Subtilase Cytotoxin-Coding Genes in Verotoxin-Producing *Escherichia coli* Strains from Sheep and Goats Differ from Those from Cattle[▽]

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Subtilase cytotoxin (SubAB) from verotoxin (VT)-producing *Escherichia coli* (VTEC) strains was first described in the 98NK2 strain and has been associated with human disease. However, SubAB has recently been found in two VT-negative *E. coli* strains (ED 591 and ED 32). SubAB is encoded by two closely linked, cotranscribed genes (*subA* and *subB*). In this study, we investigated the presence of *subAB* genes in 52 VTEC strains isolated from cattle and 209 strains from small ruminants, using PCR. Most (91.9%) VTEC strains from sheep and goats and 25% of the strains from healthy cattle possessed *subAB* genes. The presence of *subAB* in a high percentage of the VTEC strains from small ruminants might increase the pathogenicity of these strains for human beings. Some differences in the results of PCRs and in the association with some virulence genes suggested the existence of different variants of *subAB*. We therefore sequenced the *subA* gene in 12 strains and showed that the *subA* gene in most of the *subAB*-positive VTEC strains from cattle was almost identical (about 99%) to that in the 98NK2 strain, while the *subA* gene in most of the *subAB*-positive VTEC strains from small ruminants was almost identical to that in the ED 591 strain. We propose the terms *subAB1* to describe the SubAB-coding genes resembling that in the 98NK2 strain and *subAB2* to describe those resembling that in the ED 591 strain.

Verotoxin (VT)-producing *Escherichia coli* (VTEC), also referred to as Shiga toxin-producing *E. coli*, can cause diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS) in humans (9). VTEC produces two major toxin types, VT1 and VT2, as well as different variants (5, 6). Ruminants represent an important reservoir for VTEC and a source of infection for humans (3, 10–12). VT production alone is not sufficient to cause VTEC-related disease (1); the *eae* gene affects the virulence of VTEC and is required for the production of attaching and effacing lesions in the intestinal mucosa (1). In addition to the *eae* gene, a novel cytotoxin called subtilase cytotoxin (SubAB) might also contribute to the virulence of *eae*-negative VTEC strains in humans (17). It has been suggested previously that this toxin may be able to augment the effects of VT or to cause pathology in its own right (14, 15). SubAB was first described in the VTEC O113:H21 strain 98NK2, associated with an outbreak of HUS in Australia (17). SubAB in the 98NK2 strain is encoded by two closely linked, cotranscribed genes (*subA* and *subB*), located on a large, conjugative virulence plasmid designated pO113 (17). This plasmid also contains other putative virulence genes such as *saa* and *ehxA*, which encode an autoagglutinating adhesin and a hemolysin called EHEC (enterohemorrhagic *E. coli*) enterohemolysin, respectively (16–18). *subAB* genes have subsequently been identified only in VTEC strains (2, 14). However, SubAB has

recently been identified in two VT-negative *E. coli* strains (ED 591 and ED 32) isolated from unrelated cases of childhood diarrhea (21). The ED 591 and ED 32 strains were positive for the *tia* gene, a genetic determinant of invasion previously described in enterotoxigenic *E. coli* (4), but were negative for the *saa* gene (21). In both strains, *subAB* genes were located close to the *tia* gene (21).

A limited number of studies have investigated the presence of *subAB* in VTEC from healthy cattle (2, 7, 8), but to the best of our knowledge, the presence of these genes in VTEC from diarrheic calves and small ruminants has not been studied. In the present study, we therefore examined the distribution of *subAB* in a large collection of VTEC strains isolated from healthy and diarrheic cattle, sheep, and goats to determine their association with ruminant VTEC strains.

MATERIALS AND METHODS

Bacterial strains. A total of 261 VTEC strains were used in this study. All strains were isolated between 1993 and 2005 in Spain from healthy cattle (36 strains), sheep (60 strains), and goats (145 strains) and from calves (16 strains), lambs (3 strains), and goat kids (1 strain) with diarrhea. Most of these strains have previously been serotyped and tested for *vt* types and for the presence of the *eae*, *ehxA*, and *saa* genes (3, 6, 10–13). Only one strain per animal was included in the study.

Detection of virulence genes by PCR. One colony from each VTEC strain was harvested after overnight culture, suspended in sterile water, incubated at 100°C for 10 min, and centrifuged. The supernatant was used for PCR. Primer pairs RTsubABF/RTsubABR, SubHCDF/SubSCDR, and tia_lo/tia_sense were used to detect *subAB*, *subA*, and *tia* genes, respectively. The 16S rRNA housekeeping gene was used as an internal amplification control. Primer sequences and PCR conditions are given in Table 1.

Sequencing of the *subA* gene. One bovine *saa*-positive O113 VTEC strain, one bovine O8 VTEC strain, one caprine *saa*-positive O91:H14 VTEC strain, and nine VTEC strains that were considered representative of the most frequent serotypes of VTEC from sheep and goats (O5:HNM [NM indicates nonmotile], O76:H19, O81:H21, O91:HNM, O128:HNM, O146:H21, O166:H28, ONT [NT

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TABLE 1. PCR primers and conditions used for detection of *subAB* and *tia* genes and for amplification of the *subA* gene

Target gene	Primer	Nucleotide sequence (5′–3′)	Annealing conditions	Reference
subAB	RTsubABF	GCAGATAAATACCCTTCACTTG	55°C, 60 s	17
subAB	RTsubABR	ATCACCAGTCCACTCAGCC	55°C, 60 s	17
subA	SubHCDF	TATGGCTTCCCTCATTGCC	50°C, 60 s	14
subA	SubSCDR	TATAGCTGTTGCTTCTGACG	50°C, 60 s	14
tia	tia_lo	TCCATGCGAAGTTGTTATCA	56°C, 60 s	21
tia	tia_sense	TTCTCTTTTTACCCTGCTTTTTGC	56°C, 60 s	21
subA	Sub-F1	CAATACGGCGCTCTGTTGACG	60°C, 60 s	This study
subA	Sub-F2	AACATATTGACCAGCAATAC	60°C, 30 s; 50°C, 30 s	This study
subA	Sub-R	AAACATGCCATCCCGGGCATC	60°C, 60 s, with Sub-F1; 60°C, 30 s, and 50°C, 30 s, with Sub-F2	This study
16S rRNA	16SF	CATTGACGTTACCCGCAGAA	56°C, 60 s	19
16S rRNA	16SR	CGCTTTACGCCCAGTAATTCC	56°C, 60 s	19

indicates nontypeable]:HNM, and ONT:H21) were used to sequence *subA*. The sequence of the whole *subA* gene was obtained by PCR amplification using two different sets of primer pairs (Sub-F1 and Sub-R for the *saa*-positive strains and Sub-F2 and Sub-R for the *saa*-negative strains) (Table 1). The PCR products were purified using the SpinPrep PCR cleanup kit (Novagen, Darmstadt, Germany), and DNA was sequenced at the Sequencing Unit of CIB-CSIC (Madrid, Spain) and Secugen S.L. (Madrid, Spain). The VECTOR NTI program was used to align the sequences of these strains, which were compared with those of the prototype 98NK2 and ED 591 strains.

Production and detection of SubAB in CHO cells. Bacteria were grown for 20 h in Luria-Bertani medium at 37°C. Cultures were then disrupted by sonication at 4°C, and the lysates were centrifuged (15,700 × *g*) for 5 min at 4°C. In

TABLE 2. Serotypes and virulence genes in VTEC strains isolated from cattle[a]

Source and serotype	No. of strains	*vt* genotype (no. of strains)	No. of strains positive for:					
			subAB1	*subAB2*	*saa*	*tia*	*ehxA*	*eae*
Healthy cattle								
O2:HND[b]	1	*vt2* (1)	1	0	1	0	0	0
O4:HND	1	*vt2* (1)	0	0	0	1	0	0
O5:HNM[c]	1	*vt1* (1)	0	0	0	0	1	1
O8:HND	4	*vt2* (4)	0	2	0	2	0	0
O21:HND	1	*vt2* (1)	0	0	1	0	1	0
O22:HND	3	*vt2* (1), *vt1* + *vt2* (2)	0	0	2	1	3	0
O23:HND	1	*vt1* + *vt2* (1)	0	0	1	0	1	0
O74:HND	1	*vt2* (1)	0	0	1	0	1	0
O84:H2	1	*vt1* (1)	0	0	0	0	1	1
O87:HND	1	*vt1* (1)	0	0	0	0	0	0
O91:HND	1	*vt2* (1)	0	0	1	1	1	0
O98:HNM	1	*vt1* (1)	0	0	0	0	1	1
O103:H8	1	*vt1* (1)	0	0	0	0	0	1
O110:HND	1	*vt1* (1)	0	0	0	0	0	0
O111:HNM	2	*vt1* + *vt2* (2)	0	0	0	0	2	2
O113:HND	4	*vt2* (4)	3	0	3	1	3	0
O116:HND	1	*vt2* (1)	1	0	1	0	1	0
O136:HND	1	*vt1* (1)	0	0	0	1	0	0
O172:HNM	1	*vt2* (1)	0	0	0	0	0	1
O174:HND	2	*vt2* (2)	0	0	0	2	1	0
O175:HND	1	*vt2* (1)	0	0	0	1	0	0
ONT[d]:HND	5	*vt1* (2), *vt2* (3)	2	0	4	0	4	0
Diarrheic calves								
O4:HND	1	*vt2* (1)	0	0	0	1	0	0
O5:HNM	1	*vt1* (1)	0	0	0	0	1	1
O23:HND	1	*vt1* + *vt2* (1)	0	0	1	0	1	0
O26:HNM	2	*vt1* (2)	0	0	0	0	2	2
O26:H11	3	*vt1* (3)	0	0	0	1	2	3
O39:HND	1	*vt1* (1)	0	0	0	0	0	0
O91:HND	2	*vt2* (2)	0	0	0	0	1	0
O113:HND	1	*vt2* (1)	0	0	0	1	0	0
O128:H16	1	*vt1* (1)	0	0	0	0	1	1
O128:HND	1	*vt1* (1)	0	0	1	0	1	0
O171:HND	1	*vt2* (1)	0	0	0	0	0	0
ONT:HND	1	*vt1* (1)	0	0	0	1	0	0

[a] Data for the serogrouping/serotyping, *vt* genotype, and possession of *saa*, *ehxA*, and *eae* genes were reported from previous studies (6, 10, 11, 13).
[b] ND, not determined.
[c] NM, nonmotile.
[d] NT, nontypeable.

TABLE 3. Serotypes and virulence genes in VTEC strains isolated from sheep[a]

Source and serotype	No. of strains	*vt* genotype (no. of strains)	No. of strains positive for[b]:			
			subAB2	*tia*	*ehxA*	*eae*
Healthy sheep						
O5:HNM[c]	13	*vt1c* (7), *vt1c + vt2* (6)	13	3	13	0
O6:H10	3	*vt1c* (3)	0	0	0	0
O21:H21	1	*vt1c + vt2* (1)	1	1	0	0
O26:H11	1	*vt1 + vt2* (1)	0	0	1	1
O71:HNM	1	*vt1c + vt2* (1)	1	1	0	0
O75:H8	1	*vt1c + vt2* (1)	1	1	1	0
O91:HNM	6	*vt1 + vt2* (5), *vt1c + vt2* (1)	6	6	0	0
O128:HNM	6	*vt1c* (3), *vt1c + vt2* (3)	5	5	0	0
O128:H16	1	*vt1c* (1)	1	1	0	0
O141:HNM	1	*vt1c* (1)	1	1	1	0
O146:H21	8	*vt1c + vt2* (8)	8	8	8	0
O163:HNM	1	*vt1 + vt2* (1)	1	0	1	0
O163:H11	1	*vt1 + vt2* (1)	1	1	0	0
O166:HNM	1	*vt1c + vt2* (1)	1	1	1	0
O166:H28	4	*vt1c* (2), *vt1c + vt2* (2)	4	0	4	0
ONT[d]:HNM	9	*vt1c* (3), *vt1c + vt2* (6)	9	8	3	0
ONT:H4	2	*vt1c* (2)	2	0	2	0
Diarrheic lambs						
O71:HNM	1	*vt1c + vt2* (1)	1	1	0	0
O110:HNM	1	*vt1c* (1)	0	0	0	0
ONT:HNM	1	*vt1c* (1)	0	0	0	0

[a] Data for the serotyping, *vt* genotype, and possession of *saa*, *ehxA*, and *eae* genes were reported from previous studies (6, 12, 13).
[b] None of the strains analyzed was positive for the *subAB1* and *saa* genes.
[c] NM, nonmotile.
[d] NT, nontypeable.

addition, culture supernatants were obtained by centrifuging at $15,700 \times g$ for 5 min. The sonicated extracts and culture supernatants were tested for SubAB production by cytotoxicity assays in Chinese hamster ovary (CHO) cells, as previously described (17), with minor modifications. Briefly, CHO culture assays were performed on confluent cell monolayers in 96-well plates. A total of 50 μl per well of each undiluted culture supernatant or sonicated extract was added, and cells were incubated at 37°C for 60 min. After incubation, 150 μl of F-12K medium supplemented with 2% fetal calf serum was added per well. The cytopathic effect (characterized by rounding cells and detachment from the substratum) was observed after 3 days of incubation.

Nucleotide sequence accession numbers. The sequences of the *subA* gene from the *subAB2* VTEC strains B218, C20, C36, C91, C103, C137, K93, K95, K300, and K394 have been deposited into GenBank under the accession numbers JN638552 to JN638561, respectively.

RESULTS AND DISCUSSION

A total of 261 VTEC strains from ruminants were examined for the presence of *subAB* genes by PCR using the primer pairs RTsubABF/RTsubABR and SubHCDF/SubSCDR. Primers RTsubABF and RTsubABR are able to amplify a region bridging the end of *subA* and the beginning of *subB* (17, 21), while primers SubHCDF and SubSCDR are specific for two of the three critical functional domains in the SubA-coding sequence (14). The only strain from a diarrheic goat kid, 135 of the 145 (93.1%) strains from healthy goats, 55 of the 60 (91.7%) strains from healthy sheep, one of the three (33.3%) strains from diarrheic lambs, and 9 of the 36 (25%) strains from healthy cattle gave positive reactions with the two primer pairs used to detect the *subAB* genes. In contrast, the *subAB* genes were not detected in any of the 16 strains from diarrheic calves (Tables 2 to 4). In agreement with previous studies (2, 7, 8, 14, 21), *subAB* genes were found only in *eae*-negative VTEC strains. The percentage of *subAB*-positive VTEC strains from healthy

cattle in this study (25%) was identical to that found by Khaitan et al. (8) but lower than those reported by Cergole-Novella et al. (2) and Karama et al. (7) (41.2 and 54.2%, respectively). VTEC strains from sheep and goats have been considered a minor human health hazard (1, 3, 12); however, the presence of *subAB* genes in a high percentage (91.9%) of VTEC strains from small ruminants might increase the pathogenicity of these strains for humans.

Seven of the nine *subAB*-positive VTEC strains from healthy cattle were positive for *vt2* and *saa*, and all but one were also positive for *ehxA* (Table 2). Other studies (2, 7, 8) have also found an association between *subAB* and *vt2*, *ehxA*, and *saa* genes in VTEC strains from cattle. The other two *subAB*-positive VTEC strains from healthy cattle possessed the *vt2* and *tia* genes (Table 2). Although all *subAB*-positive VTEC strains from cattle possessed the *saa* or *tia* gene, 10 *saa*-positive VTEC strains and eight *tia*-positive VTEC strains from cattle were *subAB* negative. The association of *subAB* with *saa* or *tia* is probably due to the possibility that the *subAB* genes may be located on a plasmid which also contains *saa* (17) or on a putative pathogenicity island together with *tia* (21).

A total of 188 of the 192 *subAB*-positive VTEC strains from sheep and goats were *saa* negative. Of these, 133 possessed the *tia* gene and 142 possessed the *ehxA* gene (Tables 3 and 4). The other four *subAB*-positive VTEC strains from small ruminants possessed the *saa* and *tia* genes, and three of these also had the *ehxA* gene (Table 4). Most of the VTEC strains from sheep and goats produced VT1 (alone or in combination with VT2) and possessed the *vt1c* gene (6). Interestingly, the four *subAB*-positive VTEC strains from goats that were also positive for *saa* and *tia* were the only VT1-producing isolates from that

TABLE 4. Serotypes and virulence genes in VTEC strains isolated from goats[a]

Source and serotype	No. of strains	vt genotype (no. of strains)	No. of strains positive for:					
			subAB1	subAB2	saa	tia	ehxA	eae
Healthy goats								
O5:HNM[b]	9	vt1c (2), vt1c + vt2 (7)	0	9	1	2	7	0
O5:H21	1	vt1c + vt2 (1)	0	1	0	0	1	0
O7:H21	1	vt1c (1)	0	0	0	0	0	0
O18:H28	1	vt1c (1)	0	1	0	1	0	0
O58:H21	1	vt1c + vt2 (1)	0	1	0	1	1	0
O64:H21	1	vt1c (1)	0	0	0	0	0	0
O76:HNT[c]	1	vt1c (1)	0	1	0	1	0	0
O76:H19	30	vt1c (30)	0	30	0	30	30	0
O81:HNM	2	vt1c + vt2 (2)	0	2	0	1	0	0
O81:H21	9	vt1c (9)	0	9	0	8	8	0
O87:H38	1	vt1 + vt2 (1)	1	0	1	1	1	0
O91:H14	3	vt1 (3)	3	0	3	3	2	0
O119:H19	1	vt1c (1)	0	0	0	0	1	0
O126:H8	6	vt1c (6)	0	6	0	3	6	0
O128:HNM	2	vt1c (2)	0	2	0	2	1	0
O128:H2	4	vt1c (2), vt1c + vt2 (2)	0	4	0	4	3	0
O128:H19	1	vt1c (1)	0	1	0	1	1	0
O145:H21	1	vt1c (1)	0	0	0	0	0	0
O146:HNM	2	vt1c + vt2 (2)	0	2	0	2	2	0
O146:H21	10	vt1c (2), vt1c + vt2 (8)	0	10	0	10	10	0
O156:H25	1	vt1c (1)	0	0	0	0	1	1
O157:H7	3	vt2 (3)	0	0	0	0	3	3
O166:H28	11	vt1c (9), vt1c + vt2 (2)	0	11	0	4	5	0
O173:H8	1	vt1c (1)	0	1	0	1	0	0
O174:H8	1	vt1c (1)	0	1	0	1	0	0
O175:H38	1	vt2 (1)	0	1	0	1	1	0
ONT:HNM	8	vt1c (3), vt1c + vt2 (5)	0	8	0	7	3	0
ONT:H2	2	vt1c + vt2 (2)	0	2	0	2	2	0
ONT:H4	5	vt1c (5)	0	5	0	0	5	0
ONT:H19	2	vt1c (1), vt1c + vt2 (1)	0	2	0	1	2	0
ONT:H21	20	vt1c (17), vt1c + vt2 (3)	0	18	0	10	18	0
ONT:H28	1	vt1c + vt2 (1)	0	1	0	1	1	0
ONT:HNT	2	vt1c (2)	0	2	0	1	2	0
Diarrheic goat kid								
O166:H28	1	vt1c (1)	0	1	0	0	0	0

[a] Data for the serotyping, vt genotype, and possession of saa, ehxA, and eae genes were reported from previous studies (3, 6, 12, 13).
[b] NM, nonmotile.
[c] NT, nontypeable.

animal species that were negative for the *vt1c* gene (6). Although some of the *subAB*-positive VTEC strains from sheep and goats were negative for *tia* and *saa*, all the strains from small ruminants positive for *tia* and/or *saa* were also positive for *subAB*. As previously mentioned, the presence of *saa* or *tia* in the *subAB*-positive VTEC strains is probably due to a genetic linkage among these genes (17, 21).

Of the 261 VTEC strains included in this study, 21 were positive for *saa* and 151 were positive for *tia*, but only five were positive for both genes (four *subAB*-positive strains from goats and one *subAB*-negative strain from a healthy cow). Tozzoli et al. (21) also observed that the *E. coli* strains positive for the *tia* gene were negative for the *saa* gene and vice versa. These results thus demonstrate a negative association between the *saa* and *tia* genes.

All VTEC strains shown to be positive for *subAB* using the primer pair RTsubABF/RTsubABR were also positive using the primer pair SubHCDF/SubSCDR and vice versa. All *subAB*-positive VTEC strains produced bands of similar intensities with the primer pair RTsubABF/RTsubABR, but the 11

subAB-positive, *saa*-positive VTEC strains gave a more intense band than that of the 190 *subAB*-positive, *saa*-negative strains with the primer pair SubHCDF/SubSCDR (Fig. 1). The differences in intensities of amplicons with the primer pair SubHCF/SubSCDR were due to two nucleotide mismatches and not to differences in the amounts of DNA template (Fig. 1). The differences in PCRs observed with the primer pair SubHCDF/SubSCDR and in the association with the *saa* and *tia* genes suggest that the *subAB* genes in most of the VTEC strains from cattle differed from most VTEC strains from sheep and goats. We therefore sequenced the *subA* gene in 12 strains. The three critical functional domains characteristic of members of the subtilase family (17) were conserved in the predicted SubA sequences of all the strains tested. In addition, the alignment of the *subA* sequences and the translated amino acid sequences from these strains with the corresponding sequences from the prototype 98NK2 and ED 591 strains showed that the two *saa*-positive strains that gave an intense band with the SubHCDF/SubSCDR primer pair demonstrated nucleotide and amino acid homologies of 99.9% and 100%, respec-
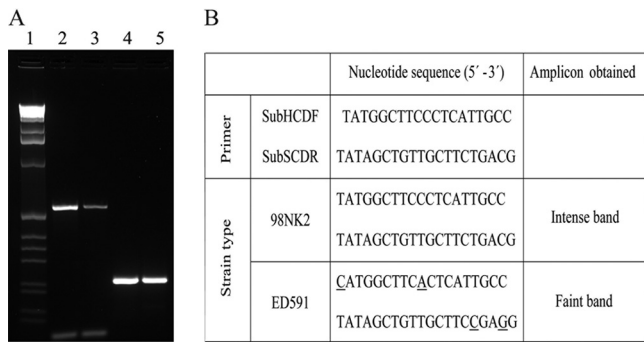
FIG. 1. (A) PCR amplifications of *subA* in B173 (VTEC O113 strain isolated from cattle) and C137 (VTEC O146:H21 strain isolated from sheep) using the primer pair SubHCDF/SubSCDR (lanes 2 and 3, respectively) and of *subAB* in the same strains using the primer pair RTsubABF/RTsubABR (lanes 4 and 5, respectively). Equal amounts of bacterial DNA template were used in each PCR amplification as is shown by the internal 16S rRNA amplification controls in lanes 2 and 3 (100 bp). Lane 1, molecular size marker X (0.07 to 12.2 kbp; Roche, Mannheim, Germany). B173 showed 99.9% homology at the nucleotide level and 100% at the amino acid level with the prototype strain 98NK2. The *subA* gene in C137 showed 99.4% homology at the nucleotide level and 99.8% at the amino acid level with the prototype strain ED 591. (B) Alignment of primers used in PCR loaded in lanes 2 and 3 and counterpart sequence from *subA* of the two reference type strains. The nucleotide mismatches according to the PCR primers used for the amplifications are underlined.

tively, with the 98NK2 strain. The 10 *saa*-negative strains that gave a faint band with the SubHCDF/SubSCDR primer pair demonstrated homologies of 97.8 to 99.9% with the ED 591 strain at the nucleotide level and 97.1 to 100% at the amino acid level. These results therefore demonstrated that the *subA* gene in the strains producing an intense band with the SubHCDF/SubSCDR primer pair was almost identical to that of the 98NK2 strain, while that in the strains producing a faint band was almost identical to that of the ED 591 strain.

SubAB production in the 12 VTEC strains with sequenced *subA* genes (2 98NK2-like and 10 ED 591-like) and in two *subAB*-negative strains was tested in CHO cells, which are susceptible to SubAB but refractory to VT (17). Sonicated extracts and culture supernatants of the *subAB*-positive but not the *subAB*-negative VTEC strains caused a cytopathic effect in CHO cells. SubAB is not secreted very efficiently from live VTEC (J. C. Paton, personal communication); however, we were able to detect SubAB production in undiluted culture supernatants of the bacterial strains.

The *subAB* genes in the 98NK2 and ED 591 strains show a nucleotide sequence homology of 90% (21). In addition, the results of the current study, as well as those of previous studies, showed that the *subAB* genes in *E. coli* strains with *subA* genes almost identical to that in the 98NK2 strain and the *subAB* genes in strains with *subA* genes resembling that in the ED 591 strain have different main hosts and different associated virulence genes. We therefore propose calling the 98NK2-like SubAB-coding genes *subAB1* and the ED 591-like SubAB-coding genes *subAB2*.

SubAB has been detected almost exclusively in VTEC strains to date, but the plasmid carrying *subAB* is capable of conjugative transmission, at least in VTEC O113:H21 (20), and Paton and Paton (15) suggested the potential for wider

dissemination among other *E. coli* pathotypes. In support of this hypothesis, Tozzoli et al. (21) found two VT-negative *E. coli* strains that produced SubAB. VTEC detection in our laboratory has been performed by testing for VT production using the Vero cell cytotoxicity assay in four isolates for each animal sampled. In addition to the 261 VTEC strains included in this study, the presence of the *subAB* genes was examined by PCR in 14 *E. coli* strains negative for the *vt* genes responsible for the cytopathic effect in Vero cells, but none of these was positive for *subAB* (data not shown). Because SubAB has a cytopathic effect in Vero cells (17), the results indicate that SubAB production was restricted to VTEC strains, at least in those strains used in the current study. The results of the present study showed that many of the VTEC strains from small ruminants and two strains from healthy cattle possessed a *subA* gene almost identical to that previously identified in two VT-negative *E. coli* strains producing SubAB (21). The two VTEC strains from healthy cattle belonged to the same serogroup (O8) as did one of the two VT-negative *E. coli* strains found by Tozzoli et al. (21), and it is thus possible that the two VT-negative strains represented strains that have lost their *vt* genes.

subAB genes in cattle were found only in VTEC strains from healthy animals, and the fact that VTEC is rarely found in diarrheic lambs and goat kids (12) suggests that SubAB is not associated with diarrhea in neonatal ruminants.

The PCR results using the primer pair SubHCDF/SubSCDR and the association with virulence genes suggest that all the human SubAB-producing VTEC strains in Australia (14, 17) possess *subAB* genes similar to that in the 98NK2 strain. However, three of the five *subAB*-positive VTEC strains found by Tozzoli et al. (21) in Italy probably possessed *subAB* genes resembling those found in the two VT-negative *E. coli* strains (ED 591 and ED 32) described in that same study. It would therefore be interesting to determine the distribution of the two *subAB* variants in other human VTEC collections and to establish if these SubAB variants display differences in potency and clinical outcome, as is the case for variants and types of VT (5).

In conclusion, the results of this study showed that *subAB* genes are widely distributed among VTEC strains from sheep and goats, suggesting that these strains might be of more pathogenic significance for humans than previously believed. Our data also showed that *subAB*-positive VTEC strains from ruminants possess two variants of the genes, associated with different hosts and virulence genes. We propose the terms *subAB1* and *subAB2* to distinguish between these variants.

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