Received: 17 October 2017

DOI: 10.1111/zph.12467

ORIGINAL ARTICLE

WILEY

Molecular epidemiology of Shiga toxin-producing O113:H21 isolates from cattle and meat

A. M. Sanso | A. V. Bustamante | A. Krüger | J. S. Cadona | R. Alfaro | M. E. Cáceres | D. Fernández | P. M. A. Lucchesi | N. L. Padola

Laboratorio de Inmunoquímica y Biotecnología, Facultad de Ciencias Veterinarias, Centro de Investigación Veterinaria de Tandil, CONICET-CIC-UNCPBA, Tandil, Argentina

Correspondence
Andrea Mariel Sanso, Laboratorio de Inmunoquímica y Biotecnología, Facultad de Ciencias Veterinarias, Centro de Investigación Veterinaria de Tandil, CONICET-CIC-UNCPBA, Tandil, Argentina.
Email: msanso@vet.unicen.edu.ar

Funding information
CONICET, Grant/Award Number: PIP939/12 and PIP365/15; Fondo para la Investigación Científica y Tecnológica (FONCYT), Grant/Award Number: PICT2666/15; SECAT-UNICEN

Summary

The serotype O113:H21 is considered one of the relevant non-O157 STEC serotypes associated with severe human infections. Due to the increased detection of O113 strains and their relationship with clinical cases, which emphasizes the importance of this serogroup as an emerging pathogen, our aim was to determine the characteristics of STEC O113:H21 strains circulating in bovine cattle and retail meat from Argentina. For this purpose, we determined the presence and combinations of various virulence genes (and their variants) related to adhesion and toxicity in a collection of 34 isolates. Their genetic relatedness using multiple-locus variable-number tandem repeat analysis (MLVA) was also studied. Subtyping of *stx* genes indicated that O113:H21 strains circulating in Argentina mainly present stx_{2a} alone or together with stx_{2c} or, less frequent, with stx_{2d}, all of which are subtypes associated with human disease. We found plasmid markers, such as *saa*, *ehxA* and *subA*, in a higher proportion than previous studies, and five variants of *saa*, two of which were novel ones. In relation to MLVA subtyping, we detected a limited diversity among the isolates considering that several loci were not discriminative and, that in some farms, the same clone seemed to remain circulating throughout the year. The O113:H21 strains studied harbour several toxin and adhesion genes (*saa*, *espP*, *fimCD*, *ehaA*, *iha*, *hcpA*, *elfA*, *lpfO113*, *ecpA*, *subA*, *cdt-V*) and Stx subtypes associated with human disease. Results also highlighted that subtyping of *stx* and *saa* is useful to discriminate O113:H21 strains that share virulence genes. In conclusion, this study shows that a number of O113:H21 strains that occur in foods and bovines could be pathogenic for humans. This situation calls for further attention in the prevention and control of foodborne disease caused by these strains.

KEYWORDS

cattle, meat, multiple-locus variable-number tandem repeat analysis, STEC O113:H21, virulence

1 | INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) is a zoonotic pathogen that can cause diarrhoea and haemolytic–uraemic syndrome (HUS) (Etcheverría & Padola, 2013). Outbreaks have been mostly attributed to STEC O157:H7, but the potential of the non-O157 serotypes as human pathogens should not be underestimated because there is growing concern over the emergence of highly virulent STEC non-O157 serotypes that are globally distributed, several of which are associated with outbreaks and/or severe human illness (Coombes et al., 2008). There is a group of STEC strains named LEE-negative that lack the LEE locus (required for the formation of Attaching and Effacing (A/E) lesions and intestinal colonization) but are also capable of causing severe human disease using different mechanisms for

colonization of intestinal epithelial cells (Paton, Srimanote, Woodrow, & Paton, 2001). STEC O113:H21 is considered one of the relevant LEE-negative non-O157 serotypes associated with severe human infections (Bettelheim, 2007; Miko et al., 2009). In 1983, this STEC serotype was involved by the first time in a case of HUS, which occurred in Canada (Karmali et al., 1985), and in 1998, STEC O113:H21 was responsible for an outbreak of HUS in southern Australia (Paton, Woodrow, Doyle, Lanser, & Paton, 1999). Since then, reports of STEC infection by STEC O113 have increased, and these strains have been frequently isolated from cattle (Fernández, Irino, Sanz, Padola, & Parma, 2010; dos Santos, Kinue, Ibelli Vaz, & Cabilio Guth, 2010) and several foods, such as beef, milk and pork products (Bosilevac & Koohmaraie, 2011; Werber, Beutin, Pichner, Stark, & Fruth, 2008).

In addition to Shiga toxin genes, STEC O113:H21 strains may have other genes encoding toxins such as subtilase cytotoxin (*sub*AB gene) (Paton, Srimanote, Talbot, Wang, & Paton, 2004; Paton et al., 2006), a toxin that distends the cytoskeleton CDT-V (Janka et al., 2003); or the EHEC toxin also called EHEC haemolysin (*ehx*A gene) (Schmidt, Beutin, & Karch, 1995). The *saa*, also present in some STEC O113:H21 strains, encodes an autoagglutinating adhesin that varies in size as a consequence of differences in the number of copies of a 37-aa repeat unit in the C-terminal region (corresponding to direct 111-bp repeats) (Paton et al., 2001). Galli, Miliwebsky, Irino, Leotta, and Rivas (2010) showed that bovine LEE-negative STEC strains, especially O113, possess genes encoding for putative adhesins and toxins present in human LEE-negative STEC strains, some of which cause severe diseases. Recently, it was proposed a novel pathogenicity island named Locus of Adhesion and Autoaggregation (LAA), associated with LEE-negative strains. It harbours several virulence genes, such as the one encoding Hes (haemagglutinin from Shiga toxin-producing *E. coli*), which participates in several colonization-associated phenotypes, including haemagglutination, adhesion and autoaggregation (Montero et al., 2017).

The increased detection of O113 strains and their relationship with clinical cases emphasize the importance of this serogroup as an emerging pathogen (Monaghan et al., 2012). Several authors, including dos Santos et al. (2010) and Feng et al. (2014), state that, particularly, O113:H21 strains represent a risk to public health and should be monitored carefully. Moreover, the knowledge on combination of virulence genes and the pathogenic mechanisms of a STEC strain are necessary to improve the efficacy of the diagnostics of human infections, the surveillance of animal reservoirs and the assessment of public health risks (Galli et al., 2010).

The aim of this study was to subtype a collection of O113:H21 STEC strains isolated from different cattle farms and foods in relation to adhesion and toxicity, and to determine their genetic relatedness.

Impacts

- Shiga toxin-producing *Escherichia coli* O113:H21 is considered one of the relevant non-O157 serotypes associated with severe human infections.
- Results showed that O113:H21 STEC strains could be differentiated mainly by the combination of *stx* and *saa* subtypes, plus the presence/absence of *cdt*-V, all of which are genes located on mobile genetic elements.
- A number of O113:H21 strains that occur in bovines and retail meat harbour several toxin and adhesion genes associated with human disease and therefore could be pathogenic for humans. This situation calls for further attention in the prevention and control of foodborne disease caused by these strains.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains

Thirty-four STEC O113:H21 isolates from the collection of the Laboratorio de Inmunoquímica y Biotecnología (UNCPBA, Tandil, Argentina) were studied. They had been collected between 1995 and 2008 from cattle, and chicken- and bovine-derived products. Cattle isolates were obtained from different dairy farms geographically separated and named A, B, C, D and E, that had herds in which no outside breeding stock cattle were introduced (closed herds), two feedlots and a grazing farm (Table 1). Strains had been previously characterized by PCR regarding the presence of stx_1, stx_2, *eae*, *ehxA* and *saa* genes (Table 1 references).

2.2 | Detection of virulence-associated genes

Virulence-associated genes assessed by PCR in this study are listed in Table S1 (Supplementary Material). The plasmid-encoded genes *ehxA*, *subA*, *katP*, *stcE and espP* were amplified by a pentavalent PCR. The remaining genes were amplified using monoplex PCRs.

2.2.1 | Determination of *espP* alleles

Detection of α, ß, γ and δ subtypes was carried out by PCR as described by Brockmeyer et al. (2007) and subtype ε as described by Bielaszewska et al. (2009).

2.2.2 | Determination of *sub*AB variants

$subAB_1$ and $subAB_2$ variants plus the *tia* gene were assessed as described by Michelacci et al. (2013).

2.2.3 | Determination of *stx* subtypes

Shiga toxin genes were subtyped according to the protocol described by Scheutz et al. (2012) to detect stx_{1a}, stx_{1c}, stx_{1d}; and stx_{2a} to stx_{2g}. As suggested by those authors, annealing temperatures were adjusted to achieve specific amplification.

2.2.4 | Determination of *saa* gene variants

Primers and PCR conditions used to amplify a region containing the repeat sequence of *saa* were those from Lucchesi, Krüger, and Parma (2006). Amplification products of the variants were sequenced (Macrogen, Korea) to determine the number of repeat units.

2.3 | Multiple-locus variable-number tandem repeat analysis (MLVA)

Nine variable number of tandem repeat (VNTR) loci were analysed, seven loci (CVN001, CVN002, CVN003, CVN004, CVN007, CVN014 and CVN015) proposed by Lindstedt, Brandal, Aas, Vardund, and Kapperud (2007), and loci CVN016 and CVN017 proposed by Løbersli, Haugum, and Lindstedt (2012). PCR conditions, detection of amplification products and identification of allelic variants were performed as described by González, Sanso, Lucchesi, and Bustamante (2014). Alleles were named according to the number of TR, and null alleles were named −2. The diversity index (D_N), based on Nei's marker diversity, was calculated for each locus as described by Noller, McEllistrem, Pacheco, Boxrud, and Harrison (2003). The Simpson diversity index (D_S) for the method was determined as described by Hunter and Gaston (1988).

3 | RESULTS

3.1 | Virulence-associated genes and their subtypes

Most isolates (91%) harboured stx_{2a} subtype, either as the unique *stx* subtype (44%) or in the combinations stx_{2a} stx_{2c} (38%), stx_{2a} stx_{2c} stx_{2d} (6%) and stx_{2a} stx_{1a} (3%). Three isolates (9%) harboured only the stx_{2d} subtype (Figure 1).

All tested isolates harboured *espP*, *fimCD*, *ehaA*, *iha*, *hcpA*, *elfA*, *lpfO113* and *ecpA*, whereas *agn43* (a gene encoding for an autotransporter protein responsible for biofilm formation) was found in all except isolate VO17-2-1. On the other hand, genes *eae*, *katP*, *stcE*, *aida-I*, *sfpA*, Z_{4326}, Z_{4332}, Z_{4333} were not found in any isolate. Z_{4321} (a gene encoding putative PagC-like membrane protein) was only absent in three isolates. The allele ß of *espP* was identified in all but one isolates (VO17-2-1), in which the allele could not be determined.

All isolates positive for *subA* exhibited allele *subA1* with the exception of two isolates (VO17-2-1 and VO89-2-2) in which the allele could not be determined. The only three strains negative for *subA* were also negative for other plasmid-encoded genes such as *ehxA*, *saa* and *epeA* (but not for *espP*).

Regarding *saa* gene, five variants which differed in the length of their repetitive region were determined. Three variants (designed 1, 2 and 4) have been previously described, but two are novel ones, one higher than variant 5 (*saa6*) and one shorter than variant 1 (*saa*0). The most prevalent *saa* allele among the 31 *saa*-positive isolates was variant 4, found in 17 isolates (55%), followed by variant 2 (26%) and variant 1 (13%). The novel variants were detected in two cattle isolates from farm A (Figure 1). Furthermore, one amplimer of each variant was sequenced and confirmed that size differences were due to a different number of copies of the direct 111-bp repeat sequence. Particularly, the novel variant named 6 showed seven complete repeat units plus a partial one, and the other, named 0, showed one complete repeat unit plus a partial one.

The toxin-encoding gene *cdt-V* was detected in over 61% of cattle strains and in only one food strain.

3.2 | MLVA typing

The isolates could be principally discriminated by alleles of loci CVN014 (D_N = 0.87) and in lesser degree CVN016 (D_N = 0.22). Loci CVN001, CVN002, CVN004, CVN007 and CVN015 had very low diversity indices. On the other hand, loci CVN003 and CVN0017 showed a null allele in all the isolates.

Multiple-locus variable-number tandem repeat analysis typing divided the total number of strains into 17 distinct genotypes (named A–Q, Table 1; Figure 2), 11 of them being unique. The Simpson diversity index, calculated for the combined typing set, showed a value of D_S = 0.93. Nevertheless, most of the MLVA profiles were highly related, differing in only one locus.

Some isolates with a spatial relationship and identical virulence profile showed the same MLVA type (VO16-5-1 and TG11-1-1 from dairy farm B, summer and spring samplings, respectively; VO19-1-1, VO22-3-1, VO36-3-2 and VO58-6-2 from dairy farm D, winter, spring and summer samplings; TR14-1-2 and TR36-2-3 from dairy farm E, summer sampling; 184-2-2 and 170-4-2 isolated from the same butchery for two consecutive days) (Table 1; Figure 2).

4 | DISCUSSION

STEC O113:H21 conforms to the features of seropathotype C that includes O91:H21 and O174:H21 (best known serotypes of clonal group STEC 1), but serotypes O8:H19, O22:H8, O91:H10, O105:H18 and O174:H2, which were also associated with HUS, have been included in this seropathotype (Girardeau et al., 2005; Karmali et al., 2003).

However, the classification of pathotypes is limited by the presence of diverse virulence factors, which are acquired by horizontal transfer, being able to convert an *E. coli* strain into a more virulent strain (Coombes, Gilmour, & Goodman, 2011).

Although *stx* is the main virulence factor, O113:H21 strains isolated in this study harboured genes encoding other toxins, adhesins and additional virulence factors such as *espP*, *fimCD*, *ehaA*, *iha*, *hcpA*, *elfA*, *lpfO113* and *ecpA*. Feng et al. (2014) also detected *ehaA* and *lpfO113* in all the O113:H21 isolates, and *esp*P, *iha*, *ehx*A, *saa*, *sub*A and *epeA* in a frequency higher than 70%.

The *stx* subtype more prevalent was stx_{2a} alone or associated with stx_{2c} or, less frequent, with stx_{2d}, all of which are subtypes associated with human disease. These results are in agreement with those of Feng et al. (2014) who reported that O113:H21 strains

TABLE 1 Epidemiological relationships of O113:H21 STEC isolates studied

Source	Origin	Year	Season	Strain name	MLVA profile[a]
Cattle	Dairy farm A	2006	Autumn	VO1-1-3[b]	D
				VO31-2-2[b]	E
				VO21-1-1[b]	B
			Spring	VO3-3-3[b]	O
				VO16-2-2[b]	H
			Summer	VO33-1-1[b]	I
				VO50-3-2[b]	A
	Dairy farm B		Spring	VO32-1-1[b]	E
				VO6-5-2[b]	J
			Summer	VO16-5-1[b]	B
			Spring	TG11-1-1[c]	B
	Dairy farm C		Summer	VO34-1-2[b]	D
				VO19-2-4[b]	A
				VO10-2-3[b]	B
		2008		TR30-2-4[c]	G
	Dairy farm D	2006		VO36-3-2[b]	C
			Spring	VO17-2-1[b]	P
			Summer	VO89-2-2[b]	L
			Winter	VO19-1-1[b]	C
			Spring	VO22-3-1[b]	C
			Summer	VO58-6-2[b]	C
		2008		TR1-3-2[c]	B
	Dairy farm E	2006		VO72-1-1[b]	K
		2008		TR14-1-2[c]	E
				TR36-2-3[c]	E
	Feedlot 1	2001	N/D	FO165[f]	C
	Feedlot 2			FC103[f]	D
	Grazing Farm	1995		AP97-3[f]	E
Food	Chicken	2007	Winter	184-2-2[d]	F
				183-1-1[e]	M
				170-4-2[e]	F
	Bovine	2003	N/D	5M[g]	Q
		1995		BE2-3[f]	N
				AP97-3[f]	E
		1997		HT7-14[f]	E

N/D, not determined.
[a]Denomination according to Figure 2 data.
[b]Fernández et al. (2010).
[c]Fernández, Sanz, Parma, & Padola (2012).
[d]Alonso, Krüger, Sanz, Padola, & Lucchesi (2016).
[e]Alonso (2012).
[f]Blanco et al., 2004.
[g]Sanz, Villalobo, Elichiribehety, & Arroyo (2007).

isolated from Argentinian patients carried stx_{2a} alone (2) or the combination stx_{2a} stx_{2c} (1), while strains isolated from cattle and foods harboured mainly stx_{2a} alone. The same PCR protocol (described by Scheutz et al., 2012) was used in that study.

Plasmid markers, such as *saa*, *ehxA* and *subA*, were found in a higher proportion than those reported by dos Santos et al. (2010) in Brazilian O113:H21 strains from bovine cattle and meat. However, the presence of genes such as *espP* and *epeA* suggests the presence

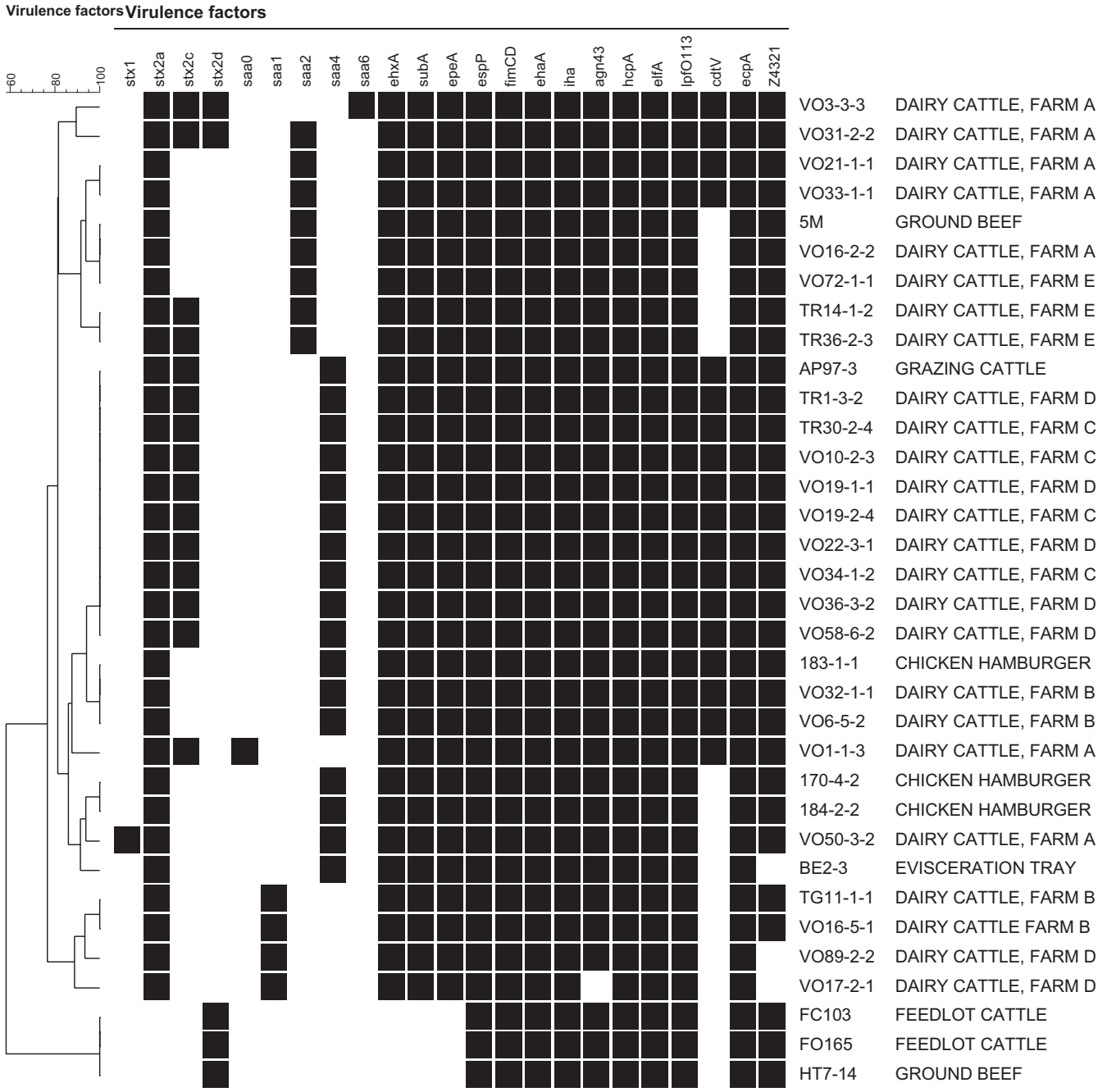
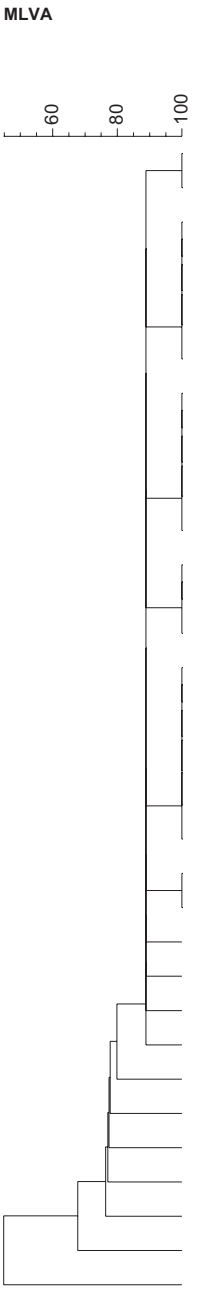


FIGURE 1 Cluster analysis of virulence-associated genes in O113:H21 isolates from cattle and food. The dendrogram was generated using the BioNumerics v.6.6 software. The presence (black) or absence (blank) of genes, the name and the origin of the strains are shown. Genes not found in any of the studied strains: *eae*, *katP*, *stcE*, *aida-I*, *sfpA*, Z_{4326}, Z_{4332}, Z_{4333}

of similar megaplasmids in O113:H21 isolates from both countries. In our study, two strains isolated from cattle from different feedlots and one from ground beef were negative for almost all plasmid genes tested (*saa*, *ehxA*, *subA*, *epeA*, *katP*) but not for *espP*, suggesting the loss of a virulence plasmid like pO113 and the fixation of this virulence profile in different clones, as showed by MLVA profiles.

Several variants of *saa* detected among O113:H21 isolates and two novel variants described in this study highlighted that *saa* subtyping can be useful for discriminating STEC O113:H21 strains.

Subtilase cytotoxin is encoded by *subA* and *subB* genes, which are organized in an operon structure and localized either on plasmid pO113 (*sub*AB1), on a pathogenicity island (SE-PAI) (*sub*AB2-1) or on another chromosomal locus termed OEP-locus (*sub*AB2-2) (Funk, Stoeber, Hauser, & Schmidt, 2013; Paton et al., 2004; Tozzoli et al., 2010). Bosilevac and Koohmaraie (2011) characterized STEC isolates from ground beef and detected *subA* in 97% of STEC O113:H21 isolates, similar to this study where *subA* gene was detected in more than 90% of the isolates. Hauser et al.



MLVA										
CVN001	CVN002	CVN003	CVN004	CVN007	CVN014	CVN015	CVN016	CVN017		
7	1	–2	12	6	6	5	2	–2	VO19-2-4	A
7	1	–2	12	6	6	5	2	–2	VO50-3-2	A
7	1	–2	12	6	5	5	2	–2	TG11-1-1	B
7	1	–2	12	6	5	5	2	–2	TR1-3-2	B
7	1	–2	12	6	5	5	2	–2	VO10-2-3	B
7	1	–2	12	6	5	5	2	–2	VO16-5-1	B
7	1	–2	12	6	5	5	2	–2	VO21-1-1	B
7	1	–2	12	6	2	5	2	–2	FO165	C
7	1	–2	12	6	2	5	2	–2	VO19-1-1	C
7	1	–2	12	6	2	5	2	–2	VO22-3-1	C
7	1	–2	12	6	2	5	2	–2	VO36-3-2	C
7	1	–2	12	6	2	5	2	–2	VO58-6-2	C
7	1	–2	12	6	7	5	2	–2	FC103	D
7	1	–2	12	6	7	5	2	–2	VO1-1-3	D
7	1	–2	12	6	7	5	2	–2	VO34-1-2	D
7	1	–2	12	6	10	5	2	–2	AP97-3	E
7	1	–2	12	6	10	5	2	–2	HT7-14	E
7	1	–2	12	6	10	5	2	–2	TR14-1-2	E
7	1	–2	12	6	10	5	2	–2	TR36-2-3	E
7	1	–2	12	6	10	5	2	–2	VO31-2-2	E
7	1	–2	12	6	10	5	2	–2	VO32-1-1	E
7	1	–2	12	6	11	5	2	–2	170-4-2	F
7	1	–2	12	6	11	5	2	–2	184-2-2	F
7	1	–2	12	6	3	5	2	–2	TR30-2-4	G
7	1	–2	12	6	1	5	2	–2	VO16-2-2	H
7	1	–2	12	6	15	5	2	–2	VO33-1-1	I
7	1	–2	12	6	9	5	2	–2	VO6-5-2	J
6	1	–2	12	6	2	5	2	–2	VO72-1-1	K
7	1	–2	12	6	1	5	7	–2	VO89-2-2	L
7	1	–2	12	2	9	5	2	–2	183-1-1	M
7	1	–2	12	6	8	5	4	–2	BE2-3	N
7	1	–2	-2	6	13	5	2	–2	VO3-3-3	O
7	7	–2	12	6	2	5	30	–2	VO17-2-1	P
7	1	–2	9	–2	6	–2	4	–2	5M	Q

FIGURE 2 Multiple-locus variable-number tandem repeat analysis-based clustering of STEC O113:H21 isolates investigated in this study. Similarities among MLVA profiles were calculated using categorical coefficients and UPGMA clustering method (BioNumerics v.6.6 software). Last column indicates profile denomination

(2016) demonstrated an important cytotoxic effect on Vero cells for $SubAB_1$, which is the subtilase variant with the highest cytotoxicity (Funk et al., 2015) and also the one that predominated in this study.

The cytolethal distending toxin V (CDT-V) is a phage-encoded toxin that causes G2/M cell cycle arrest leading to distension, inhibition of proliferation and death of human endothelial cells (Bielaszewska, Sinha, Kuczius, & Karch, 2005). This toxin was detected in a higher prevalence (61% in cattle isolates) than previous studies such as Feng et al. (2014), who found that 37% of the O113:H21 strains had *cdt-V* in samples from several sources/countries, and dos Santos et al. (2010), who detected this gene in 23% of strains from cattle in Brazil.

Regarding the putative virulence genes Z_{4321}, Z_{4326}, Z_{4332} and Z_{4333} (which are located in OI-122 in the STEC O157:H7 strain EDL933), the studied O113:H21 strains only harboured Z4321 (pagC-like), coincidently with results from Karmali et al. (2003). In addition, Shen, Mascarenhas, Rahn, Kaper, and Karmal (2004) have shown that in other O113:H21 strain (CL3), Z4321 is not part of an OI-122-like structure, but rather a part of a unique hybrid genomic island that contains segments of two EDL933 genomic O islands (OI-122 and OI-48) and other elements that show homology to putative virulence genes from *Yersinia pestis*. Recently, *pagC* together with other virulence genes such as *hes*, *iha*, *lesP* and *agn43* has been proposed as part of the novel pathogenicity island LAA (Montero et al., 2017). The presence of LAA in the studied

isolates could not be related with any virulence factor combination (data not shown).

The O113:H21 STEC strains could be differentiated mainly by the combination of *stx* and *saa* subtypes present, plus the presence/absence of *cdt*-V, all of which are genes located on mobile genetic elements such as plasmids or phages. In addition, few other strains could be discriminated by the absence of other genes (Z_{4321}; *agn43*; or the combination *saa*, *ehxA*, *subA* and *epeA*).

Multiple-locus variable-number tandem repeat analysis is highly discriminative and appears to perform as well or better than PFGE (pulsed-field gel electrophoresis) for subtyping. Unlike PFGE, the targets of MLVA are specific sequences that can be amplified by PCR using pathogen-specific primers. Thus, MLVA is easier to interpret than PFGE, because the fragments generated in MLVA are of known size and sequence (Bustamante & Sanso, 2017). In this study, the MLVA detected a limited diversity among the isolates considering that several loci were not discriminative. As the available MLVA assay for STEC no-O157:H7 is generic, it can be less discriminative for some serotypes, and for this reason, we conclude that new VNTRs have to be identified for O113:H21 typing. We suggest that CVN003, CVN017 and CVN015 should be replaced by new more variable VNTR markers. However, MLVA in combination with virulence subtype profile could be useful for epidemiological tracing. Dairy farm A was the one which had higher genetic diversity of O113:H21 isolates circulating in different seasons of the year, while in dairy farm D, one of the clones seemed to remain circulating throughout the year. Noticeably, two O113:H21 strains isolated from chicken hamburgers, which were bought for two consecutive days in the same butchery, shared an identical profile, suggesting that this strain has remained in this retail shop.

The ability to produce different toxins and the presence of other virulence genes such as *saa*, *ehx*A and *iha* in some LEE-negative STEC has been highlighted by Hauser et al. (2016). In addition, the presence of stx_{2a}, stx_{2d} and $lpfA_{O113}$ might also enhance the pathogenicity of STEC strains, increasing their risk for humans (Miko et al., 2014). In this regard, we have shown in the present study that the O113:H21 strains characterized harbour several toxin and adhesion genes. The most common virulence gene profile found in the present study, considering also the stx_2 subtypes present, was shared with the two isolates from HUS–Argentinian patients characterized by Feng et al. (2014). Furthermore, all of the strains carried Stx subtypes associated with human disease, coinciding with those authors who suggested that bovine and food strains may also be a health concern.

In conclusion, the results of this study show that a number of O113:H21 strains that occur in foods and bovines could be pathogenic for humans, based on their virulence profiles. This situation calls for further attention in the prevention and control of foodborne disease caused by O113:H21 strains. Also, they show that subtyping of *stx* and *saa* is useful to discriminate O113:H21 strains that share virulence genes.

ACKNOWLEDGEMENTS

We thank M. R. Ortiz for her technical assistance. This study was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Fondo para la Investigación Científica y Tecnológica (FONCYT) and SECAT-UNICEN.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ORCID

A. M. Sanso http://orcid.org/0000-0001-7144-3907

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Sanso AM, Bustamante AV, Krüger A, et al. Molecular epidemiology of Shiga toxin-producing O113:H21 isolates from cattle and meat. *Zoonoses Public Health*. 2018;00:1–9. https://doi.org/10.1111/zph.12467