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## Prevalence of subtilase cytotoxin in verocytotoxin-producing isolated from humans and raw meats in Belgium

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Abstract: Recently, subtilase cytotoxine (SubAB) was detected in verocytotoxin-producing Escherichia coli (VTEC) that do not carry the Locus of Enterocyte Effacement (LEE) pathogenicity island. The distribution of the subA gene in VTEC isolated from patients with the hemolytic uremic syndrome, patients with diarrheal disease and raw meats from ruminants and wildlife in Belgium was investigated with PCR. The subA gene was detected more frequently ( $\chi^2=10.2$ ; d.f.=1; P=0.001) in VTEC from raw meats (10 of 87 strains) than in those from humans (8 of 274 strains), and never in serogroups O157, O26, O103, O111 and O145. This virulence marker could play a role in the development of HUS after infection with LEE-negative VTEC but was only found in one O178:H19 isolate out of 36 HUS-associated VTEC strains.

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**Prevalence of subtilase cytotoxin in verocytotoxin-producing *Escherichia coli* isolated from humans and raw meats in Belgium**

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## Abstract

1 Recently, subtilase cytotoxine (SubAB) was detected in verocytotoxin-producing *Escherichia coli* (VTEC) that  
2  
3 do not carry the Locus of Enterocyte Effacement (LEE) pathogenicity island. The distribution of the *subA* gene  
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5 in VTEC isolated from patients with the hemolytic uremic syndrome, patients with diarrheal disease and raw  
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7 meats from ruminants and wildlife in Belgium was investigated with PCR. The *subA* gene was detected more  
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## Keywords

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19 Verotoxin, Shigatoxin, subtilase, prevalence, meat, food  
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## Introduction

1 Verocytotoxin-producing *Escherichia coli* (VTEC) cause a broad range of human illness ranging from mild  
2 diarrhea to severe disease, such as hemorrhagic colitis and the hemolytic uremic syndrome (HUS) [1]. While  
3 over 200 different VTEC serotypes have been described (<http://www.microbionet.com.au/vtetable.htm>),  
4 serotypes O157:H7/H-, O26:H11/H-, O103:H2/H-, O111:H8/H-, and O145:H28/H- have been most frequently  
5 associated with outbreaks and severe pathology. Although the bulk of VTEC virulence may be attributed to their  
6 ability to produce one or more types of verocytotoxins (VT), multiple accessory virulence factors that may play a  
7 role in VTEC pathogenesis have been described. One of the most prominent accessory virulence components is  
8 the Locus of Enterocyte Effacement (LEE) [1]. This pathogenicity island contains the genetic determinants that  
9 induce specific attachment and effacement lesions following the destruction of the microvillous brush border and  
10 actin rearrangement at the site of bacterial attachment. While LEE-positive VTEC serotypes such as those  
11 mentioned above are much more frequently associated with outbreaks and HUS, LEE-negative VTEC have also  
12 been implicated in some outbreaks and serious disease [2]. This suggests that additional factors may also  
13 contribute to the virulence potential of VTEC strains.

14 In 2004, Paton *et al.* described the presence of a new AB<sub>5</sub> cytotoxine, called subtilase cytotoxine (SubAB), in  
15 VTEC O113:H21 strain 98NK2, which was responsible for a small HUS outbreak in Adelaide, Australia [3].  
16 This LEE-negative strain was positive for VT2, enterohemolysin (*ehxA*), the STEC auto-agglutinating adhesin  
17 (*saa*) and SubAB. In 98NK2, SubAB is transcribed from two cotranscribed genes, *subA* and *subB*, located on the  
18 large conjugative virulence plasmid pO113. This may facilitate the transfer of the SubAB operon to other VTEC  
19 serotypes and other bacterial pathogens. In vivo experiments in mice have shown that intraperitoneal injection of  
20 purified SubAB is lethal, resulting in widespread microvascular injury, thrombosis and necrosis in brain, kidneys  
21 and liver. The striking resemblance between the pathology caused by SubAB in mice, and VT in humans  
22 suffering from HUS raises the question whether SubAB directly contributes to human disease, particularly in  
23 LEE-negative VTEC. Previous studies have shown the presence of *subA* in VTEC isolated from humans,  
24 animals and food sources [3-10]. However, prevalence data for this putative virulence marker are scarce. In  
25 Australia, Paton reported 11% of VTEC isolates recovered from primary fecal cultures to be positive for *subA*,  
26 whereas in the United States only approximately 2% of non-O157 strains were positive for this marker [8,10].  
27 Tozzoli *et al.* reported the presence of *subA* in *E. coli* isolates negative for VT and other diarrhea-associated  
28 virulence genes, while Irino *et al.* only found *subA* in VTEC when testing a large collection of well-characterized  
29 diarrheagenic *E. coli* [5,9].

In this study, we investigated the prevalence of *subA* in VTEC isolates from patients with HUS and gastrointestinal disease as compared to isolates from raw meats in order to determine its potential role in human disease and its spread in food.

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## Materials and methods

1 PCR was used to investigate the prevalence of *subA* and other virulence factors – *vtx1*, *vtx2*, *eaeA*, *ehxA* and *saa*  
2 – in 114 consecutively isolated VTEC strains recovered from human stool samples submitted for routine culture  
3 in our laboratory during 2008 and 2009 [8,11,12]. Moreover, we analyzed the distribution of these virulence  
4 factors in 160 stored VTEC strains representing the serogroups isolated in our laboratory, in all (N=25) available  
5 non-O157 VTEC isolates from HUS patients and in 87 VTEC isolates recovered from raw meats of ruminants  
6 and wildlife between 1991 and 2009, most being part of a study published in 1997 [13].  
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## Results and discussion

**Prevalence of subtilase cytotoxin.** The distribution of the VTEC serotypes, source of isolation and the virulence factors are shown in tables 1 and 2. Overall the *subA* gene was carried by 18 (4,9%) of the 361 strains. None of the *subA*-positive strains possessed the *eaeA* gene or belonged to serogroups O157, O26, O103, O111, O145. However, they were all positive for *ehxA* and *saa*.

**Human isolates.** Eight (2,9%) of 274 human isolates in this study were *subA*-positive (see table 1). In the group of 114 consecutively isolated human VTEC, *subA* was only detected in 3 OX183:H18 isolates out of 91 non-O157 (3.3%) but in none of the 23 O157 isolates. Among the 160 stored VTEC strains from our collection, 5 (3.1%) carried *subA*: 2 O113:H21, 1 O174:H28, 1 O178:H19 and 1 OX183:H18. All 5 were positive for *vtx2*, *ehxA* and *saa*, and lacked *eaeA*. Twenty-four out of 25 non-O157 isolates from HUS patients were negative for *subA*.

**Clinical data.** The O178:H19 isolate was recovered from a 76-year-old HUS patient, one OX183:H18 from a patient with bloody diarrhea while two other OX183:H18 and one O113:H21 were associated with uncomplicated diarrhea. Clinical data for the remaining 3 *subA*-positive strains are lacking.

**Raw meat isolates.** Ten (11,5%) out of the 87 meat isolates, all non-O157, carried the *subA* gene (see table 2). They were all positive for *ehxA* and *saa*. Seven carried *vtx2* as their only toxin type, two carried *vtx1* as well as *vtx2* and one was only positive for *vtx1*. The *subA*-positive strains originated from beef (1 Orough:H8, 1 Orough:Hunt, Ount:H18, 1 Ount:H21, 1 O73:Hunt and 1 Ount:Hunt), antelope (1 O65:Hunt, 1 O91:HNM), roe deer (1 O91:H14) and wild boar (1 O77:Hunt).

**Statistical analysis** revealed a significant difference in the frequencies of several genes between VTEC recovered from humans and from raw meats. Whereas *subA* ( $\chi^2=10.2$ ; d.f.=1; P=0.001) and *saa* ( $\chi^2=28.2$ ; d.f.=1; P<0.0001) were significantly more frequent in VTEC from raw meats, *eaeA* ( $\chi^2=89.7$ ; d.f.=1; P<0.0001) and *ehxA* ( $\chi^2=33.0$ ; d.f.=1; P<0.0001) were more frequent in human VTEC strains. Among the 131 VTEC serotypes in this study (85 among the human isolates and 58 among the raw meats isolates), only 12 (9.1%) occurred in both groups. Of these, only two meat isolates, O91:H14 and 1 O91:HNM, respectively, were identified as carriers of *subA*, but none of their human counterparts were positive for *subA* and *saa*, indicating that these strains are not clonally related.

**In conclusion**, our study shows that the prevalence of *subA* was low in VTEC from humans in Belgium, and significantly higher in VTEC recovered from raw meats. The significantly higher frequency of *saa*-positive VTEC among the raw meat isolates suggests that this adherence factor may be of greater importance for



1 attachment in the gut of animals than in humans [14]. The detection of *subA* in one VTEC O178:H19 recovered  
2 from an HUS patient suggests that this virulence factor could play a role in the development of HUS at least in  
3 some LEE-negative VTEC strains, as already indicated for O113:H21 [2].  
4

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13  
14 Paris, France (3 & 4 December 2009).  
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#### 17 **Conflict of interest**

18 The authors declare that they have no conflict of interest.  
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**Table 1: Distribution of virulence factors in VTEC isolated from humans**

Serogroup <sup>a,b</sup>	H-type <sup>c,d</sup>	Total	Source (no. of strains)					Virulence factors (no. of strains positive for)					
			HUS	Bloody diarrhea	Diarrhea	Other	Not available	<i>vtx1</i>	<i>vtx2</i>	<i>eaeA</i>	<i>ehxA</i>	<i>saa</i>	<i>subA</i>
O1	H7 (1)	1	1					0	1	0	0	0	0
O2	H6 (3)	3	1		2			0	3	2	0	0	0
O3	HNM (2)	2				1	1	0	2	0	2	2	0
O4	H16 (1); HNM (1)	2			2			1	1	1	1	0	0
O5	HNM (2)	2			2			2	0	1	2	0	0
O6	H10 (1)	1			1			1	0	0	0	0	0
O8	H9 (2); H14 (2)	4			4			0	4	0	0	0	0
O15	HNM (1)	1			1			1	1	0	0	0	0
O20	H4 (1)	1			1			1	0	0	1	0	0
O24	H10 (1)	1			1			1	0	0	0	0	0
O26	H11 (38); HNM (13)	51	3	6	28	4	10	47	6	51	45	0	0
O38	H26 (2)	2			1	1		2	2	0	2	0	0
O45	HNM (1)	1			1			0	1	1	0	0	0
O55	H12 (1); H29 (1)	2				1	1	2	0	0	0	2	0
O63	H6 (3)	3			3			0	3	3	1	0	0
O76	H19 (1)	1			1			1	0	0	1	0	0
O78	HNM (1)	1			1			1	0	0	1	0	0
O79	H14 (2)	2			2			2	0	0	0	0	0
O80	HNM (2)	2			2			0	2	2	2	0	0
O84	H28 (2); HNM (1)	3			3			3	0	3	3	0	0
O87	Hunt (1)	1				1		0	1	0	0	0	0
O91	H14 (1); HNM (4)	5			2	1	2	4	4	0	3	0	0
O98	HNM (1)	1			1			1	0	1	1	0	0
O103	H2 (15)	14	1		9	4		13	1	13	13	0	0
O104	H2 (1)	1			1			0	1	0	0	0	0
O109	H5 (1)	1				1		1	0	0	0	0	0
O111	H8 (11); HNM (5)	16	3		11	1	1	16	6	16	16	0	0
O111ac	HNM (2)	2		1	1			2	0	2	2	0	0
O113	H4 (1); H21 (2); HNM (2)	5			3	2		1	5	0	3	2	2
O115	HNM (1)	1			1			1	0	1	1	0	0
O117	H7 (2)	2			2			2	0	0	0	0	0
O118	H16 (2); H25 (1); HNM (1); Hunt (1)	5	1	1	2		1	4	1	5	4	0	0
O121	HNM (3)	3	3					0	3	3	3	0	0
O127	H40 (1)	1			1			1	0	1	0	0	0
O128ab	H2 (1); HNM (2)	3			2	1		2	3	0	2	0	0
O128ac	HNM (1)	1			1			0	1	1	0	0	0
O132	H34 (1)	1			1			0	1	1	0	0	0
O136	H20 (1)	1			1			1	0	0	0	0	0
O145	H16 (1); H28 (3); HNM (12)	16	8	1	3	3	1	6	10	16	16	0	0
O146	H21 (9); H28 (1)	10		1	7	2		7	8	0	9	0	0
O147	HNM (1)	1			1			0	1	1	1	0	0
O150	HNM (1)	1				1		1	0	1	1	0	0
O153	H19 (1); HNM (1)	2			1		1	1	1	2	1	0	0
O156	H12 (1)	1					1	1	0	1	1	0	0
O157	H7 (31); HNM (13)	44	11	9	20	1	3	15	44	44	43	0	0
O162	HNM (1)	1				1		0	1	0	0	0	0
O165	H25 (1)	1					1	0	1	1	1	0	0
O166	HNM (1)	1					1	1	1	0	1	0	0
O168	Hunt (1)	1			1			0	1	1	0	0	0
O171	H29 (1)	1			1			0	1	0	0	0	0
O172	HNM (2)	2	2					0	2	2	2	0	0
O174	H21 (1); H28 (1)	2			1	1		0	2	0	1	1	1
O176	HNM (1)	1			1			1	1	0	1	0	0
O177	H11 (1)	1			1			1	0	1	0	0	0
O178	H19 (1)	1	1					0	1	0	1	1	1
O181	H40 (1)	1			1			1	0	0	0	1	0
OX182	H34 (1)	1			1			0	1	1	0	0	0
OX183	H18 (4)	4		1	2	1		3	3	0	4	4	4
Ount	H10 (1); H21 (1); HNM (1); Hunt (3)	6		1	5			4	4	2	2	0	0
Ount	H14 (1); HNM (5); Hunt (5)	11			9	2		7	5	6	6	1	0
NT	NT	14	1	1	12			10	10	6	12	1	0

<sup>a</sup>Ount: O antigen untypable, <sup>b</sup>NT: not typed, <sup>c</sup>HNM: not motile, <sup>d</sup>Hunt: H antigen untypable

**Table 2: Distribution of virulence factors in VTEC isolated from raw meats**

Serogroup <sup>a,b</sup>	H-type <sup>c,d</sup>	Source (no. of strains)	Total	Virulence factors (no. of strains positive for)					
				<i>vtx1</i>	<i>vtx2</i>	<i>eaeA</i>	<i>ehxA</i>	<i>saa</i>	<i>subA</i>
O2	Hunt (1)	Veal (1)	1	0	1	0	1	0	0
O3	Hunt (1)	Blesbok (1)	1	1	1	0	0	0	0
O7	H1 (1); Hunt (1)	Deer (1); veal (1)	2	0	2	0	1	0	0
O8	Hunt (2)	Lamb (2)	2	0	2	0	0	1	0
O9	H21 (1)	Beef (1)	1	0	1	0	0	0	0
O15	H21 (1); H27 (1); Hunt (1)	Beef (1); deer (1); lamb (1)	3	2	2	0	0	0	0
O21	H21 (1)	Deer (1)	1	0	1	0	0	0	0
O25	Hunt (1)	Fallow deer (1)	1	1	1	0	1	0	0
O26	H11 (2)	Beef (2)	2	2	0	2	2	0	0
O32	H45 (1)	Roe deer (1)	1	0	1	0	0	0	0
O55	Hunt (3)	Veal (3)	3	3	0	0	1	3	0
O65	Hunt (1)	Antelope (1)	1	0	1	0	1	1	1
O73	Hunt (1)	Beef (1)	1	0	1	0	1	1	1
O74	H52 (1)	Wild boar (1)	1	1	0	0	0	0	0
O76	H19 (1)	Lamb (1)	1	1	0	0	1	0	0
O77	H8 (1); Hunt (1)	Deer (1); wild boar (1)	2	1	2	0	1	1	1
O87	H16 (2)	Antelope (2)	2	0	2	0	0	0	0
O91	H14 (1); H21 (1); HNM (4); Hunt (2)	Beef (2); lamb (2); pheasant (1); roe deer (2); wild boar (1)	8	5	7	0	7	5	2
O103	Hunt (2)	Beef (1); Roe deer (1)	2	2	0	2	2	0	0
O107	H3 (1)	Beef (1)	1	0	1	0	0	0	0
O110	H31 (1)	Roe deer (1)	1	0	1	0	0	0	0
O112	H21 (1)	Antelope (1)	1	1	0	0	0	0	0
O123	H10 (1)	Roe deer (1)	1	1	0	0	1	0	0
O128ab	H2 (1); H10 (1), HNM (1)	Roe deer (2); veal (1)	3	3	2	0	2	0	0
O136	Hunt (1)	Beef (1)	1	0	1	0	0	0	0
O145	H28 (1); HNM (2)	Beef (2); wild boar (1)	3	1	2	3	3	0	0
O146	H28 (2); Hunt (1)	Deer (2); lamb (1)	3	1	3	0	1	0	0
O156	Hunt (1)	Beef (1)	1	0	1	0	0	0	0
O165	HNM (1)	Beef (1)	1	1	1	1	1	0	0
O172	Hunt (1)	Beef (1)	1	1	1	0	0	0	0
O182	H25 (2)	Beef (2)	2	2	0	2	1	0	0
O"54071"	H7 (1)	Pigeon (1)	1	1	0	0	1	0	0
Orough	H7 (1); H8 (2); H12 (1); H16 (2); HNM (1); Hunt (2)	Antelope (1); beef (5); lamb (2); veal (1)	9	4	6	0	3	4	2
Ount	H2 (1); H7 (1); H8 (4); H18 (1); H19 (1); H21 (2); H25 (1); H49 (1); Hunt (10)	Beef (13); blesbok (1); deer (2); ostrich (1); partridge (1); veal (3); wild boar (1)	22	14	14	1	7	6	3

<sup>a</sup>Ount: O antigen untypable, <sup>b</sup>NT: not typed, <sup>c</sup>HNM: not motile, <sup>d</sup>Hunt: H antigen untypable