Detection of *Escherichia coli* producing the Stx2f subtype by Real-Time PCR

1. Aims and field of application

Shiga toxin is an essential factor for the development of severe symptoms like haemolytic uraemic syndrome and can be divided into two main types: Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2). Within both groups, several subtypes are distinguished. The subtype Stx2f is one of the latest described in the literature, often found in *E. coli* strains from pigeons (Schmidt H. *et al.,* 2000; Morabito S. *et al*., 2001). So far, reports of human illness due to Stx2f-producing STEC are rare, but sporadic cases of human disease associated with such strains, including Hemolytic Uremic Syndrome cases, are increasingly reported (Friesema *et al.,* 2014; Grande L. *et al.,* 2016; Prager R. *et al.,* 2009). The sequence of the genes encoding Stx2f is highly different from that of the other subtypes of and cannot be detected by the primers and probes described in the ISO/TS 13136:2012-Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (STEC) and determination of O157, O111, O26, O103 and O145 serogroups-Qualitative Real Time polymerase chain reaction (PCR)-based Method.

This Real Time PCR protocol is used to test the samples that give negative results for the presence of *stx* genes by using the first step of the ISO/TS 13136:2012, to investigate on the presence of STEC strains possessing the *stx2f* subtype.

2. Real-Time PCR amplification

The same DNA preparation assayed by ISO/TS 13136:2012 can be used as template in the Real Time PCR amplification of the *stx2f* gene. DNA extraction step is accomplished by any method in use in the laboratory for the extraction and purification of DNA from food enrichment cultures.

The present Real Time PCR protocol is based on the 5' nuclease PCR assay. Considering that Real Time PCR may use different instruments and probes labelling chemistry, the amplification conditions to be applied may vary depending on the system used. Refer to the instructions supplied with the instrument and kit of choice. A standard two-step thermal profile used at EURL-VTEC for this reaction is the following:

95 °C x 10'
35 cycles of
95 °C x 15''
60 °C x 1'

The primers and probes to be used are listed in Table 1. The chemistry of the reporter and quencher fluorophores is not indicated, being largely dependent on the Real Time PCR systems available in each laboratory.
The reaction should be assembled applying the following instructions:

Buffer 2X	to 1X ($MgCl_2$ 3mM)
Primer Fwd	500 nM
Primer Rev	500 nM
Probe	200nM
DNA	2 µl of DNA purified from 1 ml of culture and diluted 1:10 can be sufficient
Water	to final volume

Table 1.

Primers and TaqMan probe used for 5' nuclease PCR assays, developed by EURL-VTEC

Target gene	Forward primer, reverse primer and probe sequences (5'-3')	Amplicon size (bp)	Location within sequence	GenBank accession number
stx2f	ATTCTCGGAAGTGTTGCGGT CGCCCTGTCTCCAACAATCT Probe-ATTCAGTTCGTTCCGTGAGC	111	824-843 934-915 873-892	AJ010730

3. Confirmation by conventional PCR

Enrichment cultures positive for the presence of *stx2f* gene are streaked onto MacConkey agar or TBX plates or any other media suitable for *E. coli* isolation. Up to 50 isolated colonies with typical *E. coli* morphology are collected and point-inoculated on Nutrient Agar (NA) (single colonies) and H_2O (5 pools by 10 colonies each). The bacterial pools shall be boiled for 10 minutes and the presence of *stx2f* gene can be detected by conventional PCR with the primers for *stx2f* termed here stx2f-F1 and stx2f-R1 (Scheutz F. *et al*., 2012).

Primer name	Forward and reverse primers sequences (5'-3')	Amplicon size (bp)	Location within sequence	GenBank accession number
stx2f-F1	TGGGCGTCATTCACTGGTTG	419	523-542	AJ010730
stx2f-R1	TAATGGCCGCCCTGTCTCC		941-923	

The thermocycler conditions are 95 °C for 15 min followed by 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 (Scheutz F. *et al*., 2012).

4. Controls

A DNA extracted from a STEC strain possessing *stx2f* gene should be used as positive control in Real Time and conventional PCR tests. Moreover, the Real Time PCR procedure requires an inhibition/extraction control.

5. References

- Friesema I, van der Zwaluw K, Schuurman T, Kooistra-Smid M, Franz E, van Duynhoven Y, van Pelt W. Emergence of *Escherichia coli* encoding Shiga toxin 2f in human Shiga toxin-producing *E. coli* (STEC) infections in the Netherlands, January 2008 to December 2011. Euro Surveill. 2014 May 1;19(17):26-32.
- Grande L, Michelacci V, Bondì R, Gigliucci F, Franz E, Badouei MA, Schlager S, Minelli F, Tozzoli R, Caprioli A, Morabito S. Whole-Genome Characterization and Strain Comparison of VT2f-Producing *Escherichia coli* Causing Hemolytic Uremic Syndrome. Emerg Infect Dis. 2016 Dec;22(12):2078-2086.

- Morabito S, Dell'Omo G, Agrimi U, Schmidt H, Karch H, Cheasty T, et al. Detection and characterization of Shiga toxin producing *Escherichia coli* in feral pigeons. Vet Microbiol. 2001;82(3):275-83.
- Prager R, Fruth A, Siewert U, Strutz U, Tschäpe H. *Escherichia coli* encoding Shiga toxin 2f as an emerging human pathogen. Int J Med Microbiol. 2009 Jun;299(5):343-53.
- 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 Sep;50(9):2951-63.
- Schmidt H, Scheef J, Morabito S, Caprioli A, Wieler LH, Karch H. A new Shiga toxin 2 variant (Stx2f) from *Escherichia coli* isolated from pigeons. Appl Environ Microbiol. 2000;66(3):1205-8.