



Identification of the subtypes of Verocytotoxin encoding genes (*vtx*) of *Escherichia coli* by conventional PCR

1. Aim and field of application

Verocytotoxins (VT), synonymous Shiga toxins, are a toxin family characterized by an elevated degree of diversity. The VT family is divided into two branches, VT1 and VT2, based on their antigenic differences. The terms “VT1” and “VT2” were also used to describe the prototypic toxins first described in each branch. Many toxin variants have been described in either branch and it has been recommended that VT family members be classified based on phenotypic differences, biologic activity and hybridization properties (O'Brien et al. 1994).

Classification of VT variants does not represent only a taxonomic exercise: some of the variants are clinically relevant in that they are produced by strains isolated from cases of hemolytic uremic syndrome (HUS), while some others are primarily associated with milder course of disease or are probably not produced by *E. coli* strains causing human disease (Friedrich et al. 2002; Bielaszewska et al. 2006; Persson et al. 2007).

Different systems of nomenclature have been proposed and used for VT variants and their coding genes (*vtx*) (O'Brien et al. 1994). A consensus on a comprehensive proposal of nomenclature has been reached during the 7th International Symposium on VTEC held in 2009, and the nomenclature was presented in its final form at the 8th VTEC2012 Symposium in Amsterdam, The Netherlands. This sequence based nomenclature has been used to develop a protocol for detection and subtyping of *vtx* genes that has been recently published

(Scheutz et al. 2012) and represents the basis of this method. The nomenclature is based on three levels of designations:

1. Types

The two major branches VT1 and VT2 that share structure and function but that are not cross neutralized with heterologous antibodies. The terms VT1 and VT2 should only be used when the subtype is unknown.

2. Subtypes

They are suffixed with small Arabic letters. VT1a, VT1c and VT1d. The Stx of *Shigella* spp. and VT1 are grouped within the subtype VT1a. VT2 toxins include VT2a (the prototypic VT2 sequence) and VT2b (including the previously named VT2d variant), VT2c, VT2d (toxin activation potential implied by sequence), VT2e, VT2f and VT2g.

3. Variants

Variants include the subtype specific prototypic toxins or related toxins within a subtype (that differ by one or more AAs from the prototype). The variants are designated by toxin subtype, O group of the host *E. coli* strain, followed by the strain name or number from which that toxin was described. (e.g. VT1a-O157-EDL933 or VT2c-O157-E32511). Nucleotide variants within a given VT subtype are italicised e.g. *vtx2c*-O157-E32511 is a nucleotide variant that encodes VT2c-O157-E32511.

The present method concerns the identification of the 3 *vtx1* subtypes and the 7 *vtx2* subtypes of VT-coding (*vtx*) genes of *E. coli* by conventional PCR amplification. It is intended for application on isolated VTEC strains in which the presence of *vtx1* and/or *vtx2* group genes has already been assessed.

The *vtx* gene subtypes that represent the target of this method are:

For *vtx1*:

vtx1a, *vtx1c*, *vtx1d*

For *vtx2*:

vtx2a, *vtx2b*, *vtx2c*, *vtx2d*, *vtx2e*, *vtx2f*, and *vtx2g*.

2. Procedure

2.1. Principle of the method

This procedure is used to determine the *vtx* gene subtypes in isolated VTEC strains in which the presence of *vtx1* and/or *vtx2* group genes has already been assessed. Procedures for this preliminary detection step are available at the EU-RL VTEC web site (www.iss.it/vtec), laboratory methods section. The identification of the *vtx* gene subtypes is performed by specific PCR reactions, using primers designed on the basis of analyses of existing *vtx* sequences (Scheutz et al. 2012) and reported in **Table 1**.

The method is composed of the following steps:

- Template preparation
- Setting up the PCR reaction
- Determination of the PCR results by agarose gel electrophoresis.

2.2. Template preparation

Isolated strains are streaked onto solid media (e.g. TSA) and incubated over night.

A single bacterial colony is inoculated in TSB and incubated over night.

25 µl of the overnight culture are added to 975 µl Milli Q water in Eppendorf tube and boiled for 15 minutes. Centrifuge at 18.000 g 5 minutes. Upon transfer to a clean tube, the supernatant is used directly for PCR and stored at -18 °C for further analyses.

2.3. Setting up the PCR reaction

The primer sequences are listed in **Table 1**.

PCR assays are set up in a total volume of 20 µl for standard PCR and 25 µl for triplex PCR as described in **Table 1** (stock solution of primers is 5 µM) and 5 µl supernatant of boiled lysate (stock template prepared as described above) and Milli Q water up to 20 or 25 µl. The PCR conditions are indicated below. The conditions have been set up using the HotStart Taq polymerase from Qiagen. The use of alternative reagents may require adjustments.



Standard PCR in total volume of 20 µl:

2.5 µl H₂O §

10 µl Mastermix 2X

1.25 µl of each of two primers (STOCK solution of primers is 5 µM) §

5 µl supernatant of boiled lysate (STOCK)

§ **Note:** If three primers are used (*vtx2a*), H₂O volume is reduced to 1.25 µl; If four primers are used (all *vtx2d* or detection of all *vtx2* variants), H₂O is NOT added!

Triplex-PCR for subtyping of *vtx1*

PCR in total volume of 25 µl:

12 µl Mastermix

1 µl of each of the four primers for *vtx1c* and *vtx1d* (STOCK solution of primers is 5 µM)

2 µl of each of two primers for *vtx1a* (STOCK solution of primers is 5 µM)

5 µl supernatant of boiled lysate (STOCK)

The PCR amplification conditions are:

– *vtx1* subtyping (triplex PCR):

95 °C for 15 min (HotStart Taq activation)

35 cycles of 94 °C for 50 sec, 64 °C for 40 sec and 72 °C for 60 sec, ending with 72 °C for 3 min.

– *vtx2 a,b,c* subtyping (individual standard PCR reactions):

95 °C for 15 min (HotStart Taq activation)

35 cycles of 94 °C for 50 sec, 62 °C for 40 sec and 72 °C for 60 sec, ending with 72 °C for 3 min.

– *vtx2d, e,f,g* subtyping (individual standard PCR reactions):

95 °C for 15 min (HotStart Taq activation)

35 cycles of 94 °C for 50 sec, 64 °C for 40 sec and 72 °C for 60 sec, ending with 72 °C for 3 min.

PCR amplicons can be stored at 4 °C until loading on agarose gel.

In each PCR assay, a positive and a negative control must be included. The positive controls are DNA templates obtained from *E. coli* strains harboring the different *vtx* subtypes (listed in **Table 2**) that are the object of the present method (*vtx1a*, *vtx1c*, *vtx1d*, *vtx2a*, *vtx2c* and *vtx2d*), and the negative control is constituted by a sample without template added.

2.4. Agarose gel electrophoresis

Prepare agarose gel in 1X Tris/Borate/EDTA (TBE) or Tris/Acetate/EDTA (TAE). Each well of the gel is loaded with 10 µl of each reaction added with loading dye at 1X final concentration. Run the samples in 1X running buffer (TBE or TAE) in constant voltage (100 V). Use a molecular weight marker suitable for assignment of the correct molecular weights to the amplicons produced (refer to **Table 1**). Consider that a correct band assignment is a crucial point in the assessment of the presence of the target genes. Make sure that the bands produced by the reference strains match exactly the expected molecular weight.

Agarose gels should be added of ethidium bromide to allow the visualization of DNA. This reagent is a DNA intercalating agent commonly used as a nucleic acid stain in molecular biology laboratories. When exposed to ultraviolet light, it will fluoresce with a red-orange color. Ethidium bromide should be added to a final concentration of 0.5 µg/ml before pouring the agarose gel in the electrophoresis gel cast. Alternatively the agarose gel can be stained after electrophoresis in a 0.5 µg/ml ethidium bromide aqueous solution.

2.5 Safety and protection devices

Some VTEC strains can infect human beings at a very low infectious dose and can cause severe disease. Laboratory acquired infections have been reported. Therefore, working with VTEC requires good laboratory practices and the use of protection devices.

Ethidium bromide is a mutagen and toxic agent; therefore it should be used in compliance with the safety sheet provided and with protection devices (lab-coats and latex gloves). The

U.V. light may cause damage to eyes so it is mandatory the use of plexiglass shields and protective glasses.

2.6 Reference strains

VTEC strains harboring the different *vtx* subtypes object of the present method are listed in **Table 2** and should be used as positive control. The control templates can be prepared in advance as described for the test strains and stored at -20°C for eight months.

2.7 References

- Bielaszewska, M., A. W. Friedrich, T. Aldick, R. Schurk-Bulgrin, and H. Karch. 2006. Shiga toxin activatable by intestinal mucus in *Escherichia coli* isolated from humans: Predictor for a severe clinical outcome. *Clin.Infect.Dis.* 43:1160-1167
- Friedrich, A. W., M. Bielaszewska, W. L. Zhang, M. Pulz, T. Kuczius, A. Ammon, and H. Karch. 2002. *Escherichia coli* harboring Shiga toxin 2 gene variants: frequency and association with clinical symptoms. *J.Infect.Dis.* 185:74-84
- O'Brien, A. D., M. A. Karmali, and S. M. Scotland. 1994. A proposal for rationalization of the *Escherichia coli* cytotoxins, p. 147-149. *In* M. A. Karmali and A. G. Goglio (eds.), *Recent Advances in Verocytotoxin-producing Escherichia coli Infections*. Elsevier Science, B.V., Amsterdam.
- Persson, S., K. E. P. Olsen, S. Ethelberg, and F. Scheutz. 2007. Subtyping typing method for *Escherichia coli* Shiga toxin (Verocytotoxin) 2 variants and correlations to clinical manifestations. *J Clin Microbiol* 45:2020-2024
- Scheutz F, Teel LD, Beutin L, Piérard D, Buvens G, Karch H, Mellmann A, Caprioli A, Tozzoli R, Morabito S, Strockbine NA, Melton-Celsa AR, Sanchez M, Persson S, O'Brien AD. Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing Stx nomenclature. *J Clin Microbiol.* 2012;50:2951-63.

Table 1. List of primers to be used for *vtx* genes subtyping.

Primer	Sequence (5' – 3')	Position	Amplicon size (bp)	
<i>vtx1</i>				
vtx1a-F1	CCTTTCCAGGTACAACAGCGGTT	362-384	478	All 6 primers can be used in a triplex PCR for subtyping of <i>vtx1</i> ⁽¹⁾ .
vtx1a-R2	GGAAACTCATCAGATGCCATTCTGG	815-839		
vtx1c-F1	CCTTTCTGGTACAACGCGGTT	362-384	252	
vtx1c-R1	CAAGTGTGTACGAAATCCCTCTGA	588-613		
vtx1d-F1	CAGTTAATGCGATTGCTAAGGAGTTTACC	50-78	203	
vtx1d-R2	CTCTTCCTCTGGTTCTAACCCCATGATA	225-252		
<i>vtx2</i>				
vtx2a-F2	GCGATACTGRGBACTGTGGCC	754-774	349	Some <i>vtx2d</i> strains are positive for the small fragment and some for the larger fragment. The control strain C165-02 should be positive for both bands
vtx2a-R3	CCGKCAACCTTCACTGTAAATGTG	1079-1102		
vtx2a-R2	GGCCACCTTCACTGTGAATGTG	1079-1100		
vtx2b-F1	AAATATGAAGAAGATATTTGTAGCGGC	968-994	251	
vtx2b-R1	CAGCAAATCCTGAACCTGACG	1198-1218		
vtx2c-F1	GAAAGTCACAGTTTTTATATACAACGGGTA	926-955	177	
vtx2c-R2	CCGGCCACYTTTACTGTGAATGTA	1079-1102		
vtx2d-F1	AAARTCACAGTCTTTATATACAACGGGTG	927-955	179	
vtx2d-R1	TTYCCGGCCACTTTTACTGTG	1085-1105		
vtx2d-R2	GCCTGATGCACAGGTACTGGAC	1184-		280
		1206		
vtx2e-F1	CGGAGTATCGGGGAGAGGC	695-713	411	
vtx2e-R2	CTTCTGACACCTTCACAGTAAAGGT	1080-1105		
vtx2f-F1	TGGGCGTCATTCCTGGTTG	451-475	424	
vtx2f-R1	TAATGGCCGCCCTGTCTCC	856-874		
vtx2g-F1	CACCGGGTAGTTATATTTCTGTGGATATC	203-231	573	
vtx2g-R1	GATGGCAATTCAGAATAACCGCT	771-793		

⁽¹⁾ Triplex PCR for *vtx1* subtyping: 1 µl of each of the four primers for *vtx1c* and *vtx1d* (stock solution of primers is 5 µM), and 2 µl of each of two primers for *stx1a* (stock solution of primers is 5 µM)

Table 2. List of reference strains harboring the *vtx* gene subtypes

SSI collection D number	Strain	Toxin subtype	Toxin variant designation	GenBank accession No.	Results obtained using the present method
D2653	EDL933	VT1a	VT1a-O157-EDL933	M19473	VT1a + VT2a
D3602	DG131/3	VT1c	VT1c-O174-DG131-3	Z36901	VT1c *
D3522	MHI813	VT1d	Stx1d-O8-MHI813	AY170851	VT1d
D2435	94C	VT2a	VT2a-O48-94C	Z37725	VT1a + VT2a
D3428	EH250	VT2b	VT2b-O118-EH250	AF043627	VT2b
D2587	031	VT2c	VT2c-O174-031	L11079	VT2c *
D3435	C165-02	VT2d	VT2d-O73-C165-02	DQ059012	VT2d **
D3648	S1191	VT2e	VT2e-O139-S1191	M21534	VT2e
D3546	T4/97	VT2f	VT2f-O128-T4-97	AJ010730	VT2f
D3509	7v	VT2g	2g-O2-7v	AY286000	VT2g

* These two strains also encode VT2b

** Should result in both fragments at 179 bp and 280 bp