Genotypic resistance profile[b]	Phenotypic resistance profile[c]
4	B	bla_{TEM} tetB	AMP$_{(R)}$ AMC$_{(R)}$ CEF$_{(I)}$ TET$_{(R)}$
21	B	bla_{TEM} strA strB aadA1 tetA drfA1 sul1 sul2 floR	AMP$_{(R)}$ AMC$_{(I)}$ CEF$_{(I)}$ TET$_{(R)}$ SXT$_{(R)}$ CMP$_{(R)}$ FFC$_{(R)}$
23	H	strA strB sul2	
26	M	bla_{TEM} strA strB tetB sul2	AMP$_{(R)}$ AMC$_{(I)}$ CEF$_{(I)}$ S$_{(R)}$ TET$_{(R)}$ NAL$_{(R)}$ SXT$_{(R)}$
27	M	bla_{TEM} strA strB tetB sul2	AMP$_{(R)}$ AMC$_{(I)}$ CEF$_{(I)}$ S$_{(R)}$ TET$_{(R)}$ NAL$_{(R)}$ SXT$_{(R)}$

[a]B, H, and M indicate isolates obtained from bovine, human, and meat sources, respectively.
[b]The bla_{TEM} gene is associated with beta lactam resistance; strA, strB, and aadA1 with aminoglycoside resistance; tetA and tetB with tetracycline resistance; sul1 and sul2 with sulfonamide resistance; and drfA1 with trimethoprim resistance. Resistance genes studied but not detected in any of the studied isolates: vatE for streptogramin A resistance; aac_aph, aac3, aac3la, aac3lva, aac6, aac6lb, aadA2, aadA4, aadB, ant2, aphA, grm, armA, rmtA, rmtB, rmtC, rmtD, and npmA for aminoglycoside resistance; bla_{ACC}, bla_{ACT}, bla_{CMY}, bla_{KHM}, $bla_{MOX-CMY9}$, bla_{CTX-M1}/$bla_{CTX-M15}$, bla_{CTX-M2}/bla_{CTX-M8}/$bla_{CTX-M26}$, ctx_{M9}, bla_{DHA-1}, bla_{FOX}, bla_{GES-1}, bla_{GIM-1}, bla_{IMI-3}, bla_{IMP}, bla_{KPC-4}, bla_{LAP-1}, bla_{LEN-1}, bla_{MOX}, bla_{OXA-1}, bla_{OXA-2}, bla_{OXA-7}, bla_{OXA-9}, bla_{OXA-23}, bla_{OXA-40}, bla_{OXA-48}, bla_{OXA-51}, bla_{OXA-54}, bla_{OXA-55}, bla_{OXA-58}, bla_{OXA-60}, bla_{PER-1}, bla_{PER-2}, bla_{PSE-1}, bla_{SHF-1}, bla_{SHV}, bla_{SME-1}, bla_{SPM-1}, bla_{VEB-1},and bla_{VIM} for beta lactam resistance; ble for bleomycin resistance; catA1, catB3, catB8, catIII, and cmlA1 for chloramphenicol resistance; ereA and ereB for erythromycin resistance; ermB, mphA, and mrx for macrolide resistance; qepA, qnrA1, qnrB, qnrD, and qnrS for quinolone resistance; arr for rifampin resistance; sph for streptomycin resistance; sul3 for sulphonamide resistance; tet37, tetC, tetD, tetE, tetG, and tetX for tetracycline resistance; dfrA5, dfrA7, dfrA12, dfrA13, dfrA14, dfrA15, dfrA17, dfrA19 for trimethoprim resistance.
[c]Resistent (R) and intermediate (I) results for ampicillin (AMP), amoxicillin/clavulanate (AMC), cephalothin (CEF), cefotaxime (CTX), cefoxitin (FOX), gentamicin (GEN), amikacin (AKN), streptomycin (S), nalidixic acid (NAL), ciprofloxacin (CIP), chloramphenicol (CMP), trimethoprim-sulfamethoxazole (STX), nitrofurantoin (NIT), tetracycline (TET), colistin (COL), florfenicol (FFC).

1995–2013 to contribute to the global characterization of these strains.

Subtyping of stx genes indicated that O26:H11 strains in our region mainly present either stx_{1a} or stx_{2a} genotypes, similar to O26:H11 strains reported in other countries (Bielaszewska et al., 2013; Bletz et al., 2013; Bonanno et al., 2015). The stx_{2d} subtype, present in the new pathogenic O26:H11 clone circulating in France (Delannoy et al., 2015), was not detected in our collection.

Genetic characterization showed that in addition to stx genes, O26:H11 strains harbored genes encoding other toxins, adhesins, and components related to the type III secretion system that contribute to their virulence. In particular, eae-β, tir, efa, iha, espB, cif, espA, espF, espJ, nleA, nleB, nleC, and iss genes were detected in all the isolates; and all except one contained ehxA, espP, and cba genes.

The analysis based on the presence/absence of genes associated with virulence identified three main clusters, one containing the stx_{1a}-positive isolates, another the stx_{2a}-positive isolates and a third one the stx_{1a} and stx_{2a}-positive isolate (**Figure 1**). The toxB, espI, and katP genes were differentially distributed between stx_{1a}-positive and stx_{2a}-positive groups. The toxB and espI genes, previously associated with severe disease (Mundy et al., 2004; Michelacci et al., 2014), were exclusively present in stx_{2a}-positive isolates. Conversely, the katP gene encoding for catalase-peroxidase was only detected in the stx_{1a}-positive isolates.

Heterogeneity in gene composition of STEC O26:H11 plasmids has been detected by Zhang et al. (2000). Considering the plasmidic markers ehxA, katP, espP, and etpD, two major subgroups of stx_{2a}-positive strains were identified in Europe by Bielaszewska et al. (2013), one of them typical for the new virulent German clone. Noticeably, all the stx_{2a}-positive strains of our study present a distinct profile, positive for ehxA and espP, but negative for katP and etpD genes. Thus, stx_{2a}-positive strains of our Argentinian collection differ from the German clone, and also from the human-pathogenic strain recently identified in

France (Delannoy et al., 2015), which is negative for all these plasmidic markers. The plasmid profile of stx_{1a}-positive strains of our study (positive for ehxA, katP, espP, and negative for etpD) is the same that Zweifel et al. (2013) identified in the human stx_{1}-positive strains from patients with HUS or bloody diarrhea in Switzerland. On the other hand, the unique stx_{1} and stx_{2}-positive isolate was negative for all plasmidic genes (toxB, espP, katP, ehxA, etpD, saa, subAB, epeA) tested, suggesting the absence of a virulence plasmid like pO26-Vir, pO157, or pO113.

The MLVA profiles found in the present study do not coincide with any of the profiles identified by Brandal et al. (2012) among ovine and human O26:H11 isolates from Norway. Although loci CVN001, 004, and 007 were monomorphic in both studies, the alleles were different. The loci CVN002 and CVN003 presented a null allele for all tested samples in our study, in agreement with results obtained by Løbersli et al. (2012) for O26 isolates. Available epidemiological information suggests that isolates forming clusters with identical MLVA profiles were derived from the same source and constitute a single clone. However, one cluster contained human stx_{1}-positive isolates not epidemiologically related. In addition, two isolates (isolates 20 and 21) from the same farm had the same virulence and MLVA profiles but only isolate 21 had antimicrobial resistance genes. Although there is a possibility that these isolates are from the same clone and that isolate 21 acquired antimicrobial genes, it is also possible that MLVA typing is not discriminatory enough to distinguish both isolates from each other. Interestingly, major groups obtained by MLVA analysis were similar to those obtained by the analysis of virulence factors.

Antimicrobial resistance genes were detected in five isolates obtained from two calves, two meat samples and one patient with diarrhea. Except for the human isolate, all other isolates showed phenotypic resistance profiles predicted by the corresponding genotypic profile. Among the antibiotics tested, all four isolates were resistant to ampicillin, amoxicillin/clavulanate, and tetracycline and also showed intermediate resistance to
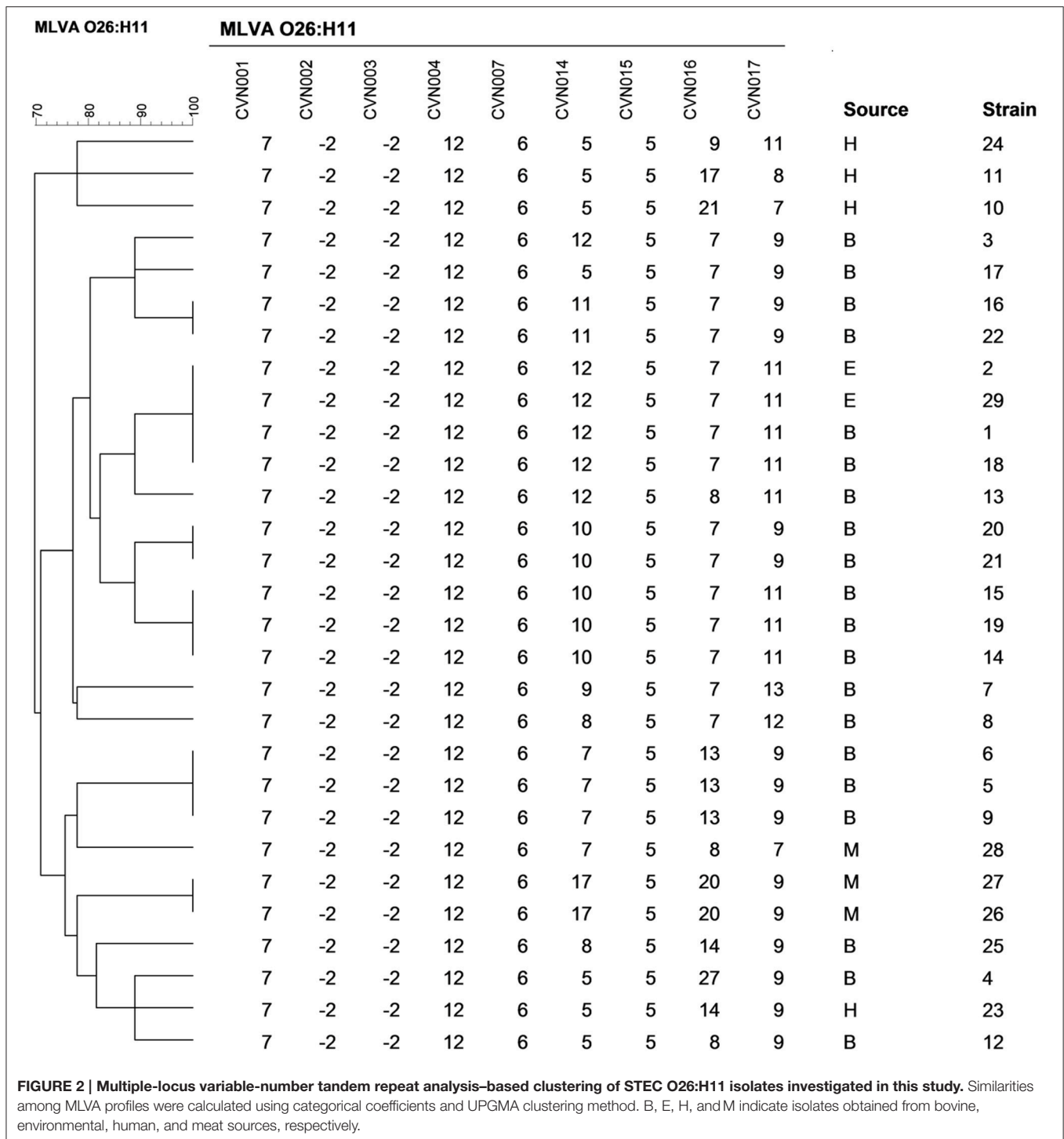
	CVN001	CVN002	CVN003	CVN004	CVN007	CVN014	CVN015	CVN016	CVN017	Source	Strain
	7	-2	-2	12	6	5	5	9	11	H	24
	7	-2	-2	12	6	5	5	17	8	H	11
	7	-2	-2	12	6	5	5	21	7	H	10
	7	-2	-2	12	6	12	5	7	9	B	3
	7	-2	-2	12	6	5	5	7	9	B	17
	7	-2	-2	12	6	11	5	7	9	B	16
	7	-2	-2	12	6	11	5	7	9	B	22
	7	-2	-2	12	6	12	5	7	11	E	2
	7	-2	-2	12	6	12	5	7	11	E	29
	7	-2	-2	12	6	12	5	7	11	B	1
	7	-2	-2	12	6	12	5	7	11	B	18
	7	-2	-2	12	6	12	5	8	11	B	13
	7	-2	-2	12	6	10	5	7	9	B	20
	7	-2	-2	12	6	10	5	7	9	B	21
	7	-2	-2	12	6	10	5	7	11	B	15
	7	-2	-2	12	6	10	5	7	11	B	19
	7	-2	-2	12	6	10	5	7	11	B	14
	7	-2	-2	12	6	9	5	7	13	B	7
	7	-2	-2	12	6	8	5	7	12	B	8
	7	-2	-2	12	6	7	5	13	9	B	6
	7	-2	-2	12	6	7	5	13	9	B	5
	7	-2	-2	12	6	7	5	13	9	B	9
	7	-2	-2	12	6	7	5	8	7	M	28
	7	-2	-2	12	6	17	5	20	9	M	27
	7	-2	-2	12	6	17	5	20	9	M	26
	7	-2	-2	12	6	8	5	14	9	B	25
	7	-2	-2	12	6	5	5	27	9	B	4
	7	-2	-2	12	6	5	5	14	9	H	23
	7	-2	-2	12	6	5	5	8	9	B	12

FIGURE 2 | Multiple-locus variable-number tandem repeat analysis–based clustering of STEC O26:H11 isolates investigated in this study. Similarities among MLVA profiles were calculated using categorical coefficients and UPGMA clustering method. B, E, H, and M indicate isolates obtained from bovine, environmental, human, and meat sources, respectively.

cephalotin. The two meat isolates (isolates 26 and 27) were also resistant to streptomycin, nalidixic acid, and trimethropim-sulfamethoxazole. As we commented below, these two isolates also shared the same virulence and MLVA profiles. Considering the epidemiological link, as they were found in meat samples from the same meat processing plant, our results suggest that both isolates correspond to the same circulating strain.

Previous reports showed that integrons can be frequently detected in STEC strains and that most of the integrons can contain the *aad*A1 gene alone, or in association with the *drf*A1 gene (Morabito et al., 2002; Cergole-Novella et al., 2011). In our study, the *int*I1 gene was detected in isolate 21 which was also positive for *aad*A1 and *drf*A1 genes. Strikingly, this isolate carrying an integron and showing multiple resistances to

antimicrobials was obtained from a newborn calf. Our results highlight the presence of multi-antimicrobial resistant STEC in cattle and meat in agreement with previous studies reporting the emergence and dissemination of antimicrobial resistance among STEC strains (Zhao et al., 2001; Li et al., 2011; Sasaki et al., 2012). Although antibiotic therapy is discouraged for treatment of STEC infections, the presence of antimicrobial resistant STEC strains in animals represent a risk for animal and human health. The genes coding for antimicrobial resistance could be transferred to other pathogens. Moreover, antimicrobial resistant STEC strains may have a selective advantage over other bacteria in intestines of animals under antibiotic treatments (Zhao et al., 2001). Taking into account that the same classes of antimicrobial agents are used both in humans and animals, joint efforts should be made to reduce the inappropriate use of antimicrobial agents in animals (Aidara-Kane, 2014).

In conclusion, we identified three different populations of native O26:H11 strains whose main differences were associated with genes present in mobile genetic elements. Although O26 strains harboring only stx_{2a} subtype have been rarely isolated from cattle and food in Europe and the United States (Pearce et al., 2006; Geue et al., 2009; Chase-Topping et al., 2012; Ison et al., 2015), stx_{2a}-positive strains have been an important proportion of O26:H11 strains circulating in farms in Argentina and showed to carry genes associated with high virulence, representing a potential risk for public health.

ACKNOWLEDGMENTS

REFERENCES

Aidara-Kane, A. (2014). Containment of antimicrobial resistance due to use of antimicrobial agents in animals intended for food: WHO perspective. *Rev. Sci. Tech.* 31, 277–287.

Aldick, T., Bielaszewska, M., Zhang, W., Brockmeyer, J., Schmidt, H., Friedrich, A. W., et al. (2007). Hemolysin from shiga toxin-negative *Escherichia coli* O26 strains injures microvascular endothelium. *Microbes Infect.* 9, 282–290. doi: 10.1016/j.micinf.2006.12.001

Bielaszewska, M., Mellmann, A., Bletz, S., Zhang, W., Kock, R., Kossow, A., et al. (2013). Enterohemorrhagic *Escherichia coli* O26:H11/H-: a new virulent clone emerges in Europe. *Clin. Infect. Dis.* 56, 1373–1381. doi: 10.1093/cid/cit055

Blanco, M., Padola, N. L., Krüger, A., Sanz, M. E., Blanco, J. E., González, E. A., et al. (2004). Virulence genes and intimin types of shiga-toxin-producing *Escherichia coli* isolated from cattle and beef products in Argentina. *Int. Microbiol.* 7, 269–276.

Bletz, S., Bielaszewska, M., Leopold, S. R., Köck, R., Witten, A., Schuldes, J., et al. (2013). Evolution of enterohemorrhagic *Escherichia coli* O26 based on single-nucleotide polymorphisms. *Genome Biol. Evol.* 5, 1807–1816. doi: 10.1093/gbe/evt136

Boerlin, P., McEwen, S. A., Boerlin-Petzold, F., Wilson, J. B., Johnson, R. P., and Gyles, C. L. (1999). Associations between virulence factors of Shiga toxin-producing *Escherichia coli* and disease in humans. *J. Clin. Microbiol.* 37, 497–503.

Bonanno, L., Loukiadis, E., Mariani-Kurkdjian, P., Oswald, E., Garnier, L., Michel, V., et al. (2015). Diversity of Shiga toxin-producing *Escherichia coli* (STEC) O26:H11 strains examined via *stx* subtypes and insertion sites of Stx and EspK Bacteriophages. *Appl. Environ. Microbiol.* 81, 3712–3721. doi: 10.1128/AEM.00077-15

Bonardi, S., Alpigiani, I., Tozzoli, R., Vismarra, A., Zecca, V., Greppi, C., et al. (2015). Shiga toxin-producing *Escherichia coli* O157, O26 and O111 in cattle faeces and hides in Italy. *Vet. Rec. Open* 2:e000061. doi: 10.1136/vetreco-2014-000061

Bosilevac, J. M., and Koohmaraie, M. (2011). Prevalence and characterization of non-O157 shiga toxin-producing *Escherichia coli* isolates from commercial ground beef in the United States. *Appl. Environ. Microbiol.* 77, 2103–2112. doi: 10.1128/AEM.02833-10

Brandal, L. T., Sekse, C., Lindstedt, B. A., Sunde, M., Løbersli, I., Urdahl, A. M., et al. (2012). Norwegian sheep are an important reservoir for human-pathogenic *Escherichia coli* O26:H11. *Appl. Environ. Microbiol.* 78, 4083–4091. doi: 10.1128/AEM.00186-12

Bustamante, A. V., Sanso, A. M., Lucchesi, P. M., and Parma, A. E. (2010). Genetic diversity of O157:H7 and non-O157 verocytotoxigenic *Escherichia coli* from Argentina inferred from multiple-locus variable-number tandem repeat analysis (MLVA). *Int. J. Med. Microbiol.* 300, 212–217. doi: 10.1016/j.ijmm.2009.08.020

Cergole-Novella, M. C., Pignatari, A. C. C., Castanheira, M., and Guth, B. E. C. (2011). Molecular typing of antimicrobial-resistant shiga-toxin-producing *Escherichia coli* strains (STEC) in Brazil. *Res. Microbiol.* 162, 117–123. doi: 10.1016/j.resmic.2010.09.022

Chase-Topping, M. E., Rosser, T., Allison, L. J., Courcier, E., Evans, J., Mckendrick, I. J., et al. (2012). Pathogenic potential to humans of bovine *Escherichia coli* O26, Scotland. *Emerg. Infect. Dis.* 18, 439–448. doi: 10.3201/eid1803.111236

CLSI (2014). *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement.* CLSI Document M100–S24.

Delannoy, S., Mariani-Kurkdjian, P., Bonacorsi, S., Liguori, S., and Fach, P. (2015). Characteristics of emerging human-pathogenic *Escherichia coli* O26:H11 strains isolated in France between 2010 and 2013 and carrying the *stx*2d gene only. *J. Clin. Microbiol.* 53, 486–492. doi: 10.1128/JCM.02290-14

EFSA. Panel on Biological Hazards (2013). Scientific opinion on VTEC-seropathotype and scientific criteria regarding pathogenicity assessment. *EFSA J.* 11:3138. doi: 10.2903/j.efsa.2013.3138

Fernández, D., Sanz, M. E., Parma, A. E., and Padola, N. L. (2012). Short communication: characterization of shiga toxin-producing *Escherichia coli* isolated from newborn, milk-fed, and growing calves in Argentina. *J. Dairy Sci.* 95, 5340–5343. doi: 10.3168/jds.2011-5140

Gerber, A., Karch, H., Allerberger, F., Verweyen, H. M., and Zimmerhackl, L. B. (2002). Clinical course and the role of shiga toxin–producing *Escherichia coli* infection in the hemolytic-uremic syndrome in pediatric patients, 1997–2000, in Germany and Austria: a prospective study. *J. Infect. Dis.* 186, 493–500. doi: 10.1086/341940

Geue, L., Klare, S., Schnick, C., Mintel, B., Meyer, K., and Conraths, F. J. (2009). Analysis of the clonal relationship of serotype O26:H11 enterohemorrhagic *Escherichia coli* isolates from cattle. *Appl. Environ. Microbiol.* 75, 6947–6953. doi: 10.1128/AEM.00605-09

Geue, L., Schares, S., Mintel, B., Conraths, F. J., Müller, E., and Ehricht, R. (2010). Rapid microarray-based genotyping of enterohemorrhagic *Escherichia coli* serotype O156:H25/H-/Hnt isolates from cattle and clonal relationship analysis. *Appl. Environ. Microbiol.* 76, 5510–5519. doi: 10.1128/AEM.00743-10

González, J., Sanso, A. M., Lucchesi, P. M., and Bustamante, A. V. (2014). Comparison of 2 proposed MLVA protocols for subtyping non-O157:H7 verotoxigenic *Escherichia coli*. *Diagn. Microbiol. Infect. Dis.* 78, 328–332. doi: 10.1016/j.diagmicrobio.2013.10.017

Gould, L. H., Mody, R. K., Ong, K. L., Clogher, P., Cronquist, A. B., Garman, K. N., et al. (2013). Increased recognition of non-O157 shiga toxin-producing *Escherichia coli* infections in the United States during 2000-2010: epidemiologic features and comparison with *E. coli* O157 infections. *Foodborne Pathog. Dis.* 10, 453–460. doi: 10.1089/fpd.2012.1401

Hunter, P. R., and Gaston, M. A. (1988). Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J. Clin. Microbiol.* 26, 2465–2466.

Ison, S. A., Delannoy, S., Bugarel, M., Nightingale, K. K., Webb, H. E., Renter, D. G., et al. (2015). Genetic diversity and pathogenic potential of attaching and effacing *Escherichia coli* O26: H11 strains recovered from bovine feces in the United States. *Appl. Environ. Microbiol.* 81, 3671–3678. doi: 10.1128/AEM.00397-15

ISPCH (2014). *Vigilancia de laboratorio de E. coli productora de toxina Shiga*. Chile, 2007–2013, Boletín Instituto de Salud Pública de Chile. Available online at: http://www.ispch.cl/vigilancia-ispch/patogenos

Käppeli, U., Hachler, H., Giezendanner, N., Beutin, L., and Stephan, R. (2011). Human infections with non-O157 shiga toxin-producing *Escherichia coli*, Switzerland, 2000–2009. *Emerg. Infect. Dis.* 17, 180–185. doi: 10.3201/eid1702.100909

Krüger, A., and Lucchesi, P. M. (2015). Shiga toxins and *stx* phages: highly diverse entities. *Microbiology* 161, 451–462. doi: 10.1099/mic.0.000003

Larkin, M. A., Blackshields, G., Brown, N., Chenna, R., McGettigan, P. A., McWilliam, H., et al. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* 23, 2947–2948. doi: 10.1093/bioinformatics/btm404

Leomil, L., de Castro, A. F. P., Krause, G., Schmidt, H., and Beutin, L. (2005). Characterization of two major groups of diarrheagenic *Escherichia coli* O26 strains which are globally spread in human patients and domestic animals of different species. *FEMS Microbiol. Lett.* 249, 335–342. doi: 10.1016/j.femsle.2005.06.030

Li, M.-C., Wang, F., and Li, F. (2011). Identification and molecular characterization of antimicrobial-resistant shiga toxin–producing *Escherichia coli* isolated from retail meat products. *Foodborne Pathog. Dis.* 8, 489–493. doi: 10.1089/fpd.2010.0688

Lindstedt, B. A., Brandal, L. T., Aas, L., Vardund, T., and Kapperud, G. (2007). Study of polymorphic variable-number of tandem repeats loci in the ECOR collection and in a set of pathogenic *Escherichia coli* and *Shigella* isolates for use in a genotyping assay. *J. Microbiol. Methods* 69, 197–205. doi: 10.1016/j.mimet.2007.01.001

Løbersli, I., Haugum, K., and Lindstedt, B. A. (2012). Rapid and high resolution genotyping of all *Escherichia coli* serotypes using 10 genomic repeat-containing loci. *J. Microbiol. Methods* 88, 134–139. doi: 10.1016/j.mimet.2011.11.003

Madic, J., Vingadassalon, N., de Garam, C. P., Marault, M., Scheutz, F., Brugère, H., et al. (2011). Detection of shiga toxin-producing *Escherichia coli* serotypes O26:H11, O103:H2, O111:H8, O145:H28, and O157:H7 in raw-milk cheeses by using multiplex real-time PCR. *Appl. Environ. Microbiol.* 77, 2035–2041. doi: 10.1128/AEM.02089-10

Michelacci, V., Grande, L., Tozzoli, R., Maugliani, A., Caprioli, A., and Morabito, S. (2014). Identification of two allelic variants of *tox*B gene and investigation of their distribution among Verocytotoxin-producing *Escherichia coli*. *Int. J. Med. Microbiol.* 304, 730–734. doi: 10.1016/j.ijmm.2014.05.009

Mohammed, M. A., Sallam, K. I., Eldaly, E., A.Z., Ahdy, A. M., and Tamura, T. (2014). Occurrence, serotypes and virulence genes of non-O157 shiga toxin-producing *Escherichia coli* in fresh beef, ground beef, and beef burger. *Food Control* 37, 182–187. doi: 10.1016/j.foodcont.2013.09.035

Monaghan, A., Byrne, B., Fanning, S., Sweeney, T., McDowell, D., and Bolton, D. J. (2011). Serotypes and virulence profiles of non-O157 shiga toxin-producing *Escherichia coli* isolates from bovine farms. *Appl. Environ. Microbiol.* 77, 8662–8668. doi: 10.1128/AEM.06190-11

Morabito, S., Tozzoli, R., Caprioli, A., Karch, H., and Carattoli, A. (2002). Detection and characterization of class 1 integrons in enterohemorrhagic *Escherichia coli*. *Microb. Drug Resist.* 8, 85–91. doi: 10.1089/107662902760190626

Mundy, R., Jenkins, C., Yu, J., Smith, H., and Frankel, G. (2004). Distribution of *esp*I among clinical enterohaemorrhagic and enteropathogenic *Escherichia coli* isolates. *J. Med. Microbiol.* 53, 1145–1149. doi: 10.1099/jmm.0.45684-0

Naylor, S. W., Gally, D. L., and Low, J. C. (2005). Enterohaemorrhagic, *E. coli* in veterinary medicine. *Int. J. Med. Microbiol.* 295, 419–441. doi: 10.1016/j.ijmm.2005.07.010

Noller, A. C., McEllistrem, M. C., Pacheco, A. G., Boxrud, D. J., and Harrison, L. H. (2003). Multilocus variable-number tandem repeat analysis distinguishes outbreak and sporadic *Escherichia coli* O157:H7 isolates. *J. Clin. Microbiol.* 41, 5389–5397. doi: 10.1128/JCM.41.12.5389-5397.2003

Oswald, E., Schmidt, H., Morabito, S., Karch, H., Marchès, O., and Caprioli, A. (2000). Typing of intimin genes in human and animal enterohemorrhagic and enteropathogenic *Escherichia coli*: characterization of a new intimin variant. *Infect. Immun.* 68, 64–71. doi: 10.1128/IAI.68.1.64-71.2000

Paddock, Z. D., Renter, D. G., Cull, C. A., Shi, X., Bai, J., and Nagaraja, T. G. (2014). *Escherichia coli* O26 in feedlot cattle: fecal prevalence, isolation, characterization, and effects of an *E. coli* O157 vaccine and a direct-fed microbial. *Foodborne Pathog. Dis.* 11, 186–193. doi: 10.1089/fpd.2013.1659

Pearce, M., Evans, J., McKendrick, I., Smith, A., Knight, H., Mellor, D., et al. (2006). Prevalence and virulence factors of *Escherichia coli* serogroups O26, O103, O111, and O145 shed by cattle in Scotland. *Appl. Environ. Microbiol.* 72, 653–659. doi: 10.1128/AEM.72.1.653-659.2006

Polifroni, R., Etcheverría, A. I., Sanz, M. E., Cepeda, R. E., Krüger, A., Lucchesi, P. M., et al. (2012). Molecular characterization of shiga toxin-producing *Escherichia coli* isolated from the environment of a dairy farm. *Curr. Microbiol.* 65, 337–343. doi: 10.1007/s00284-012-0161-0

Rivas, M., Padola, N. L., Lucchesi, P. M., and Masana, M. (2010). "Diarrheagenic *Escherichia coli* in Argentina," in *Pathogenic Escherichia coli in Latin America*, ed A. G. Torres (Oak Park, IL: Bentham Science Publishers), 142–161.

Rivero, M. A., Passucci, J. A., Rodriguez, E. M., and Parma, A. E. (2010). Role and clinical course of verotoxigenic *Escherichia coli* infections in childhood acute diarrhoea in Argentina. *J. Med. Microbiol.* 59, 345–352. doi: 10.1099/jmm.0.015560-0

Sasaki, Y., Usui, M., Murakami, M., Haruna, M., Kojima, A., Asai, T., et al. (2012). Antimicrobial resistance in shiga toxin-producing *Escherichia coli* O157 and O26 isolates from beef cattle. *Jpn. J. Infect. Dis.* 65, 117–121.

Scheutz, F., Teel, L. D., Beutin, L., Piérard, D., Buvens, G., Karch, H., et al. (2012). Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing Stx nomenclature. *J. Clin. Microbiol.* 50, 2951–2963. doi: 10.1128/JCM.00860-12

Zhang, W.-L., Bielaszewska, M., Liesegang, A., Tschäpe, H., Schmidt, H., Bitzan, M., et al. (2000). Molecular characteristics and epidemiological significance of shiga toxin-producing *Escherichia coli* O26 strains. *J. Clin. Microbiol.* 38, 2134–2140.

Zhao, S., White, D. G., Ge, B., Ayers, S., Friedman, S., English, L., et al. (2001). Identification and characterization of integron-mediated antibiotic resistance among shiga toxin-producing *Escherichia coli* isolates. *Appl. Environ. Microbiol.* 67, 1558–1564. doi: 10.1128/AEM.67.4.1558-1564.2001

Zimmerhackl, L. B., Rosales, A., Hofer, J., Riedl, M., Jungraithmayr, T., Mellmann, A., et al. (2010). Enterohemorrhagic *Escherichia coli* O26:H11-associated hemolytic uremic syndrome: bacteriology and clinical presentation. *Semin. Thromb. Hemost.* 36, 586–593. doi: 10.1055/s-0030-1262880

Zweifel, C., Cernela, N., and Stephan, R. (2013). Detection of the emerging shiga toxin-producing *Escherichia coli* O26:H11/H- sequence type 29 (ST29) clone in human patients and healthy cattle in Switzerland. *Appl. Environ. Microbiol.* 79, 5411–5413. doi: 10.1128/AEM.01728-13