Report of the 8th inter-laboratory study on the detection of VTEC belonging to the main pathogenic serogroups in water samples - 2012

1. INTRODUCTION

The duties of the EU Reference Laboratory for *E. coli* (EU-RL VTEC) include the development of methods for the detection of VTEC in food and animal samples and the organization of proficiency tests (PT) on those methods for the designated National Reference Laboratories (NRLs) for *E. coli* in the EU and EFTA Member States, EU Candidate Countries, and certain third countries.

The EU-RL VTEC has coordinated a working group that has drafted a method for the detection of VTEC in foodstuffs. On November 2012, this method has been published as an ISO Technical Specification: ISO/TS 13136 “Microbiology of food and animal feed -- Real-time polymerase chain reaction (PCR)-based method for the detection of food-borne pathogens -- Horizontal method for the detection of Shiga toxin-producing Escherichia coli (STEC) and the determination of O157, O111, O26, O103 and O145 serogroups”. Moreover, it had been recommended by EFSA for the detection of the main pathogenic serogroups in food and animal samples (Guidance on the technical specifications for the monitoring and reporting of VTEC on animals and food, *EFSA Journal* 2009; 7:1366).

The method had already been adopted and evaluated in three rounds of PT organized by the EU-RL: PT3, carried out on bovine carcass swabs, PT4, carried out on milk samples, and PT7, carried out on vegetable samples. The reports of these PTs are available in the EU-RL web site (ww.iss.it/vtec).

During the last years, a growing number of epidemic episodes have brought to the attention the role of water in the transmission of VTEC infections. Irrigation water has been often reported as the source of VTEC contamination of fresh produce involved in outbreaks. Other outbreaks have been linked to recreational water. Finally, water is largely used in the production of sprouted seeds, and testing spent irrigation water has been indicated as a possible alternative or a complement to the tests conducted on the sprouts before they are placed on the market or on the seed lots used for sprouting.

Since testing water samples of different origin for the presence of VTEC represents an important challenge for food and public health laboratories, the 8th PT was focused on the detection of the presence of VTEC belonging to the "top 5" serogroups involved in human infections (O157, O111, O26, O103, and O145) in water samples.

The results of the PT have been discussed during the 7th Annual Workshop of the EU reference laboratories for *E. coli* held in Rome on 8-9 November 2012. This document represents the full evaluation report of the study.

2. DESIGN AND OBJECTIVES OF THE STUDY

The study consisted in the assessment of the contamination of water samples with VTEC belonging to the "top 5" serogroups involved in human infections (O157, O111, O26, O103, and O145). In the absence of specific international standards for water, the method developed for the detection of VTEC in foodstuffs (CEN/ISO TS 13136) was adapted to this particular matrix. Two artificially contaminated water samples were sent to the laboratories that accepted to participate in the PT.

The **objectives** of the study were: i) to further train the NRLs in the use of the international standard for the detection of VTEC; ii) to test the effectiveness of the detection method when applied to a matrix not included in its field of application.

3. PARTICIPANTS

Thirty-two NRLs representing 23 EU Member States, Norway and Switzerland, and Egypt and Turkey participated in the study. Each NRL received its own individual laboratory numerical code, which was reported in the result tables.

The NRLs participating in the study were:

- Belgium – Scientific Institute of Public Health, Direction Opérationnelle Maladies Transmissibles et Infectieuses (also representing Luxembourg)
- Belgium – Veterinary and Agrochemical Research Centre (VAR-CODA-CERVA), Operational Directorate Bacterial Diseases
- Bulgaria – National Diagnostic and Research Veterinary Institute
- Cyprus – Laboratory for the Control of Food of Animal Origin (LCFAO), Veterinary Services
- Czech Republic – Veterinary Research Institute
- Egypt – Ministry of Agriculture, Central Lab. Of Residue Analysis of Pesticides and Heavy Metals in Foods, Giza

- Egypt – Ministry of Health and Population, Central Public Health Laboratories, Cairo
- Estonia – Veterinary and Food Laboratory
- Finland – Finnish Food Safety Authority, Evira, Microbiology Unit, Helsinki
- France – VetAgro Sup Campus Vétérinaire de Lyon, Laboratoire LMAP
- Germany – Federal Institute for Risk Assessment (BfR), Division “Microbial Toxins”
- Greece – National School of Public Health, Dept. of Microbiology
- Hungary – Central Agricultural Office Directorate Food and Feed Safety, Central Food Microbiological Laboratory
- Hungary – Central Agricultural Office Directorate Food and Safety, National Reference Laboratory for Feed Investigation
- Ireland – Central Veterinary Research Laboratory, Dept. of Agriculture & Food Laboratories
- Italy – Istituto Superiore di Sanità
- Latvia – Institute of Food Safety, Animal Health and Environment “BIOR”, Laboratory of Food and Environmental Investigation
- Netherlands – National Inst. For Public Health and the Environment (RIVM), Centre for Infectious Disease Control (Cib), Lab. For Zoonoses and Environmental Microbiology
- Netherlands – Netherlands Food and Consumer Product Safety Authority (NVWA), Microbiological Laboratory
- Norway – National Veterinary Institute, Section for food bacteriology and GMO
- Poland – National Institute of Public Health-National Institute of Hygiene, Dept. of Food and Consumer Articles Research, Warsaw
- Romania – Institute for Hygiene and Veterinary Public Health, Microbiology Dept.
- Slovakia – State Veterinary and Food Institute Dolný Kubín, Dept. of Molecular Biology
- Slovakia – Public Health Authority of Slovak Republic, UVZSR, National Reference Centre of Environmental Microbiology
- Slovenia – University of Ljubljana, National Veterinary Institute, Veterinary Faculty, Laboratory for Bacteriology and Mycology
- Spain – Laboratorio Central de Sanidad Animal
- Spain – Agencia Española de Seguridad Alimentaria y Nutrición, AESAN, Centro Nacional de Alimentación, Servicio de Microbiología Alimentaria
- Sweden – National Veterinary Institute, SVA, Dept. of Bacteriology
- Sweden – National Food Administration, SLV, Microbiology Division

- Switzerland – University of Zurich, Vetsuisse Faculty, Institute for food safety and hygiene
- Turkey – Public Health Institution of Turkey, Microbiology Reference Laboratories Dept. Nat. Ref. Lab. For Enteric Pathogens
- UK – Health Protection Agency Microbiology Services, Food Water and Environmental Microbiology Laboratory, Preston (also representing Malta).

4. MATERIALS AND METHODS

Two water samples (samples A and B, volume: 1 L) potentially contaminated with VTEC were sent in the blind to the NRLs. The NRLs were requested to identify the presence of VTEC belonging to the 5 main pathogenic serogroups (O157, O26, O103, O111 and O145) by examining the enrichment cultures for the presence of their virulence (*vtx*1 group, *vtx*2 group, and *eae*) and serogroup-specific genes. The PCR-positive samples were then subjected to a serogroup-specific enrichment procedure, based on immuno-magnetic concentration, followed by the isolation and characterization of the contaminating strains. In the absence of specific international standards for water, the method developed for the detection of VTEC in foodstuffs (ISO/TS 13136) was adapted to this particular matrix. The procedure comprised the following sequential steps:

- Filtration of water samples through membranes of mean pore size 0.45 µm, according to ISO 8199, section 8.2.5.
- Tranfer of the membranes to the enrichment medium
- Microbial enrichment
- Nucleic acid extraction
- Detection of virulence genes
- Detection of serogroup-associated genes
- Isolation of VTEC from the PCR-positive samples.

The laboratory procedure, provided by the EU-RL, is reported in the **Annex**.

4.1. Sample preparation

The characteristics of the samples are reported in Table 1 and were considered as the gold standard.

Table 1: Characteristics of the water samples included in the study

Contaminant	Sample A	Sample B
VTEC O157, *vtx1, vtx2, eae*	-	1.2 x 10^3 CFU/ml of contaminant

The uncertainty of measurement (UM) was calculated for the bacterial suspension of VTEC O157 used to spike the samples according to the ISO/TS 19036:2006. The UM value was 0.27 log CFU/ml

4.2. Assessment of the stability and homogeneity of the samples

The stability and homogeneity of the samples were evaluated according to the requirements of ISO 17043:2010.

For the stability assessment, *ad hoc* samples were prepared on 4 April 2012 using the same procedures adopted for the preparation of the test samples. One sample was analyzed on the same day of preparation, whereas three samples were stored at 5 °C ± 3 °C (ISO 7218:2007) and tested according to the study protocol on 6, 11, and 13 April. All the tests yielded the expected results.

The test samples were prepared on 16 April and labeled with randomly generated numerical codes different for each NRL. Ten sets of samples, randomly selected immediately after preparation, were tested to assess the homogeneity of the test samples. The tests were initiated on the same day of preparation and gave the expected results. The test samples were transferred into refrigerated packages that were shipped by a courier on the same day, with the exception of the samples for two laboratories, L35 and L70, which were stored at 5 °C ± 3 °C and shipped on 20 April. The NRLs were requested to start the analyses within 18 h upon receipt. They were also requested to record date and time of sample delivery, date and time the analyses start, and the sample temperature upon reception.

4.3. Collection and elaboration of the NRL results

The results were submitted directly through an on-line system, using a dedicated page in the “Restricted Area” of the EU-RL web site. The NRLs received their own user ID and password for the log-in procedure and a step-by-step procedure for the submission of the results. After the log in procedure, they had access to a dedicated section for submitting the test results. This section also contained a *Shipment form* with the list of the samples to

be analyzed and fields to provide information on the arrival date, temperature and quality of the sample, and the possibility to write notes to specify any problem with the sample delivery/packaging. At the end of the study, the participants could print their own individual reports directly from the web site.

4.4. Evaluation of the NRL performance

The NRL performance was evaluated by calculating the following parameters:

- Agreement (*Cohen's kappa*)
- Sensitivity
- Specificity

Cohen's kappa refers to the level of association between two measurements of the same item. K test estimates the level of agreement beyond chance. The threshold values described by Fleiss J.L. (*Statistical methods for rates and proportions, 1981*) were used for the evaluation of the value of Kappa. Therefore Kappa values > 0.75 were considered as excellent agreement, values between 0.4 and 0.75 as good agreement and values < 0.45 as poor agreement.

Sensitivity was defined as the proportion of positive results (detection of, *vtx1*, *vtx2, eae* and serogroup associated genes for the PCR screening step, isolation of VTEC O157 for the isolation step) correctly provided by the laboratory out of the total true positive results (gold standard). Specificity was defined as the proportion of negative results (lack of *vtx1*, *vtx2, eae* and serogroup associated genes for the PCR screening step, no isolation of VTEC O157 for the isolation step) correctly provided by the laboratory out of the total true negative results (gold standard). The 95 % confidence interval (95 %CI) was calculated for all the above-mentioned parameters.

5. RESULTS

Test samples were sent on 16 April 2012, with the exception of the samples for the laboratories L35 and L70, which were stored at 5 °C ± 3 °C and shipped on 20 April. A few laboratories reported problems occurred with the refrigerating gel bags, which were damaged, but no damage was reported for the test samples. As expected, 27 laboratories received the samples within 24 hours and 3 laboratories within 48 hours. Two laboratories, L35 and L70, received the samples on April 26 and 30, respectively. Twenty-two laboratories reported temperatures at delivery ranging between 2 °C and 8 °C; one

laboratory received the samples at temperatures < 2 °C, and 5 laboratories at temperatures > 8 °C. Four laboratories did not provide the information.

5.1. Real-time PCR detection of the virulence and serogroup-associated genes in the enrichment cultures

The Real-time PCR screening step was performed correctly by all the 32 NRLs (100%), which correctly identified the presence/absence of all the target genes in the enrichment cultures of both samples (Table 2).

Table 2. Detection of virulence and serogroup-associated genes in the enrichment cultures by Real-time PCR. The green boxes highlight the correct results, the red boxes the wrong results.

NRL	Detection of genes in:															
	Sample A								Sample B							
	vtx1	*vtx2*	*eae*	O26	O157	O103	O111	O145	*vtx1*	*vtx2*	*eae*	O26	O157	O103	O111	O145
True value	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L01	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L03	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L06	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L09	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L10	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L12	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L14	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L15	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L18	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L22	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L25	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L31	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L33	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L35	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L36	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L38	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L41	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L47	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L50	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L53	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L54	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L58	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L60	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L63	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L68	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L70	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L71	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L83	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L97	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L105	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L108	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-
L110	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-

5.2. Isolation of the VTEC strains from the PCR-positive samples.

The isolation step was carried out by all but one (L60) the 32 laboratories. After the correct identification of the presence/absence of VTEC genes in the enrichment cultures, all the 31 NRLs performed successfully the isolation step and identified correctly the genotype of the isolated strain (Table 3).

Table 3. Isolation and genotyping of VTEC strains from the water samples. The green boxes highlight the correct results, the red boxes the wrong results.

NRL	Isolation and genotyping of VTEC from:							
	Sample A				Sample B			
	VTEC	Genotype			VTEC	Genotype		
True value	None	-	-	-	O157	+	+	+
L01	-	-	-	-	O157	+	+	+
L03	-	-	-	-	O157	+	+	+
L06	-	-	-	-	O157	+	+	+
L09	-	-	-	-	O157	+	+	+
L10	-	-	-	-	O157	+	+	+
L12	-	-	-	-	O157	+	+	+
L14	-	-	-	-	O157	+	+	+
L15	-	-	-	-	O157	+	+	+
L18	-	-	-	-	O157	+	+	+
L22	-	-	-	-	O157	+	+	+
L25	-	-	-	-	O157	+	+	+
L31	-	-	-	-	O157	+	+	+
L33	-	-	-	-	O157	+	+	+
L35	-	-	-	-	O157	+	+	+
L36	-	-	-	-	O157	+	+	+
L38	-	-	-	-	O157	+	+	+
L41	-	-	-	-	O157	+	+	+
L47	-	-	-	-	O157	+	+	+
L50	-	-	-	-	O157	+	+	+
L53	-	-	-	-	O157	+	+	+
L54	-	-	-	-	O157	+	+	+
L58	-	-	-	-	O157	+	+	+
L63	-	-	-	-	O157	+	+	+
L68	-	-	-	-	O157	+	+	+
L70	-	-	-	-	O157	+	+	+
L71	-	-	-	-	O157	+	+	+
L83	-	-	-	-	O157	+	+	+
L97	-	-	-	-	O157	+	+	+
L105	-	-	-	-	O157	+	+	+
L108	-	-	-	-	O157	+	+	+
L110	-	-	-	-	O157	+	+	+

Therefore, for all the 31 NRLs the overall and individual agreement, evaluated by the Cohen's Kappa values (K), was 1 and the overall and individual sensitivity and specificity were 100%.

6. REMARKS

1. A total of 32 NRLs participated in the study. They included 27 NRLs representing 23 EU Member States, and the NRLs of Egypt (2 NRLs) Norway, Switzerland, and Turkey.
2. The participation in the PT confirmed that most NRLs are now able to perform the Real Time PCR-based procedure (ISO/TS 13136) for the detection of the main pathogenic VTEC non-O157 serogroups in food.
3. All the NRLs carried out the Real-Time PCR screening step and identified correctly the presence of the target genes in the enrichment cultures.
4. All but one the NRLs carried out the isolation step: All these 31 NRLs isolated correctly VTEC O157 from sample B and reported a negative result for sample A. The genotyping of the isolated strain was performed correctly as well by all the NRLs.
5. The PCR-based horizontal method for the detection of VTEC in food (ISO/TS 13136) was adapted to analyze the water samples, which are not included in its field of application. Notwithstanding, the results confirmed that ISO/TS 13136 represents a robust method for the detection of VTEC, allowing all the participating laboratories to detect the presence of VTEC O157 in water samples where it was present at a concentration of 10^3 CFU/ml.

Annex

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8th inter-laboratory study on the detection of Verocytotoxin-producing *E. coli* (VTEC) belonging to the serogroups most involved in human infections (O157, O111, O26, O103, and O145) in water samples

Laboratory procedure

Introduction

In the absence of specific international standards for the detection of VTEC in water, the method developed for the detection of VTEC in foodstuffs, ISO/TS 13136 "Microbiology of food and animal feed — Real-time polymerase chain reaction (PCR)-based method for the detection of food-borne pathogens — Horizontal method for the detection of Shiga toxin-producing Escherichia coli (STEC) belonging to O157, O111, O26, O103 and O145 serogroups", has been adapted to this particular matrix.

The procedure comprises the following sequential steps:

- Filtration of water samples through membranes of mean pore size 0.45 µm
- Tranfer of the membranes to the enrichment medium
- Microbial enrichment
- Nucleic acid extraction
- Detection of virulence genes
- Detection of serogroup-associated genes
- Isolation from positive samples.

1. Filtration of the water samples and transfer of the membranes to the enrichment medium

These steps are performed according to ISO 8199, section 8.2.5. Briefly, a sterile filtration apparatus connected to a source of vacuum is used. Place a sterile membrane filter of mean pore size 0.45 µm on the porous disc of the filter base, with only the outer part of the membrane filter being grasped by flat-ended sterile forceps. Pour the content of the bottle

containing the test sample and apply sufficient vacuum (about 70 kPa) to filter the water through the membrane. The membrane is then placed in a flask containing 100 ml of the enrichment liquid culture medium. This is constituted by buffered peptone water (BPW), which is indicated to analyze samples that are supposed to contain stressed target bacteria. The flask is then incubated for 18 -24 h at 37°C ± 1°C

2. Nucleic acid extraction, detection of virulence and serogroup-associated genes, and isolation of VTEC

These steps are performed according to the method CEN/ISO TS 13136 "Microbiology of food and animal feed — Real-time polymerase chain reaction (PCR)-based method for the detection of food-borne pathogens — Horizontal method for the detection of Shiga toxin-producing Escherichia coli (STEC) belonging to O157, O111, O26, O103 and O145 serogroups".

This method is described in the **Annex**. Briefly, it is based on a Real-time PCR screening of enrichment cultures to detect the presence of virulence genes (*vtx*1 and *vtx*2, and *eae*) and serogroup-specific genes for O26, O103, O111 and O145, followed by the isolation of VTEC from PCR-positive samples, accomplished by an immuno-concentration enrichment step specific for the serogroups identified in the PCR step. One ml aliquot of the enrichment culture performed as described above is used for DNA extraction and purification, accomplished according to the ISO 20837 "Microbiology of food and animal feeding stuffs - Polymerase chain reaction (PCR) for the detection of foodborne pathogens - Requirements for sample preparation for qualitative detection". The remaining culture shall be stored at 4°C for the isolation steps that will follow a positive PCR result. The method is sequential:

Step 1: Detection of the genes *vtx1*, *vtx2* and *eae.*

Step 2: Samples positive for both *vtx* and *eae* are tested for the serogroup-associated genes (molecular serogrouping).

Step 3: Isolation of the VTEC strain; samples positive at the same time for *vtx*, *eae*, and at least one of the serogroup-associated genes are submitted to a further step aimed at isolation of the VTEC strain. This requires serogroup-specific enrichment based on IMS or other immuno-capture suitable approaches. A guideline for the isolation of the different VTEC serogroups is also included in **Annex 1**.

Step 4: Characterization of the isolate i.e. identification, detection of *vtx* genes, the *eae* gene and the serogroup gene.

Annex 1

Laboratory procedure for the detection of Shiga toxin (Verocytotoxin)-producing *Escherichia coli* (STEC/VTEC) belonging to O157, O111, O26, O103 and O145 serogroups - Qualitative Method

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) cause severe disease in humans such as haemorrhagic colitis and haemolitic uraemic syndrome (HUS). Although STEC may belong to a large number of serogroups, those that have been firmly associated with severe human disease, in particular HUS, belong to O157, O26, O111, O103, O145 (1), and represent the targets of this Laboratory Procedure.

In this Laboratory Procedure, the wording Shiga toxin (Stx) is synonymous of Verocytotoxin (Vtx).
The following nomenclature has been adopted throughout the text:
stx: Shiga toxin genes (synonymous of *vtx)*
Stx: Shiga toxin (synonymous of vtx)
STEC: Shiga toxin-producing *Escherichia coli (*synonymous of VTEC: Verocytotoxin-producing *Escherichia coli).*

1. Scope

This Laboratory Procedure describes a horizontal method for the detection of (i) the major virulence genes of STEC (2,3), and (ii) the genes associated with the serogoups O157, O111, O26, O103 and O145 (3,4).

In the case of detection of these genes, the isolation of the strain is attempted, to confirm the simultaneous presence of the genes in the same live bacterial cell.
The analytical approach is based on the use of the Real-time PCR..

This Laboratory Procedure is applicable to any enrichment culture from:

- products intended for human consumption and the feeding of animals,
- environmental samples in the area of food production and food handling,
- environmental samples in the area of primary production.
- Water samples

2. Normative references

ISO/DIS 7218

General requirements and guidance for microbiological examinations

ISO/DIS 20837

Requirements for sample preparation for qualitative detection

ISO/DIS 20838

Requirements for amplification and detection of qualitative methods

ISO/DIS 22174

General method specific requirements

3. Terms and definitions

3.1 Shiga toxin-producing *Escherichia coli* (STEC): Microrganism possessing the Stx-coding genes

3.2 Shiga toxin-producing *Escherichia coli* (STEC) potentially pathogenic to humans: Microrganism possessing the Stx-coding genes and the intimin-coding gene *eae*.

3.3 Shiga toxin-producing *Escherichia coli* (STEC) highly pathogenic to humans: Microrganism possessing the Stx-coding genes, the intimin-coding gene *eae* and belonging to one of the serogroups in the scope of the present Laboratory Procedure.

4. Principle

4.1 General

The detection of STEC and of the 5 serogroups comprises the following sequential steps:

1. Microbial enrichment
2. Nucleic acid extraction

3. Detection of virulence genes
4. Detection of serogroup-associated genes in samples positive to point 3
5. Isolation from samples positive to points 3 and 4.

A flow-diagram of the whole procedure is given in Annex A.

4.1.1 Microbial enrichment

The number of STEC cells to be detected is increased by incubating the test portion in a non-selective liquid nutrient medium, either:

(a) Tryptone-soy broth (TSB) supplemented with 1.5 gr/l bile salts n. 3 and 16 mg/l of novobiocin (mTSB+N).

(b) Buffered peptone water (BPW)

c) Tryptone-soy broth (TSB) supplemented with 1.5 gr/l bile salts n. 3 and 12 mg/l of acriflavin (mTSB+A) for analysis of dairy products.

The broth mTSB is to be used when analysing matrices suspected to contain high levels of contaminating microflora. Novobiocin and acriflavin inhibit the growth of Gram-positive bacteria and promote the growth of Gram-negative cells including STEC. The broth BPW is to be used to analyse samples which are supposed to contain stressed target bacteria (such as frozen products), to resuscitate stressed STEC cells, and expected lower levels of contaminating microflora than in fresh samples.

NOTE: The addition of novobiocin is controversial and has been investigated by several authors. It has been observed that the Minimum Inhibitory Concentration (MIC) of the antibiotic for non-O157 STEC is lower than for O157 strains (5). The addition of novobiocin in the enrichment broth mTSB at the usual concentration of 20 mg/l, as specified in the ISO 16654:2001 standard, seems to inhibit the growth of about one third of non-O157 strains (6) increasing the risk of false negative results. The 16 mg/l concentration represents the most balanced option to inhibit the growth of the contaminating background microflora expected in enrichment cultures of food samples, while still allowing the growth of STEC cells.

4.1.2 Nucleic acid extraction

Bacterial cells are separated from the enrichment medium and lysed. The nucleic acid is then purified according to any protocol allowing the production of template DNA suitable for Real Time PCR.

4.1.3 Target genes

The purified nucleic acid is used for the detection of the following target genes:

- the main virulence genes for STEC: *stx* genes, encoding the Shiga toxins and the *eae* gene, encoding a 90KDa protein, the intimin, involved in the attaching and effacing mechanism of adhestion, a typical feature of the pathogenic STEC strains.
- the *rfbE (O157), wbdI(O111), wzx(O26), ihp1(O145)* and *wzx(O103)* genes, to identify the corresponding serogroups.

4.1.4 Detection

The Real-time PCR products are detected by light emission in a 5' nuclease PCR assay.

4.1.5 Isolation

Once a STEC is detected and the serogroup identified, in order to isolate the STEC strain, a serogroup-specific enrichment is performed followed by plating onto the agar Trpytone-bile-glucuronic medium (TBX) or onto a specific selective medium where available (see note in Annex A).

5. Diluent, culture media and reagents

5.1 Culture media

5.1.1 modified Tryptone- Soy broth (mTSB)

5.1.1.1.1 Basic medium

Composition

Casein peptone 17 g

Soy peptone 3 g

D(+) Glucose 2.5 g

Sodium chloride 5 g

Dipotassium hydrogen phosphate (K_2HPO_4) 4 g

Bile salts no. 3 1.5 g

Distilled water to 1 L

pH 7.4 ± 0.2

Preparation

Dissolve the components or the dehydrated medium in water. Adjust pH with a pH-meter pH 7.4 +/- 0.2 at 25°C (6.8 and sterilize by autoclaving at 121°C 15 min (6.9).

5.1.1.1.2 Novobiocin solution

Composition

Novobiocin	0,16	g
Water	10	ml

Preparation

Dissolve the novobiocin in the water and sterilize by membrane filtration.

Prepare on the day of use.

5.1.1.1.3 Acriflavin solution
Composition

Acriflavin	0,12	g
Water	10	ml

Preparation

Dissolve the acriflavin in the water and sterilize by membrane filtration.

Prepare on the day of use.

5.1.1.1.4 Preparation of the complete medium

Immediately before use, add 1 ml of novobiocin (5.1.1.1.2) or acriflavin solution (5.1.1.1.3) to 1000 ml of cooled mTSB (5.1.1.1.1)

The final concentration of novobiocin is 16 mg per litre of mTSB.

The final concentration of Acriflavin is 12 mg per litre of mTSB

5.1.2 Buffered peptone water (BPW)

Peptone	10	g
Sodium chloride	5.0	g
Disodium phosphate (Na_2HPO_4)	3.5	g
Potassium dihydrogen phosphate (KH_2PO_4)	1.5	g
Water	to 1000	ml

pH 7.2 ± 0.2

Preparation

Dissolve the components or the dehydrated powder in the water. Adjust pH with a pH-meter pH 7.4 ± 0.2 at 25 °C (6.8) and sterilize by autoclaving at 121 °C 15 min (6.9).

5.2 Reagents for nucleic acid extraction

The reagents to be used for nucleic acid extraction are not listed being dependent on the method adopted (9.3)

5.3 Reagents for PCR

See ISO 20838.

5.3.1 Oligonucleotides (primers) and detection probes

Primers and probes for specific detection of the target gene sequences by Real time PCR are listed in Annex E.

6. Equipment

Usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following:

6.1 Water bath up to 100 °C

6.2 Incubator according to ISO 7218, 37 ± 1 °C

6.3 Appatarus for nucleic acid extraction

Appropriate equipment according to the method adopted. If needed.

6.4 Pipettes for volumes between 1 µl and 1000 µl

6.5 Thin walled Real-Time PCR microtubes (0,2 ml /0,5 ml reaction tubes), multi-well PCR microplates or other suitable light transparent disposable plasticware.

6.6 Thermal cycler Several brands of apparatus are available and can be chosen according to the laboratory policies.

6.7 Apparatus for detection of the PCR product

Light emission following 5' nuclease PCR assay is detected by the Real-time PCR apparatus

6.8 pH-meter capable of measuring to an accuracy of ± 0.05 pH units and its resolution shall be 0.01 pH units

6.9 Autoclave according to ISO 7218

6.10 Stomacher peristaltic blender with sterile bags possibly with a device for adjustin speed and time

7. Sampling

It is important that the laboratory receives a sample which is truly representative and has not been damaged or changed during transport or storage. Sampling is not part of the method specified in this Technical Specificiation. See the specific International Standard dealing with the product concerned. If there is no specific International Standard dealing with sampling of the product concerned, it is recommended that the parties concerned come to an agreement on this subject.

8. Preparation of test sample

Prepare the test sample in accordance with the specific International Standard dealing with the product concerned where available, or other suitable guidelines.

9. Procedure

9.1 Test portion and initial suspension

9.1.1 General

Use the necessary quantity of enrichment medium to yield a final dilution of 1/10 of the original test portion. Filters used for membrane filtration of water samples are added to 100 ml of enrichment broth (BPW).

9.1.2 For matrix sample supposed to contain high level of annex floras

For solid matrices, aseptically transfer a test portion of sample to a stomacher bag containing the appropriate amount of mTSB added with novobiocin or acriflavin (5.1.1.1.4). Bags with filters should be preferred.

Homogenise in a stomacher (see ISO 7218) (6.10).

For liquid matrices, transfer the test portion of liquid sample, using a sterile pipette, directly into the base of the tube/bottle containing the enrichment broth mTSB added with novobiocin or acriflavin (5.1.1.1.4).

9.1.3 For matrix sample supposed to contain stressed target bacteria

In the case of frozen products, allow them to thaw at room temperature, then transfer the test portion to a stomacher bag containing the approapriate volume of BPW (5.1.2) and proceed as above.

9.2 Enrichment

9.2.1 Incubation

Incubate the stomacher bag or the tube/bottle (9.1.2) at 37 °C ± 1 °C for 16 h to18 h.

9.2.2 Process control (for Real-time PCR)

Perform process control according to ISO 22174.

Guidance on internal amplification control (IAC) and process control are given in Annex D.

9.3 Nucleic acid preparation

Use an appropriate nucleic acid extraction procedure for Gram-negative bacteria. A comprehensive collection of methods can be found in (7). Alternatively, commercial kits may be used according to the manufacturers' instructions.

9.4 Real-time PCR amplification

9.4.1 General

Follow all requirements for the PCR amplification as described in ISO 20838 "Microbiology of food and animal feeding stuffs – Polymerase chain reaction for the detection of food pathogens – amplification and detection".

Primers and detection probes for conducting the real-time PCR are described in Annex E.

9.4.2 PCR controls

In accordance with ISO 22174, examples of PCR controls are given in Annex D**.**

9.4.3 Detection of PCR products

Light emission is captured by the apparatus once generated during the amplification.

9.4.4 Interpretation of PCR results

The PCR results obtained, including the controls specified in ISO 22174 and in Annex D, are interpreted by the software linked to the apparatus. During amplification, the Software monitors 5'-nuclease PCR amplification by analysing fluorescence emissions (*Rn*) of the reporter dye for each sample. ΔRn was *Rn* minus the baseline reporter dye intensity established in the first few cycles. At the end of the PCR, a reaction was considered positive if its ΔRn curve exceeded the threshold, defined as 10 times the standard deviation of the mean baseline emission calculated between the first few cycles. The cycle threshold (*Ct*) was defined as the cycle number at which a sample's ΔRn fluorescence crossed the determined threshold value.

If the results are ambiguous, check the emission curves. Positive samples give a curve with a clear increase in fluorescence, starting from a number of cycles corresponding to the *Ct*.

If the controls yield unexpected results, repeat the procedure.

9.5 Strain isolation

The pattern of virulence genes, exhibited by STEC which are considered to be pathogenic to humans, is complex and it is possible that different strains presenting only part of the virulence gene pattern may be present in the food sample at the same time. Therefore, the isolation of the STEC strains is required to confirm that the positive PCR signals are generated from genes that are simultaneously present in the same live bacterial cell.

A serogroup-specific enrichment followed by direct plating onto suitable solid media and screening of the colonies for the presence of the virulence genesis are required.

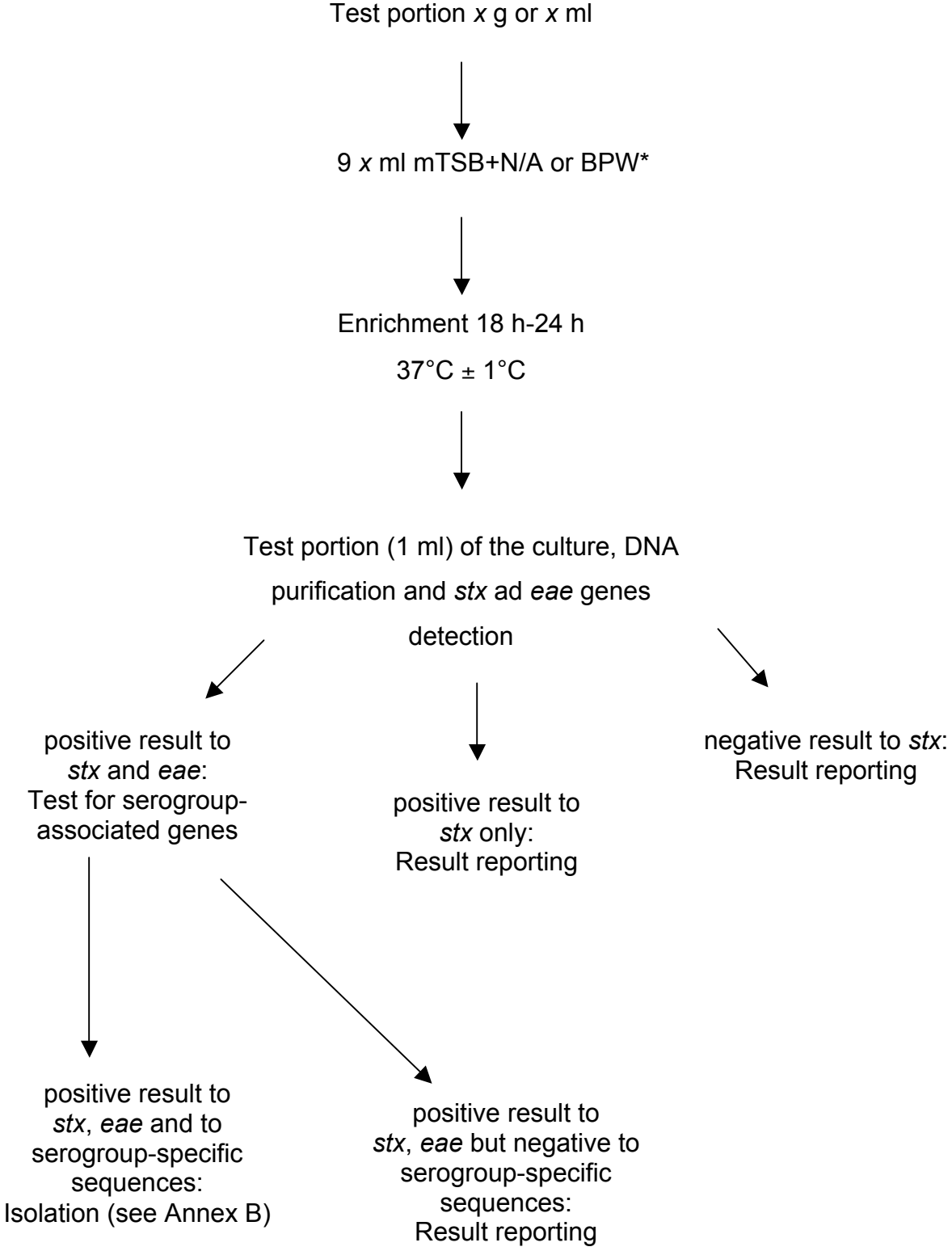
The real-time PCR protocol described in this Laboratory Procedure may be used in order to confirm the presence of the virulence genes in the isolated colonies. In alternative other equivalent PCR protocols can be downloaded from the EU RL VTEC website (http://www.iss.it/vtec/work/cont.php?id=152&lang=2&tipo=3).

STEC isolation is described in the flow chart of Annex B.

NOTE: In the absence of positive results for the presence of *eae* gene, and generally in absence of positivity to serogroup-associated genes isolation is not attempted.

Annex A

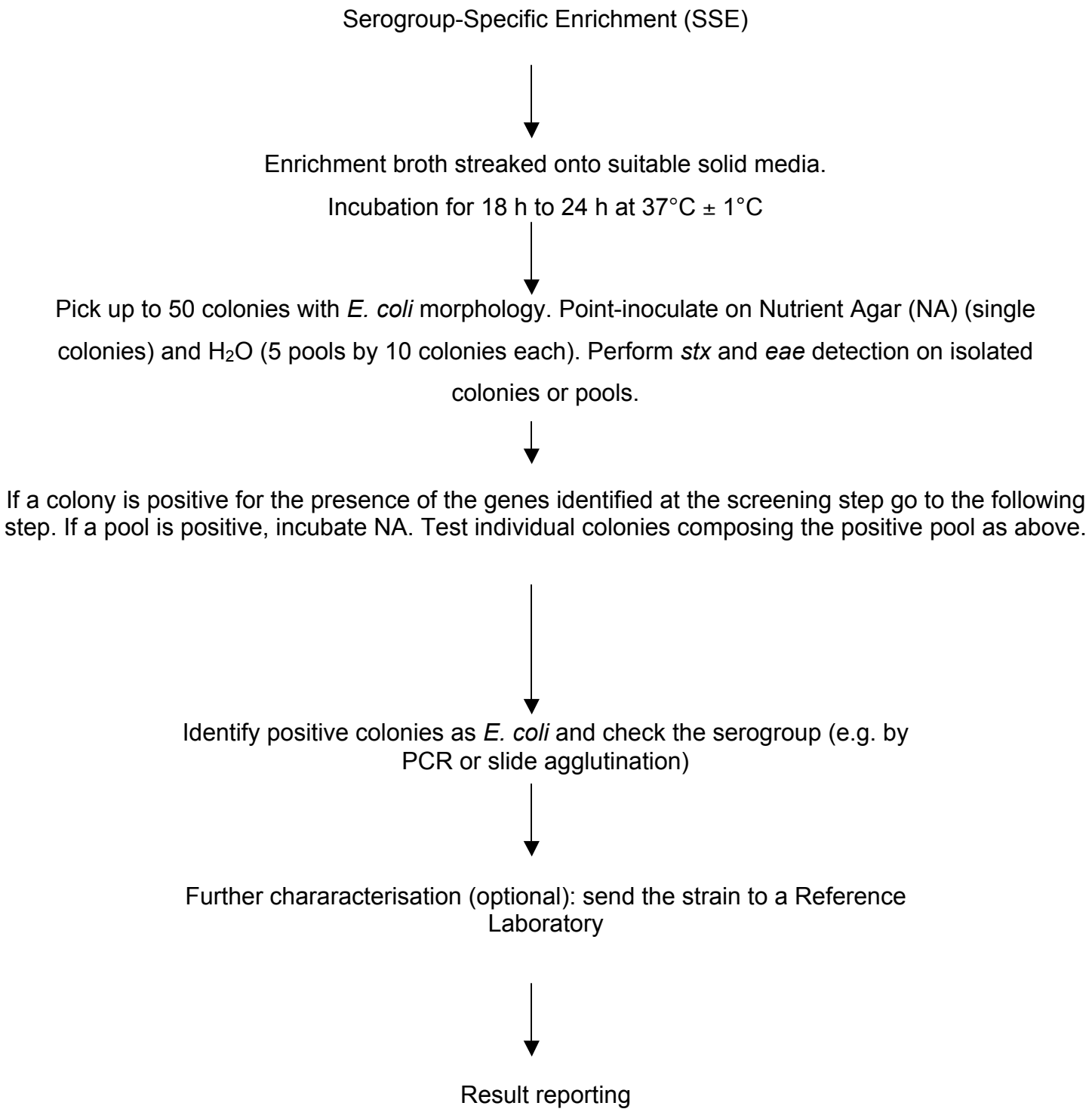
Flow-diagram of the screening procedure



*= Not applicable to water samples processed by membrane filtration method

Annex B

Flow-diagram of the isolation procedure



Annex D
Internal Amplification Control

Three different internal amplification controls (IACs) can alternatively be used in Real-time PCR:

- A commercially available TaqMan® Exogenous Internal Positive Control (Applied Biosystems, Foster City, CA, USA). The reagent kit include all reagent 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. (11). Approximately 100 copies of target DNA (pUC 19) should be used per PCR reaction. The size of the IAC was 119 bp.
- A recombinant plasmid (named pIAC-STEC) can be used in the *stx*-specific real-time PCR assay (Auvray *et al*. personal communication). This IAC contains the following DNA fragment cloned into the *Eco*RI site of pUC19:5'-ATT**TTTGTTACTGTGACAGCTGAAGCTTTACG**TGAATCGCCAGCGGCATCAGCACCTTGTCGCCTTGCGTATA**GATGTTGATCTTACATTGAACTGGGG**AATT-3' (bold letters: stx1/stx2 forward and reverse primers binding sites sequences; underlined sequence: IAC-probe binding site). The IAC is co-amplified with *stx* genes using the same primers as *stx* (Annex E), under the same conditions and in the same PCR tube (12). It is detected by a specific DNA probe (5' [Red640]-CAAGGCGACAAGGTGCTGATGCCG-[BHQ2] 3') included in the PCR mix at a concentration identical to that of the *stx1* and *stx2* DNA probes (both labelled with [Fluorescein] and [BHQ1] at their 5' and 3' ends, respectively). 64 copies of the IAC should be used per PCR reaction. The IAC PCR product is 96 bp-long. The performance of the resulting *stx*-IAC real-time PCR assay has been shown using artificially and naturally contaminated food samples (Auvray *et al*. personal communication).

The last two systems may be also used as an extraction control by adding 100 copies of the pUC 19 plasmid or of the pIAC-STEC to the sample aliquot prior to the DNA purification step.

ANNEX E

The Real-time PCR protocol described is based on the use of the following primers and probes which shall be considered as refererence reagents. However, other primers and probes may be used provided that they have been recognised equivalent to those indicated in the tables E.1 and E.2 according to the ISO 16140 rules.

Primers and probes for the PCR assays

Tables E.1 and E.2 provides respectively the primers and probes sequences for:

- the detection of *stx* and eae genes by real-time-PCR (PCR A);
- the detection of serogroup-related genes genes using real-time-PCR (PCR B).

In these tables , the chemistry of the reporter and quencher phluorophores is not indicated, being largely dependent on the real time PCR instruments available in each laboratory.

Table E.1: Degenerate primers and TaqMan probes used for 5' nuclease PCR assays. (§3 and *2)

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[§ b]	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).

[b] This combination of primer/probe recognises all the *stx2* variants but the *stx2f*

Table E.2. Primers and probes used for amplification of O antigen specific genes in 5' nuclease PCR assays. ([§]3 and [*]4)

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

Annex F

Isolation of STEC strains

Follow the procedure described below to isolate STEC strains from real time PCR positive samples:

1) Perform a serogroup-specific enrichment (SSE) on the remaining enrichment culture (see Note 1)
2) Streak SSE onto TBX or other suitable medium (see note 2). Incubate for 18 to 24 hours at 37°C
3) Pick up 10 to 50 colonies with *E. coli* morphology or with characteristic aspect (see Note 5) and point-inoculate on nutrient agar (NA) (see Note 3) and H_2O (the colonies may be pooled in water up to a number of ten per pool).
4) Perform the detection of the *stx*-coding gene and the *eae* gene on the isolated colonies or the H_2O pools (see Note 4).
5) If a pool is positive, go back to NA and assay the individual colonies forming the positive pool in order to select one single positive colony.
6) Identify the colonies as *E. coli* and confirm the serogroup the sample was positive to in the screening PCR assay (e.g. by PCR B in the Annex E), see Note 5.
7) Isolates may be sent to the a Reference Laboratory for further characterization.

NOTE 1: Serogroup-specific enrichment may be achieved by using immunocapture systems such as immuno-magnetic separation (IMS) or equivalent. Generally, refer to the instruction supplied by the manufacturer.
For O157 positive samples, use ISO 16654 or alternative methods validated according to ISO 16140.

NOTE 2: For O157 positive samples, use ISO 16654 or alternative methods validated according to ISO 16140. Sorbitol-fermenting *E. coli* O157 are susceptible to tellurite contained in the CT SMAC medium indicated in ISO 16654. Therefore the use of a second SMAC isolation plate without antibiotics is recommended. In the absence of Sorbitol-negative colonies on the plates, the screening of Sorbitol-positive colonies is suggested.
For STEC O26 isolation, a differential solid media (MacConkey) containing Rhamnose instead of lactose is commercially available (RMAC). It is very effective in distinguishing STEC O26 strains, which do not ferment Rhamnose, from other *E. coli*.

NOTE 3: There are several types of nutrient agar media commercially available either ready to use plates or prepared in house from dehydrated powders. Every type of non-

selective nutrient agar media (e.g. TSA) is suitable for the purpose of maintaining the colonies for further characterisation. Enterohaemolysin Agar, can also be used. It gives the advantage to detect the Enterohaemolysin production, which is a common feature of STEC pathogenic to humans.

NOTE 4: The Real Time PCR described in this protocol may be adopted to confirm the presence of the *stx* and *eae* in the isolated strains. Conventional PCR may be used as an alternative (http://www.iss.it/vtec/work/cont.php?id=152&lang=2&tipo=3).

NOTE 5: Colony confirmation as *E. coli* may be achieved by using any commercial biochemical multi-assay or by assessing the indole production. Confirmation of the serogroup may be achieved either by PCR or by agglutination with commercial antisera.

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