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Verotoxins in Bovine and Meat Verotoxin-Producing *Escherichia coli* Isolates: Type, Number of Variants, and Relationship to Cytotoxicity[▽]

Alejandra Krüger,* Paula M. A. Lucchesi, and Alberto E. Parma

Laboratorio Inmunoquímica y Biotecnología, Departamento de Sanidad Animal y Medicina Preventiva, Facultad de Ciencias Veterinarias, Universidad Nacional del Centro de la Provincia de Buenos Aires, Pinto 399 (7000), Tandil, Buenos Aires, Argentina

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In this study, we determined *vt* subtypes and evaluated verotoxicity in basal as well as induced conditions of verotoxin-producing *Escherichia coli* (VTEC) strains isolated from cattle and meat products. Most (87%) of the 186 isolates carried a vt_2 gene. Moreover, the vt_2 subtype, which is associated with serious disease, was present in 42% of our VTEC collection. The other *vt* subtypes detected were vt_1, vt_{1d}, vt_{2vha}, vt_{2vhb}, vt_{2O118}, vt_{2d} (mucus activatable), and vt_{2g}. A total of 41 (22%) of the isolates possessed more than one *vt* subtype in its genome, and among them the most frequent combination was vt_1/vt_2, but we also observed multiple combinations among vt_2 subtypes. Differences in verotoxicity titers were found among a selection of 54 isolates. Among isolates with a single vt_2 variant, those carrying the vt_2 subtype had high titers under both uninduced and induced conditions. However, the highest increase in cytotoxicity under mitomycin C treatment was detected among the strains carrying vt_{2vha} or vt_{2hb} variants. Notably, the isolates carrying the vt_1 subtype showed a lesser increase than that of most of the vt_2-positive VTEC strains. Furthermore, the presence of more than one *vt* gene variant in the same isolate was not reflected in higher titers, and generally the titers were lower than those for strains with only one gene variant. The main observation was that both basal and induced cytotoxic effects seemed to be associated with the type and number of *vt* variants more than with the serotype or origin of the isolate.

Verotoxin-producing *Escherichia coli* (VTEC), also known as Shiga toxin-producing *E. coli* (STEC), are important pathogens that can cause severe human diseases, including hemorrhagic colitis and hemolytic-uremic syndrome (HUS) (23, 33). Ruminants, especially bovines, are regarded as the principal reservoir of VTEC strains (22, 34). As VTEC-infected animals do not usually present signs of disease, they are not easily excluded from food production. Therefore, products of bovine origin are important sources of human infection.

The pathogenesis of VTEC in humans is considered to be multifactorial and dependent on several bacterial virulence factors in addition to host factors (8, 41), but none of them is fully understood. Among VTEC factors, verotoxins are considered to be the most critical virulence factor associated with human disease (36). Several variants of the two major types of verotoxin genes, vt_1 and vt_2, have been identified. The vt_1 group is more homogeneous than the vt_2 group, which includes a high number of variants (reviewed by Scheutz and Strockbine [50]).

Previous studies have found that the clinical outcome of VTEC infection depends on the *vt* genotype of the infecting strain. Among vt_2-positive VTEC strains, those harboring the subtypes vt_2 and mucus-activatable vt_{2d} were found to be related to higher virulence and were significantly associated with HUS (4, 6, 14, 42).

On the other hand, VTEC strains harboring vt_{2e} or vt_{2O118} (formerly vt_{2dOunt}) have not been associated with severe disease in humans but represent a significant proportion of VTEC from patients with uncomplicated diarrhea or asymptomatic carriers (4, 14, 21, 45, 53). It has been suggested that vt_{2f} and vt_{2g} genes have minimal links to pathogenicity in humans (3, 14, 28). Nevertheless, more studies are necessary to determine the role of these last vt_2 variants in VTEC infections (44).

It also has been observed that infections by VTEC harboring the vt_1 subtype can cause HUS, while VTEC containing vt_{1c} appear to be associated with either mild disease or asymptomatic carriage (4, 15, 21, 58). Although VTEC harboring vt_{1d} may infect humans, there is no information available about the clinical significance of these strains (27).

The genes encoding verotoxins are carried by bacteriophages. In general, *vt* genes are situated among genes controlled by the late promoter, suggesting that VT production is linked to the induction or progression of the phage lytic cycle (35, 56). Thus, VT phages play an important role in both the expression of VT and lateral gene transfer (31, 51). Köhler et al. (25) observed that certain antibacterials commonly used as growth promoters in animal husbandry can induce the VT phage lytic cycle and therefore may contribute to the spread of VT and the development of new VTEC pathotypes. In this way, it is important to evaluate the extent to which VTEC strains carrying inducible VT phages can be isolated from animals (31).

Despite the fact that VTEC harboring different *vt* variants differ in their association with HUS and that phage variability could play a role in disease outcome, few studies have been performed to date on the level of the expression of *vt* genes and their ability to be induced (12). The aim of this study was to

* Corresponding author. Mailing address: Facultad de Ciencias Veterinarias, Universidad Nacional del Centro de la Provincia de Buenos Aires, Pinto 399 (7000), Tandil, Buenos Aires, Argentina. Phone and fax: 54-2293-439850. E-mail: akruger@vet.unicen.edu.ar.

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TABLE 1. Virtual PCR-RFLP analysis performed with iPCR and REBsites electronic tools[a]

vt gene variant	GenBank accession no.	Predicted size of PCR products[b]	Predicted sizes of restriction fragments[c]	
			HincII	RsaI
vt_1	M19473	898	707, 158, 33	574, 162, 93, 69
vt_{1c}	AJ312232	899	708, 158, 33	354, 221, 162, 93, 69
vt_{1d}	AY170851	897	864, 33	573,162, 93, 69

[a] Predicted sizes of PCR products and restriction fragments for vt_1 and vt_2 variants are indicated in bp.
[b] Calculated with iPCR software (http://www.ch.embnet.org/software/iPCR_form.html).
[c] Calculated using REBsites, a virtual digestion tool (http://tools.neb.com/REBsites).

examine VTEC strains isolated from cattle and meat products for *vt* subtypes and to evaluate verotoxicity in basal as well as in induced conditions.

(Data were presented in part as a poster at the 7th International Symposium on Shiga Toxin [Verotoxin]-Producing *Escherichia coli* Infections, Buenos Aires, Argentina, 10 to 13 May 2009.)

MATERIALS AND METHODS

Bacterial strains and culture conditions. A total of 186 VTEC strains isolated from cattle and from bovine meat in Argentina were investigated (see Table 2). Most of them have been described previously regarding the serotype and presence of vt_1, vt_2, *eae*, and *ehxA* genes (38, 39, 49). The vt_{2g} subtype was also identified in some strains in a previous study (26).

VTEC strains or their DNA that were used as controls for vt_1 (*E. coli* EDL933), vt_{1c} (*E. coli* 6592/02), vt_2 (*E. coli* EDL933), vt_{2c} (*E. coli* E32511), vt_{2vhb} (*E. coli* 1398-152), vt_{2O118} (*E. coli* EH250), vt_{2d} (*E. coli* B2F1), and vt_{2g} (*E. coli* 7v) have been kindly supplied by A.W. Friedrich (Institut für Hygiene, Universitätsklinikum Münster, Germany), P. H. M. Leung (Queen Mary Hospital, The University of Hong Kong, People's Republic of China), J. Blanco (Laboratorio de Referencia de *E. coli*, Spain), and E. López (Hospital de Niños Ricardo Gutiérrez, Buenos Aires, Argentina). Bacteria were routinely grown overnight at 37°C in Luria-Bertani (LB) medium with shaking and stored at −70°C with 20% (vol/vol) glycerol.

vt_1 subtyping. To subtype vt_1 sequences, a PCR-restriction fragment length polymorphism (RFLP) assay was designed. Briefly, an ~900-bp fragment of the gene was amplified in vt_1-positive VTEC by using the Lin5′ and Lin3′ primers (1). The PCR products (10 μl) were digested separately with 10 U of HincII and RsaI. These enzymes were selected to distinguish between vt_1, vt_{1c}, and vt_{1d} subtypes by using the iPCR and Rebsites informatic tools (Table 1).

vt_2 subtyping. The strategy to detect vt_2 subtypes was as follows: all vt_2-positive VTEC were subjected to PCR with the primer pair VT2-c/VT2-d, and amplification products were independently digested with restriction endonucleases HaeIII, RsaI, and NciI to detect vt_2, vt_{2vha}, vt_{2vhb}, vt_{2g}, and/or vt_{2NV206} (2, 26, 55). All isolates were evaluated with the VT2-cm/VT2-f primer set (43) specific for vt_{2O118} (first termed vt_{2d} by Piérard and renamed vt_{2O118} as proposed by Scheutz and Strockbine [50]). The presence of more than one vt_2 subtype in a unique isolate was also analyzed by using the PCR-RFLP method described by Bastian et al. (1).

The presence of the vt_{2d} (mucus-activatable) subtype was evaluated in all vt_2-positive strains using a specific PCR (59).

Cytotoxic activity on Vero cells. We studied 54 VTEC strains belonging to serotypes in which strains harboring different *vt* genotypes were present. The isolates carrying the emergent vt_{2g} variant were also analyzed. A single colony of each VTEC strain was grown in 10 ml of Penassay broth for 5 h at 37°C with shaking. The cultures were then divided into two flasks, and mitomycin C was added to one of them to a final concentration of 0.5 μg/ml. After an overnight growth, cultures were centrifuged (10 min, 12,000 × *g*, 4°C) and supernatants were stored at −20°C.

Vero cells were grown at 37°C in Eagle's minimal essential medium (MEM) supplemented with 10% (vol/vol) fetal calf serum, 100 mg/liter penicillin, 200 mg/liter streptomycin, and 2.2 g/liter $NaHCO_3$ in an atmosphere of 5% CO_2.

Twofold serial dilutions of bacterial supernatants in MEM were done in 96-well plates (100 μl final volume), and 100 μl of MEM containing 4×10^4 freshly trypsinized Vero cells was added to each well. The culture plates were incubated at 37°C in a 5% CO_2 atmosphere. The cell monolayers were fixed and stained with 10% (vol/vol) formaldehyde and 0.2% (wt/vol) crystal violet in phosphate-buffered saline solution. The toxin titer was expressed as the reciprocal of the highest sample dilution that caused ≥50% Vero cells detaching from the plastic after 48 h of incubation. The fold change in verotoxicity of induced cultures compared to that of uninduced cultures was determined for each sample by dividing its mean titer under mitomycin C treatment by its mean titer in uninduced conditions, where I/U fold change = mean induced titer/mean uninduced titer.

Statistical analyses. The data were analyzed with Epi-Info version 6.04a software by the χ^2 test, except for the variable needing the two-tailed Fisher exact test. A P value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Verotoxins are the main virulence factors of VTEC and are essential for many of the pathological features and for some of the severe complications of VTEC infection (36). Several studies have shown that VTEC strains with different *vt* alleles are associated with particular reservoirs (2, 9, 10) and differ markedly in their association with HUS (14, 21). Argentina has a high incidence of HUS (47), but little is known about *vt* subtypes and cytotoxic levels of VTEC isolated from cattle and meat products.

We evaluated *vt* subtypes in a total of 186 native VTEC strains isolated from cattle and beef. The results of *vt* subtyping and verotoxicity are shown in Table 2.

Typing of *vt* genes. In the strains studied, the genes for vt_1 were identified, alone or in association with vt_2 genes, in only 45 (24.2%) VTEC isolates, while vt_2 genes were found, alone or in association with vt_1, in 161 (86.6%) isolates. Indeed, the percentage of VTEC strains harboring vt_2 genes was significantly higher in food VTEC (96.0%) than bovine VTEC (82.8%) ($P = 0.020$).

The PCR-RFLP assay designed to subtype vt_1 genes successfully detected vt_1 variants. Although the primers used in the PCR-RFLP assay also amplify vt_2, the method was able to identify vt_1 variants in strains that also were vt_2 positive. No more than one vt_1 variant was observed in vt_1-positive isolates. The vt_1 gene was the vt_1 predominant subtype and was present in 97.8% (44/45) of vt_1-positive strains. Brett et al. (10) also observed a high percentage of this subtype among vt_1-positive VTEC isolated from cattle. We found only one isolate with an RFLP pattern characteristic of the vt_{1d} subtype, but none of the VTEC strains carried vt_{1c}.

Subtypes vt_2, vt_{2vha}, and vt_{2vhb} were detected (alone or in combination with vt_1 or other vt_2 subtypes) among 49.1, 18.0, and 42.7% of the vt_2-positive isolates, respectively. Other investigators also have observed that these variants are commonly present among bovine VTEC strains in Argentina (30). Bertin et al. (2) also identified vt_2, vt_{2vha}, and vt_{2vhb} subtypes (39.0, 25.5, and 39.5%, respectively) in VTEC isolates from healthy cattle in France, but they also found the vt_{2NV206} variant (14.5%), which was absent from our VTEC collection. In Australia, Brett et al. (9) found that bovine VTEC isolates predominantly possessed vt_2 and vt_{2vhb} (81.1 and 39.3%, respectively). In accordance with those studies, we found a low prevalence (less than 5%) of vt_{2O118}.

TABLE 2. Verotoxin gene (*vt*) subtyping and verotoxicity results

Serotype[a]	No. (origin)[b]	*vt* genotype[c]	*eae*[d]	*ehxA*[d]	*saa*[d]	Verotoxicity classification	
						Uninduced condition[e]	Induced condition[f]
O2:H−	1 (h)	vt_{2vhb}	−	−	−		
O2:H5	1 (a)	vt_2	−	−	+		
O2:H25	1 (f)	$\underline{vt_{2g}}$	−	−	−	C	I
O5:H−	4 (c)	vt_1	+	+	−		
O5:H27	1 (c)	vt_1	+	+	−		
O8:H16	3 (2b, 1f)	vt_1	−	−	+		
O8:H19	4 (b)	$\underline{vt_2}$	−	+	−		
O15:H21	1 (f)	$\underline{vt_{2g}}$	−	−	−	A	I
O20:H7	2 (a)	$vt_1\ vt_{2vhb}$	−	−	−		
O20:H19	2 (a, h)	$vt_1\ vt_2$	−	+	+	C	II
O20:H19	1 (f)	$vt_1\ vt_2$	−	+	+	C	III
O20:H19	1 (c)	$\underline{vt_1\ vt_{2vhb}}$	−	+	+	C	II
O20:H19	1 (c)	$\underline{vt_1\ vt_{2vhb}}$	−	−	−	B	II
O20:H19	1 (g)	vt_2	−	+	+	C	III
O20:H19	1 (h)	vt_{2vhb}	−	−	−	B	III
O20:H?	1 (c)	vt_1	+	+	−		
O22:H8	2 (h)	$vt_1\ vt_2$	−	+	+		
O22:H8	1 (b)	vt_2	−	−	−		
O25:H19	1 (f)	$\underline{vt_2}$	−	+	−		
O26:H11	3 (c)	vt_1	+	+	−	C	I
O26:H11	2 (c)	vt_2	+	+	−	C	III
O26:H11	2 (c)	vt_1	+	+	−	C	III
O38:H39	1 (c)	vt_1	+	+	−		
O39:H49	1 (a)	$vt_1\ vt_2$	−	+	+		
O39:H49	4 (a)	vt_2	−	+	+		
O74:H28	1 (a)	$vt_2\ vt_{2vhb}$	−	+	+		
O79:H19	1 (a)	$\underline{vt_{2vhb}}$	−	+	+		
O88:H21	1 (h)	$vt_1\ vt_2$	−	+	+		
O91:H21	2 (f)	vt_2	−	+	+	C	III
O91:H21	1 (h)	$\underline{vt_2\ vt_{2vhb}}$	−	+	+	B	I
O91:H21	1 (g)	$\underline{vt_{2vhb}}$	−	+	+	B	I
O103:H−	1 (c)	$vt_1\ vt_2$	+	+	−	C	III
O103:H−	1 (a)	$vt_2\ vt_{2vhb}$	+	+	−		
O103:H2	1 (f)	vt_1	+	+	−		
O103:H2	1 (c)	$vt_1\ vt_2$	+	+	−		
O111:H−	1 (c)	vt_1	+	+	−		
O112:H2	1 (b)	$vt_{2vhb}\ vt_{2O118}$	−	−	−		
O113:H−	1 (b)	$vt_2\ vt_{2vha}$	−	−	−		
O113:H21	1 (e)	vt_2	−	+	+	B	II
O113:H21	1 (b)	vt_2	−	+	+	C	III
O113:H21	1 (g)	$vt_2\ vt_{2vha}\ vt_{2vhb}$	−	+	+	C	III
O113:H21	2 (h, f)	$\underline{vt_{2vha}}$	−	−	−	A	II
O113:H21	1 (f)	$\underline{vt_{2vha}}$	−	−	−		
O116:H21	2 (b, g)	vt_2	−	+	+		
O117:H7	5 (1a, 1g, 2h, 1f)	vt_{2vha}	−	−	−		
O117:H7	3 (f)	vt_{2vhb}	−	−	−		
O118:H16	1 (c)	vt_1	+	+	−		
O120:H19	1 (f)	vt_2	−	+	+		
O141:H7	1 (a)	$vt_1\ vt_2$	−	+	+		
O141:H8	1 (g)	$vt_1\ vt_2$	−	+	+		
O141:H8	1 (g)	vt_2	−	+	+		
O145:H−	1 (f)	vt_1	+	+	−		
O145:H−	1 (f)	vt_1	+	+	−	A	I
O145:H−	1 (f)	vt_1	+	−	−	A	I
O145:H−	2 (f)	vt_1	+	+	−	B	I
O145:H−	4 (3f, 1a)	vt_2	+	+	−	C	III
O145:H−	1 (f)	vt_2	+	−	−	C	III
O145:H−	2 (f)	vt_2	+	+	−		
O146:H−	1 (f)	vt_2	+	+	−		
O146:H21	1 (f)	vt_2	+	−	−		
O157:H7	5 (4f, 1h)	$vt_2\ vt_{2vha}$	+	+	−	C	III
O157:H7	1 (f)	$vt_2\ vt_{2vha}$	+	+	−	B	I
O162:H7	1 (h)	vt_{2vha}	−	−	−		
O165:H−	1 (c)	$vt_2\ vt_{2vha}$	+	+	−		
O168:H8	1 (a)	vt_{2vhb}	−	−	−		

Continued on following page

TABLE 2—*Continued*

Serotype[a]	No. (origin)[b]	*vt* genotype[c]	*eae*[d]	*ehxA*[d]	*saa*[d]	Verotoxicity classification	
						Uninduced condition[e]	Induced condition[f]
O171:H−	3 (f)	vt_{2vhb}	−	−	−		
O171:H−	1 (b)	vt_{2vhb} vt_{2O118}	−	−	−		
O171:H2	2 (f)	vt_{2vha}	−	−	−		
O171:H2	1 (a)	$\underline{vt_{2vha}}$ vt_{2O118}	−	−	−		
O171:H2	2 (a)	$\underline{vt_{2vhb}}$ vt_{2O118}	−	−	−		
O171:H2	7 (1b, 1a, 5f)	vt_{2vhb}	−	−	−		
O171:H?	1 (b)	vt_2 vt_{2vhb} vt_{2O118}	−	−	−		
O174:H21	1 (f)	vt_1	−	−	−	B	II
O174:H21	1 (a)	vt_1 vt_2 vt_{2vhb}	−	+	+	A	I
O174:H21	1 (a)	vt_{2vha} vt_{2vhb}	−	−	−	B	II
O174:H21	1 (a)	vt_{2vhb}	−	−	−	A	I
O174:H21	1 (f)	vt_{2vhb}	−	−	−	B	III
O174:H21	1 (f)	vt_{2vhb}	−	−	−	A	III
O174:H21	1 (f)	vt_{2vhb}	−	−	−	A	II
O174:H21	4 (1c, 2b, 1f)	vt_{2vhb}	−	−	−		
O175:H8	2 (f)	$\underline{vt_{2g}}$	−	−	−	A	I
O177:H−	2 (f)	vt_{2vha}	+	+	−		
O177:H−	1 (c)	vt_{2vhb}	+	+	−		
O178:H19	1 (b)	vt_2	−	+	+	B	III
O178:H19	2 (1b, 1f)	vt_{2vha}	−	−	−	B	III
O178:H19	1 (f)	vt_{2vhb}	−	−	−	A	II
O185:H7	1 (b)	vt_{2vhb}	−	−	−		
ONT:H−	1 (b)	vt_1 vt_2 vt_{2vhb} vt_{2O118}	−	+	−		
ONT:H−	15 (6a, 9f)	vt_2	−	−	−		
ONT:H−	1 (f)	vt_{2vhb}	−	−	−		
ONT:H7	2 (b)	vt_{2vha}	−	−	−		
ONT:H7	4 (3b, 1h)	vt_{2vhb}	−	−	−		
ONT:H7	1 (b)	$\underline{vt_{2vhb}}$	−	+	+		
ONT:H8	1 (a)	vt_{1d}	−	−	−		
ONT:H8	1 (h)	vt_1 vt_2	−	+	+		
ONT:H19	1 (b)	vt_1 vt_2	−	+	+		
ONT:H19	1 (b)	vt_2	−	+	+		
ONT:H19	2 (b)	$\underline{vt_{2vhb}}$	−	+	+		
ONT:H19	1 (b)	vt_{2vhb}	−	+	+		
ONT:H21	1 (f)	vt_1 vt_2	−	+	+		
ONT:H21	1 (e)	vt_2	−	+	+		
ONT:H21	1 (c)	vt_{2vha} vt_{2vhb}	−	−	−		
ONT:H21	10 (3a, 1c, 3b, 3f)	vt_{2vhb}	−	−	−		
ONT:H21	1 (f)	vt_{2vhb}	−	+	−		
ONT:HNT	1 (b)	vt_{2vhb}	−	−	−		
ONT:HNT	1 (h)	vt_2 vt_{2vhb}	−	+	+		
O157:H7	Reference strain[g]	vt_1 vt_2	+	+	−	C	III

[a] Isolates presenting the same serotype and virulence profile (in regard to *vt* genotype and the presence/absence of *eae* and *ehxA* genes) have been isolated from humans with bloody diarrhea and HUS in Argentina (47, 48) are in boldface.
[b] a, cattle at abattoir; b, ground beef; c, calf; e, evisceration tray (at abattoir); f, cattle in feedlot; g, grazing cattle; h, hamburger.
[c] Underlined areas indicate the mucus-activatable genotype.
[d] Presence of vt_1, vt_2, *ehxA*, and *eae* genes was determined by multiplex PCR analysis (40).
[e] Mean titers were classified in three categories: A, ≤ 16; B, 17 to 255; C, ≥256.
[f] Mean titers were classified in three categories: I, ≤4,096; II, 4,097 to 131,071; III, ≥131,072.
[g] The control strain was *E. coli* EDL933.

The vt_{1c} and vt_{2O118} subtypes have been associated previously with isolates from sheep (10, 24, 45). The absence of vt_{1c} and the low prevalence of vt_{2O118} in VTEC isolated from bovine sources in our study support the hypothesis formulated by Brett et al. (9) that different populations of VTEC inhabit the gastrointestinal tracts of cattle and sheep. Moreover, Ramachandran et al. (45) suggested that lambdoid phages carrying different vt_2 subtypes lysogenize distinct *E. coli* populations, which may be determined by their serotype. According to our results, there is no stringent relationship between *vt* genotype and serotype in bovine VTEC, as there were strains of the same serotype (O20:H19, O91:H21, O113:H21, O117:H7, and O174:H21) harboring different vt_2 variants. Other authors also described several *vt* genotypes among O91:H21 and O113:H21 VTEC isolates from Argentina (16).

Of the vt_2 subtypes found in our VTEC collection, all except vt_{2g} were present in isolates from both bovine and meat sources. Moreover, there were no significant differences in the distribution of vt_2 variants according to the origin of the isolate (cattle or meat) (vt_2, $P = 0.4377$; vt_{2vha}, $P = 0.9126$; vt_{2vhb}, $P = 0.2254$; vt_{2O118}, $P = 0.1996$; vt_{2g}, $P = 0.3161$). The observation that all VTEC strains carrying the vt_{2g} gene originated only from cattle samples could be related to the lower number of food samples analyzed and the probable low frequency of vt_{2g}.

Furthermore, Beutin et al. (3) found this variant in food samples, indicating that humans can be exposed to this vt_2 variant along the food chain.

A total of 41 (22.0%) of the 186 VTEC isolates possessed more than one *vt* variant in its genome. The most-frequent combination of subtypes was vt_1/vt_2, which was found in 14 isolates. We also observed multiple combinations among vt_2 subtypes detected by the PCR-RFLP analysis of the B subunit. All VTEC O157 strains carried the vt_2 subtype in association with vt_{2vha}. This genotype also predominated among O157 VTEC isolates from meat, cattle, and humans in other studies in Argentina (11, 29, 47).

We detected the vt_{2d} gene in 21 (13% of vt_2-positive strains) strains, always in *eae*-negative strains, in accordance with other studies (6, 18, 20, 54, 59). In contrast to the article of Tasara et al. (54), in which all vt_{2d}-positive strains were negative for *ehxA* and *saa*, we found seven strains harboring vt_{2d} in combination with *ehxA* and *saa* genes.

It has been reported that VTEC isolates harboring different *vt* variants differ markedly in their association with HUS (14, 21). In this way, it is interesting that 43.2% of our VTEC collection presented the vt_2 subtype (either alone or with another *vt* variant), which is a subtype associated with serious disease in humans in Argentina and around the world (14, 37, 42, 47). Besides, most of the remaining isolates carried at least one of the other *vt* subtypes that have been found in VTEC strains quite capable of causing severe disease. In Table 2 we highlighted isolates that belong to the same serotype and harbor the same virulence profile as VTEC isolated from humans with bloody diarrhea and HUS in Argentina (47, 48).

Cytotoxicity. The amount of VT produced by the strain may also play an important role in the clinical outcome (13, 17, 32), and several studies show that VT production is linked to phage induction (25, 31, 32).

The cultures of 54 VTEC strains were grown with and without mitomycin C treatment and were tested by a microtiter verotoxicity assay. The supernatants of all isolates were cytotoxic to Vero cells; moreover, bacteria grown in the presence of mitomycin C presented an increase in cytotoxicity. We found, however, that verotoxicity titers differed depending on the strain, both in basal and induced conditions. This variability allowed us to classify the titers obtained in uninduced conditions (absence of mitomycin C) in three categories, A to C, and the titers under mitomycin C induction in another three categories, I to III (Table 2). Although the criteria used for this classification are arbitrary, we used them as an aid to clarify the expression of the results.

Taking into account isolates with only one *vt* variant gene, the cytotoxic effect was associated more with the *vt* variant than the serotype or origin. The isolates carrying the vt_2 subtype had high titers under both uninduced and induced conditions. Supernatants of 11/13 VTEC strains carrying the vt_2 subtype had basal titers of $\geq$256 (category C) and $\geq$131,072 (category III) when were treated with mitomycin C. The strains presenting the vt_2 subtype showed a median I/U fold increase of 970 (I/U ranging from 512 to 2,730).

Half of the isolates that contained the vt_1 subtype as a unique *vt* variant showed basal titers corresponding to category C, and the others had titers corresponding to categories A and B. Notably, under mitomycin C induction, 7 (70%) of the 10 strains carrying the vt_1 subtype presented titers of $\leq$4,096 (category I), which was reflected in an I/U fold increase of no higher than 64 fold, and only two strains (of the O26:H11 serotype) presented titers of $\geq$131,072 (category III).

The isolates carrying vt_{2vha} or vt_{2vhb} variants showed low and intermediate uninduced titers (categories A and B). The cytotoxic effect of cultures treated with mitomycin C was variable, including titers of the three categories. Interestingly, we observed a high increase in cytotoxicity after mitomycin C induction in several isolates carrying vt_{2vha} or vt_{2vhb} variants and even I/U values of $\geq$10,000.

Only one of the four vt_{2g}-positive strains, belonging to O2:H25, had a high basal titer (category C) comparable to those obtained from strains carrying the vt_2 subtype. A similar result for an O2:H25 vt_{2g}-positive strain was previously described by Leung et al. (28). In addition, we also observed that vt_{2g}-positive strains showed a low response to mitomycin C induction (I/U $\leq$ 16 fold). A low level of vt_{2g} expression as well as the production of biologically inactive toxin by some vt_{2g}-positive strains has been suggested by Beutin et al. (5).

Our results are consistent with a recent study by de Sablet et al. (12), who observed that the expression of vt_2 is heterogeneous in basal and in induced conditions, depending on the strain and on the vt_2 variant. In this way, some differences reported in basal and induced VT production between disease-associated and bovine-associated VTEC bacteria (46) could be explained by the *vt* subtypes present in the strains studied.

Notably, the isolates carrying the vt_1 subtype had a lower response to mitomycin C treatment than most of the vt_2-positive VTEC strains. In a previous study, Ritchie et al. (46) also observed that mitomycin C treatment had a minimal effect on VT1; instead, VT1 production was induced by growth in low-iron medium, and they suggested that prophage induction is not as important for VT1 production as it is for VT2 production. Differences in phage induction could be one of the factors contributing to the variable clinical significance of VTEC strains. It is known that VT2-producing VTEC isolates are more frequently associated with HUS than isolates that produce VT1 (8, 19). On the other hand, vt_1-positive strains are more virulent for calves than strains harboring other *vt* genes (57). More studies based on mechanisms that influence and regulate VT production could help to understand the clinical outcome of VTEC infection.

The presence of more than one *vt* gene variant in the same isolate was not reflected in higher titers, and generally they were lower than those from strains with only one gene variant that belonged to the same serotype. For instance, among O20:H19 VTEC strains, two isolates harboring both vt_1 and vt_{2vhb} subtypes had similar basal titers but strikingly lower titers in induced conditions than the isolate carrying only vt_{2vhb}. Notably, all isolates carrying two or three *vt* variants had a $\leq$1,024 I/U fold increase.

Our results reinforce the idea that the presence of two or more VT phages within the same bacterium could alter the expression of *vt* genes. Recently, the influence of the presence of more than one VT prophage on toxin and phage production was examined by Serra-Moreno et al. (52) using recombinant phages. They found that lysogens with two phages produced less toxin and fewer phage particles than those carrying only one prophage. Furthermore, it has been reported that the

severity of disease caused by VTEC is influenced by phage-related factors (17). Muniesa et al. (32) analyzed *E. coli* O157:H7 strains isolated from a single outbreak in Spain that harbored either two kinds of phages or only one of them. Their results showed that high-phage-production isolates harbored only one phage. In Germany, a study of patients from whom SF EHEC O157:NM strains harboring one or two vt_2 genes were isolated found that HUS development was significantly associated with the presence of a single vt_2 copy in the infecting VTEC strain (7).

In summary, a broad range of variability in VTEC population of Argentine cattle, both in *vt* variants as well as in verotoxic effect, was evidenced in the present study. Taking into account that both bacterial and environmental or biological factors influence the pathogenicity of VTEC in the host, our observations have many implications. On the one hand, there is a considerable proportion of VTEC isolates in cattle that potentially are highly pathogenic for humans. On the other hand, differences in VT phage induction could enhance VT production and also the horizontal transfer of *vt* genes between bacteria in cattle, therefore contributing to the emergence of new VTEC strains.

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