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OCCURRENCE OF SUBTILASE CYTOTOXIN AND RELATION WITH OTHER VIRULENCE FACTORS IN VEROCYTOTOXIGENIC *ESCHERICHIA COLI* ISOLATED FROM FOOD AND CATTLE IN ARGENTINA

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ABSTRACT

We investigated the presence of the gene of subtilase cytotoxin (SubAB), described in certain highly virulent verocytotoxigenic *E. coli* strains, in isolates from Argentina and its relation with other virulence factors. The gene *subA* was present in *eae*-negative strains mostly associated with *saa*, *vt2* and *ehxA* genes.

Key words: subtilase; VTEC; megaplasmid; verotoxin

Verocytotoxigenic *Escherichia coli* (VTEC) are a diverse group of *E. coli* strains characterized by the production of verotoxins (VT1 and/or VT2) which are regarded as their main virulence factors (6). VTEC are an important cause of gastrointestinal disease in humans (7, 12, 17) and life-threatening complications such as haemolytic uraemic syndrome (HUS).

More recently, it has been reported that some VTEC strains also produce another toxin called subtilase cytotoxin (SubAB). It was identified by Paton *et al.* (19) from an *E. coli* O113:H21 strain, which was responsible for an outbreak of HUS in South Australia in 1998, and since then has been detected in several other VTEC serotypes (5, 8, 14, 19, 20). Furthermore, Tozzoli *et al*. (25) found the first evidence that SubAB can also be produced by *vt*-negative *E. coli* isolated from cases of childhood diarrhoea. SubAB is encoded in the megaplasmid, and is the prototype of a new family of AB5 toxins comprising a single 35 kD A subunit which is a subtilase-like serine protease and a pentamer of B subunits, which mediates binding to glycolipid receptors on the target cell surface (19, 24).

SubAB was shown to be cytotoxic to Vero cells and lethal for mice, causing extensive microvascular thrombosis as well as necrosis in the brain, kidney, and liver (11, 19). The extreme cytotoxicity of this toxin for eukaryotic cells is due to a specific single-site cleavage of the essential endoplasmic reticulum chaperone BiP/GRP78 which is a master regulator of endoplasmic reticulum function (21). Its cleavage by subtilase cytotoxin represents a previously unknown trigger for cell death.

The cytotoxin SubAB has been described in certain highly virulent VTEC strains which are negative for the locus of enterocyte effacement (LEE), but the global distribution of SubAB-encoding VTEC strains is unknown. Furthermore, non-O157 VTEC including LEE negative strains predominate in Argentina, where HUS prevalence is the highest in the world.

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Consequently, our aim was to investigate the presence of the *subA* gene in strains isolated from various sources in Argentina. The relation with other VTEC virulence factors and with the serotype was also evaluated.

A total of 95 strains were selected from a well characterized, previously described strain collection of our Laboratory. To assess potential associations among *subA*, other virulence factors and serotypes, we selected representative strains taking into account the presence of vt_1, vt_2, *eae*, *saa*, and *ehxA* genes determined in previous studies (15, 16, 22). In the present work, a previously described multiplex PCR, which detects *subA*, vt_1 and vt_2, was used for *subA* screening (20). Samples were obtained by boiling a dilution 1:25 of the bacterial culture for 10 min. Amplification products were visualized in 2% agarose gels, stained with ethidium bromide.

We found that 21 VTEC strains of diverse origins were positive for *subA* gene. They belonged to serotypes O2:H5, O20:H19, O39:H49, O79:H19, O88:H21, O113:H21, O141:H7, O141:H8, O178:H19. In O non-typable strains, *subA* was detected among those expresing H7, H8 and H19. To our knowledge this is the first time *subA* gene is found in VTEC serotypes O2:H5, O20:H19, O79:H19, O88:H21, O141:H7 and O141:H8. Within serotypes O20:H19, O113:H21, O141:H8, O178:H19 and ONT:H19 both *subA*-negative and *subA*–positive isolates were found (Table 1), in agreement with the reports of authors, such as Cergole-Novella *et al*. (1), Newton *et al*. (13) and Irino *et al*. (3).

In addition to SubAB, VTEC can present other megaplasmid-encoded virulence factors such as an enterohaemolysin (Ehx), considered as an indicator of megaplasmid presence, and the STEC autoagglutinating adhesin (Saa), only present in LEE-negative VTEC strains (2, 4, 9, 18, 23, 26). In our study, most of the *sub*A-positive strains were also *ehx*A-positive with the exception of a strain belonging to O2:H5 serotype, positive for *subA* and negative for *ehxA*. We found two combinations among subtilase-positive strains: *subA*+/*ehxA*+/*saa*+ (20 strains) and *subA*+/*ehxA*-/*saa*+ (only one strain). Although subtilase gene was always detected in *saa*-positive strains, strains positive for *saa* were not necessarily *subA*-positive. Paton & Paton (20) and Karama *et al*. (5) also found some subtilase negative strains among *saa*-positive VTEC.

We have previously reported several *saa* variants among VTEC strains in our collection (10). Subtilase gene was found in strains carrying *saa* variants 1, 2, 3, 4 or 5, and therefore there was no evident association between *saa* variants and the presence/absence of *subA*.

As we expected, *subA* was found in *eae*-negative strains (which were screened for the presence of all known *eae* variants). These results are in accordance with those of Paton & Paton (20), Osek (14), Khaitan *et al*. (8), Cergole-Novella *et al*. (1), Karama *et al*. (5) and Irino *et al*. (3). However, Newton *et al*. (13) reported one VTEC strain harboring both *subA* and *eae* genes.

All *subA*-positive VTEC isolates carried vt_2 either alone or with vt_1 gene. Of 21 VTEC strains that were tested positive for *subA*, 14 strains had the gene encoding the vt_2 toxin only and 7 strains were positive for both vt_1 and vt_2. Paton & Paton (20) demonstrated a strong association between the presence of *subA* and VTEC carrying the vt_2 gene only, although they found a few VTEC strains that were *subA*-positive vt_2-negative. Osek (14), Karama *et al.* (5) and Irino *et al*. (3) also found one vt_1-positive vt_2-negative VTEC isolate which was *subA*-positive.

The association of *subA* with vt_2 and *ehxA*, in addition to the described potential of SubAB to augment clinical manifestations of VTEC infection or to cause disease in its own right, highlights the risk of some LEE-negative VTEC.

In summary, our results reveal the presence of subtilase cytotoxin gene in Argentine VTEC isolates belonging to several non-O157:H7 serotypes, and the existence of different combinations of megaplasmid encoded factors, confirming once again the great genetic variability of these plasmids.

Table 1. Presence of *subA* and other virulence genes in the selected Argentine VTEC strains.

		Presence or absence of indicated gene					
Serotype	**Source (n° of isolates)**	***subA***	***vt*$_1$**	***vt*$_2$**	***eae***	***ehxA***	***saa***
O2:H5	S (1)	+	-	+	-	-	+
O2:H25	F (1)	-	-	+	-	-	-
O5:H-	C (1)	-	+	-	+	+	-
O8:H16	B (2); F (1)	-	+	-	-	-	+
O15:H21	F (1)	-	-	+	-	-	-
O20:H19	F (1); H (1); S (1)	+	+	+	-	+	+
O20:H19	G (1)	+	-	+	-	+	+
O20:H19	C (1)	-	+	+	-	+	+
O20:H19	C (1)	-	+	+	-	-	-
O20:H19	H (1)	-	-	+	-	-	-
O22:H8	H (2)	-	+	+	-	+	+
O22:H8	B (1)	-	-	+	-	-	-
O25:H19	F (1)	-	-	+	-	+	-
O26:H11	C (5)	-	+	-	+	+	-
O26:H11	C (2)	-	-	+	+	+	-
O39:H49	S (1)	+	+	+	-	+	+
O39:H49	S (4)	+	-	+	-	+	+
O74:H28	S (1)	-	-	+	-	+	+
O79:H19	S (1)	+	-	+	-	+	+
O88:H21	H (1)	+	+	+	-	+	+
O91:H21	F (2); G (1); H (1)	-	-	+	-	+	+
O103:H-	C (1)	-	+	+	+	+	-
O113:H21	B (1); G (1); V (1)	+	-	+	-	+	+
O113:H21	F (2); H (1)	-	-	+	-	-	-
O116:H21	B (1); G (1)	-	-	+	-	+	+
O120:H19	F (1)	-	-	+	-	+	+
O141:H7	S (1)	+	+	+	-	+	+
O141:H8	G (1)	+	-	+	-	+	+
O141:H8	G (1)	-	+	+	-	+	+
O145:H-	F (5); S (1)	-	-	+	+	+	-
O145:H-	F (4)	-	+	-	+	+	-
O145:H-	F (1)	-	+	-	+	-	-
O146:H21	F (1)	-	-	+	+	-	-
O157:H7	F (4); H (1)	-	-	+	+	+	-
O174:H21	B (2); C (1); F (4); S (2)	-	-	+	-	-	-
O174:H21	S (1)	-	+	+	-	+	+
O174:H21	F (1)	-	+	-	-	-	-
O175:H8	F (2)	-	-	+	-	-	-
O177:H-	F (1)	-	-	+	+	+	-
O178:H19	B (1)	+	-	+	-	+	+
O178:H19	B (1); F (2)	-	-	+	-	-	-
ONT:H7	B (1)	+	-	+	-	+	+
ONT:H8	H (1)	+	+	+	-	+	+
ONT:H19	B (1)	+	-	+	-	+	+
ONT:H19	B (3)	-	-	+	-	+	+
ONT:H19	B (1)	-	+	+	-	+	+
ONT:H21	V (1)	-	-	+	-	+	+
ONT:H21	F (1)	-	+	+	-	+	+
ONT:HNT	H (1)	-	-	+	-	+	+

Source: S (cattle at slaughterhouse), F (feedlot cattle), C (calf), B (ground beef), H (hamburger), G (grazing cattle), V (evisceration tray).

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REFERENCES

1. Cergole-Novella, M.C.; Nishimura, L.C.; dos Santos, L.F.; Irino, K.; Vaz, T.M.I.; Bergamini, A.M.M.; Guth, B.E.C. (2007). Distribution of virulence profiles related to new toxins and putative adhesins in Shiga toxin-producing *Escherichia coli* isolated from diverse sources in Brazil. *FEMS Microbiol. Lett*. 274, 329-334.
2. Friedrich, A.W.; Borell, J.; Bielaszewska, M.; Fruth, A.; Tschäpe, H.; Karch, H. (2003). Shiga toxin 1c-producing *Escherichia coli* strains: phenotypic and genetic characterization and association with human disease. *J. Clin. Microbiol*. 41, 2448-2453.
3. Irino, K.; Vieira, M.A.; Gomes, T.A.; Guth, B.E.; Naves, Z.V.; Oliveira, M.G.; dos Santos, L.F.; Guirro, M.; Timm, C.D.; Pigatto, C.P.; Farah, S.M.; Vaz, T.M. (2010). Subtilase cytotoxin-encoding subAB operon found exclusively among Shiga toxin-producing *Escherichia coli* strains. *J. Clin. Microbiol.* 48, 988-990.
4. Jenkins, C.; Perry, N.Y.; Cheasty, T.; Shaw, D.J.; Frankel, G.; Dougan, G.; Gunn, G.J.; Smith, H.R.; Paton, A.W.; Paton, J.C. (2003). Distribution of the *saa* gene in strains of Shiga toxin-producing *Escherichia coli* of human and bovine origins. *J. Clin. Microbiol*. 41, 1775-1778.
5. Karama M.; Johnson, R.P.; Holtslander, R.; McEwen, S.A.; Gyles, C.L. (2008). Prevalence and characterization of verotoxin-producing *Escherichia coli* (VTEC) in cattle from an Ontario abattoir. *Can. J. Vet. Res*. 72, 297–302.
6. Karmali, M.A.; Petric, M.; Lim, C.; Fleming, P.C.; Arbus, G.S.; Lior, H. (1985). The association between idiopathic hemolytic syndrome and infection by verotoxin-producing *Escherichia coli*. *J. Infect. Dis.* 151, 775-782.
7. Karmali, M.A. (1989). Infection by verotoxin-producing *Escherichia coli*. *Clin. Microbiol. Rev*. 2, 15-38.
8. Khaitan, A.; Jandhyala, D.M.; Thorpe, C.M.; Ritchie, J.M.; Paton, A.W. (2007). The operon encoding SubAB, a novel cytotoxin, is present in Shiga toxin-producing *Escherichia coli* isolates from the United States. *J. Clin. Microbiol.* 45, 1374-1375.
9. Kumar, H.S.; Karunasagar, I.; Teizou, T.; Shima, K.; Yamasaki, S. (2004). Characterization of Shiga toxin-producing *Escherichia coli* (STEC) isolated from seafood and beef. *FEMS Microbiol. Lett.* 233, 173-178.
10. Lucchesi, P.M.A.; Krüger, A.; Parma, A.E. (2006). Distribution of *saa* gene variants in verocytotoxigenic *Escherichia coli* isolated from cattle and food. *Res. Microbiol*. 157, 263-266.
11. Morinaga, N.; Yahiro, K.; Matsuura, G.; Watanabe, N.; Moss, J.; Noda, M. (2007). Two distinct cytotoxic activities of subtilase cytotoxin produced by Shiga-toxigenic *Escherichia coli. Infect. Immun*. 75, 488-496.
12. Nataro, J.P.; Kaper, J.B. (1998). Diarrheagenic *Escherichia coli*. *Clinical Microbiol. Rev*. 11, 142-201.
13. Newton, H.J.; Sloan, J.; Bulach, D.M.; Seemann, T.; Allison, C.C.; Tauschek, M.; Robins-Browne, R.M.; Paton, J.C.; Whittam, T.S.; Paton, A.W.; Hartland, E. L. (2009). Shiga toxin–producing *Escherichia coli* strains negative for Locus of Enterocyte Effacement. *Emerging Infect. Dis.* 15, 372-380.
14. Osek, J. (2006). Identification of the subtilase cytotoxin gene among Shigatoxigenic *Escherichia coli* isolated from different sources. *Bull. Vet. Inst. Pulawy* 50, 29-33.
15. Padola, N.L.; Sanz, M.E.; Blanco, J.E.; Blanco, M.; Blanco, J.; Etcheverría, A.I.; Arroyo, G.H.; Usera, M.A.; Parma, A.E. (2004). Serotypes and virulence genes of bovine Shigatoxigenic *Escherichia coli* (STEC) isolated from a feedlot in Argentina. *Vet. Microbiol*. 100, 3-9.
16. Parma, A.E.; Sanz, M.E.; Blanco, J.E.; Blanco, J.; Viñas, M.R.; Blanco, M.; Padola, N.L.; Etcheverría, A.I. (2000). Virulence genotypes and serotypes of verotoxigenic *Escherichia coli* isolated from cattle and foods in Argentina. Importance in public health. *Eur. J. Epidemiol.* 16, 757-762.
17. Paton, J.C.; Paton, A.W. (1998). Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. *Clin. Microbiol. Rev*. 11, 450-479.
18. Paton, A.W.; Srimanote, P.; Woodrow, M.C.; Paton, J.C. (2001). Characterization of Saa, a novel autoagglutinating adhesin produced by locus of enterocyte effacement-negative Shiga toxigenic *Escherichia coli* strains that are virulent for humans. *Infect. Immun*. 69, 6999-7009.
19. Paton, A.W.; Srimanote, P.; Talbot, U.M.; Wang, H.; Paton, J.C. (2004). A new family of potent AB5 cytotoxins produced by Shiga toxigenic *Escherichia coli*. *J. Exp. Med*. 200, 35-46.
20. Paton, A.W.; Paton, J.C (2005). Multiplex PCR for direct detection of Shiga toxigenic *Escherichia coli* producing the novel subtilase cytotoxin. *J. Clin. Microbiol*. 43, 2944-2947.
21. Paton, A.W.; Beddoe, T.; Thorpe, C.M.; Whisstock, J.C.; Wilce, M.C.J.;

Rossjohn, J.; Talbot, U.M.; Paton, J.C. (2006). AB5 subtilase cytotoxin inactivates the endoplasmic reticulum chaperone BiP. *Nature* 443, 548-552.

22. Sanz, M.E.; Villalobo, C.; Elichiribehety, E.; Arroyo, G. (2007). Prevalencia de *Escherichia coli* verotoxigenico en productos cárnicos de la ciudad de Tandil. *La Ind. Cárnica Lat.* 146, 56-58.
23. Srimanote, P.; Paton, A.W.; Paton, J.C. (2002). Characterization of a novel type IV pilus locus encoded on the large plasmid of locus of enterocyte effacement-negative Shiga-toxigenic *Escherichia coli* strains that are virulent for humans. *Infect. Immun*. 70, 3094-3100.
24. Talbot, U.M.; Paton, J.C.; Paton, A.W. (2005). Protective immunization of mice with an active-site mutant of subtilase cytotoxin of Shiga toxin-producing *Escherichia coli*. *Infect. Immun.* 73, 4432-4436.
25. Tozzoli, R.; Caprioli, A; Cappannella, S.; Michelacci, V.; Marziano, M.L.; Morabito, S. (2010). Production of the Subtilase AB5 cytotoxin by Shiga toxin-negative *Escherichia coli. J. Clin. Microbiol*. 48, 178–183.
26. Zweifel, C.; Blanco, J.E.; Blanco, M.; Blanco, J.; Stephan, R. (2004). Serotypes and virulence genes of ovine non-O157 Shiga toxin-producing *Escherichia coli* in Switzerland. *Int. J. Food Microbiol*. 95, 19-27.