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

1. Aim and field of application

The present method concerns the identification by 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, O80, 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.
O-Somatic antigen: Serogroups or "O" antigens are identified by numbers, counting from 1 to 187, and the serogroups list is evolving constantly.
Deoxyribonucleotides (dNTPs): nucleotidic units of DNA that are added to the amplification reaction in order to allow in vitro polymerization of DNA.
Primers: oligonucleotides used to prime the amplification of a template by DNA polymerase.
Taq polymerase: DNA polymerase enzyme that catalyzes the polymerization of a DNA tract complementary to a template.

3. Abbreviations

PCR: polymerase chain reaction
TSA: Tryptone Soy Agar
STEC: Shiga toxin-producing *Escherichia coli*

EtBr: ethidium bromide

4. Procedure

4.1 Principle of the method

The method is based on the amplification by end-point PCR of the genes associated with the following O-groups: O26, O45, O55, O80, O91, O103, O104, O111, O113, O121, O128, O145, O146 and O157. The method includes the use of specific primers selected from the literature and is composed of the following steps:

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

4.2 Template preparation

Isolated strains streaked onto solid media (*e.g.* TSA) are processed as follows:
• Pick a single bacterial colony with a sterile 1 µl loop;
• Prepare the template by suspending the bacteria in 100 µl of filter-sterilized deionized water (0.22 µm) and keeping the tube in boiling water for 10 minutes.

4.3 Setting up the PCR reaction

Set up 50-µl reactions for each O-group, according to the conditions described in **section 5**. In each PCR assay, a positive and two negative controls must be included. The positive controls are DNA templates containing the O-associated genes tested, while negative controls must include a DNA sample negative for the O-antigen-genes concerned and a sample without any DNA added.

4.4 Agarose gel electrophoresis

Prepare an agarose gel (2 % w/v) in 1X Tris/Borate/EDTA (TBE) or Tris/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 (**section 5**). Make sure that the bands

produced by the samples and the positive control match exactly the expected molecular weight. Agarose gels should be added of ethidium bromide to allow the visualization 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 1 µg/ml ethidium bromide aqueous solution. Ethidium bromide is a mutagen and should be handled according to the relevant safety sheet and or regulations. Less hazardous stains for visualization of DNA in agarose may be employed for the visualization of the DNA bands in the gel. These alternative dyes are commercially available and shall be used in the conditions indicated by the supplier.

4.5 Devices/Instruments

- Laminar flow hood for PCR
- Bacteriology sterile loops
- 37 °C +/-1 °C incubator
- Technical bench scales
- Autoclave
- Pipet-aid
- Micropipettes
- Sterile micropipette tips
- 1.5 ml microcentrifuge tubes
- 0.2 or 0.5 ml PCR tubes
- Thermalcycler
- Water deionizer
- Electrophoresis apparatus
- U.V. transilluminator
- Microwave oven

4.6 Reagents and media

- TSA plates
- Deionized water

- dNTPs stock solution
- Synthetic oligonucleotides solution
- Taq DNA polymerase and reaction buffer 10X with or without $MgCl_2$
- Electrophoresis running buffer
- Molecular weight DNA marker
- Loading dye
- Agarose
- Ethidium bromide or alternative gel staining solution

4.7 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, working with STEC requires good laboratory practices and the use of protection devices. STEC are class 3 pathogens and in some countries their handling is allowed in Class 3 laboratory only. 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 nytrile gloves). The U.V. light may cause damage to eyes so it is mandatory the use of plexiglass shields and protective glasses.

4.8 Reference strains

STEC strains belonging to serogroups O26, O45, O55, O80, 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 programs can be used as reference strains.

4.9 Interpretation of the results

Samples showing amplification fragments of the expected size (**section 5**) are considered positive.

5. Primers' sequences and amplification conditions

5.1 Primers sequences and amplicon sizes

O antigen	Target gene	Primer	Sequence	Amplicon (bp)	Ref.
O26	*wzx*	5'O26	ACTCTTGCTTCGCCTGTT	268	(5)
		3'O26	CAGCGATACTTTGAACCTTAT		
O45	*wzx*	O45 wzx2 F	TATGACAGGCACATGGATCTGTGG	255	(2)
		O45 wzx2 R	TTGAGACGAGCCTGGCTTTGATAC		
O55	*wbgN*	O55F	TGTAATTCGATGCACCAATTCAG	70	(7)
		O55R	CGCTTCGACGTTCGATACATAA		
O80	*wzy*	O80F	TGAGAGCCAAGATCCAAGCA	158	EURL-VTEC
		O80R	TGGGCCATATTCGAAGTTTGAA		
O91	*wzy*	O91F	CGATTTTCTGGAATGCTTGATG	105	(7)
		O91R	CAATACATAGTTTGATTTGTGTTTAAAGTTTAAT		
O103	*wzx*	5'O103	TATCCTTCATAGCCTGTTGTT	320	(5)
		3'O103	AATAGTAATAAGCCAGACACCTG		
O104	*rfb*	O104rfbO-f	TGAACTGATTTTTAGGATGG	351	(1)
		O104rfbO-r	AGAACCTCACTCAAATTATG		
O111	*wzx*	5'O111.3	GTTGCGAGGAATAATTCTTCA	829	(5)
		3'O111.2	CCATAGATATTGCATAAAGGC		
O113	*wzy*	O113F	AGCGTTTCTGACATATGGAGTG	593	(6)
		O113R	GTGTTAGTATCAAAAGAGGCTCC		
O121	*wzx*	5'O121	GTAGCGAAAGGTTAGACTGG	651	(5)
		3'O121	ATGGGAAAGCTGATACTGC		
O128	*wzy*	O128 13F	ATGATTTCTTACGGAGTGC	782	(3)
		O128 13R	CTCTAACCTAATCCCTCCC		
O145	*wzx*	5'O145.6	TTGAGCACTTATCACAAGAGATT	418	(5)
		3'O145.B	GATTGAATAGCTGAAGTCATACTAAC		
O146	*wzy*	O146 F	ATTCGGGTAACGACCCTGTGTTGA	378	(4)
		O146 R	AGACTGCTAATGCAAGGAACATGG		
O157	*wzx*	5'O157	GCTGCTTATGCAGATGCTC	133	(5)
		3'O157	CGACTTCACTACCGAACACTA		

5.2 Amplification conditions by serogroup

1) O26: Reaction Buffer 1X; $MgCl_2$ 3 mM; dNTPs concentration 0.3 mM each; primers' concentration 400 nM; 2.5 Units of Taq. Thermal profile: 95 °C for 5 minutes, 10 cycles of 95 °C for 30 seconds, 68–59 °C (–1 °C/cycle) for 20 seconds, 72 °C for 52 seconds, followed by 35 cycles of 95 °C for 30 seconds, 59 °C for 20 seconds, 72 °C for 52 seconds and a final extension at 72 °C for 1 minute.

2) O45: Reaction Buffer 1X; $MgCl_2$ 2 mM; dNTPs concentration 0.2 mM each; primers' concentration 500 nM, 1 Unit of Taq. Thermal profile: 95 °C for 5 minutes, 30 cycles 95 ° C for 30 seconds, 59 ° C for 30 seconds, 72 ° C for 30 seconds; and a final extension step at 72 ° C for 5 minutes.

3) O55: Reaction Buffer 1X; $MgCl_2$ 1.5 mM; dNTPs concentration 0.2 mM each; primers' concentration 500 nM; 1 Unit of Taq. Thermal profile: 95 °C for 5 minutes, 35 cycles 95 ° C for 30 seconds, 57 ° C for 30 seconds, 72 ° C for 30 seconds; and a final extension step at 72 ° C for 5 minutes.

4) O80: Reaction Buffer 1X; $MgCl_2$ 1.5 mM; dNTPs concentration 0.2 mM each; primers' concentration 500 nM; 1 Unit of Taq. Thermal profile: 95 °C for 5 minutes, 35 cycles 95 ° C for 30 seconds, 55 ° C for 30 seconds, 72 ° C for 30 seconds; and a final extension step at 72 ° C for 5 minutes

5) O91: Reaction Buffer 1X; $MgCl_2$ 1.5 mM; dNTPs concentration 0.2 mM each; primers' concentration: 500 nM; 1 Unit of Taq. Thermal profile : 95 °C for 5 minutes, 30 cycles 95 ° C for 30 seconds, 58 ° C for 30 seconds, 72 ° C for 30 seconds; and a final extension step at 72 ° C for 5 minutes.

6) O103: Reaction Buffer 1X; $MgCl_2$ 3 mM; dNTPs concentration 0.3 mM each; primers' concentration 200 nM; 2.5 Units of Taq. Thermal profile: 95 °C for 5 minutes, 10 cycles of 95 °C for 30 seconds, 68–59 °C (decrease 1 °C/cycle) for 20 seconds, 72 °C for 52 seconds, followed by 35 cycles of 95 °C for 30 seconds, 59 °C for 20 seconds, 72 °C for 52 seconds and a final extension at 72 °C for 1 minute.

7) O104: Reaction Buffer 1X; $MgCl_2$ 1.5 mM; dNTPs concentration 0.2 mM each; primers' concentration 360 nM; 1 Unit of Taq; Thermal profile: 95 °C for 5 minutes, 30 cycles 95 °C for 30 seconds, 55 °C for 1 minute, 72 °C for 1 minute; and a final extension step 72 °C for 5 minutes.

8) O111: Reaction Buffer 1X; $MgCl_2$ 3 mM; dNTPs concentration 0.3 mM each; primers' concentration 200 nM; 2.5 Units of Taq. Thermal profile: 95 °C for 5 minutes, 10 cycles of 95 °C for 30 seconds, 68–59 °C (decrease 1 °C/cycle) for 20 seconds, 72 °C for 52 seconds, followed by 35 cycles of 95 °C for 30 seconds, 59 °C for 20 seconds, 72 °C for 52 seconds and a final extension at 72 °C for 1 minute.

9) O113: Reaction Buffer 1X; $MgCl_2$ 2 mM; dNTPs concentration 0.2 mM each; primers' concentration 250 nM; 1 Unit of Taq. Thermal profile: 95 °C for 5 minutes, 30 cycles 95 °C for 30 seconds, 60 °C for 30 seconds, 72 °C for 1 minute; and a final extension step 72 °C for 5 minutes.

10) O121: Reaction Buffer 1X; $MgCl_2$ 3 mM; dNTPs concentration 0.3 mM each; primers' concentration 100 nM; 2.5 Units of Taq. Thermal profile: 95 °C for 5 minutes, 10 cycles of 95 °C for 30 seconds, 68–59 °C (decrease 1 °C/cycle) for 20 seconds, 72 °C for 52 seconds, followed by 35 cycles of 95 °C for 30 seconds, 59 °C for 20 seconds, 72 °C for 52 seconds and a final extension at 72 °C for 1 minute.

11) O128: Reaction Buffer 1X; $MgCl_2$ 1.5 mM; dNTPs concentration 0.2 mM each; primers' concentration 500 nM; 1 Unit of Taq; Thermal profile: 95 °C for 5 minutes, 35 cycles 95 °C for 30 seconds, 50 °C for 30 seconds, 72 °C for 1 minute; and a final extension step 72 °C for 5 minutes.

12) O145: Reaction Buffer 1X, $MgCl_2$ 3 mM; dNTPs concentration 0.3 mM each; Primers' concentration 200 nM; 2.5 Units of Taq. Thermal profile: 95 °C for 5 minutes, 10 cycles of 95 °C for 30 seconds, 68–59 °C (decrease 1 °C/cycle) for 20 seconds, 72 °C for 52 seconds, followed by 35 cycles of 95 °C for 30 seconds, 59 °C for 20 seconds, 72 °C for 52 seconds and a final extension at 72 °C for 1 minute.

13) O146: Reaction Buffer 1X; $MgCl_2$ 1.5 mM; dNTPs concentration 0.2 mM each; primers' concentration: 500 nM; 1 Unit of Taq. Thermal profile: 95 °C for 5 minutes, 35 cycles 95 °C for 30 seconds, 50 °C for 30 seconds, 72 °C for 1 minute; and a final extension step 72 °C for 5 minutes.

14) O157: Reaction Buffer 1X; $MgCl_2$ 3 mM; dNTPs concentration 0.3 mM each; primers' concentration: 300 nM; 2.5 Units of Taq. Thermal profile: 95 °C for 5 minutes, 10 cycles of 95 °C for 30 seconds, 68–59 °C (decrease 1 °C/cycle) for 20 seconds, 72 °C for 52 seconds, followed by 35 cycles of 95 °C for 30 seconds, 59 °C for 20 seconds, 72 °C for 52 seconds and a final extension at 72 °C for 1 minute.

The PCR reactions for the amplification of the genes associated with O26, O103, O111, O121, O145 and O157 can be performed as a multiplex PCR. In case of a faint band in the positive control for a given target it is advisable to repeat the PCR in single.

6. References

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