Identification of the subtypes of Shigatoxin encoding genes (*stx*) of *Escherichia coli* by conventional PCR

1. Aim and field of application

Shiga toxins (Stx), synonymous of Verocytotoxins (VT), are a toxin family characterized by an elevated degree of diversity. The Stx family is divided into two branches, Stx1 and Stx2, based on their antigenic differences. The terms "Stx1" and "Stx2" 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 Stx family members be classified based on phenotypic differences, biologic activity and hybridization properties (O'Brien *et al.* 1994).
Classification of Stx subtypes does not represent only a taxonomic exercise: some of the subtypes 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 (EFSA 2020, Friedrich *et al.* 2002; Bielaszewská *et al.* 2006; Persson *et al.* 2007).
Different systems of nomenclature have been proposed and used for Stx variants and their coding genes (*stx*) (O'Brien *et al.* 1994). A consensus on a comprehensive proposal of nomenclature has been reached during the 7th International Symposium on STEC held in 2009, and the nomenclature was presented in its final form at the 8th VTEC 2012 Symposium in Amsterdam, The Netherlands. This sequence based nomenclature has been used to develop a protocol for detection and subtyping of *stx* 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 Stx1 and Stx2 that share structure and function but that are not cross neutralized with heterologous antibodies. The terms Stx1 and Stx2 should only be used when the subtype is unknown.

2. Subtypes

They are suffixed with small Arabic letters. Stx1a, Stx1c and Stx1d. The Stx of Shigella spp. and Stx1 are grouped within the subtype Stx1a. Stx2 toxins include Stx2a (the prototypic VT2 sequence) and Stx2b (including the previously named VT2d variant), Stx2c, Stx2d (toxin activation potential implied by sequence), Stx2e, Stx2f and Stx2g.

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.* Stx1a-O157-EDL933 or Stx2c-O157-E32511). Nucleotide variants within a given VT subtype are italicised *e.g. stx2c*-O157-E32511 is a nucleotide variant that encodes Stx2c-O157-E32511.

The present method concerns the identification of the 3 *stx1* subtypes and the 7 *stx2* subtypes of Stx-coding (*stx*) genes of *E. coli* by conventional PCR amplification. It is intended for application on isolated STEC strains in which the presence of *stx1* and/or *stx2* group genes has already been assessed.

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

For *stx1*:

stx1a, *stx1c, stx1d.*

For *stx2*:

stx2a, stx2b, stx2c, stx2d, stx2e, stx2f and *stx2g.*

2. Procedure

2.1. Principle of the method

This procedure is used to determine the *stx* gene subtypes in isolated STEC strains in which the presence of *stx1* and/or *stx2* group genes has already been assessed. Procedures for this preliminary detection step are available at the EURL-VTEC website, laboratory methods section. The identification of the *stx* gene subtypes is performed by specific PCR reactions, using primers designed on the basis of analyses of existing *stx* 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 ºC 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 H_2O §

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 (*stx2a*), H_2O volume is reduced to 1.25 µl; if four primers are used (all *stx2d* or detection of all *stx2* variants), H_2O is NOT added!

Triplex-PCR for subtyping of *stx1*

PCR in total volume of 25 µl:

12 µl Mastermix

1 µl of each of the four primers for *stx1c* and *stx1d* (STOCK solution of primers is 5 µM)

2 µl of each of two primers for *stx1a* (STOCK solution of primers is 5 µM)

5 µl supernatant of boiled lysate (STOCK)

The PCR amplification conditions are:

– *stx1* subtyping (triplex PCR):

95 ºC for 15 min (HotStart Taq activation)

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

– *stx2 a,b,c* subtyping (individual standard PCR reactions):

95 ºC for 15 min (HotStart Taq activation)

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

– *stx2d, e,f,g* subtyping (individual standard PCR reactions):

95 ºC for 15 min (HotStart Taq activation)

35 cycles of 94 ºC for 50 sec, 64 °C for 40 sec and 72 ºC for 60 sec, ending with 72 ºC 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 *stx* subtypes (listed in **Table 2**) that are the object of the present method (*stx1a, stx1c, stx1d, stx2a, stx2c* and *stx2d*), 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 STEC 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 STEC 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

STEC strains harboring the different *stx* 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

- Bielaszewská, 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
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- Friedrich, A. W., M. Bielaszewská, 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
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- 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
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Table 1. List of primers to be used for *stx* genes subtyping.

Primer	Sequence (5' – 3')	Position	Amplicon size (bp)	
stx1				
stx1a-F1 stx1a-R2	CCTTTCCAGGTACAACAGCGGTT GGAAACTCATCAGATGCCATTCTGG	362-384 815-839	478	All 6 primers can be used in a triplex PCR for subtyping of *stx1* [(1)].
stx1c-F1 stx1c-R1	CCTTTCCTGGTACAACTGCGGTT CAAGTGTTGTACGAAATCCCCTCTGA	362-384 588-613	252	
stx1d-F1 stx1d-R2	CAGTTAATGCGATTGCTAAGGAGTTTACC CTCTTCCTCTGGTTCTAACCCCATGATA	50-78 225-252	203	
stx2				
stx2a-F2 stx2a-R3 stx2a-R2	GCGATACTG**R**G**B**ACTGTGGCC CCG**K**CAACCTTCACTGTAAATGTG GGCCACCTTCACTGTGAATGTG	754-774 1079-1102 1079-1100	349 347	
stx2b-F1 stx2b-R1	AAATATGAAGAAGATATTTGTAGCGGC CAGCAAATCCTGAACCTGACG	968-994 1198-1218	251	
stx2c-F1 stx2c-R2	GAAAGTCACAGTTTTTATATACAACGGGTA CCGGCCAC**Y**TTTACTGTGAATGTA	926-955 1079-1102	177	
stx2d-F1 stx2d-R1 stx2d-R2	AAARTCACAGTCTTTATATACAACGGGTG TT**Y**CCGGCCACTTTTACTGTG GCCTGATGCACAGGTACTGGAC	927-955 1085-1105 1184-1206	179 280	Some *stx2d* strains are positive for the small fragment and some for the larger fragment. The control strain C165-02 should be positive for both bands.
stx2e-F1 stx2e-R2	CGGAGTATCGGGGAGAGGC CTTCCTGACACCTTCACAGTAAAGGT	695-713 1080-1105	411	
stx2f-F1 stx2f-R1	TGGGCGTCATTCACTGGTTG TAATGGCCGCCCTGTCTCC	451-475 856-874	424	
stx2g-F1 stx2g-R1	CACCGGGTAGTTATATTTCTGTGGATATC GATGGCAATTCAGAATAACCGCT	203-231 771-793	573	

[(1)] Triplex PCR for *stx1* subtyping: 1 µl of each of the four primers for *stx1c* and *stx1d* (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 *stx* gene subtypes

SSI collection D number	*Strain*	*Toxin subtype*	*Toxin variant designation*	*GenBank accession No.*	*Results obtained using the present method*
D2653	EDL933	Stx1a	Stx1a -O157-EDL933	M19473	Stx1a + Stx2a
D3602	DG131/3	Stx1c	Stx1c -O174-DG131-3	Z36901	Stx1c *
D3522	MHI813	Stx1d	Stx1d-O8-MHI813	AY170851	Stx1d
D2435	94C	Stx2a	Stx2a -O48-94C	Z37725	Stx1a + Stx2a
D3428	EH250	Stx2b	Stx2b-O118-EH250	AF043627	Stx2b
D2587	031	Stx2c	Stx2c-O174-031	L11079	Stx2c *
D3435	C165-02	Stx2d	Stx2d-O73-C165-02	DQ059012	Stx2d **
D3648	S1191	Stx2e	Stx2e-O139-S1191	M21534	Stx2e
D3546	T4/97	Stx2f	Stx22f-O128-T4-97	AJ010730	Stx2f
D3509	7v	Stx2g	Stx2g-O2-7v	AY286000	Stx2g

* These two strains also encode Stx2b

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