Report of the 7th inter-laboratory study on the detection of VTEC belonging to the main pathogenic serogroups in food (vegetables) samples - 2011

1. INTRODUCTION

The duties of the EU Reference Laboratory for VTEC (EU-RL VTEC) include the development of methods for the detection of VTEC in food and animal samples and the organisation 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 belonging to the main pathogenic serogroups. This method is about to be published as an ISO Technical Specification: ISO/WD TS 13136 "Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (STEC) belonging to O157, O111, O26, O103 and O145 serogroups - Qualitative Method". Moreover, it has 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).

Such a method has already been adopted and evaluated in two rounds of PT organized by the EU-RL: the 3rd PT, carried out on bovine carcass swabs and the 4th PT, carried out on milk 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 foodborne illnesses have been traced back to fruits and vegetables, increasing the concern that plants might be more important as a vehicle for human enteric pathogens, including VTEC, than previously thought.

Therefore, the 7th 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 vegetable (spinach) samples. It is interesting to note that the PT was launched just

before the occurrence of the large outbreak of VTEC O104:H4, which has also been associated to food of vegetal origin.

The results of the PT have been discussed during the 6th Annual Workshop of the EU reference laboratories for *E. coli* held in Rome on 4 November 2011. This document represents the full evaluation report of the study.

2. OBJECTIVES AND DESIGN OF THE STUDY

The aim of the study was to assess the contamination of food of vegetal origin with VTEC belonging to the "top 5" serogroups involved in human infections (O157, O111, O26, O103, and O145), by using the Real-time PCR-based method (ISO/WD TS 13136) recommended by EFSA.

Three spinach samples potentially contaminated with VTEC and containing a natural background microflora were distributed in the blind to the NRLs who accepted to participate, together with the laboratory procedure for the analysis (**Annex 1**). The procedure consisted of two steps:

- Detection of virulence genes (*vtx1* group, *vtx2* group, and *eae*) and serogroup-associated genes in the pre-enrichment cultures by Real-time PCR;
- Isolation and characterization of the contaminating strains from the PCR-positive samples by using a serogroup-specific immuno-concentration enrichment.

3. PARTICIPANTS

Thirty NRLs representing 21 EU Member States, Norway, Serbia, Switzerland 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:

- Austria - Österreichische Agentur für Gesundheit und Ernährungssicherheit 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
- Czech Republic - Veterinary Research Institute

- Denmark - Division of Microbiology and Risk Assessment, National Food Institute, Technical University of Denmark
- Estonia - Veterinary and Food Laboratory
- Finland - Finnish Food Safety Authority, Evira, Microbiology Unit, Helsinki
- Finland - Finnish Food Safety Authority, Evira, Veterinary Bacteriology Research Unit, Kuopio
- France - VetAgro Sup Campus Vétérinaire de Lyon, Laboratoire LMAP
- Germany - Federal Institute for Risk Assessment (BfR), Division "Microbial Toxins"
- 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, Department 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
- Lithuania - National Food and Veterinary Risk Assessment Institute, Molecular biology and GMO Section
- Netherlands - RIVM, Centre for Infectious Disease Control (CIb)
- Norway – National Veterinary Institute, Section for food bacteriology and GMO
- Poland - National Veterinary Research Institute, Department of Hygiene of food of animal origin, Pulawy
- Portugal - Laboratório Nacional de Investigação Veterinária, Unidade de Higiene Pública/ Microbiologia dos Alimentos
- Serbia - University of Belgrade, Institute of Veterinary Medicine of Serbia
- Slovakia - State Veterinary and Food Institute Dolný Kubín, Department 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 - 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 - Ulusal Gida Referans Laboratuvar Müdürlügü, Microbiology Department

4. MATERIALS AND METHODS

Three spinach samples (samples A, B and C) potentially contaminated with VTEC and containing a background microflora 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 using the Real-Time PCR-based method ISO/WD TS 13136. The PCR-positive samples were then subjected to a serogroup-specific immuno-concentration enrichment procedure, followed by the isolation and characterization of the contaminating strains. The laboratory procedure, provided by the EU-RL, is reported in **Annex 1**.

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 spinach samples included in the study

Contaminant	Sample A	Sample B	Sample C
VTEC O157, *vtx1, vtx2, eae*	40 CFU/ml	0	0
VTEC O145, *vtx1, eae*	0	40 CFU/ml	0

The uncertainty of measurement (UM) was calculated for each of the bacterial suspensions used to spike the samples according to the ISO TS 19036:2006. The UM values were the following:

VTEC O157: 0.27 log cfu/ml

VTEC O145: 0.37 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 5 May 2011, using the same procedures adopted for the preparation of the test samples. These samples were stored at 5 °C +/- 3 °C (ISO 7218:2007) and tested according to the study protocol on 6, 9, and 11 May. All the tests yielded the expected results.

The test samples were prepared on 19 May and labelled with randomly generated numerical codes different for each NRL. The homogeneity of the test samples was assessed by testing 5 sets of samples, randomly selected immediately after preparation. The tests were initiated on the same day of preparation and gave the expected results. The test samples were kept stored at 5 °C+/- 3 °C until transferred into refrigerated packages that were shipped by a courier on 23 May. The NRLs were requested to begin 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, they had access to an *Entry form* to confirm their participation in the study and to a dedicated section for submitting the test results. This section also contained a *Shipment form* with the list of the samples to be analysed 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) correctly identified 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) correctly identified 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 samples were sent on 23 May. Delivery problems occurred for three laboratories (L02, L43, and L81), whose results could not be included in the evaluation. As expected, 21 NRLs received the samples within 24 hours, and five NRLs within 48 hours. One NRL (L94) received the samples on 27 May.

For 17 NRLs, the temperature at delivery ranged between 2 °C and 8 °C; for the remaining NRLs, one received the samples at temperatures < 2 °C and 7 NRLs at temperatures > 8 °C. Two NRLs 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 23 (85%) of the 27 NRLs, which correctly identified the presence/absence of all the target genes in the enrichment cultures of both samples. The results reported by each NRL for the detection of the virulence and serogroup-associated genes in the enrichment cultures are listed in Table 2.

Four NRLs made errors: L70 and L94 failed to identify the presence of VTEC virulence genes in Sample A and Sample B, respectively. L75 wrongly reported the presence of all the VTEC O157 target genes in the negative Sample C, probably due to the occurrence of cross contamination with Sample A during the analytical procedure. The NRL L69 made an error in Sample A, missing to report the presence of *vtx1*.

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 virulence and serogroup-associated genes in:																							
	Sample A								Sample B								Sample C							
	vtx1	*vtx2*	*eae*	O157	O26	O103	O111	O145	*vtx1*	*vtx2*	*eae*	O157	O26	O103	O111	O145	*vtx1*	*vtx2*	*eae*	O157	O26	O103	O111	O145
True value	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L01	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L04	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L05	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L14	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L15	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L26	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L31	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L36	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L44	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L46	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L51	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-					
L53	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L55	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L57	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L58	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L62	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L69	-	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L70	-	-	-						+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L75	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	-	-	-
L77	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L78	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L82	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L87	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L91	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L94	+	+	+	+	-	-	-	-	-	-	-									-	-	-	-	-
L95	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L98	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-

The analytical performances of the NRLs for the Real-time PCR step were evaluated in terms of agreement, sensitivity and specificity.

The level of agreement between the results of each NRL and the true values was evaluated by the Cohen's Kappa values. The overall agreement (K) was 0.96 (CI: 0.87 – 1) and the values of each NRL are reported in Table 3.

Table 3. Detection of virulence and serogroup-associated genes in the enrichment cultures by Real-time PCR: agreement (Cohen's Kappa) between the results obtained by the NRLs and the true values of the samples. The green boxes highlight the values of Kappa > 0.75 (excellent agreement); the yellow boxes the values between 0.45 and 0.75 (good agreement), and the red boxes the values < 0.45 (poor agreement).

NRL	Cohen's Kappa values for NRL:													
	L01	L04	L05	L14	L15	L26	L31	L36	L44	L46	L51	L53	L55	L57
K value	1	1	1	1	1	1	1	1	1	1	1	1	1	1
NRL	L58	L62	L69	L70	L75	L77	L78	L82	L87	L91	L94	L95	L98	
K value	1	1	0.88	0.53	0.65	1	1	1	1	1	0.70	1	1	

Most NRLs (24 of the 27) obtained an "excellent" agreement, while three NRLs (L70, L75, L94) had a lower analytical performance, even if considered "good".

The overall sensitivity of the participating NRLs in the Real-Time PCR assay for the detection of virulence and serogroup-associated genes in the enrichment cultures was 96.8 % (95 % CI: 94.3 % - 99.3 %) and the overall specificity was 98.7 % (95 % CI: 97.5 % - 100.0 %).

The sensitivity and specificity values for each NRL are reported in Table 4.

Table 4. Detection of virulence and serogroup-associated genes in the enrichment cultures by Real-time PCR: Sensitivity and Specificity for each NRL. The red boxes highlight values < 100%.

	Sensitivity (Se) and Specificity (Sp) for NRL								
NRL	**L01**	**L04**	**L05**	**L14**	**L15**	**L26**	**L31**	**L36**	**L44**
Se	100%	100%	100%	100%	100%	100%	100%	100%	100%
Sp	100%	100%	100%	100%	100%	100%	100%	100%	100%
NRL	**L46**	**L51**	**L53**	**L55**	**L57**	**L58**	**L62**	**L69**	**L70**
Se	100%	100%	100%	100%	100%	100%	100%	85.7 %	50.0%
Sp	100%	100%	100%	100%	100%	100%	100%	100%	100%
NRL	**L75**	**L77**	**L78**	**L82**	**L87**	**L91**	**L94**	**L95**	**L98**
Se	100%	100%	100%	100%	100%	100%	66.6 %	100%	100%
Sp	76.5 %	100%	100%	100%	100%	100%	100%	100%	100%

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

Among the 23 NRLs that had detected correctly the presence/absence of the target genes in the enrichment cultures, 20 (87%) also performed successfully the isolation step (Table 5).

The two NRLs (L70, L94) that had failed to identify the presence of the VTEC target genes in the enrichment cultures also failed to isolate the strains from the corresponding samples.

Other three NRLs failed to isolate both (L15) or one (L26 and L91) of the VTEC strains despite they had correctly identified the presence of the target genes in the enrichment cultures.

Table 5. Isolation and genotyping of VTEC strains from the spinach 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				Sample C			
	VTEC	Genotype			VTEC	Genotype			VTEC	Genotype		
		vtx1	*vtx2*	*eae*		*vtx1*	*vtx2*	*eae*		*vtx1*	*vtx2*	*Eae*
True value	O157	+	+	+	O145	+	-	+	None	-	-	-
L01	O157	+	+	+	O145	+	-	+	-	-	-	-
L04	O157	+	+	+	O145	+	-	+	-	-	-	-
L05	O157	+	+	+	O145	+	-	+	-	-	-	-
L14	O157	+	+	+	O145	+	-	+	-	-	-	-
L15									-	-	-	-
L26	-				O145	+	-	+	-	-	-	-
L31	O157	+	+	+	O145	+	-	+	-	-	-	-
L36	O157	+	+	+	O145	+	-	+	-	-	-	-
L44	O157	+	+	+	O145	+	-	+	-	-	-	-
L46	O157	+	+	+	O145	+	-	+	-	-	-	-
L51	O157	+	+	+	O145	+	-	+				
L53	O157	+	+	+	O145	+	-	+	-	-	-	-
L55	O157	+	+	+	O145	+	-	+	-	-	-	-
L57	O157	+	+	+	O145	+	-	+	-	-	-	-
L58	O157	+	+	+	O145	+	-	+	-	-	-	-
L62	O157	+	+	+	O145	+	-	+	-	-	-	-
L69	O157	-	+	+	O145	+	-	+	-	-	-	-
L70									-	-	-	-
L75	O157	+	+	+	O145	+	-	+	-	-	-	-
L77	O157	+	+	+	O145	+	-	+	-	-	-	-
L78	O157	+	+	+	O145	+	-	+	-	-	-	-
L82	O157	+	+	+	O145	+	-	+	-	-	-	-
L87	O157	+	+	+	O145	+	-	+	-	-	-	-
L91	-				O145	+	-	+	-	-	-	-
L94	O157	-	-	-					-	-	-	-
L95	O157	+	+	+	O145	+	-	+	-	-	-	-
L98	O157	+	+	+	O145	+	-	+	-	-	-	-

The analytical performances of the NRLs in the VTEC isolation step were evaluated in terms of agreement, sensitivity and specificity.

The overall agreement, evaluated by the Cohen's Kappa values (K), was 0.81 (CI: 0.60 – 1) and the values of each NRL are reported in Table 6.

Table 6. Isolation of VTEC strains: agreement (Cohen's Kappa) between the results obtained by the NRLs and the true values of the samples. The green boxes highlight the values of Kappa > 0.75 (excellent agreement); the yellow boxes the values between 0.45 and 0.75 (good agreement), and the red boxes the values < 0.45 (poor agreement).

NRL	Cohen's Kappa values for NRL:													
	L01	L04	L05	L14	L15	L26	L31	L36	L44	L46	L51	L53	L55	L57
K value	1	1	1	1	0	0.40	1	1	1	1	1	1	1	1
NRL	L58	L62	L69	L70	L75	L77	L78	L82	L87	L91	L94	L95	L98	
K value	1	1	1	0	1	1	1	1	1	0.40	0.40	1	1	

The overall sensitivity of the participating NRLs for the VTEC isolation step was 87.1 % (95 % CI: 78.1 % - 96.0 %) and the overall specificity was 100 % (95 % CI: 84.0 % - 100 %). The sensitivity and specificity values for each NRL are reported in Table 7.

Table 7. Isolation of VTEC strains: Sensitivity and Specificity for each NRL. The red boxes highlight values < 100%.

	Sensitivity (Se) and Specificity (Sp) for NRL								
NRL	L01	L04	L05	L14	L15	L26	L31	L36	L44
Se	100%	100%	100%	100%	na	50 %	100%	100%	100%
Sp	100%	100%	100%	100%	na	100%	100%	100%	100%
NRL	L46	L51	L53	L55	L57	L58	L62	L69	L70
Se	100%	100%	100%	100%	100%	100%	100%	100%	na
Sp	100%	100%	100%	100%	100%	100%	100%	100%	na
NRL	L75	L77	L78	L82	L87	L91	L94	L95	L98
Se	100%	100%	100%	100%	100%	50 %	50 %	100%	100%
Sp	100%	100%	100%	100%	100%	100%	100%	100%	100%

All the 25 NRLs that isolated the VTEC O157 and/or O145 strains from the enrichment cultures performed correctly their genotyping (Table 5), with the exception of L69, who failed to detect the presence of the *vtx1* gene in the VTEC O157 strain isolated from sample A. Therefore, 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% for all the labs but L69, for which a Kappa of 0.57 and a sensitivity value of 80% were reported, due to the false negative error.

6. REMARKS

1. Thirty NRLs representing 21 EU Member States participated in the study, together with the NRLs of Norway, Serbia, Switzerland and Turkey. Due to problems in the delivery of samples, the results submitted by 27 NRLs were included in the analysis.
2. The participation in the PT confirmed that most NRLs are now able to perform the Real Time PCR-based procedure (CEN/ISO TS 13136) for the detection of the main pathogenic VTEC non-O157 serogroups in food.
3. Twenty-two NRLs (81%) reported correctly the isolation of VTEC O157 and O145 from sample A and B, respectively, and a negative result for sample C.
4. Twenty NRLs (74 %) did not report any error, also including the real time PCR screening step, while 7 NRLs provided incorrect results: two NRLs (L70, L94) failed to identify the presence of the VTEC target genes and, consequently, failed to isolate the VTEC strains from the corresponding samples. Other 3 NRLs failed to isolate both (L15) or one (L26 and L91) of the VTEC strains, despite they had correctly identified the presence of the target genes in the enrichment cultures by Real-Time PCR. Two other NRLs made errors in the Real-Time PCR screening steps, but these did not hamper the correct isolation of the VTEC strains. In particular, one NRL wrongly reported the presence of VTEC O157-specific genes also in the negative Sample C. The false positive results were probably due to a cross contamination between the two samples during the analytical PCR procedure.
5. Most NRLs had an excellent or good agreement (K value ≥ 0.75) for the Real-time PCR screening step. A very good accuracy of the molecular tests was obtained in terms of sensitivity and specificity.
6. This PT further confirmed that the Real-time PCR-based horizontal Technical Specification (CEN/ISO TS 13136) represents a robust tool for the detection of non-O157 VTEC in food samples, allowing most NRLs to isolate the VTEC O145 strain from a vegetal matrix where it was present at a concentration of 40 CFU/g.

Annex 1

7th inter-laboratory study on Verocytotoxin-producing *E. coli* (VTEC): detection and identification in spinach samples

Laboratory procedure

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 Technical Specification.

In this Technical Specification, the wording Shiga toxin (Stx) is synonymous of Verocytotoxin (Vtx). The following nomenclature has been adopted in this Technical Specification:
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 Technical Specification 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 TS has been developed based on the use of the Real-time PCR as refererence technology for the detection of the virulence and serogroup-associated genes. Therefore a Real-time PCR protocol is described in detail.

This Technical Specification is applicable to:

- 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.

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 Technical Specification.

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
5. Isolation from positive samples.

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 promotes 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 the requirements of the adopted detection system.

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 *stx* genes encode a family of toxins including two main types: stx1 and stx2. The latter comprises seven recognized variants (from stx2a to stx2g). Only the variants stx2a, stx2b, and stx2c have been found to be produced by the STEC strains included in the field of application of this TS, and therefore constitute the target stx-coding genes of the proposed TS. The GenBank accession numbers corresponding to the stx2 variants-coding genes are:
  stx2a: X07865
  stx2b: AF043672
  stx2c: M59432
- the *rfbE (O157), wbdI(O111), wzx(O26), ihp1(O145)* and *wzx(O103)* genes, to identify the corresponding serogroups.

4.1.4 Detection

The detection of the target genes is performed accoding to the adopted detection system. 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.

6.4 Pipettes for volumes between 1µl and 100 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. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

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.

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 PCR amplification (for Real-time PCR)

9.4.1 General

The PCR amplification approach described is based on real-time PCR.

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.

The method is sequential (flow in Annex A):

- Step 1: detection of the Stx-coding genes and the *eae* gene (PCR A in Annex E);
- Step 2: samples positive at the first step are tested for the molecular serogrouping (PCR B in Annex E);
- Step 3: samples positives at both steps are subjected to strain isolation (flow in the Annex B).

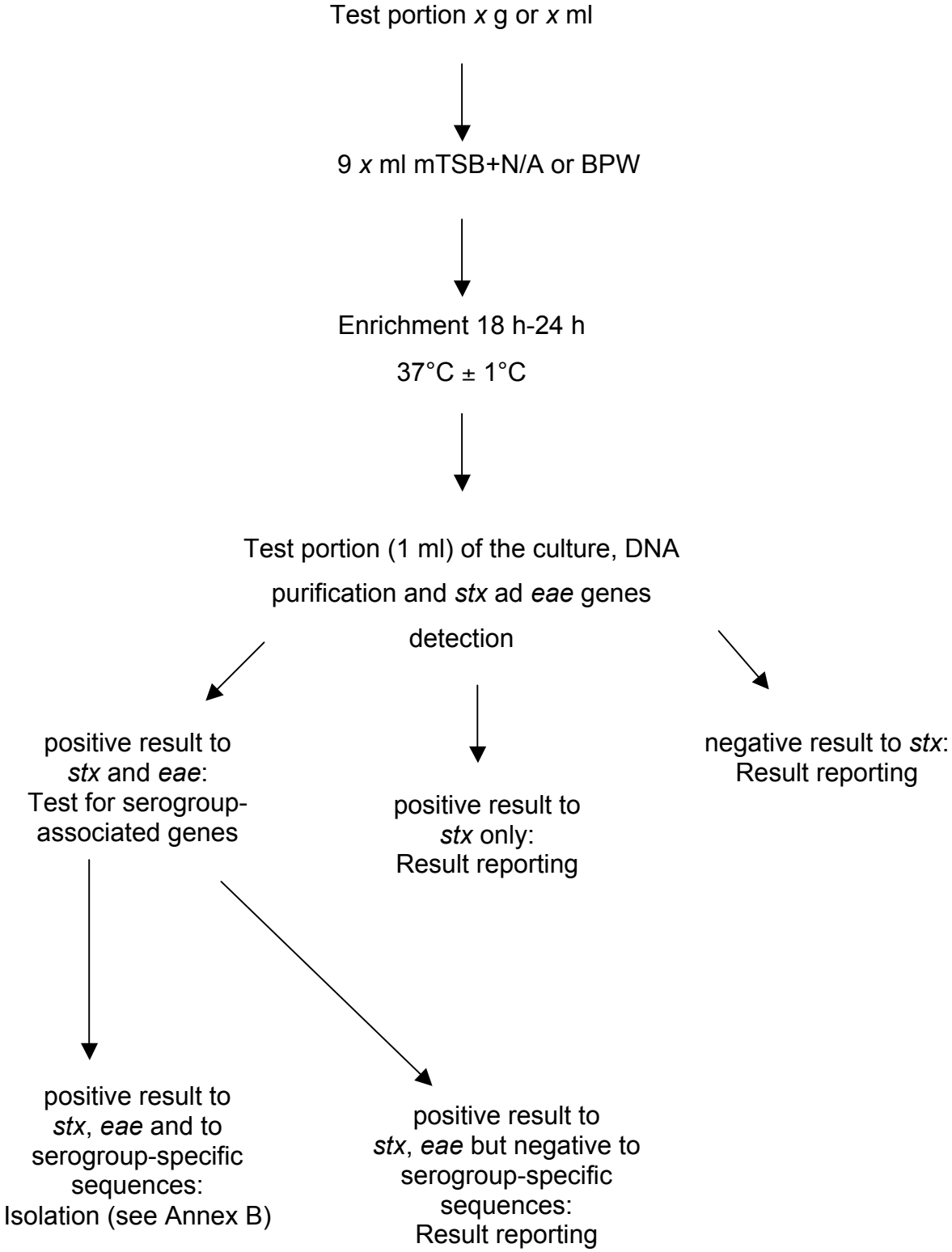
9.5 Strain isolation

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 in Annex E or any other equivalent PCR protocol (http://www.iss.it/vtec/work/cont.php?id=152&lang=2&tipo=3) may be used in order to confirm the presence of the virulence genes in the isolated colonies.
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.

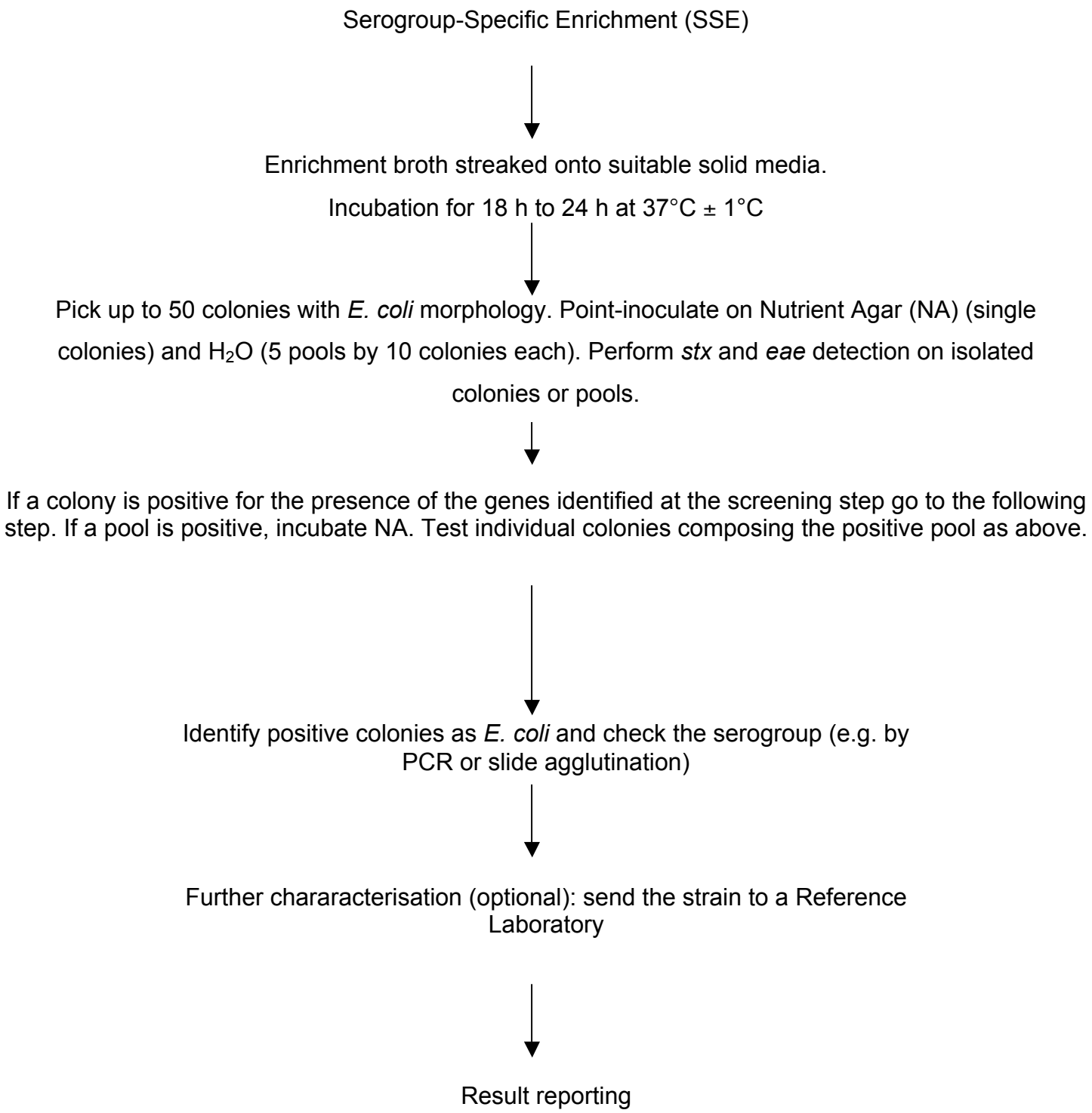
Annex A

Flow-diagram of the screening procedure



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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