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Virulence genes and genetic diversity assessment of Shiga toxin-producing *Escherichia coli* O91 strains from cattle, beef and poultry products

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) O91 has ranked in the top five of the non-O157 serogroups most frequently associated with human cases. In order to gain insight into the genetic diversity of O91 Latin American STEC strains, we analyzed their virulence properties and carried out a subtyping assay. A panel of 21 virulence genetic markers associated with human and animal infections was evaluated and the relatedness among strains was determined by a multiple-locus variable-number tandem repeats analysis (MLVA) comprising 9 VNTR loci. Twenty-two STEC O91 isolated from cattle and meat food and belonging to 5 serotypes (O91:H21, O91:H8, O91:H14, O91:H28, O91:H40) were studied. Eight virulence profiles were obtained for the O91 STEC strains: 4 for O91:H21 plus one for O91:H8, O91:H14, O91:H28 and O91:H40. All strains contained *ehxA* and $lpfA_{O113}$ genes and only both stx_1-positive strains lacked *saa*, which encodes the STEC autoagglutinating adhesin. Other genes involved in adhesion were detected: *ehaA* (91%), *elfA* and *espP* (86%), *ecpA* (82%) and, *hcpA* (77%). The gene encoding the cytolethal distending toxin type-V (CDT-V) was found only in O91:H8 and O91:H21, being present in the majority (89%) of strains of this last serotype. MLVA typing divided the total number of strains into 12 genotypes, and 9 of them were unique to a single strain. No association was observed between the virulence profiles and the source of the strains. Although they lack the *eae* gene, most of the strains have the genetic potential to adhere to host cells through other structures and possess *cdt-V*, which has been found in STEC strains involved in serious diseases. The MLVA showed clonal relatedness among strains isolated from cattle belonged to a same dairy farm and suggested that the same clone remains circulating throughout the year and, on the other hand, the need to increase the number of VNTR loci which could allow a higher discrimination among O91:H21 isolates.

1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) O91 has ranked in the top five of the non-O157 serogroups most frequently associated with human cases, and strains belonging to this serogroup are the most common human pathogenic *eae*-negative STEC strains [1]. They have been isolated from foods of different origins, such as beef, pork, lamb or poultry [2–8] as well as animals [7,9–13].

Clinical cases related to STEC O91 have been regularly reported since the 1990s [1,14–21]. Particularly, strains of the O91:H21 serotype have caused severe infections, including haemolytic uremic syndrome (HUS). However, unlike other strains that cause disease mainly in young children, these have been commonly isolated from adult patients [22–24]. The strains of this serogroup, at least in Germany, seem to be transmitted mainly by food, since these have been identified as the only risk factors for adults with sporadic infection by STEC O91 in that country between 2001 and 2003 and, in addition, because O91 is the second STEC serogroup most frequently isolated in food samples in that region [22,23].

There has been an increase in the detection of O91 strains in Germany from ~5% of all STEC strains isolated from humans in 1999 to ~15% in 2012 and 2013 [25]. Similarly from 2007 to 2012, the serogroups O91 and O113 were among the most common non-O157 serogroups associated with human disease in Netherlands [26].

Pradel et al. [27] compared strains of serogroup O91 isolated from patients, cattle and food from the central region of France and did not find any characteristic that was specific to the strains originating from HUS. Recently, Feng et al. [28] analyzed foods, environmental and

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clinical strains from United States and the European Union.

Multiple-locus variable-number of tandem-repeats analysis (MLVA) has emerged as a valuable method for subtyping foodborne pathogens and our laboratory has applied it successfully to investigate STEC diversity in several serotypes [29]. As far as we know, until now the MLVA has not been used to study O91 strains diversity.

Most studies that have characterized STEC O91 strains have focused on clinical isolates, and according to our knowledge, there is no specific study of O91 STEC strains in Latin America. For these reasons and in order to gain insight into the genetic diversity of STEC O91 strains, we analyzed virulence properties of strains isolated from cattle and food in Argentina and carried out a MLVA assay to define genetic relatedness among the isolates.

2. Materials and methods

Twenty-two STEC O91 isolates obtained from cattle and meat food in Argentina and belonging to 5 serotypes (O91:H21 (n = 18), O91:H8 (n = 1), O91:H14 (n = 1), O91:H28 (n = 1), O91:H40 (n = 1)) were studied. They had been collected between 1995 and 2010 from cattle, and beef and poultry products. Cattle isolates were obtained from 4 dairy farms (named A-C-D-E), one feedlot and one grazing farm. Strains had been previously characterized by PCR regarding the presence of *stx$_1$*, *stx$_2$*, *eae*, *ehxA*, and *saa* genes [6,30–32].

We evaluated a panel of 21 virulence genetic markers (*cdt-V*, *ecpA*, *ehaA*, *elfA*, *epeA*, *espP*, *hcpA*, *katP*, *lpfA$_{O113}$*, *sfpA*, *stcE*, *subA*, Z_{4321}, Z_{4326}, Z_{4332}, Z_{4333} plus the virulence genes mentioned in the above paragraph) associated with human and animal infections by PCR [see Table 1]. Also, we determined the genetic relatedness by a Multiple-locus Variable Number Tandem Repeat Analysis (MLVA) amplifying 9 generic VNTR loci [42,43], with the conditions described by González et al. [44].

3. Results

Eight virulence profiles were obtained for the O91 STEC strains: 4 for O91:H21 and one for O91:H8, O91:H14, O91:H28 and O91:H40, respectively. All strains contained *ehxA* (encoding for a hemolysin) and *lpfA$_{O113}$* (encoding for a fimbriae) genes and only both *stx$_1$*-positive strains lacked *saa*, which encodes the STEC autoagglutinating adhesin. Other genes involved in adhesion were detected: *ehaA* (91%), *elfA* and *espP* (86%), *ecpA* (82%) and, *hcpA* (77%). On the other hand, nine virulence genes (*eae*, *epeA*, *katP*, *sfpA*, *stcE*, *subA*, Z_{4326}, Z_{4332}, Z_{4333}) were never detected in the studied isolates. The gene encoding the cytolethal distending toxin type-V (CDT-V) was found only in O91:H8 and O91:H21, being present in the majority (89%) of strains of this last serotype (Fig. 1).

MLVA typing divided the total number of strains into 12 distinct genotypes, and 9 of them were unique to a single strain. This assay detected a limited diversity considering that several loci were little polymorphic. The isolates could be principally discriminated by alleles of locus CVN014 (Nei's Diversity index: $D_N = 0.7$) since other VNTR loci showed considerably lower diversity index values (CVN016: $D_N = 0.3$; CVN001 and CVN004: $D_N = 0.17$; CVN002, CVN007 and CVN015: $D_N = 0.09$). On the other hand, loci CVN003 and CVN0017 showed a null allele in all the isolates (Fig. 2). The value of Simpson's diversity index, related to the discrimination power of the method, was D_S: 0.86.

Some isolates obtained from the same dairy farm (dairy farm D) but in different seasons and identical virulence profile showed the same

Table 1
Virulence genes assessed by PCR in the present study. Primer sequences, annealing temperature, amplicon size and references are given. EHEC: Enterohemorrhagic *Escherichia coli*.

Gene	Encoded protein	Primer sequence (5′-3′)	Annealing temperature	Amplicon (bp)	Reference
ehxA	EHEC hemolysin	Fw- ACAGCTGCAAGTGCGGGTCTG Rv- GGGATGCACTGGAGGCTGCAC	58 °C	262	[33]
subA	Subtilase cytotoxin	Fw- TATGGCTTCCCTCATTGCC Rv- TATAGCTGTTGCTTCTGACG	58 °C	556	[34]
katP	Periplasmic catalase peroxidase	Fw- GCGCCAGTGGTGGTCAGCAA Rv- ATATCGGGCTGCCGGTCCCA	58 °C	914	[33]
stcE	Zinc metalloprotease	Fw- GGCTCCGGAGGTGGGGGAAT Rv- GAAGCCGGTGGAGGAACGGC	58 °C	399	[33]
espP	Extracellular serine protease	Fw- GCTGGCAACCAGCAACAGCG Rv- CGGTAGCCCGCTTCTGCACC	58 °C	774	[33]
ehaA	EHEC autotransporter	Fw- AGGCATGAGACACGATC Rv- AAGTCGTGCCATTGAGC	55 °C	500	[35]
lpfA$_{O113}$	Long polar fimbriae	Fw- ACTTGTGAAGTTACCTCC Rv- CGGTATAAGCAGAGTCG	55 °C	360	[35]
ecpA	*E. coli* common pilus	Fw- GCAACAGCCAAAAAAGACACC Rv- CCAGGTCGCGTCGAACT	55 °C	477	[36]
elfA	*E. coli* laminin-binding fimbriae	Fw- ACGATGAAAAAAAGTGTATTGACGG Rv- CCGCATTCACATTACCAGAA	60 °C	511	[36]
hcpA	Pilin subunit of hemorrhagic coli pilus	Fw- TCGCTAGTTGCTGACAGATTT Rv- AATGTCTGTTGTGTGCGACTG	48 °C	~680	[37]
sfpA	Sorbitol-fermenting EHEC O157 fimbriae plasmid-encoded	Fw- AGCCAAGGCCAAGGGATTATTA Rv- TTAGCAACAGCAGTGAAGTCTC	60 °C	440	[38]
epeA	Serine protease autotransporter	Fw- CACCCTGTAGAATCTTA Rv- CTGAATAAATCCAGCCC	46 °C	1259	[39]
cdt-V	Cytolethal distending toxin	Fw- TTCATTGTTCGCCTCCTG Rv- TTTATAAGCTGGTATCCTG	50 °C	755	[40]
Z_{4321}	Protein homologous to PagC membrane protein of *Salmonella* serovar Typhimurium	Fw- ATGAGTGGTTCAAGACTGG Rv- CCAACTCCAACAGTAAATCC	56 °C	521	[41]
Z_{4326}	Protein homologous to *Shigella flexneri* enterotoxin 2	Fw- GGATGGAACCATACCTGG Rv- CGCAATCAATTGCTAATGC	56 °C	551	[41]
Z_{4332}	Protein homologous to Efa1 (EHEC factor for adherence)	Fw- CTCCCAGAGATAATTTTGAGG Rv- CAACTGTATGCGAATAGTACTC	56 °C	504	[41]
Z_{4333}	Protein homologous to Efa1 (EHEC factor for adherence)	Fw- CTGTCAGACGATGACATTGG Rv- GAAGGATGGGCATTGTGTC	56 °C	547	[41]

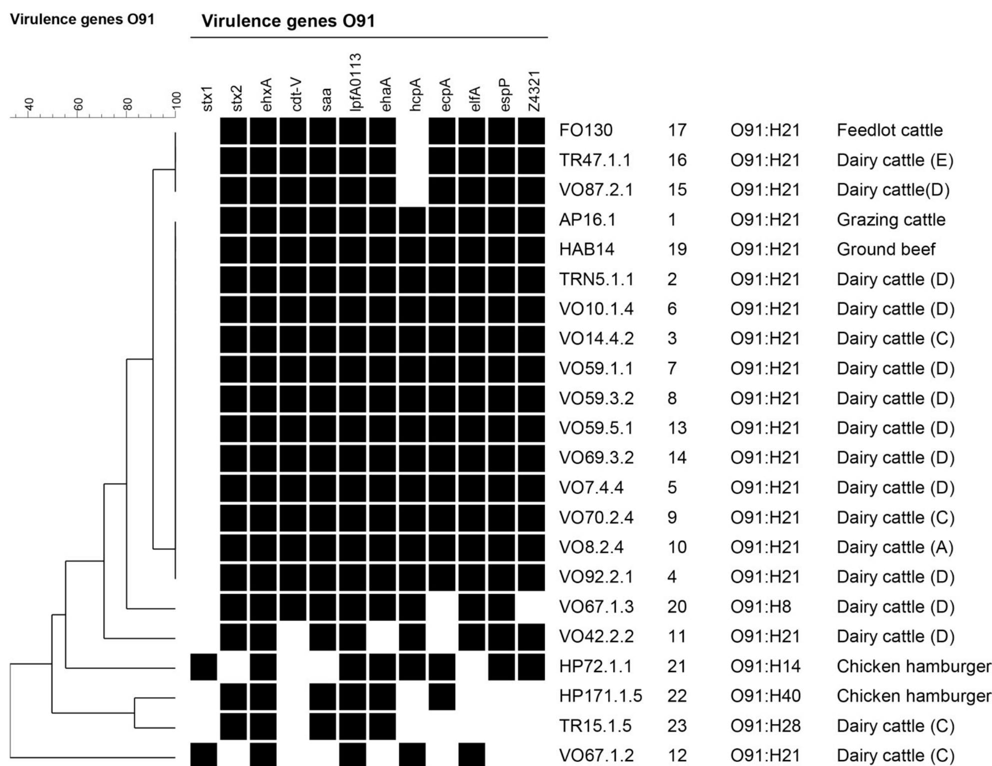

Fig. 1. Cluster analysis of STEC O91 strains isolated from cattle and food based on virulence-associated genes profiles. The dendrogram was generated using the BioNumerics v.6.6 software. The presence (black) or absence (blank) of genes, the name and the origin of the strains are shown. Genes not found in the any of the studied isolates: *eae*, *epeA*, *katP*, *sfpA*, *stcE*, *subA*, Z_{4326}, Z_{4332}, Z_{4333}.

MLVA type (isolates TRN5.1.1, VO7.4.4, VO10.1.4, VO59.1.1, VO59.3.2, and VO69.3.2).

4. Discussion

No association was observed between the virulence profiles and the source of the isolates. Strains of different serotypes within the O91 serogroup differed by the spectrum of putative virulence genes. However, it should be taken into account that although the 22 STEC isolates studied comprised 5 serotypes, mostly belonged to O91:H21, with only a single isolate represented each of the other four serotypes.

Although isolates lack the LEE pathogenicity island, most of the strains have the genetic potential to adhere to host cells through other structures such as *ehaA*, *elfA, espP*, *ecpA* and, *hcpA,* and possess the *cdt-V* gene, which has been found in STEC strains involved in serious diseases. This gene encodes a genotoxin and cyclomodulin which causes DNA damage, cell cycle arrest, and ultimately the death of the cells [45]. Also, Bielaszewska and colleagues [1] demonstrated that CDT-V

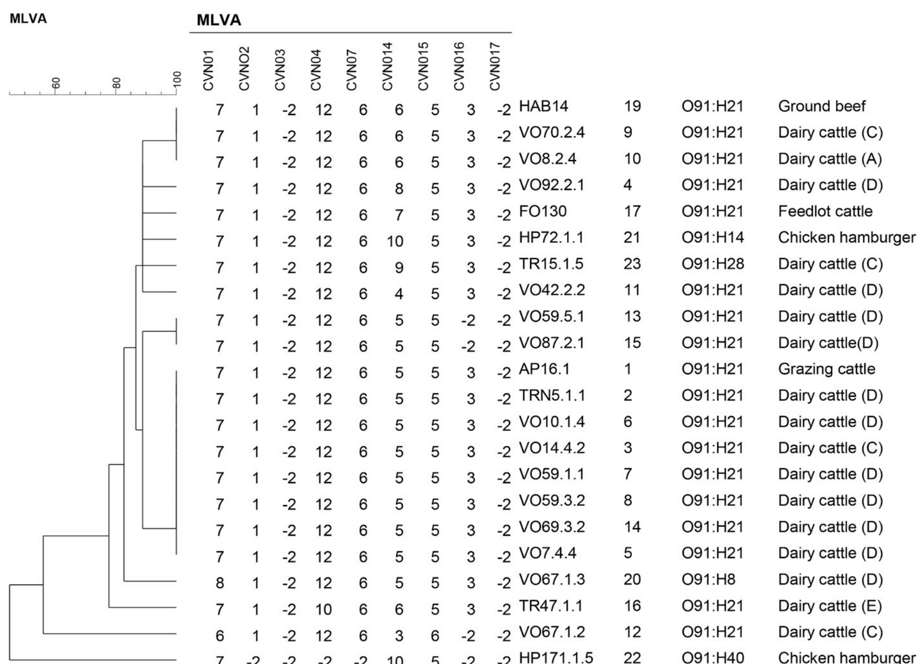

Fig. 2. Multiple-locus variable-number tandem repeats analysis–based clustering of STEC O91 isolates investigated in this study. Similarities among MLVA profiles were calculated using categorical coefficients and UPGMA clustering method.

produced by STEC O91:H21 strains causes irreversible injury to the human microvascular endothelium. In previous studies *cdt-V* was found at lower frequencies, 70% and 65% of the O91:H21 strains, respectively [1,28] than in this study (89%) and, this gene was also detected in two O91:H14 strains [28].

All isolates had *ehxA* encoding a hemolysin. This gene was also common in another O91 study, being found in 82% of the O91:H21 and 60% of the O91:H14 strains [28]. However, other common EHEC (Enterohemorrhagic *E. coli*) plasmid genes, such as *katP, epeA, stcE,* and *subA*, were not detected.

Interestingly, from the nine virulence genes which were not found in the isolates, five (*epeA*, *katP*, *sfpA, stcE, subA*) are located on plasmids. In relation to *katP* and *subA* they were previously detected in different percentages in O91:H14 [28]. It is possible that, if we could have analyzed more O91:H14 isolates, we would have detected those genes in some isolates. On the other hand, genes *sfpA* and *stcE* have been only detected in O157 strains. The remaining four genes not detected (*eae*, Z_{4326}, Z_{4332}, Z_{4333}) are located on pathogenicity islands (PAI), another kind of mobile genetic elements. The lack of *eae* is a characteristic trait of O91 serogroup. In relation to the Z genes, located on another PAI, OI-122, our results agree with those of Konczy et al. [46] and Cadona et al. [47], which showed that when *eae* was absent, only Z_{4321} was present.

The plasmidic serine protease gene *espP,* was present in 100% of the O91:H21 strains versus the 82% detected by Feng et al. [28]. On the other hand, the *saa* gene was found in all stx_2-positive isolates of this study, which represent 94% of all O91:H21 isolates. The lone O91:H14 strain of our study was stx_1-positive/*saa*-negative and it was isolated from a chicken burger. In Feng's study [28], *saa* was found in 78% of the O91:H21 strains. Results suggest the presence of megaplasmids harbouring different genes' combination. We conclude that STEC O91 isolates from Argentina harbor several toxin and adhesion genes and show variability in relation to virulence factors, with the presence of greater number of virulence traits in the O91:H21 serotype.

The MLVA results presented here showed genetic diversity within the STEC O91 serogroup. But, although different MLVA profiles were detected, the differences between profiles, in general, were given in a single locus (SLV- Single Locus Variation) and, in many of the cases, by a single repetition unit. By other hand, CVN003 and CVN017 did not amplify in any isolates, and therefore we suggest excluding them from the assay to analyze O91 genetic diversity. The MLVA showed clonal relatedness among strains isolated from cattle belonged to a same dairy farm and suggested that the same clone remains circulating throughout the year. This fact is not totally stricking since the environment has been highlighted as an important source of transmission of STEC. Once contaminated the environment has the potential to act as a reservoir of infection/re-infection for cattle [48]. Particularly, water contaminated with fecal material is one of the major source of exposure of cattle to enteric bacteria such as *E. coli* [49].

The O91:H21 strains seem to be a highly conserved group with cattle or food strains with similar or nearly identical virulence profile to those of the clinical strains that have caused HUS. Also, in relation to Multi Locus Sequence Typing (MLST) data and regardless of the scheme used, the results of previous studies are consistent, in that the O91:H21 strains are genetically uniform, sharing HUS-associated strains and environment/foods strains the same clonal profile. Two of the isolates here analyzed, one from cattle (AP 16-1) and one from food (HAB 14) had the MLST profile ST89 (CG34, CC230) [50]. Feng et al. [28] using the same MLST scheme (Whittam MLST) obtained ST89 or variants of it for all O91:H21 strains from environment, food and cattle obtained in USA. On the other hand, Mellmann and colleagues [51] detected, using the Achtman MLST scheme, that all the twenty O91:H21 from European Union patients had ST442, which describe de same clonal group [28].

The information on virulence genetic characteristics of O91 strains circulating in Latin America, until now, has been very scarce. This is a first report but further studies are needed to provide new data about this group of STEC, especially from South America region.

Conflicts of interest

The authors declare no conflict of interest.

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