The International Collaborating Centre for Reference and Research on *Escherichia* and *Klebsiella*

Identification of three *stx1* and seven *stx2* subtypes of Shiga toxin encoding genes of *Escherichia coli* by conventional PCR amplification

Version 7

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1. Aim and field of application

Shiga toxins (Stx), synonymous with 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 to classify Stx family members based on phenotypic differences, biologic activity and hybridization properties (3).
Classification of Stx 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 haemolytic uremic syndrome (HUS) (1,2,5), while others are primarily associated with milder course of disease (2,5) or are probably not produced by *E. coli* strains causing human disease (6).
Different systems of nomenclature have been proposed and used for Stx variants and their coding genes (*stx*) (3). A consensus on a comprehensive proposal of nomenclature has been reached during the 7th International Symposium on VTEC held in Buenos Aires, Argentina, VTEC2009 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 the protocol for detection and subtyping of *stx* genes and the scientific analysis and presentation has been published (7). In summary, there are three levels of designations:

1. **Types**: Toxin type 1 and 2.
2. **Subtypes**: Stx/Stx1a, Stx1c and Stx1d. The prototypic Stx (in *Shigella* spp.) and Stx1 are grouped within one new subtype, Stx/Stx1a. Stx2 toxins include two new subtypes, Stx2a (the prototypic Stx2 sequence) and Stx2b (including the previously named Stx2d variant), and the five existing subtypes i.e. Stx2c, Stx2d (activation potential implied by sequence), Stx2e, Stx2f and Stx2g from *Acinetobacter haemolyticus*, *Citrobacter freundii*, *Enterobacter cloacae*, *Escherichia albertii* (4) and *Escherichia coli*.
3. **Variants**: Variants include the subtype specific prototypic toxins or related toxins within a subtype (that differ by one or more amino acids 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 Stx 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 three *stx1* subtypes and the seven *stx2* subtypes of Stx encoding (*stx*) genes of *E. coli* by conventional PCR amplification. It is intended for application on isolated STEC strains.
The *stx* gene subtypes that represent the target of this method are:
stx1: *stx/stx1a, stx1c, stx1d*
stx2: *stx2a, stx2b, stx2c, stx2d, stx2e, stx2f* and *stx2g*.

2. Procedure

1.1. Principle of the method

The method is based on PCR amplification of specific DNA regions from a template DNA, with oligonucleotides triggering the start of the PCR reaction.
The procedure includes a first phase of identification of the *stx* gene types (*stx1* and/or *stx2*) possessed by the strain under examination. The primers for this preliminary typing step are shown in **Table 1**. The second phase concerns the determination of the *stx* gene subtypes and is performed by specific PCR reactions, using primers designed on the basis of analyses of existing *stx* sequences (7).
The method is composed of the following steps:

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

1.2. Template preparation

Isolated strains are streaked onto solid media (e.g. TSA) and incubated overnight.
A single bacterial colony is inoculated in beef broth or TSB and incubated overnight.
100 µl of the overnight culture is added to 900 µl Milli Q water in an Eppendorf tube and boiled or placed in a heating block at 100 ºC for 15 minutes. Centrifuge at 18.000 g for five minutes at room temperature. Upon transfer to a clean tube, the supernatant is used directly for PCR and stored at -18ºC for further analyses.

1.3. Setting up the PCR reaction

Standard PCR in total volume of 20 µl:
2.5 µl H_2O §
10 µl Mastermix (HotStarTaq , Qiagen),
1.25 µl of each of two primers (STOCK solution of primers is 5 µM) §
5 µl supernatant of boiled lysate (STOCK)

§ If three primers are used (as for *stx2a* subtyping or *stx2d* subtyping without primer stx2d-O55-R), H_2O volume is reduced to 1.25 µl;
If four primers are used (subtyping with all *stx2d* primers or running of the *stx2* detection system), H_2O is NOT added!

Triplex-PCR for subtyping of *stx1* in total volume of 25 µl:
12 µl Mastermix (HotStartTaq, Qiagen),
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 thermo cycler conditions are:
stx1 and *stx2* detection with primers stx1-det-F1/ stx1-det-R1; F4/R1/F4-f/R1-e/f:
95ºC for 15 min (HotStartTaq activation)
35 cycles of 94ºC for 50 sec, 56° for 40 sec and 72ºC for 60 sec, ending with 72ºC for 3 min.

stx1 and *stx2* subtyping – see **Table 1**:
95ºC for 15 min (HotStartTaq activation)
35 cycles of 94ºC for 50 sec, 64-66°C (please see the Note on the design of primers and **Table 1**) for 40 sec and 72ºC for 60 sec, ending with 72ºC 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 harbouring the different *stx* subtypes that are the object of the present method (*stx1a, stx1c, stx1d, stx2a, stx2b, stx2c, stx2d, stx2e, stx2f* and *stx2g* – reference strains listed in **Table 4**), and the negative control is constituted by a sample without template added.

1.4. 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 PCR product 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 (**Table 1**). Make sure that the bands produced by the reference strains match exactly the expected molecular weight.
Add ethidium bromide to the agarose gel to a final concentration of 0.5 µg/ml before pouring it in the electrophoresis gel cast. Ethidium bromide 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 colour. Alternatively, the agarose gel can be stained after electrophoresis in a 0.5 µg/ml ethidium bromide aqueous solution.

1.5. 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 UV light may cause damage to eyes so it is mandatory the use of plexiglas shields and protective glasses.

1.6. Reference strains

STEC strains harbouring the different *stx* subtypes are listed in **Table 4** and should be used as positive controls – see **Figure 2** illustrating *stx2a, stx2b, stx2c, stx2d, stx2e, stx2f* and *stx2g* positive strains. A complete set of strains harbouring the genes encoding all *stx* subtypes (for a total of ten isolates) are provided by the WHO Collaborating Centre for Reference and Research on *Escherichia* and *Klebsiella*, The control templates can be prepared in advance as described for the test strains and stored at -20°C for eight months.

Contact:
Flemming Scheutz, Head, Ph. D., Msc.
WHO Collaborating Centre for Reference and Research on *Escherichia* and *Klebsiella*
Department of Microbiology & Infection Control
Statens Serum Institut
Artillerivej 5
DK-2300 København S
Danmark
Tlf: +45 3268 3334
Fax: +45 3268 8238
e-mail: fsc@ssi.dk

Table 1. List of primers to be used for *stx* genes detection and subtyping. PCR conditions are described in the text below. Annealing temperatures are 56°C for sequencing and detection, and 64-66°C for subtyping of *stx/stx1* or *stx2*. Especially, the resolution of *stx2a*, *stx2c* and *stx2d* may require individual calibration of thermocyclers; see note below [a)]

Primer	Sequence (5' – 3')	Position	Amplicon size (bp)	
stx/stx1 §				
Detection				
stx1-det-F1 stx1-det-R1	GTACGGGGATGCAGATAAATCGC AGCAGTCATTACATAAGAACGYCCACT	440-462 622-648	209	
Subtyping				
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* *
stx1c-F1 stx1c-R1	CCTTTCCTGGTACAACTGCGGTT CAAGTGTTGTACGAAATCCCCTCTGA	362-384 588-613	252	
stx1d-F1 stx1d-R1	CAGTTAATGCGATTGCTAAGGAGTTTACC CTCTTCCTCTGGTTCTAACCCCATGATA	50-78 225-252	203	
stx2				
Detection				
F4 R1 F4-f R1-e/f	GGCACTGTCTGAAACTGCTCCTGT ATTAAACTGCACTTCAGCAAATCC CGCTGTCTGAGGCATCTCCGCT TAAACTTCACCTGGGCAAAGCC	606-629 1209-1232 606-629 1209-1230	627 625	For detection all 4 primers can be used in one reaction
Subtyping				
stx2a-F2 stx2a-R3 stx2a-R2	GCGATACTGRGBACTGTGGCC CCGKCAACCTTCACTGTAAATGTG GGCCACCTTCACTGTGAATGTG	754-774 1079-1102 1079-1100	349 347	
stx2b-F1	AAATATGAAGAAGATATTTGTAGCGGC	968-994	251	

stx2b-R1	CAGCAAATCCTGAACCTGACG	1198-1218		
stx2c-F1 stx2c-R2	GAAAGTCACAGTTTTTATATACAACGGGTA CCGGCCACYTTTACTGTGAATGTA	926-955 1079-1102	177	
stx2d-F1 stx2d-R1 stx2d-R2 **	AAARTCACAGTCTTTATATACAACGGGTG TTYCCGGCCACTTTTACTGTG 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 – see Figure 3
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	

Wobble bases are shown in bold.

§ These primers will also detect Shiga toxin genes from *Shigella dysenteriae* type 1 and *Shigella sonnei*.

* Triplex PCR for *stx1* subtyping: 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). See Figure 1 for gel picture of fragment sizes.

** One additional primer has been designed to specifically detect the stx2d-O55-5905 variant:

Primer	Sequence (5' – 3')	Position	Amplicon size (bp)
stx2d-O55-R	TCAACCGAGCACTTTGCAGTAG	1140-1161	235

All three reverse primers in the same reaction will result in amplicons of 179bp with 9 *stx2d* variants, 235bp with variant stx2d-O55-5905, 280bp with 5 *stx2d* variants, and finally two amplicons of 179bp and 280bp with variant stx2d-O73-C165-02.

Gradient testing by SSI on an Eppendorf thermocycler has indicated that the annealing temperatures for *stx1* triplex PCR primers should be higher than 62.4 as shown in **Table 2**. As this may vary between thermocyclers we suggest that the annealing temperature is raised to 64°C or higher depending on the thermocycler.

Table 2. Maximum annealing temperatures for *stx1* subtyping in °C:
Clear bands are seen at annealing temperatures shown in bold.
Annealing temperatures for cross reacting bands between *stx1a, stx1* and *stx1d* are shown in [].

Forward primer is **F1** in all cases; Optional reverse *stx1c* primers **R1** & **R2** are shown	***stx1a***	***stx1c***	***stx1d***
stx1a	>**66.0**	None	[56.8]
stx1c	None	>**66.0**	[59.5]
stx1	None	None	>**66.0**

One gradient was tested:
54.9 – 66.0°C in 12 wells: 54.9°C- 55.1°C- 55.8°C- 56.8°C- 58.1°C- 59.5°C- 61.0°C- 62.4°C- 63.7°C- 64.8°C - 65.6°C – 66.0°C
Thermocycler: Eppendorf.

Conclusion: Unwanted cross-reactions with control strains for *stx1a*, *stx1c* and *stx1d* can be eliminated at annealing temperature higher than 59.5°C. Recommended annealing temperature is 64°C. Should you observe two *stx1* subtypes in the same strain the annealing temperature should be raised to 66°C.

[a] NOTE on the design of primers: Stx2a, Stx2c and Stx2d are very closely related and the design of primers has been quite difficult! We have identified thirty *stx2a*, twenty-four *stx2c* and twenty-six *stx2d* nucleotide variants. The primers that we have designed should be specific for each of their respective variants. However, as we have not tested all these many variants, our design is based on the fact that the primers match all the desired sequences. Furthermore, we have noted that cross-reactions occur and are seen as ghost bands – especially between *stx2c* and *stx2d* positive strains. This is illustrated in **Figure 3**. Thus, only very clearly positive bands should be interpreted as indicative of presence of that specific subtype.
Independent laboratories have indicated that they have had difficulties with the three control strains for *stx2a*, *stx2c* and *stx2d* where they have seen unwanted cross-reactions. On some thermocyclers this problem may be resolved by careful calibration of individual brands of thermocyclers by testing annealing temperatures from 64-66°C on the test panel of reference strains.

In our hands, an additional PCR using the *stx2d* primers were run at an annealing temperature of 66°C. False positive *stx2c* fragments disappeared and true *stx2d* positive fragments persisted at this annealing temperature.

Gradient testing by ISS and SSI on different thermocyclers has indicated that the maximum annealing temperatures may vary a little in different laboratories as shown in the below **Table 3**.

Table 3. Maximum annealing temperatures for *stx2* subtyping in °C:
Clear bands are seen at annealing temperatures shown italicised top is laboratory 1 and bold is laboratory 2; weak visible bands in brackets (). Annealing temperatures for cross reacting bands between *stx2a*, *stx2c* and *stx2d* are shown in [].

	stx2a	***stx2b***	***stx2c***	***stx2d***	***stx2e***	***stx2f***	***stx2g***
stx2a	*65.6* **66.9** (67.2-68.2)		[<58]	[<58] *			
stx2b		**65** (66)					
stx2c	[<58] *		*63* **64.4-65.0** (65.6-66.0)	[60.5] *			
stx2d	[<58] *		[60.5] *	*63* **65.0-65.6** (66.9- 67.0)			
stx2e					>**67**		
stx2f						**68.1** (69.5)	
stx2g							>**67**

* One test laboratory has seen cross-reactions at 62°C using a Biometra cycler. These were resolved using an annealing temperature at 64°C.

Three different gradients were tested in two laboratories:

62 – 69.8°C in 10 wells: 62°C- 63°C- 64.7°C- 65.6°C- 66.4°C- 67.2°C- 68.1°C- 68.9°C- 69.4°C- 69.8°C
58 - 67°C in 10 wells (temperature difference between wells = 1 °C)
58 - 72°C in 12 wells (temperature difference between wells ~ 1.3 °C)

Thermocyclers: Perkin Elmer 9700, Euroclone PeQ Star, Biometra cycler and Eppendorf.

Conclusion: In order to eliminate unwanted cross-reactions with control strains for *stx2a*, *stx2c* and *stx2d* an annealing temperature 64-66°C is recommended. This should also work for all the other stx2 subtypes: *stx2b, stx2e, stx2f* and *stx2g*.

Note on the choice of Taq polymerase: During our test phase, we have noted that the (conventional) PCR's are Taq dependent and therefore, **the protocol should be followed exactly as described using the Qiagen HotStarTaq Master Mix Kit.**
HotStarTaq Master Mix Kit comes in three different "sizes" 250U, 1000U and 2500U. Cat.No.s 203443, 203445 and 203446.
It's a pre-made mastermix. You just add template, primers and water.
One participant has tested the protocol with Go Taq Green (Promega) and FastStart High Fidelity Taq (Roche) at annealing temperature at 64°C with unsatisfactory results.
We have no commercial interest in Qiagen and you are of course welcome to test other Taq polymerases – but we recommend that you do this after you have validated the control strains using the Qiagen HotStart Master Mix Kit.

Figure 1. Gel picture illustrating five strains each of *stx1a*, *stx1c* (with reverse primer stx1c-R1) and *stx1d* positive strains.

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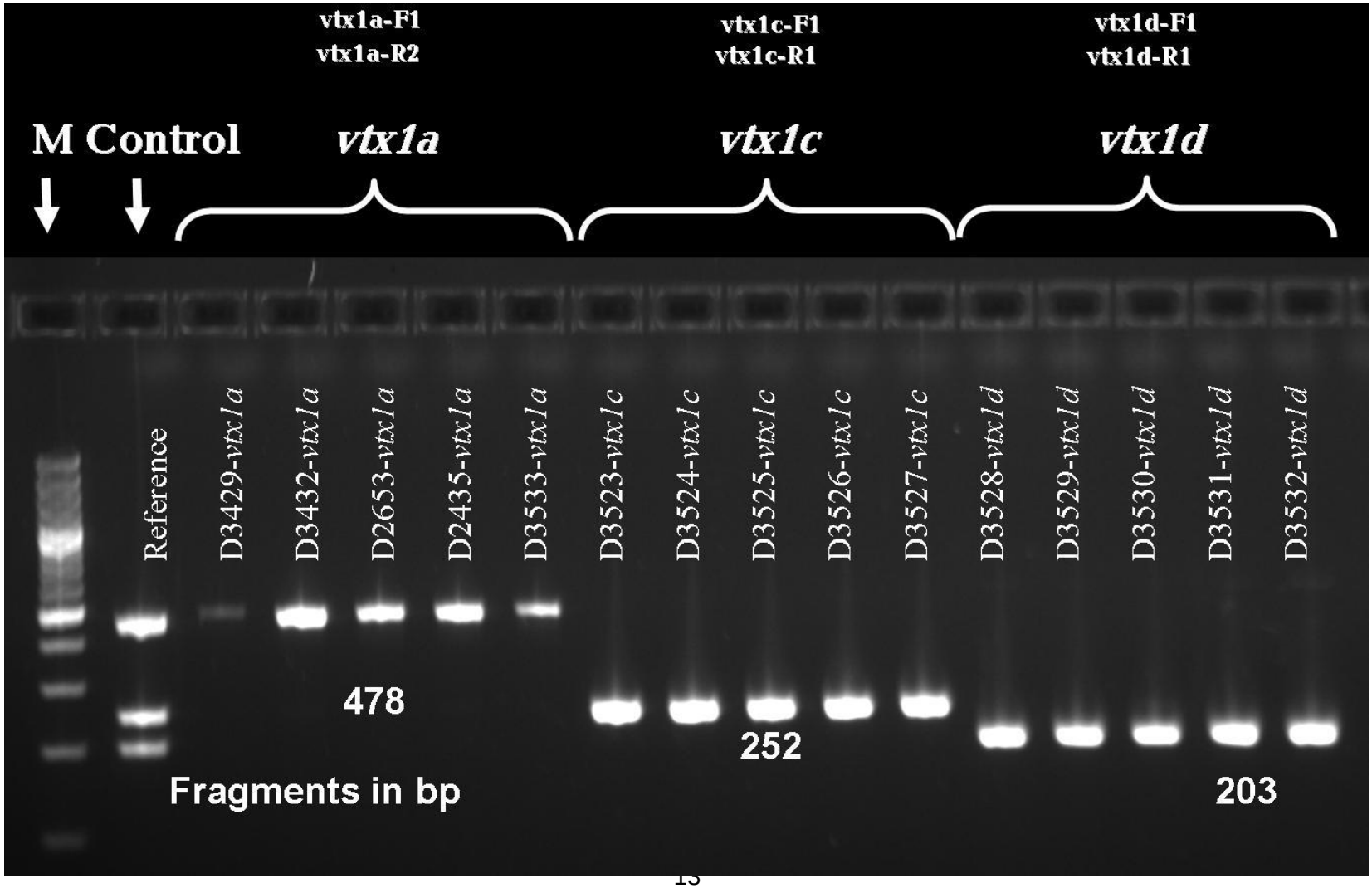

Figure 2. Gel picture illustrating *stx2a*, *stx2b*, *stx2c*, *stx2d*, *stx2e*, *stx2f* and *stx2g* positive strains.
D3435 (strain C165-02) can be positive with both reverse primers resulting in two bands of 179bp and/or 280bp respectively.

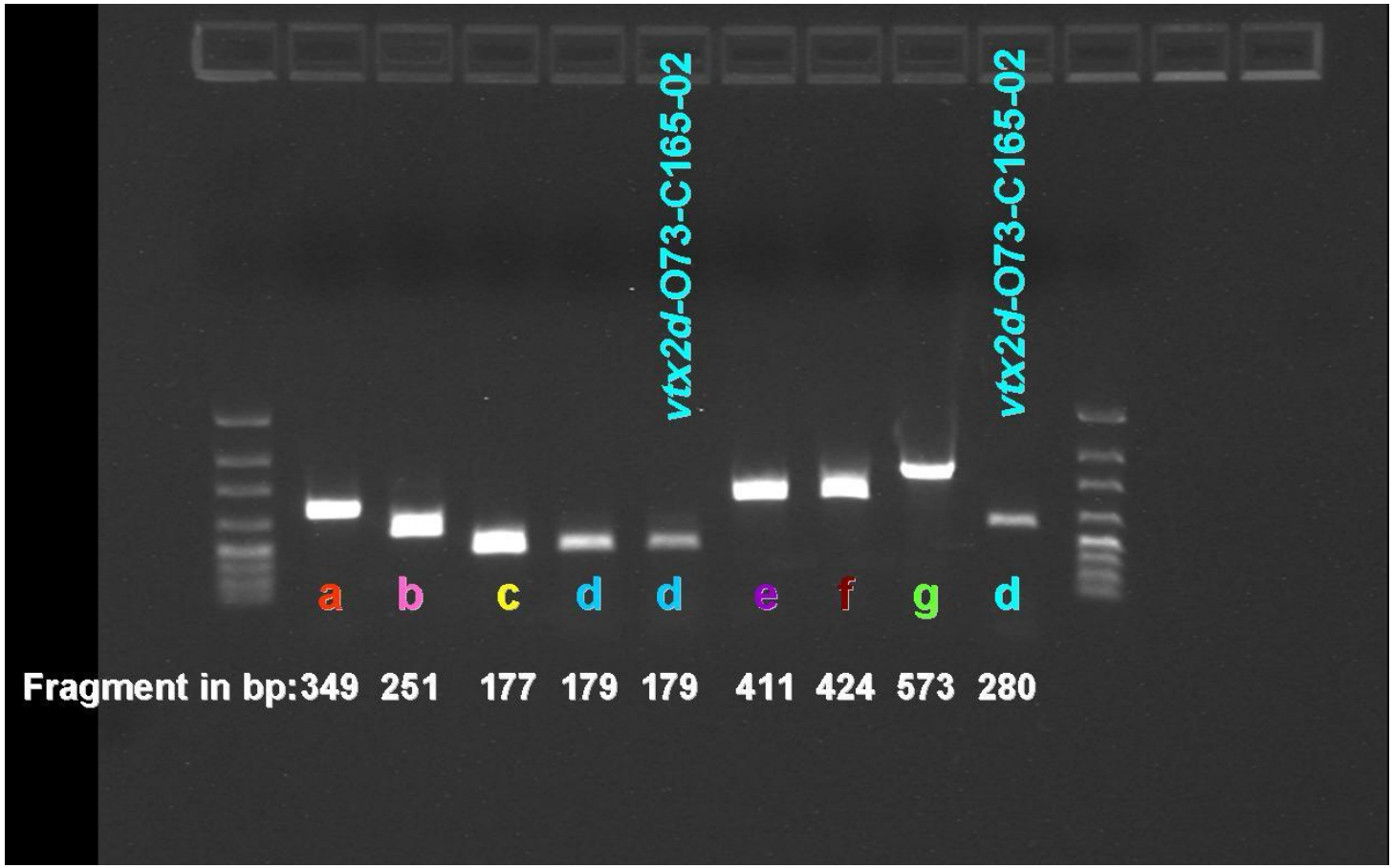

Figure 3. Gel picture illustrating *stx2a*, *stx2c* and *stx2d* positive strains.
D3435 (strain C165-02) is positive with both stx2d-R1 and stx2d-R2 resulting in two bands of 179bp and 280bp, respectively (last lane). Because of the high degree of similarity between *stx2c* and *stx2d* some *stx2d* positive strains (D3435) also give a weak *stx2c* ghost band. In order to distinguish between a ghost band and a true *stx2c* band compare with D2587 (strain 031), which is clearly positive for *stx2c* (and *stx2b*).

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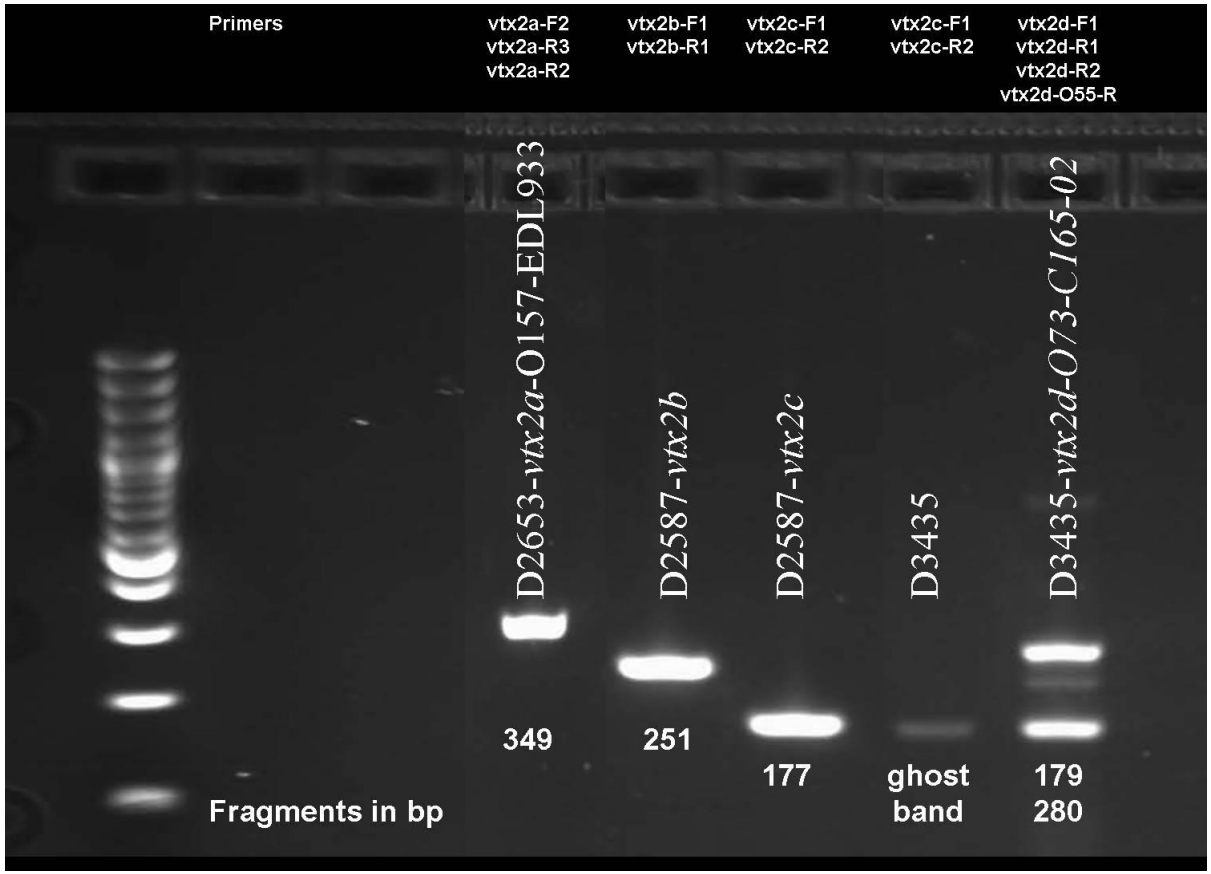

Table 4. List of reference strains harbouring the *stx* gene subtypes, their O:H serotype and additional virulence genes

SSI collection D number	Strain	Control for toxin subtype	Toxin variant designation	GenBank accession No.	Results obtained using the present method	Serotype	Additional virulence genes
D2653§	EDL933	Stx1a	Stx1a-O157-EDL933	M19473	*stx1a + stx2a*	O157:H7	*eae, ehxA, astA*
D3602	DG131/3	Stx1c	Stx1c-O174-DG131-3	Z36901	*stx1c + stx2b*	O174:H8	
D3522	MHI813	Stx1d	Stx1d-O8-MHI813	AY170851	*stx1d*	O8:K85ab:Hrough	*eae*
D2653§	EDL933	Stx1a	Stx1a-O157-EDL933	M19473	*stx1a + stx2a*	O157:H7	*eae, ehxA, astA*
D3428	EH250	Stx2b	Stx2b-O118-EH250	AF043627	*stx2b*	O118:H12	*astA*
D2587 ¤	031	Stx2c	Stx2c-O174-031	L11079	*stx2b + stx2c*	O174:H21	
D3435*	C165-02	Stx2d	Stx2d-O73-C165-02	DQ059012	*stx2d*	O73:H18	*astA*
D3648	S1191	Stx2e	Stx2e-O139-S1191	M21534	*stx2e*	O139:K12:H1	
D3546	T4/97	Stx2f	Stx2f-O128-T4-97	AJ010730	*stx2f*	O128ac:[H2]	*eae, bfpA, astA*
D3509	7v	Stx2g	Stx2g-O2-7v	AY286000	*stx2g*	O2:H25	*ehxA, astA, estAp*

§ This strain is used for both Stx1a and Stx2a

¤ This strain has been rplaced by D3431, serotype O157:H7, *eae, ehxA, astA*

* May result in both fragments at 179 bp and 280 bp; This strain has been replaced by D4134, serotype O166:H15; ESBL producing

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