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Evaluation of *vt*2-subtyping methods for identifying *vt*2g in verotoxigenic *Escherichia coli*

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Verotoxin-producing *Escherichia coli* (VTEC) are important pathogens that can cause severe human disease, including haemorrhagic colitis and haemolytic–uraemic syndrome. A new variant of verotoxin, *vt*2g, has recently been described. It was possible to find this variant for the first time in Argentina among VTEC isolated from cattle. The present study evaluated the identification of this gene with three conventional methods used for subtyping the *vt*2 gene. The results show that it is possible to screen VTEC strains for the presence of *vt*2g without the implementation of new protocols.

INTRODUCTION

Verotoxin-producing *Escherichia coli* (VTEC) are important pathogens that can cause severe human disease, including haemorrhagic colitis and haemolytic–uraemic syndrome (Karmali *et al.*, 1985). Ruminants are regarded as the principal reservoir of VTEC strains (Karmali, 1989).

Several factors contribute to the pathogenicity of VTEC, but the verotoxins (VTs) are considered to be the main virulence factors. These toxins are classified into two principal types, verotoxin 1 (VT1) and verotoxin 2 (VT2) (Nataro & Kaper, 1998). The VT1 group consists of VT1, VT1c and VT1d variants (Paton *et al.*, 1995; Zhang *et al.*, 2002; Bürk *et al.*, 2003; Friedrich *et al.*, 2003). The VT2 group is more heterogeneous and includes the prototype VT2 (VT2-EDL933) (this VT2 subtype is referred to as VT2-EDL933 in this paper, as it is the VT2 produced by the EDL933 reference strain), a considerable number of variants grouped into VT2c, VT2d, VT2e and VT2f, and the recently identified VT2g variant (Schmitt *et al.*, 1991; Piérard *et al.*, 1998; Schmidt *et al.*, 2000; Leung *et al.*, 2003). Leung *et al.* (2003) described this novel VT2 variant in a VTEC strain isolated from the faeces of healthy cattle. They found the *vt*2g gene in 0.7 % of bovine VTEC isolates. Strains carrying *vt*2g have also been identified in cattle wastewater in a study of aquatic environments (García-Aljaro *et al.*, 2005). When *vt*2g is aligned with published DNA sequences of *vt*2-EDL933 and *vt*2 variants, it exhibits the highest similarity with *vt*2 genes associated with human disease, and moreover VT2g cytotoxicity for HeLa and Vero cells is comparable to that of VT2-EDL933 (ATCC 43889) (Leung *et al.*, 2003). This potential pathogenicity and the possibility that *vt*2g may be a newly emerged variant that has not yet extensively spread among cattle (Leung *et al.*, 2003) indicate the necessity of studying the prevalence of *vt*2g in isolates from bovine, environmental and human samples.

It is known that the nomenclature of *vt*2 gene variants is confusing due to the use of different subtyping methods and designations. The continuous change of protocols to identify new variants adds more complexity. Therefore, the purpose of this study was to analyse the ability of commonly used *vt*2-subtyping methods to detect and discriminate *vt*2g, in order to facilitate its identification.

Two approaches were used for the analysis: the first was PCR amplification and PCR-RFLP subtyping, and the second was a virtual sequence analysis.

Abbreviations: VT, verotoxin; VTEC, verotoxin-producing *Escherichia coli*.

METHODS

***vt*2g subtyping by PCR and PCR-RFLP.** The 7v reference strain for *vt*2g was examined in comparison with control strains for other *vt*2 variants by three commonly used PCR-RFLP methods developed by other researchers (Tyler *et al.*, 1991; Bastian *et al.*, 1998; Piérard *et al.*, 1998). These protocols are referred to as the Tyler, Bastian and Piérard methods, respectively.

VTEC strains or their DNA used as controls for *vt*2g (*E. coli* 7v), *vt*2-EDL933 (*E. coli* EDL933), *vt*2 vha (*E. coli* E32511), *vt*2 vhb (*E. coli* 1398-152; clinical isolate) and *vt*2d-Ount (*E. coli* EH250) were kindly supplied by Dr A. W. Friedrich (Institut für Hygiene, Universitätsklinikum Münster, Germany), Dr P. H. M. Leung (Queen Mary Hospital, The University of Hong Kong, People's Republic of China), Dr J. Blanco (Laboratorio de Referencia de *E. coli*, Spain) and Dr E. López (Hospital de Niños 'Ricardo Gutiérrez', Argentina).

Bacteria were grown overnight at 37 °C in Luria–Bertani (LB) broth with shaking. An aliquot of the culture was diluted 1/10 in water to determine OD_{600}. At an OD_{600} of 0.5, a 500 µl aliquot of the stationary-phase culture was centrifuged (2 min at 12 000 ***g***) and the pellet suspended in 500 µl double-distilled water. The suspension was then boiled for 10 min and centrifuged (2 min at 12 000 ***g***). Five

microlitres of a 1/10 dilution of the supernatant were used as the template for PCR amplification.

PCR products were obtained with the Lin primer set for the Bastian method, VT2-c/VT2-d for the Tyler protocol, and VT2-e/VT2-f and VT2-cm/VT2-f for the Piérard protocol. PCR protocols and cycling conditions corresponded to those published before (Tyler *et al.*, 1991; Bastian *et al.*, 1998; Piérard *et al.*, 1998).

For each PCR-RFLP method, 10 µl of each PCR product was incubated for 4 h at 37 °C in a water bath with 10 units of the appropriate enzyme in a final volume of 25 µl (*Hae*III, *Rsa*I, *Nci*I and *Hinc*II) or 30 units in a final volume of 30 µl in the case of *Pvu*II. PCR products and restriction fragments were separated by gel electrophoresis in 2 % agarose gels with ethidium bromide in Tris/borate/EDTA buffer.

In addition, 134 VTEC isolates from cattle in Argentina were tested with the primer set specific for the *vt*2g gene variant, 209F/781R (Leung *et al.*, 2003). The serotypes and *vt* genotypes of these bacterial strains are listed in Table 1 and most of them have been described previously (Parma *et al.*, 2000; Padola *et al.*, 2004).

Strains that gave a positive result, and were therefore considered *vt*2g positive, were analysed by the Bastian, Piérard and Tyler protocols.

All the *vt*2g-positive strains were also tested with the generic primer set for *vt*2 genes (stx2F/stx2R) designed by Paton & Paton (1998). PCR cycling conditions corresponded to those published by these authors.

Virtual sequence analysis. Virtual analysis of two published sequences of the *vt*2g gene (GenBank accession nos AY286000 and AJ966782) was done to check PCR-RFLP results and assign fragment sizes. Sequences representing other *vt*2 variants were also included in this analysis to make a comparison with *vt*2g (Table 2).

Each *vt*2 sequence was amplified with the iPCR software (http://www.ch.embnet.org/software/iPCR_form.html) to obtain virtual PCR products for the three protocols studied. When a virtual PCR product was not obtained, the corresponding *vt*2 sequence and each primer were aligned to detect mismatches by using BLAST 2 Sequences (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). To simulate restriction enzyme digestion, calculated PCR products were digested using REBsites, a virtual digestion tool (http://tools.neb.com/REBsites).

RESULTS AND DISCUSSION

The results of *vt*2g subtyping by PCR and RFLP-PCR are shown in Fig. 1. Examples of other *vt* variants are also shown. The computer-predicted RFLP patterns for *vt*2g in comparison with other *vt*2 sequences are summarized in Table 2.

Table 1. Serotypes and *vt* genotypes of VTEC strains isolated from cattle in Argentina and tested in this study

Serotype	No. of strains	*vt* genotype*		Serotype	No. of strains	*vt* genotype*	
		*vt*1	v*t*2			*vt*1	v*t*2
O2 : H5	1	−	+	O117 : H7	6	−	+
O2 : H25	1	−	+	O118 : H16	1	+	−
O5 : H−	4	+	−	O120 : H19	1	−	+
O5 : H27	1	+	−	O141 : H7	1	+	+
O8 : H16	1	+	−	O141 : H8	1	+	+
O15 : H21	1	−	+	O141 : H8	1	−	+
O20 : H7	2	+	+	O145 : H−	4	+	−
O20 : H19	4	+	+	O145 : H−	8	−	+
O20 : H19	1	−	+	O146 : H−	1	−	+
O20 : H?	1	+	−	O146 : H21	1	−	+
O25 : H19	1	−	+	O157 : H7	5	−	+
O26 : H11	5	+	−	O165 : H−	1	−	+
O26 : H11	2	−	+	O168 : H8	1	−	+
O38 : H39	1	+	−	O171 : H−	3	−	+
O39 : H49	1	+	+	O171 : H2	11	−	+
O39 : H49	4	−	+	O174 : H21	1	+	+
O74 : H28	1	−	+	O174 : H21	1	+	−
O79 : H19	1	−	+	O174 : H21	7	−	+
O91 : H21	3	−	+	O175 : H8	2	−	+
O103 : H−	1	+	+	O177 : H−	2	−	+
O103 : H−	1	−	+	O178 : H19	2	−	+
O103 : H2	1	+	+	ONT : H−	17	−	+
O103 : H2	1	+	−	ONT : H8	1	+	−
O111 : H−	1	+	−	ONT : H21	9	−	+
O113 : H21	3	−	+	ONT : H21	1	+	+
O116 : H21	1	−	+				

**vt* genotype identified by a multiplex PCR developed by Paton & Paton (1998).

Table 2. Virtual PCR-RFLP analysis performed with iPCR and REBsites electronic tools

Predicted sizes of PCR products and restriction fragments for *vt*2g in comparison with other representative *vt*2 variants are indicated in bp.

Protocol	Primers and restriction enzymes	*vt*2 gene variants (GenBank accession no.)						
		***vt*2g (AY286000) (AJ966782)**	***vt*2-EDL933 (X07865)**	***vt*2vha (M59432)**	***vt*2vhb (AF479829)**	***vt*2d-Ount (AF043627)**	***vt*2d-OX3a (X65949)**	***vt*2-NV206 (AF329817)**
Tyler	VT2c and VT2d	*285**	285	285	285	–	–	285
	*Hae*III	285	285	161, 124	161, 124			285
	*Rsa*I	*285*†	216, 69	136, 80, 69	216, 69			216, 69
	*Nci*I	285	285	285	159, 126			159, 126
Piérard	VT2e and VT2f	349	348	348	348	349	349	348
	*Hae*III	349	348	216, 132	216, 132	217, 132	168, 132, 49	348
	*Pvu*II	*349*†	323, 25	323, 25	275, 73	201, 120, 28	201, 120, 28	348
	VT2cm and VT2f	–	–	–	–	256	256	–
Bastian	Lin5′ and Lin3′	906	905	905	905	906	906	905
	*Hin*cII	*819*†, 62, 25	556, 262, 62, 25	556, 324, 25	556, 349	881, 25	881, 25	556, 324, 25

*With these primers *vt*2g consistently rendered a weaker PCR product than the other variants.
†Restriction fragments that help differentiate *vt*2g from *vt*2-EDL933 are shown in italic type.

The *vt*2g gene of strain 7v was amplified with all the protocols investigated (Fig. 1a–c, lane 2). No amplification was obtained with the VT2-cm/VT2-f primer set in the Piérard protocol; this was expected, as this set is specific for *vt*2d.

Although PCR was performed with standardized bacterial suspensions, it is important to emphasize that amplification with primers VT2-c/VT2-d in the Tyler protocol resulted in a less abundant product with *vt*2g than with the other variants (Fig. 1a). This situation can be explained by a 2 bp difference in the 3′ reverse-priming region.

PCR-RFLP results with the *vt*2g-positive strain showed similarities between *vt*2g and *vt*2-EDL933 sequences (Fig. 1d–h, Table 2). Nevertheless, differences exist at some restriction sites:

(1) The *vt*2g PCR products remained unrestricted after incubation with *Rsa*I (Tyler method) and with *Pvu*II (Piérard method) (Fig. 1e, h, lane 3). The absence of these restriction sites in this *vt*2g sequence was confirmed with the virtual analysis (Table 2).

(2) In addition, when performing the Bastian method, *vt*2g lacked one of the restriction sites for *Hin*cII, in agreement with the virtual analysis. Therefore, a fragment of 819 bp was obtained instead of 556 and 263 bp fragments (Table 2).

These differences allow the identification of the *vt*2g variant with any of the analysed subtyping methods. The *vt*2g-positive VTEC strains (4/134) isolated from cattle in our country displayed the same patterns of bands as the control strain 7v in all these protocols. This variant was detected among isolates from cattle in feedlots belonging to serotypes O2 : H25, O15 : H21 and O175 : H8 (two isolates).

In addition, for all *vt*2g-positive VTEC strains, the ability of the generic primers stx2F/stx2R (Paton & Paton, 1998) to detect the *vt*2g gene was evaluated and corroborated. This is noteworthy because Leung *et al.* (2003) could not detect this variant with some *vt*2-specific primers and probes, and it is important to use initially a generic primer set to capture all possible variants before using the subtyping protocols (Ziebell *et al.*, 2002).

The present work confirms the importance of reporting the sizes of atypical restriction fragments in *vt*2 typing, in order to be able to correlate with variants identified in other studies. Also, it is interesting that a consistently lower intensity of a PCR product, under optimal conditions, can alert to the presence of a new *vt*2 variant.

A different region of the *vt*2 gene is amplified in each of the analysed subtyping methods and this situation can generate contradictory results in the identification of *vt*2 gene variants (Ziebell *et al.*, 2002). Nevertheless, all three protocols were able to identify the *vt*2g gene and differentiate it from the other *vt*2 variants.

These results show that it is possible to screen VTEC strains for the presence of *vt*2g without implementing new protocols that add further complexity to the analysis of *vt*2 variants. This variant has not been reported before in Argentina, and we found it in 3 % of VTEC strains isolated from cattle. Studies that determine the prevalence of the

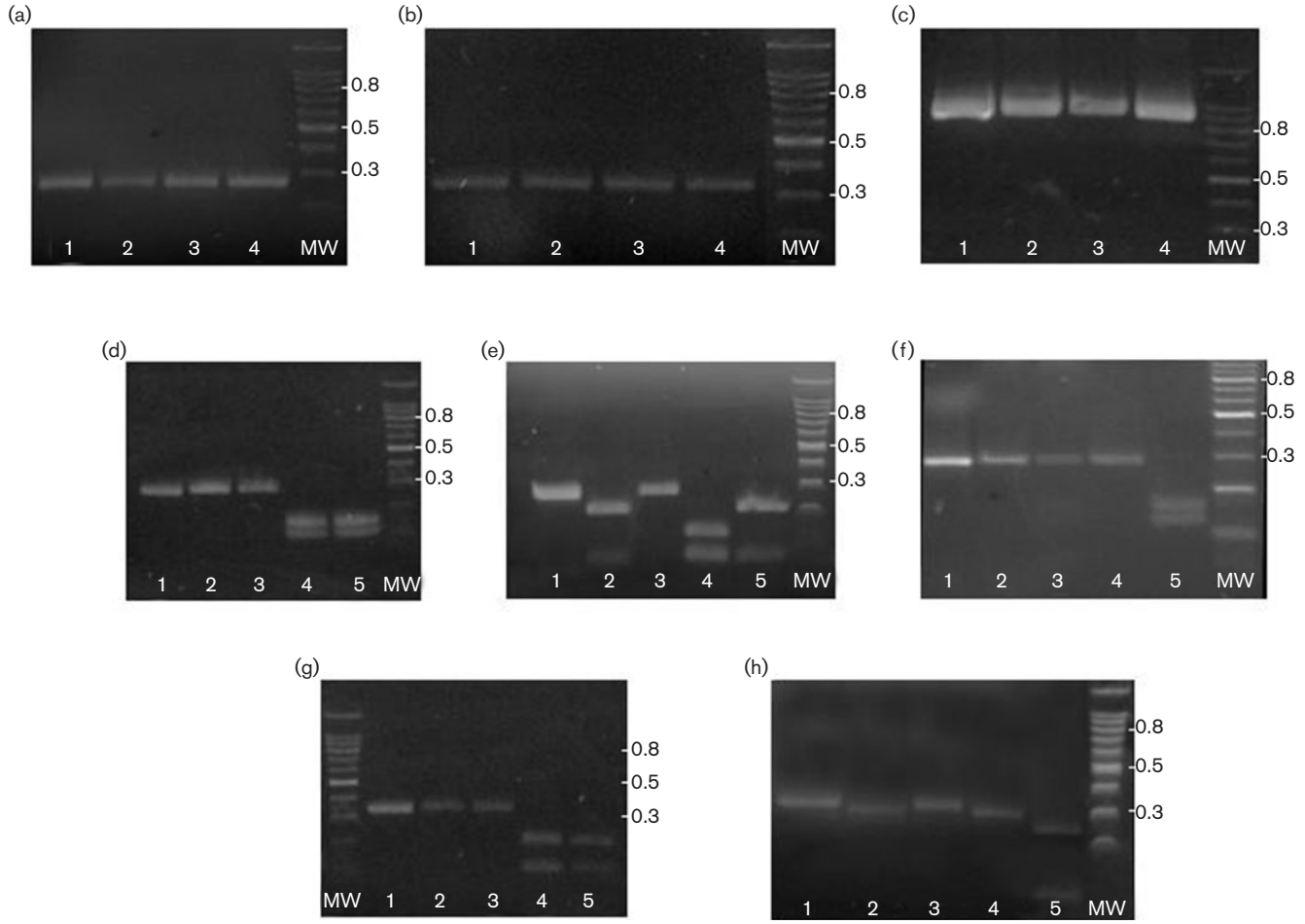

Fig. 1. Examples of PCR products and restriction fragments obtained with *vt*2g and other *vt*2 variants. (a–c) PCR products obtained with (a) primers VT2c/VT2d, Tyler method; (b) primers VT2e/VT2f, Piérard method; (c) primers Lin5′/Lin3′, Bastian method. Lanes: 1, *E. coli* EDL933 (*vt*2-EDL933); 2, *E. coli* 7v (*vt*2g); 3, *E. coli* O145 : H− (*vt*2vha); 4, *E. coli* O91 : H21 (*vt*2vhb). (d–h) PCR products obtained with the Tyler protocol restricted by the restriction enzymes *Hae*III (d), *Rsa*I (e) and *Nci*I (f), and with the Piérard protocol restricted by the restriction enzymes *Hae*III (g) and *Pvu*II (h). Lanes: 1, *E. coli* EDL933 (unrestricted PCR fragment); 2, *E. coli* EDL933 (*vt*2-EDL933); 3, *E. coli* 7v (*vt*2g); 4, *E. coli* O145 : H− (*vt*2vha); 5, *E. coli* O91 : H21 (*vt*2vhb). MW, 100 bp DNA ladder (Promega). Molecular mass (kb) is indicated at the right of each gel.

*vt*2g gene in strains isolated from bovine, human and environmental samples are important to improve the knowledge of the epidemiology and pathogenicity of VTEC strains that harbour this gene.

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