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Phenotypic and genotypic characterization of enterotoxigenic *Escherichia coli* isolated from diarrheic calves in Argentina

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Abstract
Enterotoxigenic *Escherichia coli* (ETEC) is the most common and global cause of neonatal calf diarrhea, but there is a little information regarding calf ETEC strains in Argentina. In this study, five ETEC isolates from diarrheic dairy calves (2–10 d old) from Buenos Aires and Cordoba, Argentina were characterized on the basis of virulence gene (VG) pattern, O:H serotyping, hemolytic phenotype, phylogenetic group affiliation, antimicrobial (AM) resistance profile, and presence of integron class 1 and 2. The five isolates were examined by polymerase chain reaction (PCR) for the presence of 18 bovine VGs and showed the following genotypes: $F5^+/F41^+/sta^+$ (D242), $F5^+/sta^+$ (D158), $F5^+/sta^+$ (D157), $F5^+$ (D151-9), and $F5^+/iucD^+$ (D151-5). These VGs confer pathogenic potential and most of them are associated with the ETEC pathotype. The five isolates showed a non-hemolytic phenotype, belonged to five different serotypes: $O101:H^-$, $O141:H^-$, $O60:H^-$, ONT:H10, and $ONT:H^-$, and were assigned to the phylogenetic group A by the quadruplex Clermont PCR method. The AM resistance of the three isolates D242, D157, and D151-5 was determined by agar disk diffusion method for 24 AMs and they exhibited a multi-resistance phenotype (resistance to four different AM classes: Cephalosporins, Penicillins, Macrolides, and Ansamycins). In addition, class 1 integrons were found in the isolate D151-5 containing the *dfrA17-aadA5* gene cassette and in the bovine ETEC reference strain FV10191 containing the *dfrA1-aadA1* gene cassette. The present study revealed for the first time the occurrence of multi-resistant ETEC associated with neonatal diarrhea in dairy calves in Argentina. This finding may be used for diagnostic and therapeutic purposes.
Keywords: Antimicrobial resistance, Dairy cattle, *Escherichia coli*, Neonatal diarrhea, Virulence gene.

Introduction

Escherichia coli is a commensal bacterium widespread in the intestine of warm-blooded animals and humans, and a pathogen that can induce enteric and extraintestinal diseases (Kaper *et al*., 2004). Neonatal diarrhea (ND) in calves is an acute enteric disease of economic importance which is characterized by watery diarrhea, dehydration, loss of body weight, and sometimes death of infected calves associated with multiple enteric pathogens (e.g., viruses, bacteria, and protozoa) (Cho and Yoon, 2014; Foster and Smith, 2009). Enterotoxigenic *Escherichia coli* (ETEC) is a commonly cause of ND in young calves in the first 4 d of life to 2 wk when other enteropathogens are involved (Acres, 1985; Holland, 1990; Foster and Smith, 2009). Its pathogenicity involves adherence of the pathogen to the small intestine by means of specific fimbrial adhesion factors to bind host cells receptors in newborn calves and production of one or several exotoxins responsible for the disease (Kaper *et al*., 2004). Major virulence factors of calf ETEC isolates are the fimbriae F5 (K99) and F41, and the heat-stable enterotoxin (Sta) (Acres, 1985). F5 and *sta* genes are usually located on the same plasmid that can also carry antibiotic resistance genes, while F41 is encoded on chromosome (Dubreuil *et al*., 2016).

Since 1980, studies have reported the isolation of *E. coli* strains from calves with diarrhea in Argentina. Three of them did not identify ETEC strains in any sample using immunological methods (F5) and the suckling mouse model (STa) (Bellinzoni *et al*., 1990; Campero *et al*., 1985; Mercado *et al*., 2003). Other surveys identified few ETEC strains from sick calves (Barrandeguy *et al*., 1988; Cornaglia *et al*., 1992; Odeon, 1980) but the strains found were not genetically characterized. Picco *et al*. (2015) examined samples from diarrheic and non-diarrheic dairy calves of Cordoba by PCR and they found seven virulence profiles (including the VGs F5, F41, and *sta*) compatible with ETEC in 12 isolates (n = 39). However, they did not provide information concerning the serotype, antimicrobials (AMs) resistance or phylogroup. In the last 8 yr, we analyzed the presence of 18 VGs of hundreds of samples by PCR from outbreaks and sporadic cases of ND in Argentinean farms submitted to our diagnostic laboratory and we did not detect ETEC strains up to now. The objective of this work was to report the pheno- and genotypic characterization of five ETEC isolates recovered from diarrheic dairy calves in relation to VG pattern, O:H

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serotyping, hemolytic phenotype, phylogenetic group affiliation, AM resistance profile, and presence of integron class 1 and 2.

Materials and Methods

Escherichia coli isolates

The five isolates characterized in this work (D242, D158, D157, D151-9, and D151-5) were obtained by veterinarians from samples submitted to the Bacteriology Diagnostic Laboratory at the Balcarce Experimental Station (INTA, Argentina) in 2014–2015. The isolates were obtained from feces of 2- to 10-d-old diarrheic calves (Holando-Argentino) from three dairy farms in Cordoba (D242) and Buenos Aires (D158 and D157 isolated from different calves; D151-5 and D151-9 isolated from one calf), Argentina. The isolate D242 was previously reported *E. coli* $F5^+$ by lateral flow immunoassay (Anigen Rapid BoviD-4 Ag Test Kit—Bionote, Inc., Korea). The samples were plated onto MacConkey agar (MAC, Difco, MD) and incubated at 37°C for 18 h. Afterward, the confluent bacterial growth of each sample plate was harvested and stored at the room temperature in nutrient agar 0.75% (p/v) prepared with nutritive agar (11.5 g/l; Difco, MD) and nutritive broth (4 g/l; Difco, MD) until analyzed by PCR. For this purpose, bacterial growth was plated on MAC and incubated in the above conditions. Ten individual lactose positive colonies with a phenotypic appearance consistent with *E. coli* were transferred to tripticase soy agar (TSA) (Difco, MD). The isolates were identified as *E. coli* by standard biochemical procedures according to the Bergey's Manual (positive reactions for indole and methyl red, negative reactions for Voges-P, citrate utilization, urease, and hydrogen sulfide). They were not sub-cultured more than twice before being examined for the presence of virulence genes.

Detection of hemolysin activity

Escherichia coli isolates were tested for the production of a hemolytic phenotype on Columbia Blood Agar Base (Oxoid, Basingstoke, UK) with 7% v/v defibrinated bovine blood. Production of hemolysis as a zone of lysis surrounding the bacterial growth was read after overnight incubation at 37°C. The ETEC strains FV 10189 from swine and FV 10191 from bovine were used as positive and negative control, respectively.

DNA extraction, detection, and sequencing of VGs

DNA extraction

Bacteria were harvested and suspended in 300 µl (confluent growth and five-colony pools) or 150 µl (individual colonies) of sterile distilled water and boiled for 5 min to release the DNA. After centrifugation (11,000 × g at 4°C for 3 min), the supernatant was used as a template for the PCR reaction.

Detection of VGs by PCR

The 18 VGs analyzed in this work are listed in Table 1. Amplification of bacterial DNA was performed in a total volume of 30 µl containing 7 µl of the crude lysate, 150 ng of each oligonucleotide primer, 200 µM (each) dATP, dGTP, dCTP, and dTTP (Promega, WI); 1X Green GoTaq Reaction Buffer (1.5 mM $MgCl_2$, pH 8.5), and 1 U of Go Taq DNA polymerase (Promega, WI). Primer nucleotide sequences used in this study to test the presence of VGs and predicted amplicon sizes are shown in Table 1. General conditions for the single PCR were initial DNA denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 40 sec, primer annealing at 48°C–66°C (Table 1) for 1 min and extension for 72°C for 3 min followed by a final extension at 72°C for 3 min (except for *afa8*, *clpG*, and EHEC*hlyA* where the conditions described by Le Bouguenec *et al*. (2001), Bertin *et al*. (1998), and Schmidt *et al*. (1995), respectively were followed) performed in a Px2 Thermal Cycler (Thermo Electron Corporation, MA).

One DNA from two *E. coli* reference strains (kindly provided from LREC, University of Santiago de Compostela, Lugo, Spain) was included in every PCR procedure for the detection of VGs as a positive control. Reference *E. coli* strains used as positive controls included: EDL933 (*stx1*, *stx2*, *eae*, and EH*hlyA*), 146N (*stx1*, *stx2*, and *eae*), FV 10187 (*stx2*e, *sta*, *stb*, and F18), FV 10188 (*eltA*, *stb*, K88, and *east1*), FV 10189 (*eltA*, *sta*, *stb*, F18, and *east1*), FV 10191 (*sta*, F5, and F41), B41 (F5 and F41), FV 10192 (*f17G*), FV 10193 (*papC*, *afa8*, and *iucD*), FV 10194 (*afa8* and *iucD*) FV 10195 (*cnf1*, *pap*, *sfa, HlyA*, and *iucD*), FV 10196 (*cdt*, *sfa*, K1, and *iucD*), S5 (*cnf2*, *cdt*-III, and *f17G*), FVL16 (*papC* and *sfa*), 31A (*clpG*), J96 (*cnf1*, *cdt*-IV, and *papC*), 239KH89 (*cnf1*, *papC*, and *afaE-8*), EPECM (*bfp* and *eae*), 02/02 (EHEC*hlyA and eae*). *Escherichia coli* DH5α and distilled water were used in every PCR as a negative control.

From each PCR positive confluent growth for particular VG, 10 *E. coli*-like colonies were sub-cultured from a MAC agar plate to a TSA agar plate. At first, two five-colony pools were analyzed for different VG and a subsequent PCR was performed to analyzed individual colonies from positive pools.

The amplified products were analyzed by standard gel electrophoresis on 1.6% agarose gels with SYBR® Safe DNA Gel Stain and visualized under the UV light. The sizes of the amplified products were compared with those of positive controls and DNA molecular marker (100–1,000 pb).

Sequencing

The identity of the F5, F41, *sta*, and *iucD* PCR products was confirmed by DNA sequencing. Nucleotide sequence of VGs and integron PCR products was determined on both DNA strands using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA) and a Genetic Anlyzer 3130xl (Applied Biosystems) according to the manual and were submitted to GenBank database (Table 2). Reaction conditions and the primers used for the PCR and sequencing reactions are shown in Table 1.

Table 1. PCR primers sequence, amplicon size and conditions for amplifications used in this study.

Target	Primer	Nucleotide sequence (5′-3′)	Amplicon size (bp)	Annealing (ºC),(sec)	Reference
afa8	AFAE8F	CTAACTTGCCATGCTGTGACAGTA	302	65, 60	Le Bouguenec *et al*. (2001)
	AFAE8R	TTATCCCCTGCGTAGTTGTGAATC			
clpG	clpG1	GGGCGCTCTCTCCTTCAAC	402	55, 60	Bertin *et al*. (1998)
	clpG2	CGCCCTAATTGCTGGCGAC			
cnf	CNFF	CAATGGCAACAAAAATATCTTCT	1,147	56, 40	Blanco, J. (pers. comm.)
	CNFR	GAACGACGTTCTTCATAAGTATC			
cdt	CDT-s1	GAAAGTAAATGGAATATAAATGTCCG	466	58, 40	Toth *et al*. (2003)
	CDT-s2	GAAAATAAATGGAACACACATGTCCG			
	CDT-as1	AAATCACCAAGAATCATCCAGTTA			
	CDT-as2	AAATCTCCTGCAATCATCCAGTTA			
eae	EAEV3F	CATTGATCAGGATTTTTCTGGT	510	55, 40	Mora *et al*. (2011)
	EAEMBR	TCCAGAATAATATTGTTATTACG			
EHEC*hlyA*	hlyA1	GGTGCAGCAGAAAAAGTTGTAG	1,551	57, 90	Schmidt *et al*. (1995)
	hlyA4	TCTCGCCTGATAGTGTTTGGTA			
eltA	LTFN	CGTTCCGGAGGTCTTATGCC	660	55, 40	Frankel *et al*. (1989)
	LTB	CCGAATTCTGTTATATATGTC			
east1	east 11a	CCATCAACACAGTATATCCGA	111	55, 120	Yamamoto and Echeverria (1996)
	east 11b	GGTCGCGAGTGACGGCTTTGT			
f17G	F17F	GGGCTGACAGAGGAGGTGGGGC	411	60,40	Vu-Khac *et al*. (2006)
	F17R	CCCGGCGACAACTTCATCACCGG			
F41	F41A	GGCTATGGAAGACTGGAGAGGG	546	55, 40	Blanco *et al*. (2006)
	F41RN	GACTGAGGTCATCCCAATTGTGG			
hlyA	HLY1	AACAAGGATAAGCACTGTTCTGGCT	1,177	56, 40	Yamamoto *et al*. (1995)
	HLY2	ACCATATAAGCGGTCATTCCCGTCA			
iucD	AER1	TACCGGATTGTCATATGCAGACCGT	602	60,40	Yamamoto *et al*. (1995)
	AER2	AATATCTTCCTCCAGTCCGGAGAAG			
F5	K99A	CCAGCGCCCGGCAGTAATGACTGC	278	60,40	Blanco *et al*. (2006)
	K99B	CCACCATTAGACGGAGCGCGG			
papC	PAP1	GACGGCTGTACTGCAGGGTGTGGCG	328	66,40	Le Bouguenec *et al*. (1992)
	PAP2	TCCTTTCTGCAGGGATGCAATA			
Sta	STa1	ATTTTTATTTCTGTATTGTCTTT	178	48,40	Penteado *et al*. (2002)
	STa2	GGATTACAACACAGTTCACAGCAGT			
Stb	STb1	ATCGCATTTCTTCTTGCATC	172	55,40	Blanco *et al*. (1997)
	STb2	GGGCGCCAAAGCATGCTCC			
stx1/vtx1	VT1-F	TCGCTGAATGTCATTCGCTCTGC	539	50, 40	Mora *et al*. (2011)
	VT1-R	TCAGCAGTCATTACATAAGAAC			
stx1/vtx2	VT2-F1	TTTCTTCGGTATCCTATTCCC	538	50, 40	Mora *et al*. (2011)
	VT2-F2	TGTCTTCAGCATCTTATGCAG			
	VT2-R	CTGCTGTCCGTTGTCATGGAA			

Table 1. (continued).

Target	Primer	Nucleotide sequence (5′-3′)	Amplicon size (bp)	Annealing (ºC),(sec)	Reference
intI1	Inti1F	CGAGGCATAGACTGTAC	892	52, 30	Orman *et al*. (2002)
	Inti1R	TTCGAATGTCGTAACCGC			
intI2	Inti2F	GCAAATGAAGTGCAACGC	467	54, 30	Orman *et al*. (2002)
	Inti2R	ACACGCTTGCTAACGATG			
vr-15′	CS (F)	GGCATCCAAGCAGCAAG	NA	52, 30	Levesque *et al*. (1995)
vr-13′	CS (R)	AAGCAGACTTGACCTGATAG	NA	52, 30	Orman *et al*. (2002)
dhfrA1	DhfrA1	CCTGAAATCCCCAGCAA	NA	52, 30	Orman *et al*. (2002)
sulI	SulIR	TTTGAAGGTTCGACAGC	NA	52, 30	Barbolla *et al*. (2004)

(*vr*): variable region; (NA): not apply (the amplicons differ in expected size depending on their locations in the variable region).

Table 2. Phenotypic and genotypic characterization of ETEC isolates.

Isolate/ host	Serotype	Genotype	Hemolysis	Phylogroup	Resistance profile	Integron/*vr*
D242 Cattle	O101:H$^-$	F5 F41 *sta*[a]	-	A	AMC-CL-OB-ERY-RFA-TIL	-
D158 Cattle	O141:H$^-$	F5 *sta*[b]	-	A	nt	-
D157 Cattle	O60:H$^-$	F5 *sta*[c]	-	A	AM-AMC-BC-CEC-ERY-OB-PEN-RFA-S-TIL	-
D151-9 Cattle	ONT:H10	F5[d]	-	A	nt	-
D151-5 Cattle	ONT:H$^-$	F5 *iucD*[e]	-	A	AM-AMC-CEF-CL-CMP-CNM-DO-ENR-ERY-NA-NEO-NOR-OB-OT-PEN-RFA-S3-TET-TIL-TMS	*dhfrA17 aadA5*[f]
FV10191 Cattle	O9:H9	F5 F41 *sta*	-	A	AMC-CEC-CEF-CL-CNM-OB-S3-ERY-NA-RFA-TIL-TMS	*dhfrA1 aadA1*
FV10189 Swine	O116:H39	F18 *lt sta stb*	-	A	nt	-

GenBank no.: ([a]): MF955845, MF955843 and MF955844 resp.; ([b]): KX461932 and KX463637 resp.; ([c]): KX461931 and KX463636 resp.; ([d]): KX461930; ([e]): KX356660 and KX461933 resp.; ([f]): KX461934 and KX463635 resp. (NT): nontypeable; (H$^-$): nonmotile; (nt): not tested; (*vr*): variable region.

Determination of phylogenetic group

Affiliation of *E. coli* isolates to phylogenetic group A, B1, B2, C, D, E, and F was determined by quadruplex PCR method as described by Clermont *et al*. (2013).

Detection of integrase gene and integron analysis

The presence of class 1 and 2 integrase was tested by PCR using primers specific for the integrase genes *intI1* and *intI2*, respectively (Table 1). The DNA templates for PCR were prepared as described previously. PCR amplification was performed in 25 µl volumes containing 5-µl bacterial template DNA, 0.4 µM each primer, 200-µM dNTP (Promega, WI), 1X Green GoTaq Reaction Buffer (1.5 µM $MgCl_2$, pH 8.5), 1.5 µM $MgCl_2$ and 1 U of Go Taq DNA polymerase (Promega, WI). The PCR conditions were initial denaturation at 94°C for 3 min, 35 cycles of denaturation for 1 min at 94°C, annealing for 30 sec at 52°C, and extension for 2 min at 72°C, and a final extension for 5 min at 72°C. To characterize the gene cassettes found in the variable region of class 1 integrons from the isolate D151-5 and the reference strain FV10191, PCRs were performed using the primers 5′CS/3′CS and *dhfrA1*/*sul1R* (Table 1) as described previously. PCRs products were detected by electrophoresis on agarose gels as mentioned previously. The gene cassettes were identified by nucleotide sequencing.

Determination of serotype

O and H types were determined by microagglutination technique in plates and tubes as described by Guinée *et*

al. (1981) and modified by Blanco *et al*. (1996) using all available O (O1–O175) antisera plus six putative new O antigens (OX176–OX181) and H (H1–H56) antisera (Pradel *et al*., 2000).

AM susceptibility testing

AM susceptibilities of the isolates were evaluated on Mueller–Hinton agar (Oxoid, Basingstoke, UK) by the Kirby–Bauer disk-diffusion method (Bauer *et al*., 1966) according to the Clinical and Laboratory Standards Institute (2010) guidelines (including the AM disk content). Three isolates D151-5, D157, and D242 (one from each dairy farm) were tested for resistance to 24 AMs including the most prescribed by veterinarians in the region where the samples were collected. The AM used were Amicacin, Amoxicillinum-Clavulanic Acid (AMC), Cefaclor (CEC), Cefotaxime, Cephalexin (CL), Cephalonium (CNM), Cephalothin (CEF), Cloxacillin (OB), Colistin, Compound Sulphonamides (S3), Enrofloxacin (ENR), Erythromycin (ERY), Florfenicol, Gentamicin, Nalidixic Acid (NA), Norfloxacin (NOR), Ofloxacin, Oxytetracycline (OT), Polymyxin B, Rifampicin, Streptomycin (S), Tetracycline (TET), Tilmicosin (TIL), and Trimethoprim-Sulphamethoxazole (TMS). All AM susceptibility discs were provided from OXOID, Basingstoke, UK. *Escherichia coli* ATCC 25922 was used as the reference strain for quality control of the AMs tested. Multi-resistant strain was defined as resistant to three or more AM classes (Magiorakos *et al*., 2012).

Results and Discussion

Five isolates from diarrheic dairy calves were characterized in relation to the presence of 18 VGs. The VG profiles for the five isolates are shown in Table 2. The identity of the F5, F41, *sta*, and *iucD* PCR products was confirmed by DNA sequencing (Table 2). The five isolates were found to carry the fimbrial gene F5 which has been long considered to be the major adherence factor in bovine ETEC strains (Acres, 1985; Foster and Smith, 2009). Three of them (D242, D158, and D157) carried both the fimbriae F5 and the enterotoxin *sta* (*stap*, the porcine *sta* variant) genes, including one isolate (D242) with the $F5^+/F41^+/sta^+$ genotype which is considered a typical VG combination for bovine ETEC strains (Güler *et al*., 2008; Mainil *et al*., 1990, 1992; Nagy and Fekete, 1999). The isolate D151-9 was positive only for the F5 fimbrial gene. In addition, the isolate D151-5 carried both F5 and *iucD* genes. *iucD* gene is part of the aerobactin operon (*iucABCD iutA*) encoding the siderophore aerobactin and its receptor which is normally found on plasmids (Herrero *et al*., 1988). IucD protein catalyzes the initial step in aerobactin biosynthesis (Herrero, *et al*., 1988) and it is frequently found in extra-intestinal *E. coli* strains (ExPEC) (Köhler and Dobrindt, 2011). This unusual combination of the F5 fimbrial gene and the *iucD* aerobactin gene observed in this hybrid strain could be explained on the basis of horizontal gene transfer between cells, which enables the exchange of genetic material located on mobile elements (transposons, integrons, or plasmids) among related or unrelated bacterial species (Ahmed *et al*., 2008; Hacker and Kaper, 2000). The potential for a given *E. coli* strain to possess different combinations or sets of virulence genes could lead to the emergence of new pathotypes. Consistent with this hypothesis, we found this hybrid strain (ETEC/ExPEC) with a combination of VGs from different pathotypes. No enterotoxin gene was detected in the isolates D151-9 and D151-5 which may suggest its loss during infection or laboratory culture. The loss of plasmid borne genes is not uncommon in ETEC (Francis, 2002).

The ETEC isolates belonged to five different serotypes: $O101:H^-$, $O141:H^-$, $O60:H^-$, ONT:H10, and $ONT:H^-$ (Table 2). The serotypes are diverse containing combinations of VGs associated with the $F5^+$ ETEC pathotype. The serogroup O101 was reported previously in bovine ETEC strains in other countries as France, China, Iran, and Japan (Acres, 1985; Du *et al*., 2005; Mainil *et al*., 1990; Shams *et al*., 2012; Yamamoto and Nakazawa, 1997) which is considered the most common serogroup of $F5^+$ $F41^+$ ETEC strains from calves together with the serogroup O9 (Acres, 1985; Nagy and Fekete, 1999). This serogroup was also determined in this study in the reference strain FV101191 (F5/F41/*sta*) isolated from bovine in Spain (Table 2). The serotypes $O141:H^-$ and $O60:H^-$ identified in this work were not previously reported among bovine ETEC strains. These two serotypes have been detected in strains from piglets with post-weaning diarrhea in several countries (Garabal *et al*., 1996; Klemm, 1994; Vu-Khac *et al*., 2006, 2007). The $O141:H^-$ porcine isolate contained the F18, *sta*, and *stb* (Vu-Khac *et al*., 2006), while the $O141:H^-$ bovine isolate contained the F5 and *sta*. In the case of the $O60:H^-$, porcine isolate contained the F18 and *east1* (Vu-Khac *et al*., 2006), while the $O60:H^-$ bovine isolate contained the F5 and *sta*. These differences suggest that these two serotypes can host a variety of VGs including F5 and F18 fimbrial genes associated to receptors which are expressed in at different animal ages (Acres, 1985; Francis, 2002; Nagy and Fekete, 1999). This novel association between $O141:H^-$ and $O60:H^-$ serotypes and calves reported here support the idea that the process to originate pathogen strains is present in the particular sampled environment (a dairy farm). The same idea could be extended to the non-typeable serogroups of D151-5 ($ONT:H^-$) and D151-9 (ONT:H10).

The production of hemolysis (particularly β-hemolysis) is a trait often associated with pathogen *E. coli* strains from animals (Smith, 1963). Thus, the routine diagnosis of pathogenic *E. coli* strains includes the hemolytic phenotype as presumptive evidence of pathogenic potential. No hemolytic activity on CBA was detected

in the five isolates studied in this work. This feature correlates with the negative result for the PCR detection of *HlyA*.

The phylogenetic relationship was determined according to the Clermont phylogroup method based on chromosomal markers. The quadruplex genotype of the five isolates was 242 (+ - + -, C-), D158 (+ - - -), D157 (+ - - -), D151-9 (+ - + -, C-), and D151-5 (+ - + -, C-) (Table 2). Based on these quadruplex genotypes obtained, all the isolated were assigned to the phylogenetic group A, which is associated with commensal *E. coli* strains (Clermont *et al*., 2013). These isolates may have acquired the VGs by movil genetic elements (except for the chromosomal F41 adhesin gene) through horizontal gene transfer and became pathogens from commensal bacteria (Ahmed *et al*., 2008; Hacker and Kaper, 2000). The bovine gastrointestinal tract is a natural reservoir for commensal and pathogenic *E. coli* strains with high phylogenetic and genotypic diversity. To be considered as ETEC, the presence of toxin and/or fimbrial genes (Acres, 1985; Foster and Smith, 2009; Leimbach *et al*., 2017; Rodas *et al*., 2011), it is more relevant than the chromosomal background. In humans, results have indicated that ETEC may have acquired virulence plasmids at several independent occasions during evolution and that the chromosomal genetic background is diverse and not specific for ETEC (Steinsland *et al*., 2010). Our results with bovine isolates may indicate that the chromosomal background, as measured by serotyping and phylotyping, is diverse and it is associated to commensal strains but further analyses including multilocus sequence typing (MLST) or whole genome sequencing are needed to determine chromosomal relationships.

Among the 24 AMs tested, the three isolates D242, D157, and D151-5 exhibited resistance at least to five AMs (AMC-ERY-OB-RFA-TIL) which can be included in four different AM classes (Cephalosporins, Penicillins, Macrolides, and Ansamycins). In addition, the isolate D151-5 exhibited resistance to Quinolones, Tetracyclines, and Sulphonamides (Table 2). Three isolates showed resistance to TIL that is commonly used in veterinary practice and, the isolates in our study showed resistance to AMC, ERY, OB, and RFA which are unusual AMs in veterinary practice. The isolates shared resistances to more than three AM classes and for this reason are classified as multiresistants according to Magiorakos *et al*. (2012). This multi-resistant phenotype pointed out the possible presence of integrons which are considered to play the main role in the emergence of multiresistance (Mazel, 2006). Among the five ETEC isolates and reference strains (FV10191 and FV10189), the isolate D151-5 and the reference strain FV10191 were positive for the presence of integrase class 1 and the five isolates and reference strains (FV10191 and FV10189) were negative for the integrase class 2. Gene cassettes for the two class 1 integrons detected were amplified and sequenced. Both integrons included the *dfr* and *aad* gene alleles encoding dihydrofolate reductases and adenyltransferase, respectively (Table 2). The integron of D151-5 included the genes *dhfrA17* that confer resistance to trimethoprim and *aadA5* that confer resistance to spectinomycin but not to streptomycin (White *et al*., 2000) in the variable region, and the gene *Sul1* (resistance to sulfonamide) in the 3'-conserved segment (3′CS) (White *et al*., 2000). Consistent with this result, the isolate D151-5 is trimethoprim and sulfonamide resistant and streptomycin susceptible. This gene cassette array *dhfrA17-aadA5* was detected in Europe, Africa, Asia, and Australia from cattle in the last years (Stokes and Gillings, 2011; INTEGRALL database http://integrall.bio.ua.pt/), and this cassette array was recently identified in America from a calf in Uruguay (Umpiérrez *et al*., 2017). The integron identified in the reference strain FV10191, harbored the cassette array *dhfrA1-aadA1* which is frequently found in *E. coli* (Stokes and Gillings, 2011; INTEGRALL database http://integrall.bio.ua.pt/). The resistances to trimethoprim-sulfamethoxazole and sulfonamides exhibited by the strain FV10191 correlated with the presence of *dhfr* and *sul1* in the class 1 integron, but the susceptibility to streptomycin (300 μg) showed in this strain was not concordant to the *aadA1* gene identified in the cassette arrays. This could be related to the integron location, the distance of the *aadA1* gene to the promoter or the low-level streptomycin resistance conferred by the *aadA* gene compared with the high-level streptomycin resistance conferred by the *strA-strB* genes (Sunde and Norström, 2005).

Molecular studies allow for a comprehensive characterization of the isolates involved in bovine diseases. The current study provides evidence that the ETEC pathotype was isolated from diarrheic dairy calves in the central region of Argentina based on diagnostic cases but not represent the situation in dairy calves in Argentina. We found a high level of AM resistance which may be associated with the selective pressure caused by antibiotic use on the dairy farms where the isolates were collected. The broad use of AMs in animal production may lead to the emergence and dissemination of AM-resistant bacterial pathogens which could be considered a reservoir of virulence and resistance genes in the environment and pose a potential threat to public health. This evidence emphasizes the urgent need for effective surveillance and control. Further research on a large sample size is proposed to attribute an effect of the ETEC pathotype in diarrheic neonatal calves in our country.

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Conflict of interest

The authors declare that there is no conflict of interest.

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