Identification and characterization of Verocytotoxin-producing *Escherichia coli* (VTEC) by Real Time PCR amplification of the main virulence genes and the genes associated with the serogroups mainly associated with severe human infections

1. Aim and field of application

VTEC are *Escherichia coli* strains harboring lysogenic bacteriophages carrying genes encoding the production of Verocytotoxins (VTs). The strains associated with severe infections in humans, in particular the hemolytic uremic syndrome (HUS), frequently possess the intimin-coding *eae* gene and belong to a restricted number of serogroups. The present method is applied to detect by Real Time PCR the presence of the main virulence genes in *E. coli* strain cultures, for their identification as VTEC, and of the genes associated with five O serogroup causing the majority of HUS cases in Europe.

The *E. coli* genes comprised in the field of application of this method include:

1) *vtx* genes (*vtx1* and *vtx2* groups) encoding the Verocytotoxins (Shiga toxins), the main virulence factors of VTEC;

2) The *eae* gene, encoding a 90KDa protein, the intimin, which is the key factor for the induction of the “attaching and effacing” lesion on the enterocyte, a typical feature of the pathogenic VTEC strain;

3) Serogroup-associated genes. The method is aimed at the identification of the VTEC serogroups causing the majority of HUS cases in Europe: O157, O26, O111, O103, O145. This is achieved by Real-Time PCR amplification of fragments of genes comprised in the

operons encoding the different lipopolysaccharides (LPS) constituting the O antigens or anyhow associated to each serogroup in a unique manner (Table 1).

This method is applied to DNA purified from isolated bacterial strains as the matrix, for the confirmation of the presence of the virulence genes and serogroup identification.

The target genes and the primers and probes used in this method are the same included in the international standard for the detection of VTEC in food ISO/TS 13136: 2012.

2. Procedure

2.1. DNA extraction and purification

One ml of an overnight culture in a non-selective medium suitable for *E. coli* is used for DNA extraction. This step is accomplished by any method in use in the laboratory for the extraction and purification of DNA from food enrichment cultures.

When dealing with cultures from solid media, an isolated colony is suspended in 1 ml of distilled water and used for DNA extraction.

2.2 Real-Time PCR amplification

The 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.

The primers and probes to be used are listed in the tables below. The chemistry of the reporter and quencher phluorophores is not indicated being largely dependent on the Real-Time PCR systems available in each laboratory. The bibliographic references for the primers and probes sequences are indicated in the table captions.

Table 1. Degenerate primers and TaqMan probes used for 5’ nuclease PCR assays. ([§]Perelle S. et al. Mol Cell Probes 2004 **18**:185–192 and [*]Møller Nielsen E. and Thorup Andersen M. J clin Microbiol 2003 **41:**2884-2893)

Target gene	Forward primer, reverse primer and probe sequences (5’-3’)[a]	Amplicon size (bp)	Location within sequence	GenBank accession number
stx1[§]	TTTGTYACTGTSACAGCWGAAGCYTTACG CCCCAGTTCARWGTRAGRTCMACRTC **Probe-**CTGGATGATCTCAGTGGGCGTTCTTATGTAA	131	878–906 983–1008 941–971	M16625
stx2[§]	TTTGTYACTGTSACAGCWGAAGCYTTACG CCCCAGTTCARWGTRAGRTCMACRTC **Probe-**TCGTCAGGCACTGTCTGAAACTGCTCC	128	785–813 785–813 838–864	X07865
*eae**[*]	CAT TGA TCA GGA TTT TTC TGG TGA TA CTC ATG CGG AAA TAG CCG TTA **Probe-**ATAGTCTCGCCAGTATTCGCCACCAATACC	102	899-924 1000-979 966-936	Z11541

[a] In the sequence Y is (C, T), S is (C, G), W is (A, T), R is (A, G), M is (A, C).

Table 2. Primers and probes used for amplification of O antigen-specific genes in 5' nuclease PCR assays. ([§]Perelle S. et al. Mol Cell Probes 2004 **18**:185–192 and [*]Perelle S. et al. J Appl Microbiol 2005 **98**:1162–1168)

Target gene (serogroup)	**Forward primer, reverse primer and probe sequences (5'-3')**	**Amplicon size (bp)**	**Location within sequence**	**GenBank accession number**
[§]*rfbE* (O157)	TTTCACACTTATTGGATGGTCTCAA CGATGAGTTTATCTGCAAGGTGAT **Probe-** AGGACCGCAGAGGAAAGAGAGGAATTAAGG	88	348–372 412–435 381–410	AF163329
[§]*wbdI* (O111)	CGAGGCAACACATTATATAGTGCTTT TTTTTGAATAGTTATGAACATCTTGTTTAGC **Probe-** TTGAATCTCCCAGATGATCAACATCGTGAA	146	3464–3489 3579–3609 3519–3548	AF078736
[§]*wzx* (O26)	CGCGACGGCAGAGAAAATT AGCAGGCTTTTATATTCTCCAACTTT **Probe-** CCCCGTTAAATCAATACTATTTCACGAGGTTGA	135	5648–5666 5757–5782 5692–5724	AF529080
[§]*ihp1* (O145)	CGATAATATTTACCCCACCAGTACAG GCCGCCGCAATGCTT **Probe-**CCGCCATTCAGAATGCACACAATATCG	132	1383–1408 1500–1514 1472–1498	AF531429
[*]*wzx* (O103)	CAAGGTGATTACGAAAATGCATGT GAAAAAAGCACCCCCGTACTTAT **Probe-**CATAGCCTGTTGTTTTAT	99	4299–4323 4397–4375 4356–4373	AY532664

2.3. Controls

A positive and two negative controls are included in each PCR assay. The positive control is a DNA template obtained from an *E. coli* strain possessing the target gene tested, while a negative control is the DNA from a non-pathogenic *E. coli* isolate (no virulence genes harbored, not belonging to the 5 serogroups) and the other is constituted by a sample without the template added.

The Real-Time PCR procedure requires an **inhibition/extraction control**. In particular, two different internal amplification controls (IACs) can alternatively be used:

- A commercially available TaqMan® Exogenous Internal Positive Control (Applied Biosystems, Foster City, CA, USA). The kit includes all reagents necessary (primers, a Vic™probe, IAC target DNA and blocking solution). The IAC target DNA must be diluted 10 times to achieve a copy number of approximately 100 per PCR reaction. The PCR product length is not declared to the customer.
- The open formula pUC 19 based internal amplification control IAC developed by Fricker et al. (2007. Appl Environ Microbiol **73:**1892-1898). Approximately 100 copies of target DNA (pUC 19) should be used per PCR reaction. The size of the IAC was 119 bp.

The latter may be also used as an extraction control by adding 100 copies of the pUC 19 plasmid to the sample aliquot, prior to the DNA purification step.