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ORIGINAL ARTICLE

Non-O157 Shiga toxin-producing *Escherichia coli* with potential harmful profiles to humans are isolated from the faeces of calves in Uruguay

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ABSTRACT. Shiga toxin-producing *Escherichia coli* (STEC) infections are responsible for acute illnesses and deaths in humans. Cattle and humans are exposed to STEC through faeces and contaminated food and water. The big six and O157 STEC serogroups are important food and water-borne human pathogens. Additionally, Stx1a, Stx2a and Stx2c subtypes are highly associated with the haemolytic uremic syndrome. This study aimed to determine Shiga toxin-subtypes, the presence of antigen 43 families, the genotypic and phenotypic antimicrobial susceptibility profiles, O-serogrouping, phylotypes and phylogenetic relatedness of STEC of calf origin. Sixteen STEC isolates from calf origin were analysed. PCR was performed to determine Stx subtypes, serogroups, the presence of *ag43* I and II and phylotypes. The antimicrobial profile was evaluated and the presence of PMQR and fosfomycin genes was determined by PCR. The clonal relatedness of STEC was studied by PFGE. The genotypes *stx1a+c, stx1a+*, *stx1a+/stx2e+*, *stx1a+c/stx2e* and *stx2a* were detected. Ag43 II was the most prevalent among subfamilies. STEC isolates were serotyped as O103 (*n*=5) and O111 (*n*=6). Fifty per cent of the isolates were classified as B1 phylogroup, 4/16 as E, 1/16 as C, and 1/16 as F. Non-O157 STEC isolates showed a high level of diversity, independent of the geographical and farm-origin. Isolates were resistant to ampicillin, ciprofloxacin, gentamicin, and fosfomycin-trometamol. The gene *fosA7* was detected in 1 isolate. The virulence profiles, including Shiga toxin-subtypes and serogroups, denote the potential harm of non-O157 STEC isolates to humans. We also confirmed that circulating non-O157 STEC from cattle present genetic heterogeneity and are susceptible to antibiotics.

Key words: Non-O157 STEC, Shiga toxin subtypes, antimicrobial resistance.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) is a bacterial pathogen with a defined zoonotic potential (Gyles & Fairbrother, 2010). The main natural reservoir of STEC is the bovine intestine, although it can be isolated from other domestic animals (Gyles & Fairbrother, 2010). Infections in humans are usually caused by the consumption of undercooked meat, contaminated vegetables, dairy products and contact with contaminated water. Some infections are also caused by the contact with the environment of animals and ruminants in the farm and person-to-person contact (Kintz *et al.*, 2017). Even though some individuals infected with STEC recover without significant complications, some STEC strains are highly virulent to humans (Majowicz *et al.*, 2014). STEC infections cause over 2.8 million illnesses annually, including haemorrhagic colitis (HC), haemolytic uraemic syndrome (HUS), renal failure and even in some cases haemorrhagic cystitis around the world (Gadea *et al.*, 2012, Majowicz *et al.*, 2014). The incidence of STEC infections differs between countries alongside South America. In Uruguay, cases of HUS and HC are sporadic and have an incidence of 4 to 5 per 100,000 children, whereas Argentina, considered the country with the highest incidence of HUS in children under 5 years, has 300 to 400 HUS cases per year (Blanco *et al.*, 2004, Pérez *et al.*, 2014).

STEC main virulence factors are Shiga toxin type 1 and type 2 (Stx1 and Stx2, respectively). They are encoded in the genome of tempered double-stranded lambdoid prophages (Scheutz, 2014). The number of Stx1 and Stx2 subtypes is continuously upgrading. The Stx1 group is conserved and has four subtypes, a, c, d, and e, while Stx2 is more heterogeneous, and 11 subtypes have been distinguished so far: a-k, with some of them been reported in severe disease in humans (Probert *et al.*, 2014, Scheutz, 2014, Bai *et al.*, 2018, Yang *et al.*, 2020a). In addition, some STEC isolates possess the locus of enterocyte effacement (LEE) pathogenicity island. STEC LEE+ strains are defined by the expression of Intimin, and the translocated intimin receptor (Tir), among other virulence factors. Together, they are responsible for the attaching and effacing (A/E) lesions induced in intestinal epithelial cells (Torres *et al.*, 2018).

Despite the great diversity of phenotypes and combinations of virulence factors, *E. coli* presents a clonal structure. So far, commensal *E. coli* strains, diarrheagenic *E. coli* from the diarrheagenic *E. coli* group (DEC), and those causing extraintestinal infections (ExPEC) can be grouped into eight phylogenetic groups: A, B1, B2, C, D, E, F, and clade I (Clermont *et al.*, 2013). ExPEC strains

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have been mainly assigned to group B2 and a lesser extent to groups D and F, while commensals and diarrheagenic *E. coli* strains have been assigned mostly to groups A, B1, and E (Tenaillon *et al.*, 2010). At the same time, *E. coli* strains are distinguished by their lipopolysaccharide (O) and flagellum (H) composition and antigenicity (Rivas *et al.*, 2014). Currently, in addition to *E. coli* O157:H7, other serogroups have been associated with severe HUS outbreaks. Big six serotypes O26, O45, O103, O111, O121, and O145, together with *E. coli* O157, are the most prevalent within STEC LEE+ strains (Rivas *et al.*, 2014).

As aforementioned, bovines are the main reservoir of STEC and besides the severe damage caused to humans, it can survive imperceptibly in both the bovine intestinal epithelium and in the environment. STEC abundance is low in cattle intestine, however, it can manage to be viably transmitted to the farm environment and from there, even in low abundances, to infect other animals or contaminate water courses (Sapountzis *et al.*, 2020). Persistence in such different places is associated with the phenotypes and plasticity to adapt to the ecological niches and usually involves the ability to form biofilms or have thigh adhesion conditions. Autoaggregation, cell-cell adhesion to the host and biofilm formation of STEC have been associated with the presence of autotransporter proteins like Antigen 43 (Ag43). This adhesin has been more frequently linked to pathogenic STEC LEE+ strains like *E. coli* O157:H7 than to commensals ones (Kjaergaard *et al.*, 2000).

The emergence of multidrug-resistant bacteria has been recognised as a global health issue. Antimicrobial misuse in humans and animals over the decades has determined the occurrence of non-effective treatments for several infectious diseases (Marshall & Levy, 2011). Even antibiotic treatment in STEC infections is not recommended, it is important to keep in mind the association of resistance mechanisms to mobile genetic elements, such as transposons, integrons and plasmids, that give bacteria the capability to rapidly transfer resistance genes (Marshall & Levy, 2011).

In Uruguay, the proportion of STEC in the faeces of calves is low, according to previous studies of our group (Umpiérrez *et al.*, 2017, 2021). However, given the severity and outcome of the illness, the regional context of HUS cases in children, and the ecology of STEC transmission and persistence in the environment it is required to find out the potentially harmful effects of circulating STEC isolates.

This study aimed to determine the Shiga toxin subtypes and the presence of the Antigen 43 gene, the antimicrobial phenotype, O-serogrouping, phylotypes and phylogenetic relatedness of STEC isolates of calf origin.

MATERIAL AND METHODS

ISOLATES AND GROWTH CONDITIONS

Sixteen STEC strains isolated from bovine faeces were collected between 2014 and 2017. As previously described, faeces collection and biochemical and molecular characterisation of *E. coli* were performed (Umpiérrez *et al.*, 2017, 2021). Briefly, all isolates except one were collected from faeces of seven dairy calves with signs of neonatal calf diarrhoea (NCD) and from faeces of dairy calves without NCD signs under 35 days old (table 1), whereas one isolate was collected from an ileum sample of a dead calf affected with NCD (table 1). All isolates were previously characterised by PCR, regarding the presence of *stx1*, *stx2*, and *eae* among other *E. coli* virulence genes, and classified as STEC LEE+ (*stx*+/*eae*+) (Umpiérrez *et al.*, 2017, 2021). For the routine cultivation of STEC, isolates were grown on trypticase soy agar (TSA) plates (OXOID) for 18-24 h at 37 ºC.

SHIGA TOXIN GENES SUBTYPING

Subtyping of Stx was performed according to the protocol described by Scheutz *et al.*, (2012). Three *stx1* subtypes (*stx1a*, *stx1c*, *stx1d*) and 7 *stx2* subtypes (*stx2a, stx2b, stx2c, stx2d, stx2e, stx2f, stx2g*) were evaluated by multiplex PCR. When required, the whole *stx* operon was amplified and sequencing analysis was performed using the free software BioEdit (version7.2.5) to assign the Stx subtype to each isolate (Scheutz *et al.*, 2012).

ANTIGEN 43 GENE DETECTION

The presence of Ag43 was evaluated by PCR. Partial amplification of the *ag43* gene was performed as previously described (Kjaergaard *et al.*, 2000). Amplicon size of the two Ag43 subfamilies was determined by agarose gel electrophoresis (subfamily I amplicon size: 1569pb; subfamily II amplicon size: 1839pb) (Kjaergaard *et al.*, 2000).

SEROGROUP

The O26, O45, O103, O111, O113, O121, O145, and O157 serogroups were determined by PCR according to the procedure described by Paddock *et al.*, (2012). PCR positive controls were included in each amplified serogroup. *E. coli* O157:H7 EDL933 strain was used as a positive control for O157 serogroup. The rest of the DNA controls were extracted from clinical isolates from the "Pathogenic *Escherichia coli* Laboratory" pathogenic strains collection at Universidad de Chile.

PHYLOGENETIC ANALYSIS

To assign STEC isolates into any of the phylogroups, partial amplifications of *chuA, yjaA*, *tspE4.C2* and *arpA* genes by PCR was performed according to Clermont *et al.*, (2013). The multiplex PCR method uses the amplification profile of these genes to assign isolates to eight different phylogroups: A, B1, B2, C, D, E, F, and Clade-I.

Table 1. Virulence profile, antimicrobial phenotype, and resistance genes, O-serogrouping and phylotypes of STEC isolates recovered from animals with signs of NCD and from healthy calves.

Animal	Isolate	Year of isolation	Animal signs	Virulence profile	Antibiotic resistance profiles	Resistance genes	Serogroup	Phylogroup
1	1 (74.2)	2014	NCD signs	*stx1a, ag43(II)*	---		O111	B1
2	2 (16.16)	2015	NCD signs[(n)]	*stx1a+c*	AMP / CN / FOT	*fosA7*	n/d	A
3	3 (AG2.1)	2016	NCD signs	*stx2a*	FOT		n/d	A
4	4(AD1.5)	2016	NCD signs	*stx1a+c, ag43(I)*	AMP/CIP		n/d	E
	5 (AD1.6)			*stx1a, ag43(I)*	AMP/CIP		O111	F
	6 (AD1.7)			*stx1a+c*	AMP		n/d	B1
	7 (AD1.9)			*stx1a+c*	AMP		O111	E
5	8 (AD3.2)	2016	NCD signs	*stx1a+c, ag43(II)*	AMP		O111	B1
6	9 (AD7.2)	2016	NCD signs	*stx1a*	AMP		O111	B1
	10 (AD7.5)			*stx1a*	AMP		O111	C
	11 (AD7.10)			*stx1a, ag43(II)*	---		n/d	E
7	12 (AC3.1)	2016	Calf without signs	*stx1a+c, ag43(II)*	CIP		O103	B1
	13 (AC3.10)			*stx1a, ag43(II)*	---		O103	B1
8	14 (BJ1.3)	2017	NCD signs	*stx1a/stx2e, ag43(II)*	---		O103	E
	15 (BJ1.5)			*stx1a+c, stx2e, ag43(II)*	AMP		O103	B1
	16 (BJ1.10)			*stx1a, stx2e, ag43(II)*	AMP		O103	B1

[(n)] *E. coli* isolated from an ileum sample of a dead calf with NCD. n/d: none determined serogroup. *ag43(I)* corresponds to Ag43 subfamily I, and *ag43(II)* corresponds to Ag43 subfamily II. AMP, Ampicillin; FOT, fosfomycin-trometamol; CN, gentamicin.

PULSED-FIELD GEL ELECTROPHORESIS

Clonality of the isolates was evaluated by XbaI Pulsed-Field Gel Electrophoresis (PFGE) according to PulseNet protocol. *Salmonella* Braenderup H9812 and *Staphylococcus aureus* subsp. *aureus* (strain NCTC 8325) were used as reference strains. Band patterns were analysed with BioNumerics v.6.6 software (Applied Maths, Sint-Martens-Latem, Belgium). A dendrogram was generated by the UPGMA method, using the Dice coefficient with a 1.0% of band position tolerance.

ANTIMICROBIAL SUSCEPTIBILITY ANALYSES

The Kirby-Bauer disc-diffusion method was used to analyse antimicrobial susceptibility, according to the Clinical Laboratory Standard Institute (2017). Grown isolates in Mueller-Hinton (MH) agar plates (OXOID) for 18-24 h at 37 ºC were tested for 11 different antibiotics: ampicillin (AMP), cefuroxime (CXM), ceftazidime (CAZ), ceftriaxone (CRO), nalidixic acid (NA), ciprofloxacin CIP, enrofloxacin (ENR), gentamicin (CN), amikacin (AK), trimethoprim-sulfamethoxazole (SXT), and fosfomycin trometamol (FOT). All antibiotic discs were purchased from Oxoid. Quality control was performed with *E. coli* ATCC 25922. The interpretation of results was performed according to CLSI 2017, except for ENR, which was interpreted using Veterinary Antimicrobial Susceptibility Testing (VAST) (Patel, 2014). Considering the One Health concept and the zoonotic potential of these isolates, all antibiotics were selected based on the frequency with which they are employed in the medical practice of human infectious diseases.

FOSFOMYCIN AND PLASMID MEDIATED QUINOLONE RESISTANCE GENES

With regard to resistance genes, we searched for the main mechanisms of transferable resistance to antibiotics considered critical by the World Health Organization and previously detected in our country. The presence of PMQR and fosfomycin genes was evaluated by PCR. PMQR genes *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, and *qepA* were partially amplified as previously described (Umpiérrez *et al.*, 2017). On the other hand, the search for fosfomycin resistance genes *fosA*, *fosB*, *fomA*, *fomB*, *fosA3*, *fosC2*, and *fosA7* was performed following protocols and using primers from literature (Cóppola *et al.*, 2020). Primers to detect *fosA7* gene were designed in this study: fosA7F 5'- ATGCTTCAATCTCTGAACCAC -3', fosA7R 5'- CCGAAACGCATTCCAGAGTA -3'. All PCR products were confirmed by direct sequencing.

RESULTS

A collection of 16 STEC isolates (13 isolates from calves with NCD signs, two isolates from a calf without signs of NCD and one isolate from an ileum sample of a dead calf with NCD) were characterised.

SUBTYPES OF STX1 AND STX2

Four Shiga toxins subtypes were detected by PCR. Some STEC isolates presented more than one variant of subtypes simultaneously. All *stx1+* isolates were typed as *stx1a+* (15 isolates), 6 of them were also *stx1c+* and one presented a *stx1a+*/*stx1c+*/*stx2e+* genotype (table 1). In addition, 2 isolates were *stx1a+*/*stx2e+* simultaneously and 1 isolate was *stx2a+* (table 1). Gene variants *stx1d*, *stx2b*, *stx2c*, *stx2d*, *stx2f* and *stx2g* were not detected.

PRESENCE OF ANTIGEN 43 GENE

Ag43 subfamily II, was predominant among STEC isolates (*n*=8), while only 2 isolates were assigned to Ag43 subfamily I (table 1). The rest of the isolates (*n*=6) were *ag43* negative (table 1).

O-SEROGROUPING AND PHYLOTYPES

Two *E. coli* serogroups were detected by PCR. Five STEC isolates were ascribed to O103 serogroup, whereas 6 isolates were ascribed to O111 (table 1). The remaining 5 isolates were not assigned to any of the 8 serogroups evaluated (31.3% of the isolates) (table 1).

On the other hand, STEC phylotyping showed high diversity. Fifty per cent of the isolates were classified as B1 (*n*=8) (table 1), and the rest were classified as follows: 4 belonged to E phylogroup, 2 to A phylogroup, 1 to F phylogroup and one to C phylogroup (table 1).

CLONAL RELATEDNESS OF STEC ISOLATES

A total of 13 distinct restriction patterns of the 16 STEC isolates were detected using ≥85% of similarity of the Dice coefficient (figure 1). A high level of diversity amongst isolates was observed, however, isolates with the same herd-origin were more genetically similar to each other and as a consequence showed more similar restriction patterns. Two isolates from 1 animal with symptoms of NCD (isolates AD1.5 and AD1.6) were considered indistinguishable from each other (figure 1). STEC isolates AC3.1 and AC3.10 from an asymptomatic animal were considered indistinguishable from each other (figure 1). Additionally, isolates AD1.7 and AD 7.2 coming from different animals but from the same farm, were considered identical (figure 1).

PHENOTYPIC AND GENOTYPIC ANTIMICROBIAL RESISTANCE OF STEC

STEC isolates were mostly susceptible to antibiotics. Ten out of 16 (62.5%) of the isolates were resistant to AMP, whereas 4/16 (25%) were susceptible to all antibiotics tested. Ten STEC isolates were resistant to beta-lactams (10 isolates were resistant to ampicillin), 3 STEC isolates were resistant to ciprofloxacin and 2 STEC isolates were resistant to fosfomycin trometamol (table 1). The isolate from the ileum sample was the only one resistant to 3 antibiotics: ampicillin, gentamicin and fosfomycin trometamol, whereas 2 STEC isolates were ciprofloxacin- ampicillin-resistant (table 1).

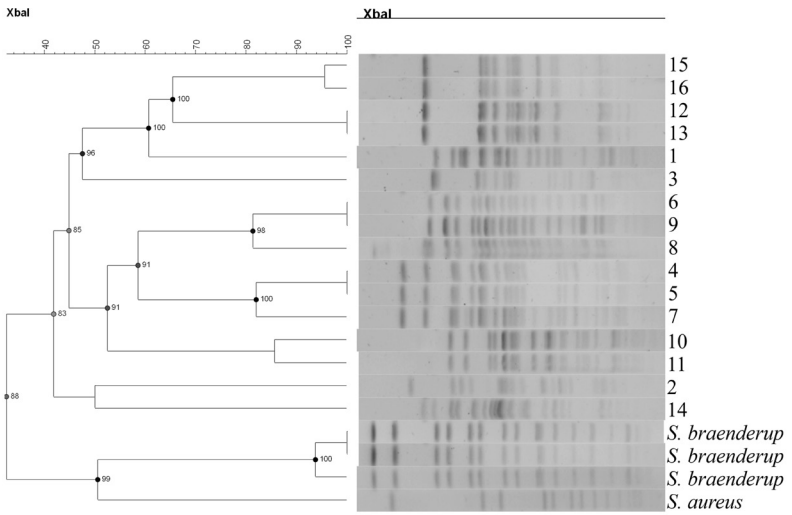
PMQR genes were evaluated in 3 isolates that showed resistance or intermediate susceptibility to CIP: AD1.5, AD1.6 and AC3.1 (table 1). None of the 7 PMQR genes was detected. Otherwise, FOT gene *fosA7* was detected in 1 STEC isolate: 16.16 (table 1).

DISCUSSION

STEC infections in humans constitute a global health concern and are endemic in Latin America, accounting for almost 2% of acute diarrhoea cases and 20%-30% of bloody diarrhoea (Torres *et al.*, 2018). STEC colonises the gastrointestinal tract of cattle, which are mainly asymptomatic carriers, and only under specific circumstances can develop diarrhoea. It can adapt and survive in different environments such as soil and water and contaminates food, meat, and dairy products, through which it can reach and infect other animals and humans (Daly & Hill, 2016). Non-O157 STEC isolates have increasingly been reported associated with human outbreaks (Bettelheim & Goldwater, 2014).

The severity of STEC infections is determined by the interaction of both host and microorganism factors. Regarding the bacterial virulence profile, all detected Stx subtypes in the present study are linked to illness in humans (EFSA BIOHAZ Panel, 2020). We detected *stx1a+c* (*n*=8) and *stx1a* (*n*=6) genotypes in high proportion amongst isolates, which were similar to previous reports in non-O157:H7 STEC isolates from cattle faeces in the United States (Shridhar *et al.*, 2017) and the occurrence of *stx1a* together with *stx2a*, *stx2c*, or *stx2d* have also been described in STEC isolates from bovine origin in China (Fan *et al.*, 2019). In this study, *stx1a/stx2e* and *stx1a+c/stx2e* genotypes were detected. Stx2e is associated with mild gastroenteritis in humans, while it is the most frequent Stx subtype in pigs, wild boars, and their meat products (Beutin *et al.*, 2008). Additionally, *stx2a* gene variant, widely associated with HUS, was detected in one isolate from an animal with signs of NCD. This is the first report of the presence of this variant in non-O157 STEC isolates from calf origin in our country.

Autotransporter protein Ag43 has been linked to pathogenic O157 and non-O157 *E. coli* strains and is associated with autoaggregation, cell-cell interaction with the host and biofilm formation (Matheus-Guimarães *et al.*, 2014). It has been observed that the main role of Ag43 depends on the presence of other virulence factors like adhesins, and the genetic background of the strain (Carter *et al.*, 2017). In this work, both Ag43 subfamilies were detected. Subfamily II (the Calcium-binding Antigen43



Isolate	Signs	Virotyping	Phylogroup	Serogroup	Antibiotic resistance profiles	Resistance genes
15 (BJ 1.5)	NCD	*stx1a+c, stx2e, ag43(II)*	B1	O103	AMP	---
16 (BJ 1.10)	NCD	*stx1a, stx2e, ag43(II)*	B1	O103	AMP	---
12 (AC 3.1)	No NCD signs	*stx1a+c, ag43(II)*	B1	O103	Sensitive	CIP
13 (AC 3.10)	No NCD signs	*stx1a, ag43(II)*	B1	O103	---	---
1 (74.2)	NCD	*stx1a, ag43(II)*	B1	O111	---	---
3 (AG 2.1)	NCD	*stx2a*	A	N/D	FOT	*fosA7*
6 (AD 1.7)	NCD	*stx1a+c*	B1	N/D	AMP	---
9 (AD 7.2)	NCD	*stx1a*	B1	O111	AMP	---
8 (AD 3.2)	NCD	*stx1a+c, ag43(II)*	B1	O111	AMP	---
4 (AD 1.5)	NCD	*stx1a+c, ag43(I)*	E	N/D	AMP/CIP	---
5 (AD 1.6)	NCD	*stx1a, ag43(I)*	F	O111	AMP/CIP	---
7 (AD 1.9)	NCD	*stx1a+c*	E	O111	AMP	---
10 (AD 7.5)	NCD	*stx1a*	C	O111	AMP	---
11 (AD 7.10)	NCD	*stx1a, ag43(II)*	E	N/D	---	---
2 (16.16)	NCD_n	*stx1a+c*	A	N/D	AMP/CN/FOT	---
14 (BJ 1.3)	NCD	*stx1a, stx2e, ag43(II)*	E	O103	---	---
S. braenderup						
S. braenderup						
S. braenderup						
S. aureus						

Figure 1. Phylogenetic relationships of STEC isolates. The phylogenetic tree was generated using the UPGMA method, using the Dice coefficient with a 1.0% of band position tolerance (BioNumerics v.6.6, Applied Maths, Sint-Martens-Latem, Belgium). *Salmonella* Braenderup and *Staphylococcus aureus* were used as reference strains.

Homologue, Cah) was detected in 14 isolates, while two STEC isolates were Ag43 subfamily I, positive. It has been observed that the gene that codes for subfamily II (*cah*) has a high mutation rate, which is associated with the adaptability of STEC to different environments (Carter *et al.*, 2017). On the other hand, Ag43 subfamily I, is not present in LEE+ strains since it is encoded in LAA pathogenicity island (Locus of Adhesion and Autoaggregation) (Montero *et al.*, 2017). Primers to detect both subfamilies used in this work were designed based on a difference of 270bp between them, and subfamily I could not be found in STEC LEE+ isolates. Therefore, it is probable that our STEC LEE+ isolates present heterogenicity among this gene, which may be due to distinct functions that could favour an in-host or environmental status.

The major serogroup associated with STEC infections in humans still is *E. coli* O157:H7, the first serogroup recognised causing enteric bloody diarrhoea (EFSA BIOHAZ Panel, 2020). However, other STEC serogroups are now recognised as important food and water-borne pathogens. Among them, the "big six" group of strains is frequently detected in HUS cases (Shridhar *et al.*, 2017). In this work, O103 and O111 were the only serogroups detected. Five STEC isolates were ascribed to the O103 serogroup (three isolates from a bovine with NCD signs and two isolates from a calf without signs) whereas six isolates were ascribed to O111. The remaining five isolates could not be assigned to any of the evaluated serogroups (31.3% of the isolates). Considering that there are over 1150 published STEC serotypes, these isolates probably could be assigned to other than the "big six". O103 and O111 serogroups have been previously determined in cattle (Thomas *et al.*, 2012, Bibbal *et al.*, 2015, Jajarmi *et al.*, 2017, Rivelli Zea *et al.*, 2020), their faeces (Blanco *et al.*, 2004, Cernicchiaro *et al.*, 2013) and carcasses (Cap *et al.*, 2019), in countries from South America and other regions. On the other hand, the isolation of STEC O111 in bloody diarrhoea cases has been reported in our country (Varela *et al.*, 2008), which demonstrates a previous circulation of this serogroup. Also, a positive correlation between the presence of the virulence-marker gene *eae* in non-O157 STEC and the occurrence of HUS have been established (Yang *et al.*, 2020b), an affirmation that agrees with the assumption that non-O157 STEC LEE+ isolates from this study could be harmful to human.

It is well known that *E. coli* isolates from different sources of isolation usually belong to different phylogroups (Clermont *et al.*, 2013). Therefore, it would be expected that the STEC isolates of bovine origin of this work all belong to the same phylogroup. However, five different phylogroups were determined. According to the molecular assignment, 50% (8/16) of the isolates were classified as B1. This observation is consistent with reports which indicate that B1 is mainly present in the microbiota of domestic animals, often associated with intestinal commensal and pathogenic *E. coli* (Tenaillon *et al.*, 2010). The second most frequently detected phylogroup was E (4/16). It has recently been reported that this phylogroup predominantly includes O157:H7 strains (Tenaillon *et al.*, 2010). The A phylogroup has been also associated with commensal/ intestinal pathogens, and in this work was assigned to 2/16 STEC isolates. Finally, C and F phylogroups were each represented with 1/16 of the isolates. Both phylogroups have been proposed to be sister groups of B1 and B2, respectively (Clermont *et al.*, 2013).

When the clonal relatedness of the isolates was analysed, a high level of diversity amongst isolates was observed. PFGE profiles showed 13 distinct restriction patterns out of 16 STEC isolates. We only found two indistinguishable band patterns within animals (the pair of isolates: AD1.5 and AD1.6 / AC3.1 and AC3.10 came each from one animal), and within a bovine herd (isolates AD1.7 and AD 7.2 came from different animals but from the same herd). Further, more than one band pattern was detected within bovine herds. When we looked out serogroups, we detected that STEC isolates ascribed to the O103 were closely related; and those ascribed to the O111 serogroup were closely related too. Similar results from PFGE analyses were previously determined in STEC isolates from cattle origin (Bibbal *et al.*, 2015, Bumunang *et al.*, 2019), which reaffirms the role of bovine as non-O157 STEC strains reservoirs.

It has been observed that antibiotic resistance in non-O157 STEC isolates from animal origin is higher than in O157 STEC strains (Mir & Kudva, 2019). Likewise, multidrug resistance is frequently associated with non-O157 STEC strains with *eae*+/*stx1*+ virulence profiles (Mora *et al.*, 2015). In the present study, 11 antibiotics were evaluated using the Kirby-Bauer method. AMP resistance was the most frequent amongst isolates (62.5%), and it was determined in the following arrangements: AMP (*n*=7), AMP/CIP (*n*=2), and AMP/CN/FOT (*n*=1) being the last one the only isolate considered MDR. Finally, one isolate was only FOT^R and 1 isolate was only CIP^R. The high percentage of resistance to AMP agrees with the fact that β-lactams are the most used antibiotics in animals and with previous reports by our group, although the antibiotic resistance profiles then were considerably higher in numbers and included resistance to cephalosporins and trimethoprim-sulfamethoxazole among others (Umpiérrez *et al.*, 2017, 2021). Even though isolates were in general susceptible to the tested antibiotics, 5/12 of the resistant isolates were also classified as O111 and 3/12 were classified as O103. In previous works, O111 serogroup has been associated with multidrug-resistant non-O157, *eae*+ STEC isolates (Mora *et al.*, 2015). Particularly it has been associated with resistance levels higher than 25% to ampicillin, amoxicillin-clavulanic acid, cephalothin, trimethoprim-sulfamethoxazole, tetracycline, chloramphenicol and streptomycin. Meanwhile, O103 STEC isolates have been associated with low resistance levels, except for trimethoprim-sulfamethoxazole, tetracycline and streptomycin (Schroeder *et al.*, 2002, Amézquita-López *et al.*, 2016). Even though non-O157 STEC isolates of this

study were mainly susceptible to antibiotics, it is important to state that they were screened from a large collection of *E. coli* isolates, in which the percentage of antibiotic resistance is significant (Umpiérrez *et al.*, 2017). In that collection, MDR isolates represented a substantial source for antibiotic resistance genes. In this study, we also detected the presence of the *fosA7* gene (STEC isolate 16.16), which confers resistance to fosfomycin. The presence of this gene has been reported in environmental *E. coli* isolates and *Salmonella* spp. from animal and human origin (Rehman *et al.*, 2017, Balbin *et al.*, 2020). Resistance to fosfomycin is a major concern in human health. In our country, on the one hand, it is within the scarce therapeutic resources available for infections of multi-resistant microorganisms (Seija *et al.*, 2015) and, on the other, it is one of the first therapeutic options for urinary tract infections (García-Fulgueiras *et al.*, 2021). Recently, we have reported the presence of *fosA3* in animals belonging to the food-production chains (Coppola *et al.*, 2020). With this scenario, the detection of *fosA7* in production chain animals reinforces the need to monitor the presence of this mechanism with a One Health concept. To the best of our knowledge, this is the first study to report on the *fosA7* gene in STEC from bovines in Uruguay. Considering the critically important antimicrobials for human medicine list published periodically by WHO (World Health Organization & WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance, 2017), dissemination of resistance to such antibiotics through non-O157 STEC isolates of calf origin did not seem to be of human or animal health concern.

It is concluded that studies encompassing molecular characterisation of non-O157 STEC of calf origin are on the rise, due to an increase in detecting these serogroups associated with severe disease in humans. Although in this study the number of evaluated isolates from cattle was low, the virulence profiles, including the confirmation of Shiga toxin subtypes *stx1a* and *stx2a*, and serogroups O103 and O111 (the last one, has been reported in human infections in our country), denote the harmful potential of them for humans. We also observed some genetic heterogeneity among the Ag43 gene, which could be associated with adaptation to different niches. Finally, circulating non-O157 STEC LEE+ from cattle faeces were not from one cluster only and showed high genetic heterogeneity, being in general susceptible to antibiotics. Further investigations with a higher number of STEC are needed to confirm these observations.

The spread of STEC isolates to the environment through bovine faeces is always a source of concern to human health, but also represents a way to contaminate the dairy environment, which increases the probability of bovine infections within a herd. Results from this study call for attention regarding the virulence profile and transferable resistance genes of non-O157 STEC LEE+ isolates, that could cause severe disease to humans and denote the essentiality of determining how they persist and transmit in the dairy environment.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing interests.

ETHICS STATEMENT

No ethical approval was required in this work, as this is an original article with only bacterial isolates and data. No animal or human samples were employed.

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