Identification of the STEC serogroups mainly associated with human infections by Real-Time PCR amplification of O-associated genes

1. Aim and field of application

The present method concerns the identification by Real-Time PCR amplification of the genes associated with the O-antigens of the STEC serogroups mainly associated to severe human disease. The method is intended for the identification of the serogroup of *E. coli* strains isolated in pure culture. The serogroups in the field of application of the present method are: O26, O45, O55, O91, O103, O104, O111, O113, O121, O128, O145, O146 and O157. All these serogroups have been frequently reported in human infections.

2. Definitions

STEC: strains possessing the genes encoding the Shiga-toxins. The majority of STEC 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.

O-antigen: serogroups or “O” antigens are identified by numbers, counting from 1 to 187, and the serogroups list is evolving constantly.

Primers: oligonucleotides used to prime the amplification of a template by DNA polymerase.

Taqman probes: oligonucleotides labelled with a fluorophore covalently attached to the 5’-end and a quencher at the 3’-end, used to increase the specificity of Real-Time PCR.

3. Procedure

3.1 DNA extraction and purification

An appropriate nucleic acid extraction procedure for Gram-negative bacteria should be used to prepare DNA, from a pure culture from liquid or from solid media, according to the selected procedure.

3.2 Real-Time PCR amplification

The protocol is based on the 5' nuclease PCR assay. Considering that Real-Time PCR may use different probes labelling chemistry and run on different instruments, 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 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 bibliographic references for the primers and probes sequences are indicated in the table. The Real-Time PCR procedures for the detection of the genes associated to the top-5 serogroups (O157, O26, O103, O111 and O145) and to serogroup O104 correspond to those illustrated in the following two EURL-VTEC methods, respectively:

- "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", available at the link: http://www.iss.it/binary/vtec/cont/EU_RL_VTEC_Method_02_Rev_0.pdf;

- "Detection and identification of Verocytotoxin-producing *Escherichia coli* (VTEC) O104:H4 in food by Real Time PCR", available at the link: http://www.iss.it/binary/vtec/cont/EU_RL_VTEC_Method_04_Rev_1.pdf.

As stated above, amplification conditions will depend on the system used and need to be fine-tuned in each laboratory. However, a standard two-step thermal profile used at EURL-VTEC is the following:

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

The following thermal profile applies to the detection of O103 serogroup-associated gene only:

95 °C X 10'

35 cycles of

95 °C X 15''

55 °C X 1'

The reaction should be assembled applying the following instructions:

Buffer 10X	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 probes used for Real Time PCR assays.

Target gene (Ref)	Forward primer, reverse primer and probe sequences (5'-3')	Amplicon size (bp)	Location within sequence	GenBank accession number
wzxO26 **(Perelle et al., 2004)**	**FWD:** CGCGACGGCAGAGAAAATT **REV:** AGCAGGCTTTTATATTCTCCAACTTT **Probe:** CCCCGTTAAATCAATACTATTTCACGAGGTTGA	135	5648–5666 5757–5782 5692–5724	AF529080
wzxO45 **(USDA, 2012)**	**FWD:** CGTTGTGCATGGTGGCAT **REV:** TGGCCAAACCAACTATGAACTG **Probe:** ATTTTTTGCTGCAAGTGGGCTGTCCA	72	7472-7489 7543-7522 7494-7519	AY771223
wzxO55 **(EURL-VTEC)**	**FWD:** AATTAACGAACATAACACCCAACC **REV:** ATATCTCTTCGTTACTGTGTGTATTTC **Probe:** ACCTCCCGCTAAAACCCCAACTCTAGTAG	101	11516-11493 11416-11442 11489- 11461	AF461121
wzyO91 **(Perelle et al., 2004)**	**FWD:** CGATTTTCTGGAATGCTTGATG **REV:** CAATACATAGTTTGATTTGTGTTTAAAGTTTAAT **Probe:** CCTGGGTTGTTAGGAACAATTTCAGCACTTC	105	9433–9454 9504–9537 9457–9487	AY035396

wzxO103 **(Perelle et al., 2005)**	**FWD:** CAAGGTGATTACGAAAATGCATGT **REV:** GAAAAAAGCACCCCCGTACTTAT **Probe:** CATAGCCTGTTGTTTTAT	99	4299–4323 4397–4375 4356–4373	AY532664
wzxO104 **(Bugarel et al., 2010)**	**FWD:** TGTCGCGCAAAGAATTTCAAC **REV:** AAAATCCTTTAAACTATACGCCC **Probe:** TTGGTTTTTTTGTATTAGCAATAAGTGGTGTC	100	2,333,750–2,333,730 2,333,673–2,333,651 2,333,724–2,333,693	CU928145
wbdIO111 **(Perelle et al., 2004)**	**FWD:** CGAGGCAACACATTATATAGTGCTTT **REV:** TTTTTGAATAGTTATGAACATCTTGTTTAGC **Probe:** TTGAATCTCCCAGATGATCAACATCGTGAA	146	3464–3489 3579–3609 3519–3548	AF078736
wzyO113 **(Perelle et al., 2004)**	**FWD:** GAGCGTTTCTGACATATGGAGTGA **REV:** TTGCTATAAATGGAAGCCATTCTTT **Probe:** TGCATGAAATGTTTAAATGCAGCGGGT	107	3689–3712 3771–3795 3738–3764	AF172324
wzxO121 **(USDA, 2012)**	**FWD:** AGGCGCTGTTTGGTCTCTTAGA **REV:** GAACCGAAATGATGGGTGCT **Probe:** CGCTATCATGGCGGGACAATGACAGTGC	189	6839-6860 7027-7008 6898-6925	AY208937

wzxO128 **(Lin et al., 2011)**	**FWD:** TCGATCGTCTTGTTCAGGTT **REV:** GAATGCAATGGGCAATTAAC **Probe:** GGGTTGCACAATTGGCCTCC	196	8857-8876 9052-9033 8918-8937	AY217096
ihp1O145 **(Perelle et al., 2004)**	**FWD:** CGATAATATTTACCCCACCAGTACAG **REV:** GCCGCCGCAATGCTT Probe-CCGCCATTCAGAATGCACACAATATCG	132	1383–1408 1500–1514 1472–1498	AF531429
wzyO146 **(EURL-VTEC)**	**FWD:** ACATTCGGCGTTTTTATCTCGT **REV:** GGTCAAATCTCGTGCCCATAGA Probe- AATTTCAAGGTGCCAACTTTTCA	106	9144-9165 9228-9249 9205-9227	DQ465249
rfbEO157 **(Perelle et al., 2004)**	**FWD:** TTTCACACTTATTGGATGGTCTCAA **REV:** CGATGAGTTTATCTGCAAGGTGAT **Probe:** AGGACCGCAGAGGAAAGAGAGGAATTAAGG	88	348–372 412–435 381–410	AF163329

3.3 Controls

DNA extracted from cultures of STEC strains belonging to serogroups O26, O45, O55, O91, O103, O104, O111, O113, O121, O128, O145, O146 and O157 should be used as positive control. The isolates provided by EURL-VTEC in the framework of the proficiency testing program can be used as reference strains. Moreover, the Real-Time PCR procedure requires an inhibition control.

3.4 Safety and protection devices

STEC strains can infect human beings at a very low infectious dose and can cause severe disease. Laboratory acquired infections have been reported. Therefore, handling STEC strains requires compliance to safety procedures in place (including the use of protection devices) and good laboratory practices. STEC are class 3 pathogens and in some countries their handling is allowed in CL 3 laboratory only.

4. References

- **Bugarel M, Beutin L, Martin A, Gill A, Fach P.** Micro-array for the identification of Shiga toxin-producing *Escherichia coli* (STEC) seropathotypes associated with Hemorrhagic Colitis and Hemolytic Uremic Syndrome in humans. Int J Food Microbiol. 2010 Sep 1;142(3):318-29.
- **Lin A, Sultan O, Lau HK, Wong E, Hartman G, Lauzon CR.** O serogroup specific real time PCR assays for the detection and identification of nine clinically relevant non-O157 STECs. Food Microbiol. 2011 May;28(3):478-83.
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- **United States Department of Agriculture.** Primer and Probe Sequences and Reagent Concentrations for non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) Real-Time PCR Assay. MLG 5B Appendix 1.01, https://www.fsis.usda.gov/wps/wcm/connect/0330211c-81ab-4e97-a9f3-d425f5759ee1/MLG_5B_Appendix_1_01.pdf?MOD=AJPERES