Identification and characterization of Verocytotoxin-producing *Escherichia coli* (VTEC) by PCR amplification of the main virulence genes

1. Aim and field of application

VTEC are *Escherichia coli* strains harbouring lysogenic bacteriophages carrying genes encoding the production of Verocytotoxins (VTs).

The present method is applied to detect by multiplex PCR the presence of VT-coding genes in *E. coli* cultures, for their identification as VTEC. The determination of the presence of the intimin-coding gene *eae* is also included, since it is considered an hallmark of VTEC strains highly pathogenic to humans.

The primer pairs used, stx1F/stx1R and stx2F/stx2R (Paton & Paton, 1998), are able to detect the genes belonging to the *vtx1* and *vtx2* groups, respectively. The latter recognise all the variants of *vtx2* except *vtx2f*. The amplification of the *vtx2f* variant is obtained by an individual primers set: 128-1/128-2 (Schmidt et al, 2000).

The primers used for the detection of *eae* (Paton & Paton, 1998) recognise all the reported polymorphic variants of this gene.

The method does not allow the discrimination of the *vtx2* variant genes, with the exception of *vtx2f*, and is intended for use with pure bacterial cultures only. A specific method for the identification of the *vtx* gene subtypes is available at the EU-RL VTEC web site (www.iss.it/vtec), section Methods.

2. Normative references

- ISO 6887:1993, Microbiology of food and animal feeding stuff - General guidance for the preparation of test samples, initial suspension and dilutions for microbiological examinations.

- ISO 7218:1996, Microbiology of food and animal feeding stuff - General rules for microbiological examinations.
- EN ISO 22174:2005 Microbiology of food and animal feeding stuffs -- Polymerase chain reaction (PCR) for the detection of food-borne pathogens -- General requirements and definitions.
- EU Directive 2003/99/CE
- ISO/CEN 20837:2006 Microbiology of food and animal feeding stuffs -- Polymerase chain reaction (PCR) for the detection of food-borne pathogens -- Requirements for sample preparation for qualitative detection.
- ISO/CEN 20838:2006 Microbiology of food and animal feeding stuffs -- Polymerase chain reaction (PCR) for the detection of food-borne pathogens -- Requirements for amplification and detection for qualitative methods.

3. Definitions

VTEC strains are pathogenic bacteria able to cause disease in humans. They possess the genes coding for VT type 1 and 2 and produce the toxin itself. The majority of VTEC strains isolated from cases of severe human disease possess also the *eae* gene, coding for the adhesin "intimin", involved in the "attaching and effacing" mechanism of adhesion to the intestinal mucosa.

Deoxyribonucleotides (dNTPs): nucleotidic units of DNA that are added to the amplification reaction in order to allow *in vitro* polymerisation of DNA.

Primers: synthesised oligonucleotides used to start the amplification of a template by DNA polymerase from 3'-OH end of the primers in *in vitro* amplification.

***Taq* polymerase:** DNA polymerase enzyme that catalyses the polymerisation of a new DNA molecule complementary to a template, in the presence of Mg2+, starting from the primers trigger region and using dNTPs for the extension.

MilliQ water: water provided by a Millipore (or equivalent) deionizer, whose conductivity is no less than 180 μS.

4. Abbreviations

PCR: polymerase chain reaction

S.U.: Sample unit

TSB: Tryptone Soy Broth

VT: verocytotoxin

VTEC: Verocytotoxin-producing *Escherichia coli*

eae: intimin-coding gene

vtx: VT-coding gene

EtBr: ethidium bromide

5. Procedure

5.1 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.

Detection of *vtx1* and *vtx2* gene groups, and *eae* gene is performed by a multiplex PCR reaction using specific primers (**Annex 1**). The method is composed of the following steps:

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

5.2 Template preparation

Cultures streaked onto solid media (e.g. TSA) are processed as follows:

- pick a single bacterial colony up with a sterile 1 µl loop;
- prepare the template by suspending the bacteria in 100 µl of 0.22 µm filter-sterilised MilliQ water and boil for 10 minutes.

5.3 Setting up the PCR reaction

For each sample, set up a 50 µl reaction (reaction buffer 1X, $MgCl_2$ 1.2 mM, dNTPs 0.2 mM each, 50 pmoles of each primer, 2 Uts of *Taq* polymerase and 10 µl of DNA template). The volume of the reagents can be scaled according to the final volume of reaction. MilliQ water must be used for PCR reactions.

In each PCR assay, a positive and two negative controls must be included. The positive controls are DNA templates obtained from *E. coli* strains possessing the virulence genes tested, while a negative control is the DNA from a non-pathogenic *E. coli* isolate (no virulence genes harboured) and the other is constituted by a sample without template added.

The reactions are incubated in a thermal cycler programmed with the thermal profile described by Paton & Paton (1998) (**Annex 1**).

5.4 Agarose gel electrophoresis

Prepare a 2.5% (w/v) agarose gel in 1X Tris/Borate/EDTA (TBE) or Trs/Acetate/EDTA (TAE). Each well of the gel is loaded with 15 µl of each reaction added with loading die 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 the table in **Annex 1**). Consider that a correct band assignment is a crucial point in the assessment of the presence of the virulence genes. Make sure that the bands produced by the reference strains match exactly the expected molecular weight. The use of 2.5% agarose gels is needed to get a satisfactory resolution between the bands referring to *eae* (384 bp) and *stx2f* (428 bp) genes.

Agarose gels should be added of ethidium bromide to allow the visualisation 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.

6. 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.

7. Reference strains

A VTEC strain harboring *vtx1*, *vtx2* and *eae* genes should be used as positive control for all these genes. An example is the *E. coli* O157 EDL933 reference strain (ATCC no 43895) (O'Brien *et al*. 1984; Perna *et al*., 2001).
A VTEC strain harboring *vtx2f* gene should be used as positive control for this gene. An example is the *E.coli* O18ab strain ED-378, which also possess the *eae* gene (Morabito et al, 2001).
Any *Escherichia coli* K12 strain such as LE392 can be used as negative control.
PCR controls are prepared as described in section 5.2 (Template preparation). The control templates can be prepared in advance and stored in 10 µl ready to use aliquots at -20°C for eight months.

8. Interpretation of the results

Samples showing amplification fragments of the expected size (See 5.4 and **Annex 1**) are considered as positive for related target genes.
Positive and negative controls must be included in each reaction and give positive and negative results, respectively.

9. References

- Morabito S., G. Dell'Omo, U. Agrimi, H. Schmidt, H. Karch, T. Cheasty, A. Caprioli. Detection and characterization of Shiga toxin-producing *Escherichia coli* in feral pigeons. Vet Microbiol 2001; 82: 275-283.
- O'Brien AD, Newland JW, Miller SF, Holmes RK, Smith HW, Formal SB. Shiga-like toxin-converting phages from Escherichia coli strains that cause hemorrhagic colitis or infantile diarrhea. Science. 1984; 226: 694-6.
- Paton AW, Paton JC. Detection and characterization of Shiga *toxigenic* *Escherichia coli* by using multiplex PCR assays for stx1, stx2, eaeA,

enterohemorrhagic *E. coli* hlyA, rfbO111, and rfbO157. J Clin Microbiol. 1998;36: 598-602.

- Perna N.T. *et al.* Genome sequence of enterohaemorrhagic Escherichia coli O157:H7. Nature 2001 409: 529-533.
- Schmidt H., J. Scheef, S. Morabito, A. Caprioli, L. Wieler, H. Karch. A new Shiga toxin 2 variant (Stx2f) from *Escherichia coli* isolated from pigeons. Appl Environment Microbiol 2000; 66: 1205-1208.

Annex 1.

Table: Primer sequences and amplicon sizes.

Target gene	Primer Name (ref.)	Primer Sequence	Amplicon Size (bp)
eae	eaeAF (Paton & Paton, 1998)	GACCCGGCACAAGCATAAGC	384
	eaeAR (Paton & Paton, 1998)	CCACCTGCAGCAACAAGAGG	
vtx1	stx1F (Paton & Paton, 1998)	ATAAATCGCCATTCGTTGACTAC	180
	stx1R (Paton & Paton, 1998)	AGAACGCCCACTGAGATCATC	
vtx2	stx2F (Paton & Paton, 1998)	GGCACTGTCTGAAACTGCTCC	255
	stx2R (Paton & Paton, 1998)	TCGCCAGTTATCTGACATTCTG	
vtx2f	128-1 (Schmidt et al. 2000)	AGA TTG GGC GTC ATT CAC TGG TTG	428
	128-2 (Schmidt et al. 2000)	TAC TTT AAT GGC CGC CCT GTC TCC	

Thermal profile (from Paton & Paton, 1998):

35 PCR cycles, each consisting of 1 min of denaturation at 95°C; 2 min of annealing at 65°C for the first 10 cycles, decrementing to 60°C by cycle 15; and 1.5 min of elongation at 72°C, incrementing to 2.5 min from cycles 25 to 35