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High prevalence of clade 8 *Escherichia coli* O157:H7 isolated from retail meat and butcher shop environment

Lucía Galli[1*], Victoria Brusa[1*], Pallavi Singh[2], Angel Adrián Cataldi[3], Shannon Manning[2], Pilar Peral García[1], Gerardo Aníbal Leotta[1]

[*]These authors contributed equally to this article.

Author affiliations:

[1]IGEVET-Instituto de Genética Veterinaria (UNLP-CONICET LA PLATA), Facultad de Ciencias Veterinarias UNLP, 60 y 118 s/n, La Plata, Argentina

[2]Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI, 48824, USA.

[3]Instituto de Biotecnología, CICVyA, Instituto Nacional de Tecnología Agropecuaria, Los Reseros y Nicolás Repetto s/n, Hurlingham, Argentina

e-mail: lgalli@igevet.gob.ar; vbrusa@igevet.gob.ar; pallavis@msu.edu, cataldi.angeladrian@inta.gob.ar, Shannon.Manning@ht.msu.edu, direccion@igevet.gob.ar, galeotta@igevet.gob.ar

Corresponding Author:

Lucía Galli, IGEVET, 60 y 118 s/n, CC:296, CP:1900, La Plata, Argentina. Tel.: +54 (0221) 423 6663 int 422; Fax: +54 (0221) 421 1799; e-mail: lgalli@igevet.gob.ar

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Abstract

Escherichia coli O157:H7 is an enteric pathogen associated with food safety threats and with significant morbidity and mortality worldwide. In Argentina, post-enteric hemolytic uremic syndrome (HUS) is endemic, with more than 70% of cases associated with *E. coli* O157 infection. To date the biological basis behind the severity among *E. coli* O157 infections is unknown. However, single nucleotide polymorphism (SNP) typing has helped to define nine *E. coli* O157:H7 clades, of which clade 8 strains are associated with severe disease cases. The aim of this study was to characterize a collection of 20 STEC O157:H7 strains isolated between 2011 and 2013 from ground beef and different environmental samples from butcher shops of Argentina. All strains harbored the *eae*, *ehx*A, *fli*C_{H7}, *efa*, *iha*, and *tox*B genes, with stx_{2a}/stx_{2c} as the predominant genotype (75%). The *Xba*I-PFGE analysis showed that the *E. coli* O157 strains had high genetic diversity. Nine strains were grouped in four *Xba*I-PFGE clusters, whereas 11 strains showed unique *Xba*I-PFGE patterns. In contrast, the SNP analysis allowed us to separate the strains in two distinct lineages representing clade 8 (70%) and clade 6 (30%). Our results show the molecular characterization of *E. coli* O157 strains isolated from ground beef and environmental samples from Argentinean butcher shops.

Keywords: O157:H7, clade 8, meat, environment, butcher shops, Argentina

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1. Introduction

Escherichia coli O157:H7 is an important food and waterborne pathogen associated with a wide spectrum of human diseases, mainly with severe cases of hemorrhagic colitis and the life threatening hemolytic uremic syndrome (HUS) (Mead and Griffin, 1998). In Argentina post-enteric HUS is endemic and represents the leading cause of acute kidney failure in children and the second leading cause of chronic renal failure, with more than 70% of these cases associated with *E. coli* O157 infection (Pianciola et al., 2014).

Shiga toxin-producing *Escherichia coli* (STEC) O157 strains are genotypically diverse, as evidenced by different methods. Molecular subtyping methods, such as pulsed field gel electrophoresis (PFGE), reveal extensive genomic diversity mainly associated with insertions, deletions or duplications of discrete genome segments containing *Xba*I restriction sites (Zhang et al., 2006). However, this method is not appropriate to perform phylogenetic analysis. In contrast, the single nucleotide polymorphism (SNP) analysis can resolve closely related bacterial genotypes and provide insights into the microevolutionary history of genome divergence, and thus contribute to an epidemiologic assessment of associations between bacterial genotypes and disease (Manning et al., 2008).

Using phylogenetic analysis, Manning et al. (2008) identified 39 SNP genotypes (SG) in a strain collection of 500 *E. coli* O157 isolates. These genotypes differed in 20% of the SNP loci and were grouped into nine different clades. The *stx* profiles varied among strains of different clades and particular clades were associated with clinical symptoms. HUS patients, for example, were significantly more likely to be infected with clade 8 strains, whose frequency increased over a 5-year period in Michigan (Manning et al., 2008). The biological basis accounting for the severity of

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the disease among *E. coli* O157 infections is not known yet. However, the differential expression of some genes may be responsible for this characteristic. In fact, previous studies support the hypothesis that the increased severity related to clade 8 strains may be partly explained by the overexpression of the stx_2 gene, among others (Abu-Ali et al., 2010; Neupane et al., 2011).

Several research groups have previously identified clade 8 strains isolated from humans and cattle (Amigo et al., 2015; Manning et al., 2008; Mellor et al., 2012; Pianciola et al., 2014; Pianciola et. al, 2016), and associated them with a high incidence of human disease. Nevertheless, this is the first time that STEC O157 strains isolated from butcher shops were sought for this potentially hypervirulent clade.

The aim of this study was to characterize a collection of STEC O157:H7 strains isolated between 2011 and 2013 from ground beef and different environmental samples from butcher shops of Argentina.

2. Materials and Methods

2.1. Bacterial strains

A total of 20 STEC O157:H7 strains isolated in previous studies from butcher shops were studied (Brusa et al., 2012; Brusa et al., 2015). The samples were collected between 2010 and 2013 from the city of Berisso, which has 135 km^2 and 83,123 inhabitants. The strains were isolated from meat and environmental samples corresponding to ground beef (N=13), food handler's hands (N=3), meat mincing machines (N=2), knife (N=1) and meat table (N=1). Briefly, 65 g of ground beef and one portion of each environmental sponge sample were incubated in 585 ml and 100 ml modified Trypticase Soy Broth plus novobiocin (Acumedia), respectively, for 20 h at 41.5ºC. Subsequently, a

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specific O157-concentration was made using immunomagnetic separation (Dynal Biotech, Oslo, Norway), then streaked onto SD-39 agar (Acumedia) and sorbitol Mac Conkey agar (SMAC) plus cefixime telurite (Oxoid, Hampshire, UK) and incubated for 20 h at 37°C. After incubation, presumptive colonies were selected and screened for rfb_{O157}, stx_1 and stx_2 genes by multiplex-PCR (Leotta et al., 2005). The isolates were stored in Brain Heart Infusion (BHI, Biokar Diagnostics, Zac De Ther, Allonne, Beauvais) with 30% glycerol at −70°C until use.

2.2. Phenotypic and genotypic characterization of strains

Confirmation of *E. coli* isolates was performed through biochemical tests according to Edwards and Ewing (1972). The *E. coli* O157:H7 biotype was determined by the ability of the strains to ferment raffinose, dulcitol, and rhamnose (Krishnan et al., 1987). Serotyping was conducted with somatic and flagellar antisera produced by Denka Seiken Co., Ltd (Tokyo, Japan), following the manufacturer´s instructions.

DNA was extracted from pure culture cells for genotypic characterization. Briefly, colonies from isolates grown on SMAC were suspended in 150 µL 1% Triton buffer in TE 1X (10 mM Tris: 1 mM ethylenediaminetetraacetic acid, pH 8) and boiled according to Leotta et al. (2005). In all STEC O157 isolates, the stx_1, stx_2, and rfb_{O157} genes were detected by multiplex PCR (Leotta et al., 2005), while the *eae* (intimin), *ehxA* (enterohemolysin), and $fliC_{H7}$ (H7 flagellin) genes were assessed as described by Karch et al. (1993), Schmidt et al. (1995), and Gannon et al. (1997), respectively. Additional virulence factors and putative virulence determinants, including *efa*, *toxB*, *iha*, *saa*, *subAB*, *cdt-V* and *astA,* were tested as previously described by Galli et al. (2010). The *aggR* (plasmid-encoded regulator) and *aaiC* (secreted protein of EAEC) genes were determined using the protocol described by the EU Reference Laboratory for *E. coli* (ISS, 2013).

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2.3. Molecular subtyping of strains

The analysis of stx_1 and stx_2 variants was done according to Scheutz et al. (2012).

PFGE was performed using the one day (24-26 h) PulseNet standardized laboratory protocol for molecular subtyping of *E. coli* O157:H7 (CDC, 2013). Restriction digestion of DNA in agarose plugs was carried out with *Xba*I and *XmaJ*I (*Bln*I) as primary and secondary enzymes, respectively (Thermo Scientific). PFGE images of gels were obtained by MaestroGen slider® imager (Maestrogen Inc., Nevada, USA). TIFF image analysis was carried out with BioNumerics, version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium) using the dice coefficient and the unweight pair group method with arithmetic mean (UPGMA) to generate dendrograms with 1.5% band matching tolerance. Two or more isolates were grouped in a cluster when they showed identical *Xba*I-PFGE pattern (100% similarity).

2.4. SNP typing for clade determination

A set of 70 previously identified SNPs was used to classify strains into SNP genotypes as described previously (Manning et al., 2008) by using the GoldenGate genotyping assay (Illumina; San Diego, CA) (Fan et al., 2006). A subset of 10 strains with known SNP genotypes identified in the original SNP genotyping study was included as control (Manning et al., 2008); three of the most common SNP genotypes representing clade 8 were also included. All 70 SNPs were concatenated in MEGA6 (Tamura et al., 2013) and evolutionary relationships were examined using the Neighbor-Joining algorithm (Saitou and Nei, 1987). Bootstrapping with 1,000 replicates was used to identify clusters, or clades (Felsenstein, 1985) and evolutionary distances were computed using the p-distance method (Nei and kumar, 2000). The evolutionary distances are expressed in the units of the number of base differences per site.

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3. Results

3.1. Characterization of STEC O157 isolates

All STEC O157 isolates were sorbitol and β-glucuronidase negative. Biotype C (rhamnose^{+}/dulcitol^{+}) was predominant and only two strains belonged to biotype D (rhamnose^{+}/dulcitol^{-}).

The genotypic characterization proved that all STEC O157 harbored the *stx*$_2$, *eae*, *ehx*A and *fli*C$_{H7}$ genes, whereas only three strains had *stx*$_1$. Coincidentally, these three strains had been isolated from different sources of the same butcher shop. The prevalent *stx* genotype was *stx*$_{2a}$/*stx*$_{2c}$ (n=15, 75%), followed by *stx*$_{1a}$/*stx*$_{2a}$ (n=3, 15%), and only two strains belonged to the *stx*$_{2a}$ genotype (Table 1).

The expression of somatic and flagellar antigens by slide agglutination was also demonstrated for all isolates, except for one that was non-motile.

Table 1 displays the distribution of additional virulence factors and putative virulence determinants. As expected, the *efa*, *iha* and *tox*B genes were present in all the studied strains, whereas *saa*, *sub*AB, *cdt*-V, *ast*A, *agg*R and *aai*C were absent.

3.2. Subtyping of STEC O157 isolates

The clonal relatedness of STEC O157 strains was established by PFGE of genomic DNA after digestion with *Xba*I and *Bln*I as first and second enzymes, respectively. STEC O157 generated 15 different patterns with at least 75% similarity with *Xba*I-PFGE (Figure 1).

Nine strains were grouped in four *Xba*I-PFGE clusters, whereas 11 strains showed unique *Xba*I-PFGE patterns. Cluster I grouped two strains: one isolated from the hands of meat

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manipulator of the butcher shop (B) 4 -B4- and the other from ground beef of B45. Coincidentally, both retail stores had the same meat supplier and were sampled the same day. Cluster II consisted of the only two biotype D strains isolated from ground beef of B31 and B52. Nevertheless, strains from cluster II were distinguished by *stx* genotyping and *Bln*I-PFGE (data not shown). Cluster III grouped three strains isolated from different sources (ground beef, table and mincing machine) of the same butcher shop. This finding suggests a possible cross contamination, as they were sampled on the same date. Finally, cluster IV included two strains: one isolated from the mincing machine at B44 and the other from the hands of meat manipulator of the B56. Again, both retail stores had the same meat supplier and were sampled with one week of difference.

The strains isolated from a knife and the hands of meat manipulator of the B27 did not cluster together, but they were close to each other, and only differed in one band.

3.3. STEC O157 clades and SNP genotype determination

Among the 20 strains examined by SNP genotyping targeting 70 SNP loci, 14 (70.0%) were classified as clade 8 in the neighbor-joining phylogeny (Figure 2). Within clade 8, 68.7% of the strains (N=11) belonged to the previously defined SG 30, whereas 18.7% (N=3) belonged to SG 31 and clustered together with over 60% bootstrap support. The remaining six strains (30%) clustered into clade 6 with SG 24.

Interestingly, the four strains isolated from the same butcher shop (B4), but from a different source (ground beef, table, mincing machine, hands), grouped into the same cluster III and belonged to clade 6, except for the strain isolated from the hands of meat manipulator. This strain clustered with another strain isolated from ground beef of another butcher shop (B45), and belonged to clade 8.

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Two isolates from B44 did not cluster together and despite the same genetic profile belonged to different clades.

4. Discussion

In Argentina, HUS is endemic and more than 70% of cases are associated with *E. coli* O157:H7 infection (Rivas et al., 2010). Over the past decade, numerous studies have reported associations between lineages or clades and the host source of *E. coli* O157. These studies have progressively become more complex and comprehensive as they aim to identify increasingly meaningful host associations.

Recently published studies analyzing Argentinean O157 strains demonstrated that in our country, clade 8 isolates dominate in both cattle and humans, but are more frequent in human strains ($p < 0.0001$). Additionally to clade 8, clades 4/5 and 3 were present in isolates obtained from humans and bovines, respectively (Pianciola et al., 2016). Another study suggested that the high HUS incidence was associated to the circulation of a highly pathogenic *E. coli* O157 lineage present in the province they studied (Pianciola et al., 2014). Nevertheless, although Pianciola et al. (2016) assessed a broad collection of Argentinean strains, they did not find a significant association between clade 8 and HUS. In their study, the SG frequency of clade 8 strains also differs with the results reported in our study. In the present work, SG 30 (68.7%) dominated over SG 31 (18.7%), whereas in Pianciola et al.'s study (2016) SG 31 was more frequent in both groups (57.6% and 56.3% in clinical and bovine isolates, respectively).

In addition, Amigo et al. (2015) found that some virulence properties such as an enhanced Shiga toxin production, epithelial cell adherence and cell lysis, which are related to type three

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secretion system were associated with clade 8 and clade 6 strains isolated from cattle in Argentina. The clade 6 lineage was also associated with severe forms of the disease (Iyoda et al., 2014).

With this in mind, in order to identify the clades of the strains present in our county, in this study we characterized 20 Argentinean strains isolated from food (ground beef) and environmental samples from butcher shops. This work, however, was circumscribed to a small area of the country.

The *Xba*I-PFGE results showed that the *E. coli* O157 strains studied in this work had high genetic diversity (15 different patterns). This finding is in line with previous reports from the rest of the country (Pianciola et al., 2016) and from other countries (Manning et al., 2008; Kudva et al., 2002). We identified circulating clones isolated from different sources of the same and different butcher shops; which suggests a probable common origin. Indeed, the retail markets where the same circulating clones were isolated shared the same meat supplier. Thus, the source of contamination could be at the slaughter house. In contrast, the SNP analysis separated the strains in two distinct lineages representing clades 8 and 6. A total of 12 (85.7%) clade 8 strains were positive for stx_{2a}/stx_{2c}. This *stx* subtype is the profile associated with clade 8 isolates reported in prior studies (Manning et al., 2008; Pianciola et al., 2016).

PFGE- and SNP-derived genotyping are complementary methods. Indeed, PFGE primarily detects insertion/deletion variation within genomic regions specific to STEC O157 (Kudva et al., 2002), whereas the polymorphism set primarily targets a 4.1 Mb backbone conserved among *E. coli* serogroups. Although the combined *Xba*I and *Bln*I PFGE patterns revealed greater genetic diversity than the polymorphism-derived genotypes identified in this study, the combined patterns are not sufficient to infer genetic relationships in the absence of epidemiological data (Davis et al., 2003). Thus, polymorphism-derived genotyping combined with PFGE could be useful in assessing strain

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diversity and evolutionary relatedness between epidemiologically unrelated strains (Bono et al., 2012).

In The Netherlands, clade 8 isolates were not more prevalent among human clinical isolates compared to bovine isolates; for this reason, they are not suitable markers to identify isolates with increased risk of causing clinical infections (Franz et al., 2012), as previously suggested in other studies (Pianciola et al., 2014; Mellor et al., 2012). Furthermore, in our study, clade 8 strains were the predominant circulating strains in butcher shops from Berisso. This finding suggests that in the geographical area we studied there should be severe cases of human diseases. However, HUS epidemiological data do not reveal the hypothesized statement (Mattarolo, pers. comm.).

Despite great efforts to identify possible virulence factors responsible for greater virulence in humans (Manning et al. 2008; Kulasekara et al. 2009), the factors involved in the greater intrinsic virulence among clade 8 strains and other O157 genotypes are yet unknown. It seems clear that clade 8 (Kulasekara et al., 2009) and clade 6 (Amigo et al., 2015) produce more Stx toxin than other lineages. Additional virulence factors carried by stx2 lambdoid phages of clade 8 strains have been suggested to be responsible for the enhanced virulence (Kulasekara et al., 2009). However, a full mechanism for hypervirulence has not been fully understood yet. Finally, Amigo et al. (2015) found that the behavior of the clade 8 strains they studied was not uniform. This group of isolates may also have its own unique SNPs.

5. Conclusions

In this study we demonstrate the presence of STEC O157:H7 from clade 8 in butcher shops (ground beef and environmental samples) from Berisso, Argentina. We describe the almost

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exclusive circulation of strains belonging to this clade, as previously reported in Argentinean human and bovine strains (Pianciola et al., 2016) and demonstrate the presence of these bacteria in the whole beef chain production. However, as other authors point out (Franz et al. 2012), we consider that it is not suitable to reduce the pathogenic potential of strains only to the clade type. This is mainly because what accounts for the greater virulence among clade 8 strains and other O157 genotypes has not been fully understood yet.

We also found, for the first time, strains from clade 6, which had not been described in Argentina before in this type of samples. This finding is particularly interesting because the sampling in our study was later (2010-2013) than in the Pianciola et al.'s (2016) study (2006-2008). Thus, although the samples were taken in different geographical regions of the country, the continuing evolution of *E. coli* strains could be demonstrated. Mellman et al. (2005) remarked that bacterial evolution is an ongoing process that leads to the emergence of other successful pathogenic clones of *E. coli* in the future.

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7. Competing Interests

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The authors have declared that no competing interests exist.

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Table 1. Source of isolation, sampling date, serotype, clade, Shiga toxin genotypes and other virulence markers of the strains studied.

Strain	Source	Butcher Shop	Sampling date	Serotype	Clade	*stx* genotype	Virulence markers										
							saa	*ehxA*	*eae*	*agg*R	*aai*C	*efa*	*tox*B	*iha*	*sub*AB	*cdt*-V	*ast*A
Argentina 1	GB	52	16/11/2010	O157:H7	8	stx_{2a}	-	+	+	-	-	+	+	+	-	-	-
Argentina 2	GB	94	17/06/2011	O157:H7	8	stx_{2a}/stx_{2c}	-	+	+	-	-	+	+	+	-	-	-
Argentina 4	K	27	14/12/2010	O157:H7	8	stx_{2a}/stx_{2c}	-	+	+	-	-	+	+	+	-	-	-
Argentina 5	MH	27	14/12/2010	O157:H7	8	stx_{2a}/stx_{2c}	-	+	+	-	-	+	+	+	-	-	-
Argentina 6	GB	20	29/12/2010	O157:H7	8	stx_{2a}/stx_{2c}	-	+	+	-	-	+	+	+	-	-	-
Argentina 7	GB	45	20/01/2011	O157:H7	8	stx_{2a}/stx_{2c}	-	+	+	-	-	+	+	+	-	-	-
Argentina 8	GB	4	20/01/2011	O157:H7	6	stx_{1a}/stx_{2a}	-	+	+	-	-	+	+	+	-	-	-
Argentina 9	MT	4	20/01/2011	O157:H7	6	stx_{1a}/stx_{2a}	-	+	+	-	-	+	+	+	-	-	-
Argentina 10	MM	4	20/01/2011	O157:H7	6	stx_{1a}/stx_{2a}	-	+	+	-	-	+	+	+	-	-	-
Argentina 11	MH	4	20/01/2011	O157:H7	8	stx_{2a}/stx_{2c}	-	+	+	-	-	+	+	+	-	-	-
Argentina 12	GB	32	28/01/2011	O157:H7	8	stx_{2a}/stx_{2c}	-	+	+	-	-	+	+	+	-	-	-
Argentina 13	GB	31	01/03/2011	O157:H7	8	stx_{2a}/stx_{2c}	-	+	+	-	-	+	+	+	-	-	-
Argentina 14	GB	69	07/04/2011	O157:H7	8	stx_{2a}/stx_{2c}	-	+	+	-	-	+	+	+	-	-	-
Argentina 15	MM	44	24/05/2013	O157:H7	6	stx_{2a}/stx_{2c}	-	+	+	-	-	+	+	+	-	-	-
Argentina 16	GB	47	03/06/2013	O157:H7	8	stx_{2a}	-	+	+	-	-	+	+	+	-	-	-
Argentina 17	MH	56	03/06/2013	O157:H7	6	stx_{2a}/stx_{2c}	-	+	+	-	-	+	+	+	-	-	-
Argentina 18	GB	85	19/05/2011	O157:H7	8	stx_{2a}/stx_{2c}	-	+	+	-	-	+	+	+	-	-	-
Argentina 19	GB	44	12/07/2011	O157:H7	8	stx_{2a}/stx_{2c}	-	+	+	-	-	+	+	+	-	-	-
Argentina 20	GB	57	10/06/2013	O157:H7	8	stx_{2a}/stx_{2c}	-	+	+	-	-	+	+	+	+	-	-
Argentina 21	GB	64	10/06/2013	O157:H7	6	stx_{2a}/stx_{2c}	-	+	+	-	-	+	+	+	+	-	-

GB: ground beef, K: knife, MH: hands of the meat manipulators, MT: meat table, MM: meat mincing machine

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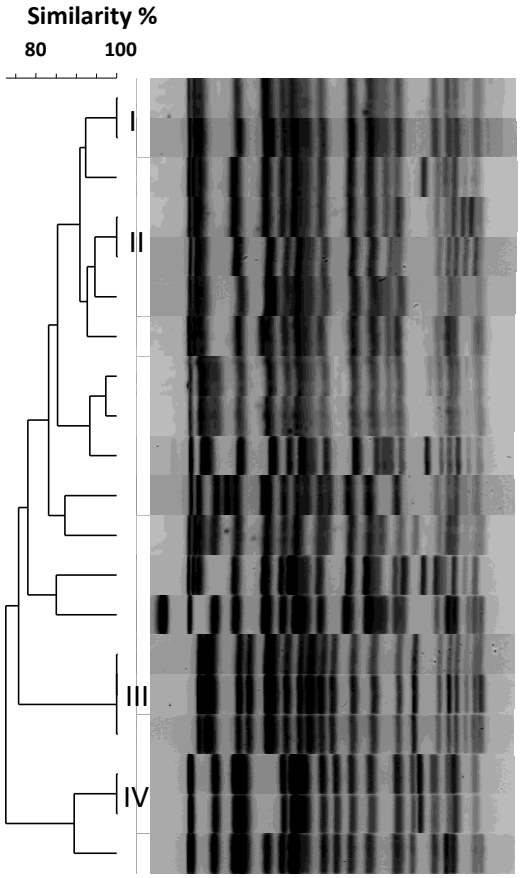
Figure 1.



strain	source	serotype	clade
Argentina 11	B4-MH	O157:H7	8
Argentina 7	B45-GB	O157:H7	8
Argentina 14	B69-GB	O157:H7	8
Argentina 13	B31-GB	O157:H7	8
Argentina 1	B52-GB	O157:H7	8
Argentina 2	B94-GB	O157:H7	8
Argentina 6	B20-GB	O157:H7	8
Argentina 4	B27-K	O157:H7	8
Argentina 5	B27-MH	O157:H7	8
Argentina 16	B47-GB	O157:H7	8
Argentina 12	B32-GB	O157:H7	8
Argentina 18	B85-GB	O157:H7	8
Argentina 19	B44-GB	O157:H7	8
Argentina 20	B57-GB	O157:H7	8
Argentina 8	B4-GB	O157:H7	6
Argentina 9	B4-MT	O157:H7	6
Argentina 10	B4-MM	O157:H7	6
Argentina 15	B44-MM	O157:H7	6
Argentina 17	B56-MH	O157:H7	6
Argentina 21	B64-GB	O157:H7	6

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Figure 2.

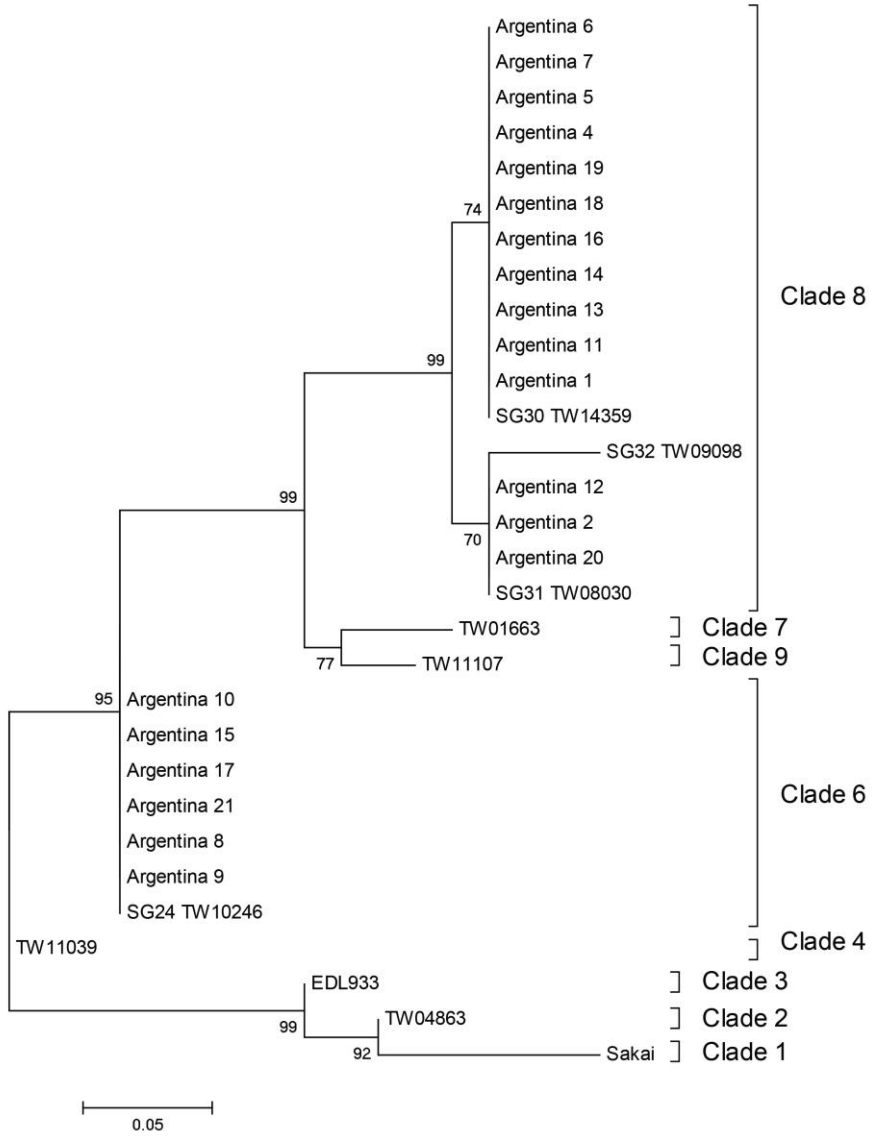

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Figure Legends

Figure1 caption. Comparison of the clonal relationship by *Xba*I-PFGE and single nucleotide polymorphisms analysis of the O157:H7 strains

Footnote:

Source nomenclature:

B: butcher shop – n° of butcher shop - GB: ground beef, K: knife, MH: hands of meat manipulators, MT: meat table, MM: meat mincing machine

Figure 2 caption. Neighbor-joining phylogeny of *E. coli* O157 isolates recovered from ground beef and environmental samples in Argentina. Clade designations were derived after constructing a phylogenetic tree based on 70 single nucleotide polymorphisms (SNPs) and assessing relationships with a subset of O157 control strains representing known clades. Control strains belonging to the multiple SNP genotypes within clade 8 were also included. The numbers at the nodes indicate the bootstrap support for each cluster.

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Highlights

- Characterization of O157:H7 strains from beef and environment from butcher shops.
- PFGE analysis showed high genetic diversity than SNP
- SNP analysis separated the strains in two distinct lineages 8 and 6.
- The almost exclusive circulation of clade 8 strains in the type of samples analyzed.
- Not suitable to reduce the pathogenic potential of strains only to the clade type.

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