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PII: S0882-4010(16)30430-2

DOI: [10.1016/j.micpath.2016.11.022](https://doi.org/10.1016/j.micpath.2016.11.022)

Reference: YMPAT 2013

To appear in: *Microbial Pathogenesis*

Received Date: 2 August 2016

Revised Date: 28 October 2016

Accepted Date: 29 November 2016

Please cite this article as: González J, Sanso AM, Cadona JS, Bustamante AV, Virulence traits and different *nle* profiles in cattle and human verotoxin-producing *Escherichia coli* O157:H7 strains from Argentina, *Microbial Pathogenesis* (2016), doi: 10.1016/j.micpath.2016.11.022.

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Virulence traits and different *nle* profiles in cattle and human verotoxin-producing *Escherichia coli* O157:H7 strains from Argentina

Running Title: VTEC O157:H7 from Argentina

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Keywords:

Verotoxin- producing *Escherichia coli*, O157:H7, subtyping, virulence factors

Abstract

Verotoxin-producing *E. coli* (VTEC) O157:H7 is the dominant serotype isolated from patients with HUS and, Argentina has the highest rate of HUS in the world. Molecular typing had allowed to identify subpopulations related to the origin and virulence of O157:H7 strains. Our aim was to perform a genetic characterization of 43 O157:H7 strains isolated in Argentina mostly from cattle and humans in order to establish the potential public health risk. For it, we used a combination of molecular subtyping methods in order to identify clade 8_1 (C3468G), LSPA-6 and virulence profiles and, a cytotoxicity assay on Vero cell. All isolates carried the clade 8 SNP variant and 98% of them belonged to lineage I/II (2% lineage II). Isolates were grouped into eleven *nle* profiles, 46% were positive for all *nle* genes, while the remaining isolates, except two, showed incomplete OI-71, particularly lacked *nleF*. All isolates showed the plasmid profile *ehxA-espP-katP-stcE* and harbored *ehaA*, *elfA*, *iha* and *lpfA* variants *lpfA1-3* and *lpfA2-2* and, ECSP_0242. The frequencies of the remaining ECSP genes were 95% ECSP_2687, 88% ECSP_3286, 86% ECSP_3620, 53% ECSP_2870/2872 and 44% ECSP_1733. All O157:H7 strains, except the isolate identified as lineage II, were cytotoxic on Vero cells. Among Argentinean strains, most genetic markers occur at equal relative frequencies among clinical and bovine isolates, showing diversity mostly in *nle* genes profiles. The belonging of the isolates to hypervirulent clade 8 and lineage I/II, the high prevalence of *nle* and putative virulence factors genes, would allow assigning most O157:H7 strains of this region a high risk to public health.

Introduction

Verotoxin-producing *E. coli* (VTEC) O157:H7 is a zoonotic pathogen associated with food and waterborne illnesses around the world. It has been implicated in large outbreaks as well as in sporadic cases of haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) [20] and is the dominant serotype isolated from patients with HUS in Argentina [44]. In this country, approximately 500 HUS cases are annually reported, with an incidence that has ranged between 7.8 and 17 cases per 100.000 children less than 5 years of age [44]. This syndrome is the most common cause of acute renal failure in children and the second highest cause of chronic renal failure [7,13].

Ruminants, especially cattle, are the main reservoirs of VTEC and Argentina has a high prevalence of VTEC in cattle, as well as in derived food [12, 31, 37, 38].

Outbreaks of VTEC O157:H7 vary dramatically in the severity of the illness and the frequency of the most severe complication, HUS [30, 40, 43]. The factors that contribute to the variation in disease severity are poorly understood [30]. Distinguishing VTEC O157:H7 isolates which constitute a high risk to human health from isolates with a lower association with clinical symptoms is an important aspect of risk-based monitoring and surveillance [14].

Phylogenetic studies have determined that O157:H7 strains integrated subpopulations of global dispersion, which differ in relation to their association with diseases in humans, possessing different types and levels of expression of virulence factors [21, 22, 61].

Manning *et al.* [30] developed a system that detects 32 single nucleotide polymorphisms (SNPs) allowing split VTEC O157:H7 strains into 9 distinct clades. The isolates of clade 8 are more virulent than other isolates. The acquirement of new virulence factors and the overexpression of others, would contribute to the increased severity related to this clade [1, 30, 36]. In order to identify strains belonging to clade 8, Franz *et al.* [14] developed a restriction digestion assay which targets one SNPs in the *rhsA* gene (SNP position 3468) [29].

On the other hand, lineage-specific polymorphic assay (LSPA-6) is a PCR-based high throughput fingerprinting method that can characterize isolates based on polymorphisms in six genetic markers [61]. By LSPA6, VTEC O157:H7 population can be divided into two primary phylogenetic lineages, termed lineage I and lineage II, and have been suggested there could be differences in their propensity to cause disease in

humans or to be transmitted to humans [21, 22]. Later, a third lineage designated lineage I/II was described [64]. The lineage I strains are most frequently identified in cattle and humans samples, the lineage II comprised predominantly bovine and nonpathogenic human-derived isolates [21], and the intermediate lineage I/II strains are less well characterized with respect to phenotype and host distribution [17, 25, 64].

Epidemiological evidence suggests that the potential of *E. coli* O157:H7 to cause HUS is due to the presence of either the Shiga toxin (encoded by *stx* gene) or Shiga toxin and intimin (encoded by *eae* gene). However, Kulasekara *et al.* [24] identified seven putative virulence determinants in the genome of strain responsible of the spinach-associated outbreak in the U.S (TW14359). These coding sequences include two putative type III secretion system effector proteins, candidate genes that could result in increased pathogenicity or, alternatively, adaptation to plants, and an anaerobic nitric oxide reductase gene.

Other important virulence properties are carried by mobile genetic elements such as pathogenicity islands (PAIs) and plasmids. The most studied PAI, “locus of enterocyte effacement” (LEE), governs the capability of VTEC to colonize the intestinal mucosa of the host [32, 33]. However, other PAIs, termed O islands, contain non-LEE effector (*nle*) genes that encode translocated substrates of type III secretion system [4, 8, 23]. Some of these effectors contribute to the colonization and persistence of VTEC in cattle and interfere with the human inflammatory response [9, 56].

On the other hand, there are virulence factors encoded in plasmids, as a protein with hemolytic activity, EhxA [48], a catalase-peroxidase (KatP), serine proteases (EpeA and EspP), a zinc metalloprotease (StcE), among others [5, 6, 26, 28, 39]. Several other virulence determinants, adherence and colonization factors, have been described in O157 strains. Among them, Lpf (long polar fimbriae), which constitutes an important colonization factor and the expression of *lpf* operons 1 and 2 has been associated with increased adherence to cells [53, 54, 55]; EhaA, an autotransporter protein (AT) involved in adhesion and biofilm formation [57]; the *E. coli* laminin-binding fimbriae (ELF) which mediates adherence to human intestinal epithelial cells [46] and Iha (*Vibrio cholerae* IrgA homologue adhesin) [50].

Previous data revealed that in Argentina there was a high proportion of hypervirulent strains and recommended to provide data on a more extensive strain collection [35, 15, 16, 2, 42]. In the present study, we used a combination of molecular subtyping methods in order to identify clade 8_1 *rhsA* (C3468G), LSPA-6 and virulence profiles (including determinants of virulence, non-LEE effector *-nle-* genes, plasmid-encoded

genes and adhesive factors-encoded genes) and, a cytotoxicity assay on Vero cell to phenotypically and genetically characterize and compare cattle and human VTEC O157:H7 strains isolated in Argentina..

Materials and methods

Bacterial strains

A total of 43 VTEC O157:H7 strains isolated in Argentine Pampa region were analyzed. They had been obtained from cattle (n=19), humans (n=23) and food (n=1) between 2000 and 2014. All the strains have been analyzed previously, detecting by PCR genes coding for verotoxins (*vtx*₁, *vtx*₂), intimin (*eae*) and enterohemolysin (*ehxA*). All isolates have the profile *vtx*₂, *eae*, *ehxA* [10, 11, 37, 45].

Identification of strains belonging to clade 8

The identification of the strains belonging to clade 8 was performed by detecting a SNP, *rhsA* (C3468G), by PCR-RFLP [14]. Reference strains (clade 8 and non-clade 8, Anguil 9.1 and EDL933 respectively) were used to assign clade.

LSPA6 lineage typing

Six genomics *loci* (*fold-sfmA*, Z5935, *yhG*, *rtcB*, *rbsB* and *arp-iclR*) were amplified and used to assign LSPA6 genotypes using a six-digit binary code [17, 61, 64]. Strains with genotype 111111 were classified as lineage I, 211111 as lineage I/II and all deviations from those genotypes as lineage II [63, 64]. To confirm the results, reference strains K12 and EDL933 were used and representative alleles of each lineage were sequenced and analyzed using Chromas 2.32 (Technelysium Pty. Ltd., Tewantin, Australia) and Clustal W [27].

Virulence typing

-Detection of putative virulence determinants:

Genes encoding putative virulence determinants (described in the TW14359 O157 strain associated with the raw spinach outbreak in the U.S. in 2006), ECSP_3620, ECSP_242, ECSP_3286, ECSP_2870/72, ECSP_2687 and ECSP_1773 were detected by PCR [24].

-Detection of non-LEE effector (*nle*) genes

The *nle* genes, *nleB2*, *nleC*, *nleH1-1*, *nleD*, encoded in genomic island O-I 36, *nleG2-3*, *nleG5-2*, *nleG6-2*, O-I 57, *nleA*, *nleF*, *nleG*, *nleG2-1*, *nleG9*, *nleH1-2*, O-I 71, and *ent/espL2*, *nleB*, *nleE*, O-I 122, were amplified according to Coombes *et al.* [8].

- Detection of plasmid-encoded genes

Plasmid-encoded genes, *espP* (extracellular serine protease), *katP* (periplasmic catalase-peroxidase), *stcE* (zinc metalloprotease), and *subA* (subtilase cytotoxin) were amplified by a multiplex PCR [3] and, *epeA* (serine-protease), by a monoplex PCR [28].

- Detection of adhesive factors-encoded genes

Genes *ehaA* (autotransporter protein of enterohaemorrhagic *E. coli* O157:H7), *elfA* (*E. coli* laminin-binding fimbriae), *iha* (IrgA homologue adhesion) and *lpfA* (long polar fimbriae), variants *lpfA1* (*lpfA1-3*) and *lpfA2* (*lpfA2-2*), encoding adhesive factors were amplified according to Wu *et al.* [59], Hernandez *et al.* [18], Szaloi *et al.* [50] and Torres *et al.* [52], respectively.

Cytotoxicity assay

The cytotoxicity of the isolates was evaluated by an assay on Vero cell monolayers [12]. The supernatants of each O157:H7 isolate were assayed in two different dilutions (1/10 and 1/5) and each one by duplicated. EDL933 was used as positive control strain. Wells having partial or total cell destruction were considered positive.

Data analysis

The statistical analysis of the frequencies of genetic markers between strains from different sources were made using the software Epi InfoTM 7.1.5.2 by 2x2 contingency tables, chi-square test (χ^2) and Fisher exact test, with confidence level of 95%.

The clustering analysis and the Minimum Spanning Tree (MST) were performed using the software BioNumerics vs. 6.6 (Applied-Maths).

Results

All VTEC O157:H7 strains presented the characteristic PCR-RFLP banding pattern associated to clade 8.

A total of 42 strains showed the LSPA profile 211111, belonging to the lineage I/II (98%). Only one strain from human (isolate 28) presented the LSPA profile 221211, belonging to the lineage II.

Distribution of virulence-associated genes is showed in Figure 1. All strains carried ECSP_0242 gene, 95% of the strains carried ECSP_2687, 88% carried ECSP_3286, 86% carried ECSP_3620, 53% carried ECSP_2870/2872 and 44% carried ECSP_1733. The distribution of putative virulence genes in strains of different sources is shown in Table 1. ECSP_1773 and ECSP_2870/2872 were overrepresented among cattle strains (63% and 84% respectively vs 30% and 26%, in the same genes in human strains, Table 1). The Odd ratios indicated that these genes (ECSP_1773 and ECSP_2870/2872) were associated with the bovine source strains (OR=3.91, $P<0,05$ and OR=15.11, $P<0,05$ respectively) (Table 1).

Regarding the presence of *nle* genes, strains were grouped into eleven profiles (Figure 2). The Minimum Spanning Tree (MST) showed that the most prevalent *nle* profile was that positive for all *nle* genes (46%). The second prevalent profile showed incomplete OI-71, lacking *nleF* (26%). Each one of the remaining profiles was detected in one or two isolates. The two profiles that grouped most of the isolates were shared by cattle and human isolates (Figure 2). These results are in accordance with the Odd ratios, which indicated no association of the *nle* genes with strain sources (Table 1).

All VTEC O157:H7 showed the plasmid profile *ehxA-espP-katP-stcE* and were positive for the studied adhesine encoding genes (*ehaA*, *elfA*, *iha* and *lpfA* variants *lpfA1* and *lpfA2*).

In relation to the cytotoxicity assay, the supernatants of O157:H7 strains, except the one belonging to isolate identified as lineage II (isolate 28), demonstrated cytotoxic effect after 48h post inoculation on Vero cells. Thirty eight isolates (88%) showed total cell destruction (100% of cytotoxicity) and four isolates (10%), partial cell destruction [80% of cytotoxicity (isolates 36 and 37), or 20% of cytotoxicity (isolates 6 and 35)] (Figure 3). No significant association was observed between cytotoxic effect and the origin of the strains (OR=0.84, $p>0.05$).

Discussion

Knowledge about population structure and the identification of virulence characteristics present in VTEC O157:H7 strains that are circulating in the region are useful for the quick identification and for taking preventive public health measures. In this study, we performed a genetic characterization of VTEC O157:H7 strains mostly isolated from cattle and humans in Argentine Pampa region during 14 years in our laboratory.

All O157:H7 strains (100%) were classified as clade 8. Previous studies also detected dominance of clade 8 among O157:H7 Argentinean isolates. Mellor *et al.* [35] found prevalence of 80% and 50%, in human and cattle isolates respectively; Pianciola *et al.* [41] detected 92% among human isolates from Neuquén Province, Pianciola *et al.* [42], 88% and 59%, among clinical and bovine isolated from different provinces and, Amigo *et al.* [2], who analyzed two human isolates as well as eight cattle isolates from four locations sited in Buenos Aires, La Pampa and Santa Fe provinces, 100% and 50% respectively. Although our result is the higher percentage reported in Argentina, it must stated that studied strains were not evaluated by the set of 96 SNP loci proposed by Manning *et al.* [30], and further studies are now in progress.

In relation with the LSPA6, the most frequent profile was 211111, characteristic of lineage I/II. These results are consistent with those found by other authors [17, 25, 29, 42], who detected that all O157:H7 strains were LSPA6 211111 (lineage I/II) and classified as clade 8. In the present study, only one strain belonged to the lineage II, very frequently detected in The Netherland [14], but detected in this study by the first time in Argentina.

Clade 8 VTEC O157:H7 isolates were suggested to be more virulent than isolates from other clades [30]. Our results show the almost exclusive presence of VTEC O157:H7 strains belonging to the hypervirulent clade 8 and lineage I/II, confirming on the circulation of this highly pathogenic lineage in Argentina.

In contrast with our results and with the ones previously cited from Argentina, Franz *et al.* [14], Yokoyama *et al.* [62] and Söderlund *et al.* [49] found a high genetic diversity (lineage/clade typing) among *E. coli* O157:H7 isolated in The Netherland (from cattle, humans and food), Japan (from humans) and Swedish (from humans and cattle), respectively.

The high prevalence of putative virulence genes, particularly ECSP_0242 (encoding a putative virulence factor common in eukaryotes that facilitates protein-protein interactions) and ECSP_2687 (encoding a protein

involved in the decline of the host immune response), coincides with previous data collected from O157:H7 strains isolated in Argentina [41]. In the present study, ECSP_1773 (encoding a protein that reduces the expression of cytokines) and ECSP_2870/2872 (gene of regulating adaptation to plant host cells) were significantly associated with cattle strains (Table 1). In relation with this last putative gene, Pianciola *et al.* [42] did not find a significant association between it and some source.

Regarding the *nle* genes, we found a high prevalence of them in the studied O157:H7 strains. These genes are genetic determinants of a variety of functions that enable the pathogen to colonize and persist in cattle and cause disease in humans. The effector proteins coded by *nle* genes are injected into the host cell cytoplasm through a Type III secretion system (T3SSs). These are complex multiprotein assemblages common to many plant and animal pathogens or symbionts that allow bacteria to subvert eukaryotic cell biology [51]. In our study the OI-122 was the most prevalent complete pathogenicity island, carrying genes with a demonstrable role in virulence, like *nleB*, *nleE*, and *ent/espL2*, as well *nleA* at OI-57 [59]. In the case of *nleB*, this gene encodes an important virulence determinant with a central role in the disease of attaching and effacing pathogens. Wickham *et al.* [58] reported that *nleB* decreases the infectious dose required for colonization and disease in the *C. rodentium* mouse model. On the other hand, Coombes *et al.* [8] could associate *nleF* and *nleH1-2*, which was found in this study with a high prevalence (98%), with epidemic potential and severe human disease.

The plasmid profile *ehxA- espP- katP- stcE* was the only one detected. The plasmids from O157:H7 have been reported to be highly similar [19]. In previous studies, where strains from different serotypes were included, the cited profile was exclusively found among O157:H7 strains, being *stcE* only present in this serotype [47, 60]. Genes responsible for bacterial adhesions to intestine, *ehaA*, *elfA*, *iha* and *lpfA* variants *lpfA1-3* and *lpfA2-2*, were detected in all isolates.

As a correlate of virulence, we evaluated the cytotoxicity in bacterial culture supernatant. In general, there was agreement between the levels of verocytotoxic activity and the Stx production. We found that all isolates, except the one belonged to lineage II, showed cytotoxic effect.

According to our results, but without an analysis of complete genomes, O157:H7 strains circulating in this region of Argentina are a homogeneous group in relation to clades and lineage assignment, but nevertheless showing diversity in *nle* genes profiles.

Among Argentinean strains, most genetic markers occur at equal relative frequencies among clinical and bovine isolates (with the exception of ECSP_1773 and ECSP_2870/2872, two putative virulence genes). The emergence of predominant and sometimes exclusive genotypes in several countries suggests that isolates may have responded to regional selective pressure to form divergent evolutionary populations, though it is equally possible that geographic divergence may be a product of genetic drift or founder effects [34]. The belonging of the Argentinean isolates to hypervirulent clade 8 and lineage I/II, the high prevalence of *nle* and putative virulence factors genes and their exhibited cytotoxic effects, would allow assigning most O157:H7 strains of this region, and in agreement from previous studies, a high risk to public health and explain at least partly, why Argentina is the country with the highest incidence of HUS in the world.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP939/12) and Fondo para la Investigación Científica y Tecnológica (FONCYT, PICT 590/10). We thank Dr. Mariana Rivero for kindly provide us information on human strains, Dr. Angel Cataldi for reference strains, and M. Rosa Ortiz for technical assistance.

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Table 1 Distribution of putative virulence determinants and *nle* genes among VTEC O157:H7 strains isolated in Argentina, and association analysis between virulence gene presence and source (cattle vs human).

Virulence gene		No. (%) of positive-strains				Association analysis between virulence gene presence and source	
		Total	Cattle (n=19)	Human (n=23)	Food (n=1)	Cattle	
						OR ^a (95% CI)	P
Putative virulence determinants	ECSP-0242	43 (100)	19 (100)	23 (100)	1 (100)	-	-
	ECSP-1773	19 (44)	12 (63)	7 (30)	-	3,91	<0,05
	ECSP-2687	41 (95)	19 (100)	21 (91)	1 (100)	Undefined	>0,05
	ECSP-2870/2872	23 (53)	16 (84)	6 (26)	1 (100)	15,11	<0,05
	ECSP-3286	38 (88)	19 (100)	18 (78)	1 (100)	Undefined	<0,05
	ECSP-3620	37 (86)	18 (95)	18 (78)	1 (100)	Undefined	>0,05
O-I 36	<i>nleB2</i>	43 (100)	19 (100)	23 (100)	1 (100)	-	-
	<i>nleC</i>	43 (100)	19 (100)	23 (100)	1 (100)	-	-
	<i>nleH1-1</i>	41 (95)	17 (89)	23 (100)	1 (100)	0	>0,05
	<i>nleD</i>	41 (95)	17 (89)	23 (100)	1 (100)	0	>0,05
O-I 57	<i>nleG2-3</i>	40 (93)	16 (84)	23 (100)	1 (100)	0	>0,05
	<i>nleG5-2</i>	42 (98)	18 (95)	23 (100)	1 (100)	0	>0,05
	<i>nleG6-2</i>	40 (93)	17 (89)	22 (96)	1 (100)	0,3863	>0,05
O-I 71	<i>nleA</i>	39 (91)	17 (89)	21 (91)	1 (100)	0,8095	>0,05
	<i>nleF</i>	20 (46)	9 (47)	10 (43)	1 (100)	1,17	>0,05
	<i>nleG</i>	39 (91)	18 (95)	20 (87)	1 (100)	2,7	>0,05
	<i>nleG2-1</i>	39 (91)	15 (79)	23 (100)	1 (100)	0	<0,05
	<i>nleG9</i>	41 (95)	17 (89)	23 (100)	1 (100)	0	>0,05
	<i>nleH1-2</i>	42 (98)	18 (95)	23 (100)	1 (100)	0	>0,05
O-I 122	<i>ent/espL2</i>	43 (100)	19 (100)	23 (100)	1 (100)	-	-
	<i>nleB</i>	41 (95)	17 (89)	23 (100)	1 (100)	0	>0,05
	<i>nleE</i>	41 (95)	17 (89)	23 (100)	1 (100)	0	>0,05

^aOdds ratios: indicate whether a gene or allelic variant is more (>1), less (<1) or is not (0) associated with the bovine source compared to the human strains.

Fig. 1. Cluster analysis of virulence-associated genes in O157:H7 isolates from Argentina. The presence of genes is indicated in black and absence, in white. Genes *epeA* and *subA* were not detected in any of the studied strains. On the right, isolate number, isolation period and source are indicated.

Fig 2. Minimum spanning tree of the 43 Verotoxigenic *Escherichia coli* (VTEC) O157:H7 isolates based on 17 *nle* genes (*nleB2*, *nleC*, *nleH1-1*, *nleD*, encoded in genomic island O-I 36, *nleG2-3*, *nleG5-2*, *nleG6-2*, O-I 57, *nleA*, *nleF*, *nleG*, *nleG2-1*, *nleG9*, *nleH1-2*, O-I 71, and *ent/espL2*, *nleB*, *nleE*, O-I 122). The strains were grouped into eleven profiles. Seven profiles were unique. Each circle represents a *nle* profile; sizes of each circle corresponds to the number of isolates and the partitions into each circle represent the number of isolates for each profile (White=human, Black=cattle, Blue=food). The *nle* profile connected by a solid line differ from one another by 1 *nle* gene, whereas the *nle* profile connected by a thin solid line differ by 2 *nle* gene. The *nle* profile that differ from each other by 3 or more *nle* gene are connected by a thinnest lines

Fig. 3. Cytotoxic effect of VTEC O157:H7 strains isolated from human and cattle in Argentina.

Most genetic markers occur at equal relative frequencies among clinical and bovine VTEC O157:H7 Argentinean isolates

nle- profiling discriminated 43 O157:H7 isolates into 11 genotypes.

Our results reaffirm the circulation almost exclusive in Argentina of strains belonging to the hypervirulent clade 8- lineage I/II and, harboring virulence factors in high frequencies.