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Serotype Distribution of Plasmid-encoded Virulence Profiles, and Identification of *espP* and *subAB* Alleles in Verotoxigenic *Escherichia coli*

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Authors' contributions

This work was carried out the collaboration of all authors. Authors AMS and AVB participated in the planning of the study, drafting and critical review of the manuscript and supervised the laboratory work. Authors AMS, AVB, TF, JG and JSC participated in the data collection and data entry. Author PMAL participated in the planning of the study and critical review of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study was designed to characterize verotoxigenic *Escherichia coli* (VTEC), important food-borne pathogens, in relation to virulence genes found in large plasmids harboured by disease-associated strains. Our aim was to detect these genes and possible combinations of them, and to establish if some kind of relationship exists between these profiles and different serotypes.
Study Design: Amplification of genes and allelic variants by PCR.
Place and Duration of Study: Laboratorio de Inmunoquímica y Biotecnología (FCV-UNCPBA, Argentina), between June 2010 and July 2013.
Methodology: 208 VTEC isolates belonging to 49 serotypes were characterized for the presence of plasmid-encoded genes: *epeA* (serine-protease), *espP* (extracellular serine protease) and its variants, *katP* (periplasmic catalase-peroxidase), *stcE* (zinc metalloprotease) and *subA* (subtilase

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cytotoxin) and its variants.
Results: The most frequently detected gene was *espP* (87%), followed by *ehxA* (47%), *saa* (20%) and *katP* (17%), whereas *epeA*, *subA* and *stcE* ranged from 7 to 12%. Taking into account these genes, twelve profiles were observed, ranging from the presence of zero to five of the genes. Some serotypes presented only one plasmid profile whereas others, such as O20:H19, showed up to four different profiles. Functional differences have been previously found among EspP subtypes, and we found that several VTEC isolated from bovines or foods contained the *espP*α, which is one of the alleles possibly associated with severe human disease. Considering subtilase cytotoxin gene subtypes, only *subAB*$_1$ was identified.
Conclusion: Our results show that VTEC strains can possess different combinations of plasmid-encoded virulence genes and even strains belonging to the same serotype can differ in relation to their plasmid genetic composition. In relation to allelic variants, LEE-positive serotypes showed for *espP* gene the allele α or the *γ,* and the detected *subAB* allele differed from the prevalent allelic variant *subAB*$_2$ carried by strains circulating in sheeps and humans in European countries.

Keywords: Plasmid profiles; verotoxigenic Escherichia coli; virulence; espP alleles; subAB alleles.

1. INTRODUCTION

The verotoxigenic *Escherichia coli* (VTEC) group, also called Shiga toxin-producing *Escherichia coli* (STEC), is very diverse, being *E. coli* O157:H7 the most common serotype associated with sporadic cases and large outbreaks of hemolytic uremic syndrome (HUS). However, there is growing concern about the risk to human health associated with non-O157 VTEC serotypes. Mobile genetic elements (MGEs) have had a profound influence on the emergence and spread of pathogenic bacteria. Examples of MGEs are plasmids, which confer determinants of pathogenicity. All clinical isolates of *E. coli* O157:H7 harbor a large plasmid called pO157, and plasmids that share some virulence factors are commonly found in disease-associated non-O157 VTEC, including O103:H2, O26:H11, O26:NM, O111:NM and O113:H21 serotypes [1-3].

Among the recognized plasmid-encoded virulence markers is the EHEC (enterohemorraghic *Escherichia coli*)-enterohemolysin (EHEC-HlyA or EhxA), which was the first described virulence factor of pO157 [4]. Furthermore, other plasmid-encoded proteins have been proposed as virulence factors in VTEC.

The STEC autoagglutinating adhesin encoded on pO113 by *saa*, has been suggested to be unique to LEE (locus for enterocyte effacement)-negative VTEC isolates [5-7].

Another gene, *katP*, encoding for a catalase-peroxidase was identified on pO157 [8]. This enzyme may help *E. coli* O157:H7 to colonize host intestines by reducing oxidative stress. It has been demonstrated that KatP is a peroxide scavenger that may protect *E. coli* O157:H7 EDL933 from oxidative damage during aerobic growth in an OxyR (peroxide-inducible transcriptional regulator)-dependent manner [9].

The protein EspP belongs to the serine protease autotransporters of Enterobacteriaceae (SPATE) family, able to cleave pepsin A and human coagulation factor V, contributing to the pathology of hemorrhagic colitis [10]. Dziva and colleagues [11] reported that EspP influences intestinal colonization of calves and adherence to bovine primary intestinal epithelial cells. In addition, it has been reported that this protease is able to cleave complement factors, and therefore could interfere with host innate immune response [12]. EspP can be subtyped into five groups and functional differences have also been found among them, being EspPα and EspPγ proteolytically active [13,14].

The zinc metalloprotease StcE is secreted through the type II secretion system encoded on pO157 [15]. StcE cleaves the C1 esterase inhibitor, which is a host regulator of the classical complement pathway. Furthermore, it has been demonstrated that it can contribute to intimate adherence of *E. coli* O157:H7 to Hep2 cells in vitro [16].

The subtilase cytotoxin (SubAB) is an AB_5 toxin described in certain VTEC strains that usually lack the LEE and has shown to be cytotoxic to Vero cells and lethal for mice [17,18]. This toxin was first described in a VTEC O113:H21 strain implicated in an outbreak of HUS in Australia, and it was encoded by two cotranscribed genes (*subA* and *subB*), on a large virulence plasmid (pO113) [17], which also carries the *saa* gene.

But recently, it has been reported that some SubAB-positive strains harbor *subAB* genes in the chromosome and next to the gene *tia*, which encodes an invasion factor previously described in enterotoxigenic *E. coli* [19].

The gen *epeA*, found in the pO113 transfer region, encodes an SPATE protein that may aid colonization and adherence to the host intestine through mucinolytic activity. The presence of *epeA* appeared to be also specifically associated with LEE-negative isolates of EHEC [2].

Our aim was to characterize a selection of VTEC strains isolated mainly from cattle and meat and representing many serotypes, in relation to several plasmid-borne virulence factors genes, to analyze the different combinations of these genes and to establish if some kind of relationship exists between these profiles and the serotypes. Also, in relation with the *espP* and *subA* genes, our aim was to investigate the occurrence of different subtypes.

2. MATERIALS AND METHODS

A total of 208 VTEC isolates obtained from cattle and raw meat, with the exception of two O157:H7 human ones, were investigated. The isolates belonging to 49 serotypes are part of the culture collection of the Laboratorio de Inmunoquímica y Biotecnología (FCV-UNCPBA, Argentina). They had all been previously analyzed in relation to the presence of genes encoding for chromosomal virulence factors, verotoxin 1 and 2 (*vtx1*, *vtx2*), intimin (*eae*), and to plasmid-encoded factors, enterohaemolysin (*ehxA*) and STEC autoagglutinating adhesin (*saa*) [6, 20-22]. In the present study, the isolates were characterized for the presence of plasmid-encoded genes: *epeA* (serine-protease), *espP* (extracellular serine protease) and its variants, *katP* (periplasmic catalase-peroxidase), *stcE* (zinc metalloprotease) and *subA* (subtilase cytotoxin) and its variants. The *epeA* gene was detected by a monoplex PCR [2] and the remaining ones, by a pentavalent PCR assay [23] which also detects *ehxA*. PCR amplification of *epeA* was performed under the following conditions: denaturing step of 5 min at 94°C and 30 cycles of 30 s at 94°C, 1 min at 46°C, and 1 min at 72°C, followed by 5 min at 72°C for elongation. Plasmid virulence profiles were defined taking into account the results obtained in the present study and those previously found for *saa*.

The strains positive for *espP* or *subA* were further characterized as follows. Determination of *espP* alleles α, ß, γ and δ was done by PCR as described by Brockmeyer et al. [13] and subtype ε, as described by Bielaszewska et al. [14]. For *subA*-positive strains, the presence of subtilase cytotoxin alleles ($subAB_1$ and $subAB_2$) plus the *tia* gene were assessed as described by Michelacci et al. [24].

3. RESULTS

In order to characterize the plasmid virulence profile of 208 VTEC strains isolated mainly from cattle and raw meat, we determined the presence of particular virulence genes that have been reported to be located on large plasmids of VTEC.

The most commonly found gene was *espP*, present in 182 (87%) of the VTEC strains tested, and the second most frequent gene was *ehxA* (47%). Both were detected either alone or in combination with other plasmid genes. The gene *katP* was found in 36 strains (17%) whereas *epeA*, *subA* and *stcE* were present in frequencies ranging from 7 to 12%, but they were never detected as the only plasmid gene. Those strains that were positive for *subA* harbored neither *stcE* nor *katP; stcE* was present only in O157:H7 isolates and *katP* appeared only in LEE-positive strains, never simultaneously with *saa*.

Taking into account the seven encoded-plasmid genes, twelve virulence profiles were observed, ranging from the presence of zero to the presence of five of the genes, being the profile *espP* (negative for the other 6 genes) the most prevalent one (98 isolates), followed by *katP espP ehxA* (22 isolates) and *epeA espP subA ehxA saa* (19 isolates) (Fig. 1). Most LEE-positive non-O157 serotypes were grouped in two profiles: *katP espP ehxA* and *espP ehxA*, whereas the profile *katP espP stcE ehxA* was characteristic of O157:H7 and only present in those isolates. Some serotypes presented only one plasmid profile whereas others, such as O20:H19, showed up to 4 different profiles. In nine VTEC isolates it was not possible to detect any of the 7 plasmid genes (Table 1). Most of the studied isolates harbored *vtx2,* alone or in combination with *vtx1* (data previously obtained). Some of the few isolates which carried only *vtx1* are those belonging to O8:H16 and these ones presented a particular profile, *espP saa*.

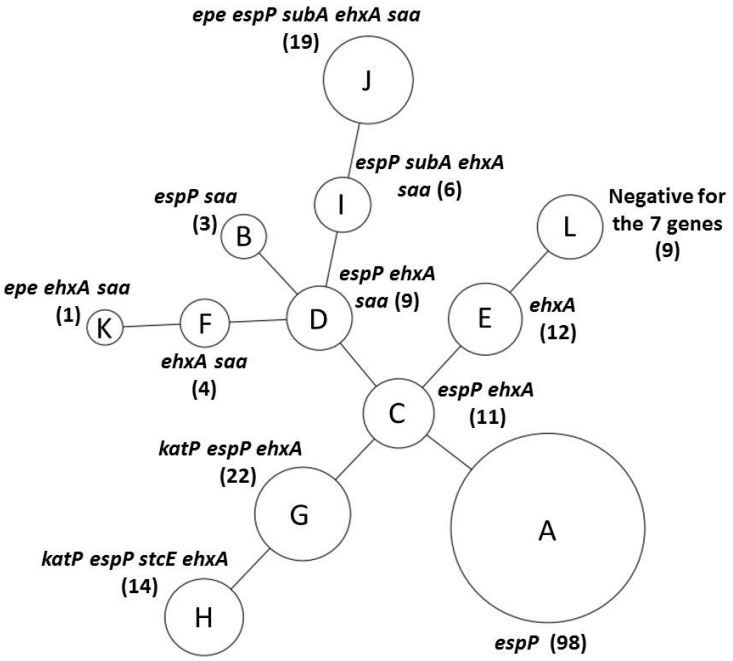

Fig. 1. Relationships of plasmid virulence profiles for VTEC isolates

Minimum-spanning tree of plasmid profiles generated with Bionumerics software v6.6 with single and double locus variance priority rules and the Dice similarity coefficient calculated for binary characters. Each circle represents a different virulence profile, and the letter in the circle indicates the virulence profile type. The circle size corresponds to the number of isolates with an identical virulence profile. Number of isolates included in each profile is indicated in parentheses.

Table 1. Plasmid virulence profiles of VTEC, frequencies and serotypes

Profiles	%	Serotypes*
*espP (*A)	47.1	O15:H21 (1), O20:H7 (2), O20:H19 (2), O112:H2 (1), O113:NM (7), O113:H21 (3), O117:H7 (11), O162:H7 (1), O171:NM (5), O171:H2 (11), O171:HNT (1), O174:H21 (13), O178:H19 (5), O185:H7 (1), ONT:NM (16), ONT:H7 (4), ONT:H8 (1), ONT:H21 (12), ONT:HNT (1)
espP saa (B)	1.4	O8:H16 (3)
espP ehxA (C)	5.3	**O5:NM** (4), O8:H19 (1), **O20:HNT** (1), **O26:H11** (2), **O103:NM** (2), **O103:H2** (1)
espP ehxA saa (D)	4.3	O20:H19 (1), O22:H8 (1), O88:H21 (1), O91:H21 (4), O174:H21 (1), ONT:H19 (1)
ehxA (E)	5.8	O8:H19 (8), O25:H19 (1), **O111:NM** (1), O175:H8 (2)
*ehxA saa (*F)	1.9	O22:H8 (1), ONT:H21 (2), ONT:HNT (1)
*katP espP ehxA (*G)	10.6	**O26:H11** (5), **O38:H39** (1), **O118:H16** (1), **O145:NM** (10), **O146:NM** (1), **O165:NM** (1), **O177:NM** (3)
katP espP stcE ehxA (H)	6.8	**O157:H7** (14)
espP subA ehxA saa (I)	2.9	O20:H19 (1), O116:H21 (1), O141:H7 (1), O141:H8 (1), O178:H19 (1), ONT:H8 (1)
epe espP subA ehxA saa (J)	9.1	O2:H5 (1), O20:H19 (4), O39:H49 (3), O79:H19 (3), O113:H21 (3), O120:H19 (1), O141:H8 (1), O178:H19 (1), ONT:H7 (1), ONT:H19 (1)
epe ehxA saa (K)	0,5	ONT:H19 (1)
Negative for the 7 genes (L)	4.3	O2:NM (1), O22:H8 (1), O113:NM (3), **O145:NM** (2), **O146:H21** (1), O174:H21 (1)

**LEE-positive serotypes are in bold. Number of isolates is indicated in parentheses after each serotype*

All *espP* and *subA*-positive strains were subjected to further analysis to identify particular subtypes. With regard to *espP*, the γ subtype was the most frequently found, being present in 62 isolates belonging to 25 serotypes, including both LEE-positive and LEE-negative ones. The α subtype was found in 35 isolates from 9 serotypes, all of them LEE-positive; the ß subtype, in 36 isolates of 14 serotypes meanwhile subtype δ was not detected in our strain collection and subtype ε was only found in the unique O15:H21 isolate. It is noticeable that it was not possible to assign alleles to 48 isolates, in particular, to most of the isolates belonging to the serogroup O171 and to serotype O174:H21 (Table 2).

Taking into account the serotypes that comprised more than one isolate and the *espP* alleles identified, several of them (O5:NM, O39:H49, O79:H19, O141:H8, O157:H7 and O177:NM) presented a same subtpype among the isolates. On the contrary, others, such as O8:H16, O20:H19, O26:H11, O113:NM, O113:H21, O117:H7 and O178:H19, presented more than one known *espP* allele. Within each serotype, no association could be found between the origin of the isolates and the *espP* subtype (data not shown).

In 23 of the 25 *subA*-positive strains, the *subAB* allele could be identified. All of them (n = 23) were positive for the prototype of SubAB *(SubAB$_1$)*, and, conversely, negative for the recently identified new variant of *SubAB* (SubAB$_2$) and for *tia*, both genes proposed to be located in a same PAI. The remaining two isolates, in which the *subAB* subtype could not be identified, belonged to O79:H19 and O120:H19 serotypes.

4. DISCUSSION

Our results showed that virulence plasmids of VTEC are highly variable. No isolates contained all seven markers, but isolates belonging to O2:H5, O20:H19, O39:H49, O79:H19, O113:H21, O120:H19, O141:H8, O178:H19, ONT:H7 and ONT:H19 did possess 5 of the genes, *epeA, espP, subA, ehxA* and *saa*. On the other hand, in some isolates belonging to serotypes O2:NM, O22:H8, O113:NM, O145:NM, O146:H21 and O174:H21 we could not detect any of these plasmid genes. It is plausible that these isolates had lost the large virulence plasmid since MGEs can be easily exchanged among strains or lost, and other isolates belonging to some of these serotypes did have one or more plasmid genes. As a consequence, VTEC clones with different genetic assets in regard to virulence genes are generated.

The most prevalent genes were *espP* and *ehxA, s*imilarly to Bosilevac et al. [25], but in contrast to other studies in which all VTEC isolates that were positive for *espP* were also positive for *ehxA* [26], we detected the profile *espP* as the most frequent.

The profile *katP espP stcE ehxA* was exclusively found among O157:H7 strains, being *stcE* only present in this serotype, in concordance with other studies [27]. The plasmids from O157:H7 have been reported to be highly similar [28], but a diversity of marker combinations has been found among plasmids from O103:H2 [29-31],

Table 2. Distribution of *espP* alleles in VTEC serotypes

Serotype (*)	*espP* subtype (**)	Serotype	*espP* subtype	Serotype	*espP* subtype
O2:H5 (1)	γ	**O103:NM** (2)	α (1) n (1)	O162:H7 (1)	γ
O5:NM (4)	γ	**O103:H2** (1)	α	**O165:NM** (1)	γ
O8:H16 (3)	β (1) γ (2)	O112:H2 (1)	γ	O171:NM (5)	γ (1) n (4)
O8:H19 (1)	n	O113:NM (7)	β (2) γ (5)	O171:H2 (11)	γ (2) n (9)
O15:H21 (1)	ε	O113:H21 (6)	β (4) γ (2)	O171:HNT (1)	n
O20:HNT (1)	γ	O116.H21 (1)	n	O174:H21 (14)	γ (5) n (9)
O20:H7 (2)	n	O117:H7 (11)	β (4) γ (6) n (1)	**O177:NM** (3)	α
O20:H19 (8)	β (5) γ (1) n (2)	**O118:H16** (1)	α	O178:H19 (7)	β (2) γ (2) n (3)
O22:H8 (1)	γ	O120:H19 (1)	γ	O185:H7 (1)	β
O26:H11 (7)	α (4) γ (2) n (1)	O141:H7 (1)	n	ONT:NM (16)	β (4) γ (4) n (8)
O38:H39 (1)	α	O141:H8 (2)	β	ONT:H7 (5)	β (2) γ (3)
O39:H49 (3)	β	**O145:NM** (10)	α (9) n (1)	ONT:H8 (2)	β (1) n (1)
O79:H19 (3)	β	**O146.NM** (1)	α	ONT:H19 (2)	γ (1) n (1)
O88:H21 (1)	γ	**O157:H7** (14)	α	ONT:H21 (12)	β (2) γ (10)
O91:H21 (4)	γ (3) n (1)			ONT:HNT (1)	γ

(*) number of analyzed espP-positive isolates, (**) number of isolates is only indicated when not all the isolates of a serotype presented the same espP allele; n: non identified espP subtype. LEE-positive serotypes are in bold

and we detected the profile *espP ehxA,* not common for this serotype.

SubAB, the subtilase cytotoxin, has been detected mostly in LEE-negative VTEC strains [32 and papers cited in it]. The *subAB* genes may be mobilized by a plasmid that also carries *saa* gene, or alternatively, by a putative pathogenicity island (PAI) together with *tia* [19]. Allelic variants of *subAB*, differentially associated with these two MGEs have been described but there are few studies that identify them and analyze their distribution among VTEC strains. In Spain, the variant $subAB_2$ was found to predominate among VTEC isolated from large game animals, their meats, small ruminants and possibly related strains from human [33-35,24]. Furthermore, this allele was also detected in almost all *subAB*-positive human strains from Denmark [24], and in deer meat strains from Germany [36]. However, all the *subAB*-positive isolates that could be subtyped in this study (obtained from cattle and derived meat) possessed $subAB_1$. This allele was also detected in most of the *subAB*-positive VTEC strains isolated from cattle in Spain [33]. Recently, two allelic variants for $subAB_2$ have been described ($subAB_{2\text{-}1}$ and $subAB_{2\text{-}2}$) [36]. Further studies from different geographical areas and VTEC reservoirs are needed to determine if there exists an association between specific animal reservoirs and *subAB* alleles.

EspP is a serine protease expressed during human infection that can degrade human coagulation factor V. From the five subtypes of *espP* identified in VTEC [13,14], only EspPα and EspPγ were able to cleave coagulation factor V [37]. This study revealed an association among some serogroups and particular *espP* subtype(s). LEE-positive serotypes showed the allele α or the *γ.* Despite that in previous studies *espP*α was found particularly in isolates from HUS patients, we detected this allele in several strains isolated from bovines or foods. Particularly interesting is the observation that serogroup O103, considered to be highly pathogenic and reported as frequently negative for *espP* [13,38], resulted *espP*-positive and carried the allele α. On the other hand, *espP*γ was detected both in LEE-positive and in LEE-negative isolates, which was also found by other authors [13]. The subtype δ was not detected in our strain collection, neither in isolates from Austria [37]. In a significant proportion of *espP*-positive isolates we were not able to determine the *espP* allele, especially in O171 and O174:H21 strains, suggesting the presence of novel alleles.

Before many of the VTEC plasmid genes were described, Brunder et al. [39] detected a considerable variation among megaplasmids. They are clearly mosaics, and most putative virulence genes are flanked by mobile genetic determinants, which probably facilitate their transfer. However, partial deletions of these mobile elements, as shown with the regions that flank the *sfp* operon in pO165, can stabilize particular virulence loci [14].

VTEC virulence is a multifactorial trait and the genetic markers to define it have yet to be determined [25]. Brandt et al. [40] remarked that serotyping is not an absolute predictor of risk to human health, and instead suggested to examine the virulence gene content of a strain.

5. CONCLUSION

In the present study, the characterization of VTEC strains belonging to 49 serotypes revealed a great diversity of virulence profiles in regard to plasmid encoded genes and, noticeably, intraserotype differences were also identified. The finding of different combinations of virulence factors in non-O157:H7 serotypes traditionally not associated with disease should alert on the desirability to search for these specific virulence factors in routine diagnostic.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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