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Distribution of additional virulence factors related to adhesion and toxicity in Shiga toxin-producing *Escherichia coli* isolated from raw products in Argentina

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Significance and Impact of the Study: Meat products are one of the main vehicles of Shiga toxin-producing *E. coli,* and the presence of genes coding for additional adhesins and toxins could increase their pathogenic potential. There is a need for a more detailed characterization of the strains in regard to these extra virulence factors.

Keywords
adhesion, bacterial toxin, food, Shiga toxin-producing *Escherichia coli*, virulence genes.

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Abstract

A total of 73 Shiga toxin-producing *Escherichia coli* (STEC) isolates, belonging to 25 serotypes and isolated from raw products in Argentina, were examined for the occurrence of genes responsible for bacterial adhesions to intestine, *ehaA* (EHEC autotransporter), *lpfAO113* (long polar fimbriae), *sab* (STEC autotransporter [AT] contributing to biofilm formation), *ecpA* (*E. coli* common pilus), *hcpA* (haemorrhagic *coli* pilus), *elfA* (*E. coli* laminin-binding fimbriae), *sfpA* (sorbitol-fermenting EHEC O157 fimbriae plasmid-encoded) and of the toxigenic gene *cdt-V* (cytolethal distending toxin). Our study showed different adhesin profiles that are not linked to one specific serotype and that all analysed isolates possess, besides *stx* genes, some adherence genes. Several of the isolates contained also multiple toxin genes. The results of the present work alert the presence of genes coding for additional adhesins and *cdt-V* toxin in LEE-negative STEC strains that occur in foods, and this traits could increase their pathogenic potential.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are a cause of foodborne infections, and foods of high risk for transmission are minced meat and other meat products. To establish and maintain an infection, STEC strains are equipped with a diverse array of virulence factors. Although the production of Shiga toxins is the main virulence trait of STEC, the capacity to colonize the host intestinal epithelium is a crucial step in pathogenesis. An additional virulence factor, the locus of enterocyte effacement (LEE), a pathogenicity island, has been shown to correlate with the presence of haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS). This pathogenicity island encodes intimin (*eae*), responsible for intimate attachment of the bacteria to the host intestinal cells (McDaniel *et al.* 1995). However, many STEC isolates from cases of severe disease, including HUS, lacking the LEE locus yet are clearly capable of efficient colonization of the human gut (Paton *et al.* 1999). Several candidate adhesins have been identified in these strains. EhaA was identified as a novel autotransporter protein (AT) of enterohaemorrhagic *E. coli* (EHEC) O157:H7, which is involved in adhesion and biofilm formation (Wells *et al.* 2008). Herold *et al.* (2009) described the identification and characterization of Sab, another member of the AT

family, produced by a hypervirulent LEE-negative O113:H21 STEC strain. This factor confers adherence to human epithelial cells and mediates also biofilm formation, and its gene is located on megaplasmid pO113, approximately 1·3 kb downstream of the haemolysin locus *ehx*. Furthermore, the *lpfA*O113 gene, located within a novel fimbrial gene cluster, was initially reported in a LEE-negative EHEC O113:H21 strain isolated from a HUS patient, and it was shown that deletion of the fimbrial subunit gene $lpfA_{O113}$ resulted in decreased adherence of these bacteria to epithelial cells *in vitro* (Doughty *et al.* 2002). Other examples of fimbrial adhesins, which may represent mechanisms of host cell adherence and colonization, are the *E. coli* common pilus (ECP) (Rendón *et al.* 2007); the haemorrhagic *coli* pilus (HCP, also called type IV pili) capable of mediating several phenomena associated with pathogenicity such as adherence to human and bovine epithelial cells, invasion of epithelial cells, haemagglutination of rabbit erythrocytes, biofilm formation, twitching motility, and specific binding to laminin and fibronectin (Xicohtencatl-Cortes *et al.* 2007, 2009) and the release of proinflammatory cytokines (Ledesma *et al.* 2010); the *E. coli* laminin-binding fimbriae (ELF) that mediates adherence to human intestinal epithelial cells and to cow and pig gut tissue *ex vivo* (Samadder *et al.* 2009) and a fimbria described in EHEC strains as part of a cluster called sorbitol-fermenting protein (*sfp*) encoded by a large plasmid in sorbitol-fermenting O157 strains (Brunder *et al.* 2001).

Besides Shiga toxins and subtilase cytotoxin (SubAB), a phage-encoded toxin, the cytolethal distending toxin V (CDT-V), occurs in *E. coli* O157:H7, O157:NM and non-O157 strains (Janka *et al.* 2003). CDT-V causes G2/M cell cycle arrest leading to distension, inhibition of proliferation and death of human endothelial cells (Bielaszewska *et al.* 2005).

The prevalence of genes encoding putative adhesins and newly identified toxins have been reported mostly in STEC strains corresponding to clinical or veterinary isolates, whereas only limited data are available about STEC strains from foods. In this study, we examined the distribution of various virulence genes related to putative adhesion, *ehaA*, $lpfA_{O113}$, *sab, ecpA*, *hcpA*, *elfA*, *sfpA* and to toxicity, *cdt-V*, among current STEC food isolates from Argentina. Correlations between the presence of the studied factors, other STEC associated virulence genes and serotypes were also analysed.

Results and discussion

The results obtained in relation to the detection of adhesin genes associated with host cell adherence and biofilm formation, *ehaA*, *sab*, $lpfA_{O113}$, *ecpA*, *hcpA*, *elfA*, *sfpA* and a toxin-encoding gene, *cdt-V*, in STEC isolates from meat products are shown in Table 1. *EhaA* and *elfA* genes were found both in non-O157 and O157:H7 isolates, whereas *sab*, $lpfA_{O113}$, *ecpA*, *hcpA* and *cdt-V* genes were detected only in non-O157:H7.

The most prevalent genes identified among all strains were *elfA* (97·3%), *ehaA* (80·8%) and $lpfA_{O113}$ (76·7%). *HcpA* gene was detected in 27·4%, *ecpA* in 17·8%, meanwhile *sab* and *cdt-V* genes were detected in 4 (5·5%) and 6 isolates (8·2%), respectively. All the isolates resulted negative for *sfpA* gene.

The gene *elfA,* which encodes a putative adherence factor present ubiquitously in *E. coli,* was found in almost all of our studied isolates. *In vitro* observations have suggested that ELF might have a role in STEC adherence to cells; however, the environmental conditions triggering the production of this fimbria and its specific role in the colonization of the human or animal intestinal tract need further investigation (Farfan and Torres 2012).

The autotransporter EhaA has been implicated in attachment to biological and abiotic surfaces (Wells *et al.* 2008). The high prevalence of *ehaA* and its detection in both groups, O157:H7 and non-O157:H7 serotypes, is in agreement with Wells *et al.* (2008) and Biscola *et al.* (2011). Only it was absent in serotypes O2:NM and O8:H16 and in a few isolates of other serotypes. Wu *et al.* (2010) found even higher percentages of *ehaA*-positive strains in samples from Japanese cattle and patients with diarrhoea (95 and 98%, respectively). On the other hand, Wells *et al.* (2008) did not detect the gene in any of the 11 strains O117:H7 they studied, so that they assumed that this gene was lacking in this serotype. However, the four O117:H7 isolates here analysed were *ehaA*-positive. Regarding to the relationship of *ehaA* with other virulence factors, we found that isolates that lacked this gene did not harbour *sab* or *cdt-V* and the co-occurrence with *saa* was variable. Moreover, all *ehaA*-positive isolates were also stx_2-positive. The only two stx_2-negative isolates, belonging to O8:H16 serotype, were *ehaA*-negative.

The presence of LPF_{O113} has been related with adherence to epithelial cells and interbacterial interaction (Doughty *et al.* 2002), and $lpfA_{O113}$ appears to be widely distributed among LEE-negative strains (Doughty *et al.* 2002; Osek *et al.* 2003; Galli *et al.* 2010). In the present study, it was detected in 78% of the LEE-negative strains, being absent in serotypes O157:H7, O2:NM, O88:H21 and ONT:H19, with variable occurrence in other serotypes. Galli *et al.* (2010) reported higher prevalences of this gene in LEE-negative strains, 100% for those of bovine origin and 98% for human origin, while Wu *et al.* (2010) detected 99% prevalence among Japanese strains of bovine origin. Furthermore, Cergole-Novella *et al.* (2007) identified $lpfA_{O113}$ in both, *eae*-negative and

Table 1 Distribution of additional virulence factors related to adhesion and toxicity in STEC isolated from foods

Serotype (No.)	*ehaA*	*sab*	$lpfA_{O113}$	*ecpA*	*hcpA*	*elfA*	*sfpA*	*cdt-V*	Virulence profile*
O2:NM (1)	–	–	–	–	–	+	–	–	stx_2
O8:H16 (1)	–	–	+	–	–	+	–	–	stx_1, *saa*
O8:H16 (1)	–	–	+	+	–	+	–	–	stx_1, *saa*
O8:H19 (1)	+	–	–	–	–	+	–	–	stx_1, stx_2, *ehxA*
O8:H19 (2)	–	–	+	–	+	+	–	–	stx_2, *ehxA*
O8:H19 (1)	–	–	–	–	–	+	–	–	stx_2, *ehxA*
O8:H19 (2)	–	–	+	–	–	+	–	–	stx_2, *ehxA*
O8:H19 (3)	–	–	+	+	+	+	–	–	stx_2, *ehxA*
O20:H19 (1)	+	–	+	–	–	+	–	–	stx_2, *ehxA*
O20:H19 (1)	+	+	+	–	+	+	–	+	stx_1, stx_2, *ehxA, saa, subA*
O22:H8 (1)	–	–	–	–	–	+	–	–	stx_1, stx_2, *ehxA, saa*
O22:H8 (1)	+	–	+	–	+	+	–	+	stx_1, stx_2, *ehxA, saa*
O22:H8 (1)	+	–	+	–	+	+	–	–	stx_2
O79:H19 (1)	+	+	+	–	+	+	–	–	stx_2, *ehxA, saa, subA*
O79:H19 (1)	+	+	+	–	–	+	–	–	stx_2, *ehxA, saa, subA*
O88:H21 (1)	+	–	–	–	+	+	–	–	stx_1, stx_2, *ehxA, saa*
O91:H21 (1)	+	–	+	–	–	+	–	+	stx_2, *ehxA, saa*
O112:H2 (1)	+	–	+	–	–	+	–	–	stx_2
O113:H21 (1)	+	–	+	–	–	–	–	–	stx_2, *ehxA, saa, subA*
O113:H21 (1)	+	–	+	–	–	+	–	–	stx_2, *ehxA, saa, subA*
O113:H21 (1)	+	–	+	–	+	+	–	–	stx_2
O113:NM (5)	+	–	–	–	–	+	–	–	stx_2
O113:NM (1)	+	–	+	–	+	+	–	–	stx_2
O113:NM (4)	+	–	+	–	–	+	–	–	stx_2
O116:H21 (1)	+	–	+	–	–	+	–	+	stx_2, *ehxA, saa, subA*
O117:H7 (2)	+	–	+	–	+	+	–	–	stx_2
O117:H7 (2)	+	–	+	–	–	+	–	–	stx_2
O157:H7 (1)	+	–	–	–	–	+	–	–	stx_2,*eae, ehxA*
O171:H2 (1)	+	–	+	–	–	+	–	–	stx_2
O171:HNT (1)	+	–	+	+	–	+	–	–	stx_2
O171:NM (1)	+	–	+	+	–	+	–	–	stx_2
O171:NM (1)	+	–	+	+	+	+	–	–	stx_2
O174:H21 (3)	+	–	+	–	–	+	–	–	stx_2
O174:H21 (1)	+	–	+	+	–	+	–	–	stx_2
O174:H21 (1)	+	–	+	–	+	+	–	–	stx_2
O174:H21 (1)	+	–	+	+	+	+	–	–	stx_2
O178:H19 (3)	+	–	+	–	–	+	–	–	stx_2
O178:H19 (1)	+	–	–	–	–	+	–	–	stx_2, *ehxA, saa, subA*
O178:H19 (1)	+	–	–	+	–	+	–	–	stx_2, *ehxA, saa, subA*
O185:H7 (1)	+	–	+	–	–	+	–	–	stx_2
ONT:H7 (2)	+	–	+	–	–	+	–	–	stx_2
ONT:H7 (1)	+	–	–	+	–	+	–	–	stx_2
ONT:H7 (1)	+	–	+	–	–	+	–	–	stx_2, *ehxA, saa, subA*
ONT:H7 (1)	+	–	+	+	+	+	–	–	stx_2
ONT:H8 (1)	+	–	+	–	–	+	–	+	$svtx_1$, stx_2, *ehxA, saa, subA*
ONT:H19 (1)	+	+	–	–	+	+	–	–	stx_2, *ehxA, saa*
ONT:H19 (1)	–	–	–	–	–	+	–	–	stx_1,stx_2, *ehxA, saa*
ONT:H21 (1)	+	–	+	–	–	+	–	–	stx_2
ONT:H21 (1)	+	–	+	+	+	–	–	–	stx_2
ONT:H21 (1)	+	–	+	–	–	+	–	–	stx_2, *ehxA, saa*
ONT:H21 (1)	+	–	–	–	–	+	–	–	stx_2
ONT:HNT (2)	+	–	+	–	–	+	–	–	stx_2
ONT:HNT (1)	–	–	+	–	–	+	–	–	stx_2, *ehxA, saa, subA*
ONT:HNT (1)	+	–	+	–	–	+	–	+	stx_2, *ehxA, saa*

STEC, Shiga toxin-producing *Escherichia coli.*
*****Examined in previous studies.

eae-positive strains. However, different frequencies were detected between those groups (98% among *eae*-negative strains versus 80% among *eae*-positive strains), but the presence or absence of $lpfA_{O113}$ was independent of *saa* gene, which is absent in *eae*-positive strains.

Hemorrhagic coli pilus appears to be an important virulence factor in STEC O157:H7 that might participate not only in the adherence of the bacteria to the host cell but also in the inflammation caused by STEC (Farfan and Torres 2012). According to Hernandes *et al.* (2011), *hcpA* was the gene most frequently amplified in a collection of Brazilian and Australian atypical enteropathogenic *E. coli* (aEPEC) strains (98·6%); however, they failed to detect the production of HCP in those strains. They hypothesized it is likely that in some *E. coli* strains, *hcpA* gene expression, like that of many other virulence factors, requires specific growth conditions that trigger or repress regulatory elements that lead to successful gene expression. In all isolates tested by us in which we detected *hcpA*, we also found *elfA*, except in one.

Escherichia coli common pilus is a pilus of EHEC O157:H7 with a potential role in host epithelial cell adhesion (Rendón *et al.* 2007). Regarding to the co-occurrence of *ecpA* with other adherence genes, all *ecpA*-positive isolates were also $lpfA_{O113}$-positive, with the exception of two isolates.

In relation to Sfp fimbriae, correlated with increased adherence to human intestinal epithelial cell lines (Müsken *et al.* 2008), we failed to detect the presence of its gene in our collection. However, it did not result surprising because it was previously detected only in sorbitol-fermenting EHEC O157:NM (Friedrich *et al.* 2004; Lee and Choi 2006), and in O165:H25/NM strains (Bielaszewska *et al.* 2009a), both isolated from humans and cattle.

Shiga toxin-producing *E. coli* strains of different serotypes carry large plasmids, which are highly heterogeneous in their genetic composition and arrangement (Brunder *et al.* 1999; Johnson and Nolan 2009). Among the virulence factors encoded in these plasmids is Sab, another autotransporter that was involved in adherence of STEC to abiotic surfaces and epithelial cells (Herold *et al.* 2009), found, so far, only in LEE-negative strains. In this study, the gene was detected in the serotypes O20:H19 and ONT:H19 and, for the first time, in O79:H19. All isolates positive for *sab* ($n = 4$) were also positive for other plasmid-encoded genes, *ehxA*, *saa*, and *subA*, with the exception of an isolate that was *subA*-negative. However, other strains which harboured other plasmidic genes were negative for *sab*, consistent with observations made by Herold *et al.* (2009). In the cited paper, which refers to the original description of the gene, the authors reported the detection of *sab* in several O113 isolates one O23 and one O82:H8 isolate. Furthermore, Polifroni *et al.* (2010) found the gene in a bovine STEC isolate belonging to O178:NM and Dos Santos *et al.* (2012) in 11% of Brazilian strains, which is a slightly higher prevalence than previously reported. Despite the original description of *sab* in STEC O113:H21, occurrence in this serotype was not found in this present study or in the study of Dos Santos *et al.* (2012).

The *cdt* gene was detected in isolates belonging to serotypes O20:H19, O22:H8, O91:H21, O116:H21, ONT:H8 and ONT:HNT. The *cdt-V*-positive isolates were also positive for *ehaA*, $lpfA_{O113}$ and *elfA*. Fourteen isolates contained multiple toxin-encoding genes and distinct virulence profiles related with *cdt-V* and other toxin-encoding genes were found: *cdt-V* stx_1 stx_2 (one isolate), *cdt-V* stx_2 (two isolates), *cdt-V* stx_2 *subA* (one isolate) and *cdt-V* stx_1 stx_2 *subA* (two isolates). The only two isolates stx_2-negative, belonging to serotype O8:H16, were also *cdt-V*-negative. Cergole-Novella *et al.* (2007) reported for the first time the simultaneous presence of subtilase toxin and Cdt-V genes in four strains belonging to O113:H21 and ONT:HNT. Subsequently, Wu *et al.* (2010) detected both genes in O17:H11 and O113:NM. In our study, three of six strains *cdt-V*-positive isolates were also positive for *subA* and belonged to serotypes O20:H19, O116:H21 and ONT:H8. Furthermore, Galli *et al.* (2010) also found this association in strains isolated from patients with severe disease, and therefore, it should not be ignored that this would have clinical relevance.

Considering only non-O157:H7 LEE-negative strains, Cergole-Novella *et al.* (2007) found the *cdt-V* gene in 12·9% of the strains isolated from cattle and beef and Allué-Guardia *et al.* (2011), in 8·6% of isolates from the environment. On the other hand, Wu *et al.* (2010) found a higher prevalence (49%) among LEE-negative cattle isolates, and Orth *et al.* (2006) detected it in O91:H21 and O76:H21. In this study, we detected a frequency of *cdt-V* of 8·3%, and we found it for the first time in the serotype O20:H19. Particularly, in relation to serotype O91:H21, Bielaszewska *et al.* (2009b) detected *cdt-V* in 70% of the strains studied, similarly to Allué-Guardia *et al.* (2011). We also detected it in the unique isolate of this serotype analysed. Noticeably, we did not detect *cdt-V* neither in O157:H7 isolated from food nor in other O157:H7 strains isolated from bovines and humans (data not shown), despite the fact the *cdt-V* gene cluster was originally described in O157:NM and O157:H7 strains (Janka *et al.* 2003). Subsequent to that publication, this gene was found again in other O157:H7 strains, isolated either from humans (Friedrich *et al.* 2006) or water (Allué-Guardia *et al.* 2011).

Taking into account all the genes detected in this study, 12 different virulence profiles were identified among the

Table 2 Virulence profiles, frequencies and serotypes in STEC isolated from foods

Profiles	%	Serotypes
ehaA/lpfA$_{O113}$/elfA	32·9	O20:H19; O112:H2; O113:H21; O113:NM; O117:H7; O171:H2; O174:H21; O178:H19; O185:H7; ONT:H7; ONT:H21; ONT:HNT
ehaA/elfA	12·3	O8:H19; O113:NM; O157:H7; O178:H19; ONT:H21
ehaA/lpfA$_{O113}$/hcpA/elfA	8·2	O22:H8; O113:H21; O113:NM; O117:H7; O174:H21
ehaA/lpfA$_{O113}$/elfA/cdt-V	5·5	O91:H21; O116:H21; ONT:H8; ONT:HNT
lpfA$_{O113}$/elfA	5·5	O8:H16; O8:H19; ONT:HNT
elfA	5·5	O2:NM; O8:H19; O22:H8; ONT:H19
ehaA/lpfA$_{O113}$/ecpA/elfA	4·1	O171:HNT; O171:NM; O174:H21
ehaA/lpfA$_{O113}$/ecpA/hcpA/elfA	4·1	O171:NM; O174:H21; ONT:H7
lpfA$_{O113}$/ecpA/hcpA/elfA	4·1	O8:H19
ehaA/ecpA/elfA	2·7	O178:H19; ONT:H7
lpfA$_{O113}$/hcpA/elfA	2·7	O8:H19
ehaA/sab/lpfA$_{O113}$/hcpA/elfA	1·4	O79:H19
ehaA/sab/lpfA$_{O113}$/hcpA/elfA/cdt-V	1·4	O20:H19
ehaA/sab/lpfA$_{O113}$/elfA	1·4	O79:H19
ehaA/sab/hcpA/elfA	1·4	ONT:H19
ehaA/lpfA$_{O113}$	1·4	O113:H21
ehaA/lpfA$_{O113}$/ecpA/hcpA	1·4	ONT:H21
ehaA/lpfA$_{O113}$/hcpA/elfA/cdt-V	1·4	O22:H8
ehaA/hcpA/elfA	1·4	O88:H21
lpfA$_{O113}$/ecpA/elfA	1·4	O8:H16

STEC, Shiga toxin-producing *Escherichia coli.*

73 strains studied (Table 2) and 34 when also previously examined virulence genes were taken into account. The combination *ehaA lpfA$_{O113}$ elfA* was the most prevalent (32·9%), followed by *ehaA elfA* (12·3%) and *ehaA lpfA$_{O113}$ hcpA elfA* (8·2%). Intraserotype differences in regard to the virulence profiles were observed in all serotypes with more than one isolate. Five isolates (6·8%) had a different combination of five virulence factors of the eight examined and one isolate belonging to O20:H19 harboured six virulence factors (plus five previously detected ones).

The strains studied here, with the exception of one belonging to O157:H7, lack intimin encoded by the *eae* gene, which is considered an important virulence factor. However, different LEE-negative serotypes are also associated with human disease (reviewed in Bettelheim 2007), and it has been suggested that these strains may compensate for this lack by a multiple adherence mechanism. Our study showed different adhesin profiles that are not linked to one specific serotype and that all the analysed isolates possess besides *stx* genes, some adherence genes and several ones contained also multiple toxin genes. The simultaneous production of several of these factors could play a synergistic role during the colonization of the host intestinal epithelium and the biofilm formation.

The results of the present work alert the presence of genes coding for additional adhesins and *cdt-V* toxin harboured in different combinations by LEE-negative STEC strains that occur in foods, and this traits could increase their pathogenic potential.

Materials and methods

Bacterial strains

Seventy-two non-O157:H7 and one O157:H7 STEC isolates belonging to a larger collection of the Laboratorio de Inmunoquímica y Biotecnología (UNCPBA, Tandil, Argentina) were investigated. They had been collected between 1998–2003 in the Pampeana region, Argentina, from minced meat, hamburgers and viscera (Parma *et al.* 2000; Sanz *et al.* 2007). The isolates belonged to 25 serotypes and have been previously analysed by PCR for the presence of genes encoding for Shiga toxin 1 and 2 (*stx1* and *stx2*), intimin (*eae*), enterohaemolysin (*ehxA*), STEC autoagglutinating adhesin (*saa*) and subtilase cytotoxin (*subA*) (Parma *et al.* 2000; Blanco *et al.* 2004; Lucchesi *et al.* 2006; Sanz *et al.* 2007; Granobles Velandia *et al.* 2011).

Detection of genes and virulence profiles

The DNA template was obtained by boiling frozen bacteria suspended in sterile water for 10 min. All the genes were amplified using monoplex PCRs. The primers used for identification of gene sequences related to *ehaA* and *lpfA$_{O113}$* were those proposed by Wu *et al.* (2010); to *sab*, those from Herold *et al.* (2009); to *ecpA* and *elfA*, those from Hernandes *et al.* (2011); to *hcpA*, those from Xicohtencatl-Cortes *et al.* (2007); to *sfpA*, those from Brunder *et al.* (2001) and the ones used for *cdt-V*, those proposed by Cergole-Novella *et al.* (2007). Each PCR was carried out in a 25 μl volume containing, 1× PCR buffer (50 m mol l^{-1} KCl, 10 m mol l^{-1} Tris pH9, 0·1% Triton X-100), 2 m mol l^{-1} $MgCl_2$, 200 μ mol l^{-1} of each dNTP, 25 pmol of each primer for *sab, lpfA$_{O113}$, hcpA, elfA* and *sfpA* genes, 5 pmol of each primer for *ehaA, ecpA* and *cdt-V* genes, 0,5 U Taq and 2·5–4 μl (depending on the gene) of crude DNA extract. After an initial denaturation step of 5 min at 94°C, the samples were subjected

to 30 cycles (*ehaA, lpfA*$_{O113}$, *ecpA, hcpA, elfA, sfpA* and *cdt-V*) or 35 cycles (*sab*), each one consisting of 30 s or 60 s (*cdt-V*) at 94°C (denaturing), 30 s at 48°C (*hcpA*), 55°C (*ehaA, lpfA*$_{O113,}$ *ecpA*), 54°C (*sab*), 60°C (*elfA*, *sfpA*) or 60 s at 50°C (*cdt-V*) (annealing) and 60 s at 72°C (extension). The reaction was completed with a final extension step of 10 min at 72°C. Amplification products were resolved by electrophoresis on 2% agarose gels stained with SYBR Safe (Invitrogen, Carlsbad, CA, USA).

Taking into account the combinations of the genes detected in the present study, virulence profiles were defined.

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