Report of the 9th inter-laboratory study on the detection of VTEC belonging to the main pathogenic serogroups in seeds intended for sprout production - 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 four rounds of PT organized by the EU-RL: PT3, carried out on bovine carcass swabs, PT4, carried out on milk samples, PT7, carried out on vegetable samples, and PT8, carried out on water samples. The reports of these PTs are available in the EU-RL web site (www.iss.it/vtec).

During the last years, a growing number of epidemic episodes have brought to the attention the role of sprouts intended for direct consumption (ready to eat) in the transmission of Salmonella and VTEC infections. In particular, sprouts have been implicated as the source of the outbreak of VTEC O104:H4 infections occurring in Europe in 2011 and an amendment to Regulation (EC) No 2073/2005 as regards microbiological criteria for sprouts shall enter into force within 2013. Contaminated seeds represented the

source of sprout contamination in most of the epidemic episodes described in the literature, and this underlines that testing of seed samples for the presence of VTEC represents an important issue for food and public health laboratories. Therefore, the 9th 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 seed samples. This study complemented the previous studies conducted within the network of Reference Laboratories for *E. coli* on matrices possibly involved in the control of the sprouts production processes: vegetable matrices (PT7) and water (PT8). 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 seed samples with VTEC belonging to the "top 5" serogroups involved in human infections (O157, O111, O26, O103, and O145). Although seeds are not included in the field of application of the standard ISO/TS 13136 for the detection of VTEC in foodstuffs, this method was adapted to this particular matrix. Two artificially contaminated seed 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-seven NRLs representing 26 EU Member States, Norway, Switzerland, Russia, Turkey, and Egypt 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:

- Austria - Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH, Bereich Humanmedizin Institut für Medizinische Mikrobiologie und Hygiene
- 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
- Denmark - National Food Institute, Technical University of Denmark, Division of Food Microbiology and Risk Assessment
- 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 (the laboratory staff involved in the analyses did not have any role in the organization of the inter-laboratory study).
- Latvia – Institute of Food Safety, Animal Health and Environment “BIOR”, Laboratory of Food and Environmental Investigation
- Lithuania - National Food and Veterinary Risk Assessment Institute, Molecular biology and GMO Section
- Netherlands – National Inst. For Public Health and the Environment (RIVM), Centre for Infectious Disease Control (Cib), Lab. for Zoonoses and Environmental Microbiology
- 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

- Poland - National Veterinary Research Institute, Dept. Hygiene of food of animal origin, Pulawy
- Romania – Institute for Hygiene and Veterinary Public Health, Microbiology Dept.
- Russia - State Research Center for Applied Microbiology and Biotechnology, Obolensk
- 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, Madrid
- Spain – Agencia Española de Seguridad Alimentaria y Nutrición, AESAN, Centro Nacional de Alimentación, Servicio de Microbiología Alimentaria, Madrid
- Spain - University of Santiago de Compostela, Dept. Microbiology and Parasitology, Faculty of Veterinary, Lugo
- 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 samples (samples A and B, each consisting of 50 g of beet seeds) potentially contaminated with VTEC were sent in the blind to the NRLs. The laboratories 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 genes (*vtx*1 group, *vtx*2 group, and *eae*) and, if positive for at least one of the toxin genes and for *eae*, for the presence of 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 and included the mashing of the seeds to allow the release of possibly internalized bacteria. The laboratory procedure, provided by the EU-RL, is reported in the **Annex**.

4.1. Sample preparation

The artificial contamination of the samples was obtained by soaking a number of beet seeds in an exponential liquid culture of a VTEC strain positive for *vtx1* and *eae* genes and belonging to serogroup O157. The seeds were allowed to air dry under a laminar flow hood overnight, then a single seed was placed in each stomacher bag containing the 50 g of seeds representing the positive test samples. To evaluate the bacterial load, single contaminated seeds were placed in 5 ml of PBS and thoroughly shaken. The bacteria released were enumerated by plating serial dilutions of the PBS suspension on Sorbitol MacConkey agar plates and the average contamination level recorded was 4 x 10^6 CFU/seed. 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, eae*	8 x 10^4 CFU/g	-

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.

The negative samples, corresponding to 50 g of seeds in stomacher bags, were prepared on 13 November and 12 samples were tested for homogeneity on 14 November. Seeds spiking was performed on 13 November and the test samples were assembled on 14 November. A total of 12 stomacher bags corresponding to sample B were randomly selected for homogeneity testing and analyzed on 15 November. All the homogeneity tests gave the expected results.

For the stability assessment, *ad hoc* artificially contaminated samples were prepared as described in the sample preparation section, by assembling 50 g of seeds with a single

contaminated seed and testing the samples in a period of time of 17 days since their preparation. Single seeds were spiked on 18 September 2012 and added to 50 g of uncontaminated seeds on 19 September. Five aliquots were assembled and tested on 24 and 26 September, and on 1, 3 and 4 October. In all tests the contaminant VTEC strain was detected as expected.

The test samples were labeled with randomly generated numerical codes different for each NRL and transferred into safety packages that were shipped by a courier on 19 November. The NRLs were requested to start the analyses within 18 h upon receipt.

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 and the relative confidence intervals refer 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 point 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

The test samples were sent on 19 November 2012. A laboratory (L118) did not receive the samples since it was not able to solve problems arisen with custom procedures. Another laboratory (L22) did not perform the analyses for problems occurred in the PCR equipment and that could not be solved in a suitable time. Therefore, the analytical results were submitted by 35 laboratories.

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 33 (94%) of the 35 NRLs that provided the results. These laboratories identified correctly the presence/absence of all the target genes in the enrichment cultures of both samples (Table 2), with individual sensitivity and specificity of 100% and a Cohen's Kappa value (K) of 1, with confidence limits 0.41-1.0.

A laboratory (L60) identified correctly the presence of virulence genes but did not perform the tests for the serogroup-associated genes. Therefore, its individual sensitivity and specificity were both 100% and the K value was 1, with confidence limits 0.20-1.0 due to the lower number of assays carried out. Another NRL (L26) provided a negative result for the positive sample A and, *vice versa*, identified the presence of *vtx1*, *eae*, and O157 genes in the negative sample B. This was likely due to an exchange of samples during the PCR procedures. The individual sensitivity and specificity for L26 were 0% and 63%, respectively, and the K value was 0, with confidence limits 0-0.28.

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

All the 34 NRLs that had identified the presence of VTEC in sample A, including L60, performed successfully the isolation step and identified correctly the genotype of the isolated strain (Table 3).

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. The empty boxes indicate that the corresponding test has not been performed, either appropriately (white boxes) or not appropriately (red boxes), in the case the corresponding test should have been performed.

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	+	-	+	-	+	-	-	-	-	-	-					
L02	+	-	+	-	+	-	-	-	-	-	-					
L03	+	-	+	-	+	-	-	-	-	-	-					
L06	+	-	+	-	+	-	-	-	-	-	-					
L09	+	-	+	-	+	-	-	-	-	-	-					
L10	+	-	+	-	+	-	-	-	-	-	-					
L12	+	-	+	-	+	-	-	-	-	-	-					
L13	+	-	+	-	+	-	-	-	-	-	-					
L14	+	-	+	-	+	-	-	-	-	-	-					
L15	+	-	+	-	+	-	-	-	-	-	-					
L18	+	-	+	-	+	-	-	-	-	-	-					
L25	+	-	+	-	+	-	-	-		-	-					
L26	-	-	-						+	-	+	-	+	-	-	-
L31	+	-	+	-	+	-	-	-	-	-	-					
L33	+	-	+	-	+	-	-	-	-	-	-					
L35	+	-	+	-	+	-	-	-	-	-	-					
L36	+	-	+	-	+	-	-	-	-	-	-					
L38	+	-	+	-	+	-	-	-	-	-	-					
L41	+	-	+	-	+	-	-	-	-	-	-					
L47	+	-	+	-	+	-	-	-	-	-	-					
L48	+	-	+	-	+	-	-	-	-	-	-					
L50	+	-	+	-	+	-	-	-	-	-	-					
L53	+	-	+	-	+	-	-	-	-	-	-					
L54	+	-	+	-	+	-	-	-	-	-	-					
L58	+	-	+	-	+	-	-	-	-	-	-					
L60	+	-	+						-	-	-					
L63	+	-	+	-	+	-	-	-	-	-	-					
L68	+	-	+	-	+	-	-	-	-	-	-					
L70	+	-	+	-	+	-	-	-	-	-	-					
L71	+	-	+	-	+	-	-	-	-	-	-					
L97	+	-	+	-	+	-	-	-	-	-	-					
L105	+	-	+	-	+	-	-	-	-	-	-					
L108	+	-	+	-	+	-	-	-	-	-	-					
L110	+	-	+	-	+	-	-	-	-	-	-					
L117	+	-	+	-	+	-	-	-	-	-	-					

Table 3. Isolation and genotyping of VTEC strains from the seed samples. The green boxes highlight the correct results, the red boxes the wrong results. The empty boxes indicate that the corresponding test has not been performed, either appropriately (white boxes) or not appropriately (red boxes), in the case the corresponding test should have been performed.

NRL	Isolation and genotyping of VTEC from:							
	Sample A				Sample B			
	VTEC	Genotype			VTEC	Genotype		
True value	O157	*vtx1*	*vtx2*	*eae*	None	*vtx1*	*vtx2*	*eae*
		+	-	+		-	-	-
L01	O157	+	-	+				
L02	O157	+	-	+				
L03	O157	+	-	+				
L06	O157	+	-	+				
L09	O157	+	-	+				
L10	O157	+	-	+				
L12	O157	+	-	+				
L13	O157	+	-	+				
L14	O157	+	-	+				
L15	O157	+	-	+				
L18	O157	+	-	+				
L25	O157	+	-	+				
L26					None			
L31	O157	+	-	+				
L33	O157	+	-	+				
L35	O157	+	-	+				
L36	O157	+	-	+				
L38	O157	+	-	+				
L41	O157	+	-	+				
L47	O157	+	-	+				
L48	O157	+	-	+				
L50	O157	+	-	+				
L53	O157	+	-	+				
L54	O157	+	-	+				
L58	O157	+	-	+				
L60	O157	+	-	+				
L63	O157	+	-	+				
L68	O157	+	-	+				
L70	O157	+	-	+				
L71	O157	+	-	+				
L97	O157	+	-	+				
L105	O157	+	-	+				
L108	O157	+	-	+				
L110	O157	+	-	+				
L117	O157	+	-	+				

6. REMARKS

1. A total of 37 NRLs participated in the study. They included 31 NRLs representing 26 EU Member States, and the NRLs of Norway, Switzerland, Turkey, Russia, and Egypt (2 NRLs). Two laboratories did not perform the analyses: one for problems occurred in their PCR equipment that could not be solved in a suitable time; the other one was not able to solve problems with custom procedure and did not receive the samples.
1. The participation in the PT confirmed that nearly all the European NRLs are now able to perform the standard Real Time PCR-based method ISO/TS 13136 for the detection of VTEC in food, regardless their serotype.
2. Of the 35 NRLs that carried out the Real-Time PCR screening step, 33 (94%) identified correctly the presence of the target genes in the enrichment cultures and 34 (97%) isolated correctly VTEC O157 from sample A. All of them performed correctly the genotyping of the isolated strain.
3. The PCR-based standard method ISO/TS 13136 for the detection of VTEC in food was adapted to analyze the seed samples, which are not included in its field of application, and the results confirmed that it represents a robust method for the detection of VTEC even in non-food matrices.

9th 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 samples of seeds intended for sprout production

Laboratory Guideline

Introduction

In the absence of specific international standards for the detection of VTEC in seeds, the method developed for the detection of VTEC in foodstuffs, 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) and the determination of O157, O111, O26, O103 and O145 serogroups", has been adapted to this matrix. In particular, the following aspects have been considered:

1. Seeds are generally contaminated at very low levels. Nonetheless, the sprouting process is characterized by conditions (humidity, heat) favoring the pathogen's enrichment. Therefore, 50 gr of seeds are analyzed instead of the usual 25 gr of food items, in order to increase the sensitivity of the assay.

– Seeds are generally dried. Therefore, the contaminating pathogens are supposed to be stressed.

– The contamination may occur on the surface of the seeds as well as inside their body.

– The enrichment cultures of seeds may contain inhibitors of the DNA polymerase used for the PCR screening of the samples.

The procedure comprises the following sequential steps:

- Smashing of the seeds to allow the release of possibly internalized bacteria;
- Transfer of the sample to the enrichment medium;
- Microbial enrichment;
- Nucleic acid extraction;

- Detection of virulence genes;
- Detection of serogroup-associated genes;
- Isolation from positive samples.

1. Treatment of the seed samples

The samples are constituted by 50 gr of seeds placed in a stomacher bag.

1. The seeds are smashed directly in the stomacher bag, using a mortar with pestel or other similar tools, before adding the enrichment broth. It is advisable to put the bag with the sample into another sterile stomacher bag, to limit the possibility of spill over due to cuts in the bag.
2. The smashed seeds are added with 450 ml BPW and incubated for 24 hrs at 37°C (either static or in agitation). Check carefully for the integrity of the stomacher bag after smashing the seeds. In case of damages evidence transfer the smashed seeds aseptically to a sterile container (flask or a new stomacher bag) before adding the culture medium. This operation must be done under a Biohazard laminar flow hood.
3. A 5 ml aliquot of the enrichment culture is taken, mixed by vortex (in order to detach as much as possible the bacteria possibly adhering to seeds), centrifuged at 500 X g 1 min to sediment the seeds' debris.
4. One ml aliquot of the supernatant is taken at this stage and used for DNA preparation.

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

This procedure is described in the **Annex 1**. 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 and treated as described in the previous paragraph 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.

To perform the Real-time PCR, the DNA sample is diluted 1:10 before use. In the case of absence of amplification of the internal amplification control (IAC), the DNA template is used at the dilution of 1:30.

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 the **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 in seed samples - 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 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 enrichment cultures from seed 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 the non-selective liquid nutrient medium Buffered peptone water (BPW).

The BPW is to be used to analyse the seeds samples since they are supposed to contain stressed target bacteria (dryed matrix).

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 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 1µl 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

In the absence of specific International Standards 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 (50 gr seeds + 450 ml BPW).

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.

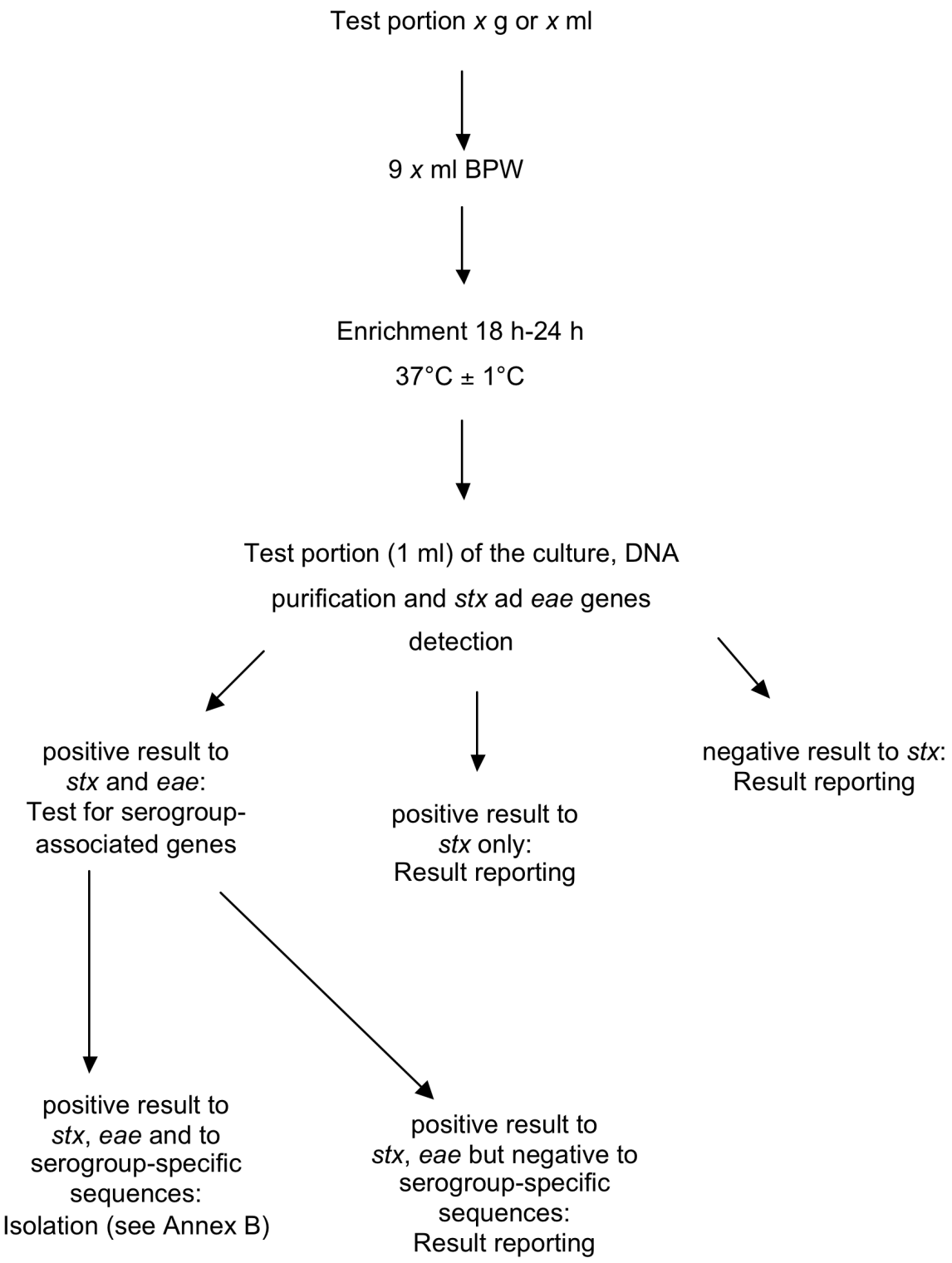
A serogroup-specific enrichment followed by direct plating onto suitable solid media and screening of the colonies for the presence of the virulence genesis are required.

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

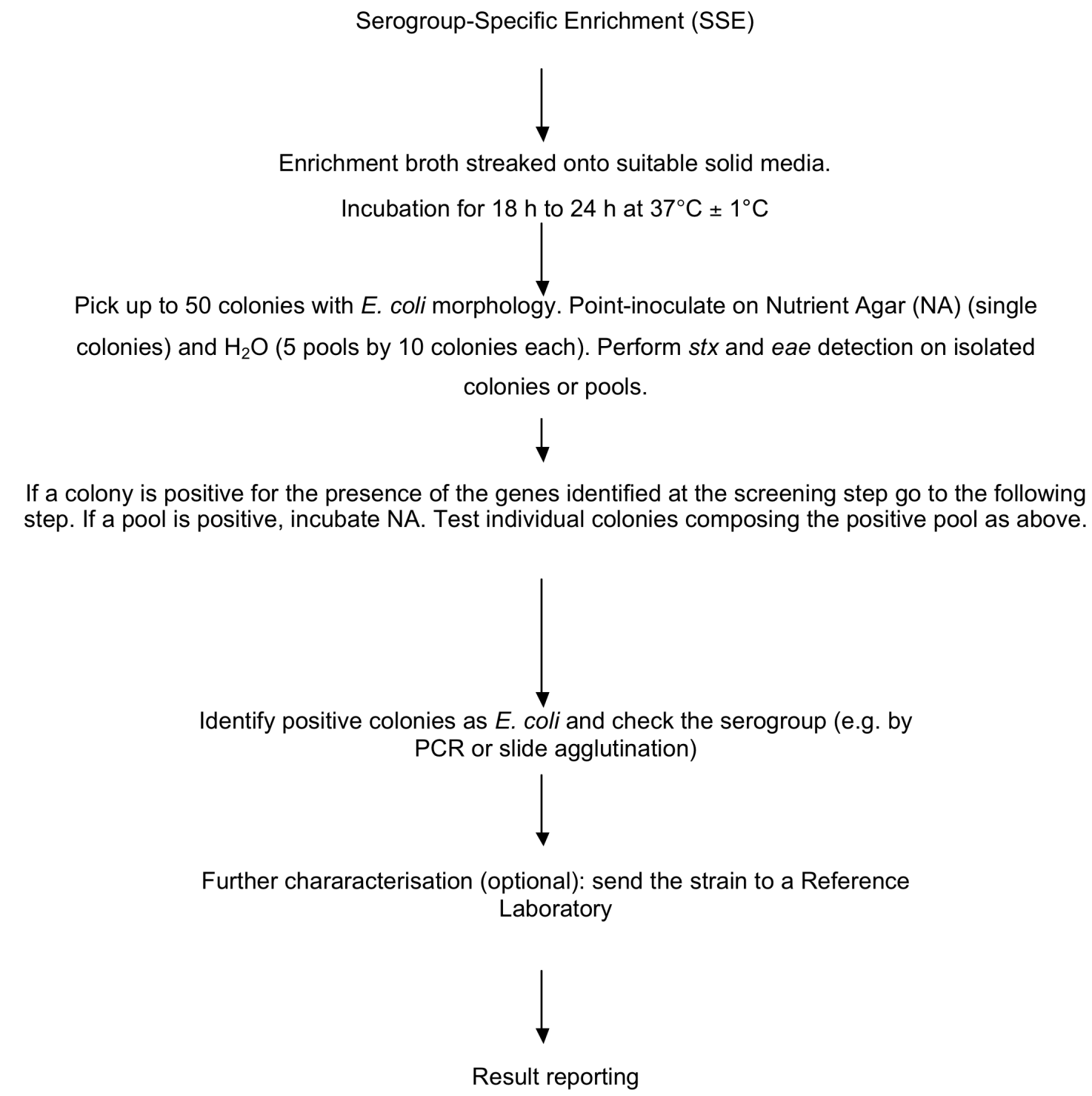
Annex A

Flow-diagram of the screening procedure



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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