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Distribution of *saa* gene variants in verocytotoxigenic *Escherichia coli* isolated from cattle and food

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Abstract

The pathogenesis of verocytotoxigenic *Escherichia coli* (VTEC) infection in humans is multifactorial, given that verocytotoxins are the principal virulence factor. Most strains causing serious diseases possess the *eae* gene that encodes the adhesin intimin, but its presence is not essential for virulence as some cases are caused by *eae*-negative strains. An autoagglutinating adhesin designated Saa was found in some *eae*-negative strains. This protein varies in size as a consequence of variation in the number of copies of a 37-aa repeat unit in the C-terminal region. Based on these findings, we designed PCR primers to amplify the region coding for these differences to detect *saa* gene variants present in VTEC strains isolated in Argentina from cattle and meat. The gene *saa* was detected in 36 (31.6%) *eae*-negative strains and 5 variants were found. Strains isolated from cattle possessed 4 *saa* variants, whereas 2 variants were present in isolates from meat. *Saa* variant 1 predominated (18 strains) and was distributed in strains isolated both from cattle and from meat. Our study revealed the existence of two novel *saa* variants, termed 4 and 5, which have a higher number of 111-bp repeats than *saa* genes previously studied.

Keywords: Escherichia coli; STEC autoagglutinating adhesin; Variants; Verocytotoxin; Food; Cattle

1. Introduction

Verocytotoxigenic *Escherichia coli* (VTEC), also called Shiga-toxin-producing *E. coli* (STEC), can cause diarrhea, hemorrhagic colitis and hemolytic–uremic syndrome (HUS) in humans [6,13]. Verocytotoxins (encoded by vt_1 and vt_2 genes) are considered to be the cardinal virulence factors but the pathogenesis of VTEC infection in humans is not fully understood. It is considered to be multifactorial and dependent on several bacterial virulence factors such as enterohemolysin (EHEC-Ehly, encoded by *ehxA* gene) and the *eae* gene, in addition to host factors [2,13]. The *eae* gene encodes intimin, an adhesin that enables an intimate attachment of the bacterium to epithelial cells of the intestine.

Although most strains causing serious cases (those that develop HUS) are *eae*-positive, the presence of this gene is not essential for VTEC virulence [12]. Recently, Paton et al. [11] described a gene, designated *saa*, which encodes an outer membrane protein which appears to function as an autoagglutinating adhesin. The introduction of cloned *saa* confers a semilocalized adherence phenotype upon *E. coli* K-12 strains. Gene s*aa* was originally isolated from the large plasmid (megaplasmid) of an *eae*-negative O113:H21 VTEC strain responsible for an outbreak of HUS in Adelaide, South Australia, in 1998 [12]. Moreover, Srimanote et al. [14] found that the HEp-2 cell adherence conferred by this megaplasmid appears to be largely attributable to *saa*.

This gene is also present in other *eae*-negative VTEC isolates from humans, including strains isolated from sporadic HUS cases, patients with diarrhea and asymptomatic carriers, and in *eae*-negative bovine, ovine and seafood isolates [4,5,7, 17,18].

Furthermore, Paton et al. [11] reported that Saa proteins from diverse VTEC strains showed marked variation in size, due largely to differences in the number of copies of a 37-aa repeat unit in the C-terminal region (corresponding to direct

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111-bp repeats). Interestingly, VTEC strains which carried the shorter *saa* genes exhibited lower adherence to HEp-2 cells.

Based on these findings, we designed PCR primers to amplify the region coding for these differences. We then investigated the distribution of *saa* gene variants in a collection of VTEC strains isolated from cattle and meat in Argentina.

2. Materials and methods

2.1. Bacterial strains

VTEC strains used in this study were isolated in Argentina from 1996 to 2003, from cattle (132 strains) and bovine meat (22 strains), and have been described previously [1,8,9]. When two or more isolations from the same sample did not differ in either one virulence factor or the serogroup, only one of them was analyzed in this study. All *E. coli* strains were routinely grown in Luria–Bertani (LB) medium at 37 °C and stored at −70 °C with 20% glycerol.

2.2. Detection of the saa gene

These strains were tested for the presence of *saa* by PCR with primers SAADF and SAADR [10]. These primers direct the amplification of a 119-bp portion of the *saa* gene which is conserved among diverse VTEC strains, and avoid the region containing the 111-bp repeat sequence.

2.3. Detection of saa gene variants

Two primers (VSAAF: 5′-ACTCGCATAATTGGTGGTG-3′ and VSAAR: 5′-ATCATTGGTATTGCTGTCAT-3′) were designed (PrimerSelect, DNASTAR) to amplify, by PCR, a region containing the repeat sequence (nucleotides 7001–7668 in the sequence deposited in GenBank [accession number AF399919]). Crude DNA extracts were amplified in 50 µl reaction mixtures containing 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 2 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 0.01% (m/v) gelatine, 200 µM each dNTP, 0.5 µM each primer, and 1 U *Taq* DNA polymerase. PCR amplifications were performed as follows: one cycle of denaturation at 94 °C for 120 s, annealing at 52 °C for 60 s, extension at 72 °C for 90 s followed by 30 cycles of denaturation at 94 °C for 60 s, annealing at 52 °C for 60 s, extension at 72 °C for 90 s, and a final extension at 72 °C for 5 min. Reaction products were analyzed in a 1.5% agarose gel with ethidium bromide.

2.4. DNA sequencing

For DNA sequencing, one amplimer of each *saa* variant (obtained with primers VSAAF and VSAAR) was cloned in pCR4-TOPO following manufacturer's instructions (TOPO TA Cloning Kit for Sequencing, Invitrogen). The sequences were determined using dye-terminator chemistry and M13 reverse and T7 promoter universal primers on an Applied Biosystems model 377 automated DNA sequencer. The sequences were analyzed using software available through wEMBOSS interface [15].

3. Results

Forty *eae*-positive and 114 *eae*-negative VTEC strains isolated from cattle and raw meat were screened by PCR for the presence of the *saa* gene. These strains belonged to a broad spectrum of serotypes and most of them had been characterized in previous studies [1,8,9].

Gene *saa* was detected in 36 (31.6%) *eae*-negative VTEC strains, 10 of them isolated from bovine meat and the other 26 from cattle (Table 1). It was not detected in *eae*-positive strains.

Saa-positive strains belonged to serotypes O2:H5, O8:H16, O20:H19, O22:H8, O39:H49, O74:H28, O79:H19, O88:H21, O91:H21, O113:H21, O116:H21, O120:H19, O141:H7, O141:H8, and O174:H21, and 5 strains could not be serogrouped using the current serotyping scheme and belonged to ONT:HNT, ONT:H8, ONT:H16, and ONT:H21.

PCR with primers VSAAF and VSAAR allowed us to amplify the repeat domain in order to study the presence of variants of the *saa* gene. We were able to detect five variants of the gene which were designated 1–5, according to amplimer size (Fig. 1). Furthermore, we sequenced one amplimer of each variant and confirmed that these size differences were due to a different number of copies of the direct 111-bp repeat sequence. Amplimer sizes were 500, 611, 722, 833 and 944 bp.

Saa variants 1, 2, 3 and 4 were detected in VTEC strains isolated from cattle and only two variants (1 and 5) were found in isolations from food (Table 1).

Saa variant 1 was present in 18 strains belonging to serotypes O8:H16, O22:H8, O88:H21, O91:H21, O116:H21, O141:H7, O141:H8, ONT:H8, ONT:H16, ONT:H21 and ONT:HNT. We found variant 2 in isolates of serotypes O20:H19, O74:H28, O79:H19, O116:H21 and O120:H19. Variant 3 was found in isolates which belonged to serotypes O2:H5, O20:H19, and O174:H21, and variant 4 in strains of serotypes O39:H49 and O113:H21. Variant 5 was found in only one strain (serotype O20:H19).

4. Discussion

Gene *saa* was detected in several *eae*-negative VTEC strains, but it was not found in *eae*-positive strains, in agreement with other authors' results [5,7,11,16–18].

Our results showed the presence of several variants of the *saa* gene in VTEC strains isolated in Argentina both from cattle and raw meat. We were able to detect five variants of this gene which differ in the number of repeat units present in the 3′ coding region.

Some of the *saa* variants (1, 2 and 3), with the lowest number of repeat units, corresponded to *saa* genes from strains MW5, B2F1, MW10, 94CR and 97MW1, characterized by Paton et al. [11]. Variant 1 has the same number of repeat units as those present in strains MW5 and B2F1 (one partial repeat and two 111-bp repeats); variant 2 corresponded to the *saa* gene from strain MW10 (one partial repeat and three 111-bp repeats) and variant 3 to the *saa* gene from strains 94CR and 97MW1 (one partial repeat and four 111-bp repeats). Variants 4 and 5 have not been described before and have one and two more

Table 1
Saa-positive strains isolated from cattle and food

Serotype	No. of strains (origin)*	Genotype			*saa* variant
		vt_1	vt_2	*ehxA*	
O2:H5	1 (g)	−	+	−	3
O8:H16	2 (1f, 1b)	+	−	−	1
O20:H19	3 (2g, 1f)	+	+	+	3
O20:H19	1 (g)	−	+	+	2
O20:H19	1 (h)	+	+	+	5
O22:H8	2 (h)	+	+	+	1
O39:H49	1 (g)	+	+	+	4
O39:H49	4 (g)	−	+	+	4
O74:H28	1 (g)	−	+	+	2
O79:H19	1 (g)	−	+	+	2
O88:H21	1 (h)	+	+	+	1
O91:H21	4 (1g, 2f, 1h)	−	+	+	1
O113:H21	2 (1g, 1e)	−	+	+	4
O116:H21	1 (g)	−	+	+	2
O116:H21	1 (b)	−	+	+	1
O120:H19	1 (f)	−	+	+	2
O141:H7	1 (g)	+	+	+	1
O141:H8	1 (g)	+	+	+	1
O141:H8	1 (g)	−	+	+	1
O174:H21	1 (g)	+	+	+	3
ONT:H8	1 (h)	+	+	+	1
ONT:H16	1 (b)	+	−	−	1
ONT:H21	1 (e)	−	+	+	1
ONT:H21	1 (f)	+	+	+	1
ONT:HNT	1 (h)	−	+	+	1

All isolates were *eae*-negative.
* Origin: g, grazing cattle; f, cattle in feedlot; b, ground beef; h, hamburger; e, evisceration tray (at slaughterhouse).

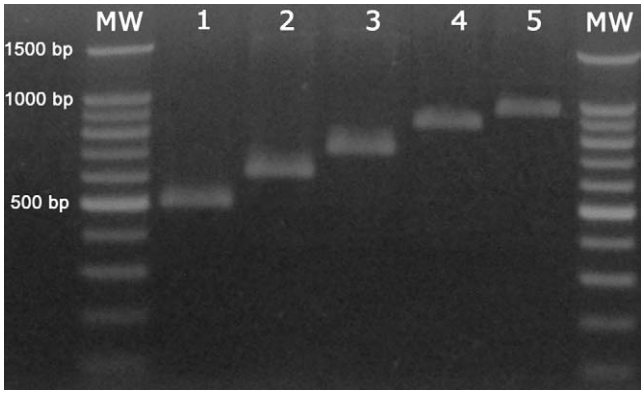

Fig. 1. Agarose gel electrophoresis of PCR products amplified with primers VSAAF and VSAAR. Lanes 1, 2, 3, 4 and 5 correspond to *saa* gene variants 1, 2, 3, 4 and 5, respectively. Lane MW contains DNA molecular size marker (100-bp DNA ladder, Promega, Madison, WI).

copies of the 111-bp repeat sequence than variant 3, respectively.

We did not find any *saa* variant with the same repeat units as the ones present in strain 98NK2 (a shorter partial repeat and four 111-bp repeats) studied by Paton et al. [11].

Variant 1 was detected in strains belonging to several serotypes, four of them being O91:H21, in which Paton et al. [11] have also found this variant. Although Paton et al. [11] could find 3 different *saa* variants in strains from serotype O113:H21, we only found a novel variant (which we called 4) in strains belonging to this serotype.

When we analyzed groups of strains belonging to the same serotype, we noticed that in some of them all the strains were *saa*-positive. This was the case for strains belonging to serotypes O8:H16 (2 strains, all with variant 1), O39:H49 (5 strains, all with variant 4), O91:H21 (4 strains, all with variant 1), O116:H21 (one strain with variant 1 and another one with variant 2), and O141:H8 (2 strains, all with variant 1).

It was interesting that in other groups of strains belonging to the same serotype, some of the strains were *saa*-positive, whereas others were *saa*-negative. This was the situation for strains of serotypes O20:H19 (2 *saa*-negative strains, 5 *saa*-positive strains with variants 2, 3 and 5), O113:H21 (3 *saa*-negative strains, 2 *saa*-positive strains with variant 4) and O174:H21 (9 *saa*-negative strains and one *saa*-positive strain with variant 3).

Furthermore, some *saa*-positive strains of the same serotype could be differentiated by *saa* variant analysis. This occurred in isolates belonging to serotypes O20:H19 and O116:H21.

Almost all *saa*-positive strains were also *ehxA*-positive, as expected, since both genes are encoded in the megaplasmid. Considering isolates belonging to serotypes O20:H19, O113:H21 and O174:H21, we observed that *ehxA*-positive strains were also *saa*-positive, and all *ehxA*-negative strains were also *saa*-negative.

Only 4 strains were *saa*-positive but *ehxA*-negative and belonged to serotypes O2:H5 (1 strain), O8:H16 (2 strains), and ONT:H16 (1 strain). This could suggest a different localization of the gene or, more likely, could be due to the known variabil-

ity of the megaplasmid of VTEC strains [3,14]. Other authors also detected a low proportion of strains that were *saa*-positive and *ehxA*-negative [4,5,17] but a higher proportion of isolates with these characteristics appear in results reported by Zweifel et al. [18].

Among *eae*-negative strains, we observed that all those that were vt_1-positive and vt_2-negative were also *ehxA*-negative, in agreement with results obtained by Zweifel et al. [18]. In addition, we found that all those harboring the *saa* gene corresponded to variant 1.

Our study revealed the existence of 2 novel *saa* variants, termed 4 and 5, which have a higher number of repeat units present in the 3′ coding region than those previously studied. Variant 4 was found in 7 strains isolated from cattle, and variant 5 was found in one strain obtained from raw meat (hamburger). These novel *saa* variants, with a higher number of repeat units, could confer greater adherence to HEp-2 cells, which deserves further investigation.

Our results show that the study of the *saa* gene can be useful for characterizing *eae*-negative strains, as isolations from the same serotype may not only differ in the presence or absence of this gene but could also possess a different variant of *saa*. It is noteworthy that the detection of *saa* variants allowed us to differentiate strains of the same serotype that shared their virulence genes.

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