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Report of the 3rd inter-laboratory study on the detection of VTEC in carcass swabs - 2009

1. INTRODUCTION

The duties of the Community Reference Laboratory for VTEC (CRL-VTEC) include the organisation of proficiency tests on detection and typing 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 inter-laboratory studies on VTEC identification and typing among the NRLs for *E.coli* in the EU Member States conducted in 2007 and 2008 aimed at the evaluation and the implementation the capability of the NRLs to identify *E.coli* strains as VTEC by the identification of their virulence genes and the VTEC serogroups most involved in severe human infections.

The NRL performance was in general good (reports available at http://www.iss.it/vtec) and, based on these successful results, the aim of the 2009 study was the detection of the "Top 5" VTEC pathogenic serogroups (O157, O26, O103, O111, O145) in animal samples, with the aim of preparing the NRLs to properly assist their competent authorities in carrying out the monitoring of VTEC foreseen by EFSA, possibly for the years 2010 or 2011. In this perspective, EFSA has issued a Guidance on the technical specifications for the monitoring and reporting of VTEC on animals and food. This guidance, now approved and published in the *EFSA Journal* (2009; 7(11):1366), was presented as a draft to the NRLs during the 2008 CRL Annual Workshop in Rome. It details a whole survey design, focusing primarily on the estimation of the prevalence of VTEC O157 contamination on hide/fleece of cattle/sheep at slaughter, using a detection method derived from the standard ISO 16654:2001 for the detection of *E. coli* O157 in food. The Guidance also proposes, as

secondary objective of the monitoring, to investigate on the presence of the other VTEC serogroups mostly involved in severe human infections: O26, O103, O111 and O145. For this purpose, the Real-time PCR-based horizontal Technical Specification developed in the framework of CEN TC275/WG6 was indicated.

Since the monitoring on cattle should be based on the examination of carcass hide swabs taken at slaughter using moisten sponges, the 2009 study consisted in the examination of a set of 5 sponges containing the organisms of interest, including both VTEC O157 and non-O157, together with background microbial flora.

The NRLs were requested to perform the study by using the methods described in the EFSA draft Guidance: i) the method derived from the standard ISO 16654:2001 for *E. coli* O157 in food for the detection of VTEC O157; ii) the Real-time PCR-based horizontal Technical Specification for the detection of non-O157 VTEC. A similar Real-time PCR protocol had been proposed to the NRLs for the characterization of isolated strains in the 2008 ring test.

The results of this proficiency test have been discussed during the 4th Annual Workshop of the EU reference laboratories for *E. coli* held in Rome on 30 October 2009, (agenda and presentations available at www.iss.it/vtec in the 'Events' session). This document represents the full evaluation report of the study.

2. OBJECTIVES AND DESIGN OF THE STUDY

The aims of the study were:

1. The isolation and characterization of VTEC O157 from bovine carcass swabs using a modification of the ISO 16654:2001 procedure intended for food;
2. The detection of the main pathogenic VTEC non-O157 serogroups in the same samples using the Real-time PCR-based Technical Specification (O26, O103, O111, O145), followed by their isolation and characterization.

A set of five sponges containing VTEC strains at different concentrations and background microflora was distributed in the blind to the NRL who accepted to participate, together with the laboratory procedure for the analyses (**Annex 1**).

Therefore, the study consisted of 3 parts:

1. Mandatory: isolation of VTEC O157 from sponge samples by using the modified ISO 16654:2001 for *E. coli* O157 in food;

2. Facultative: from the same pre-enrichment cultures, detection and isolation of the main VTEC non-O157 serogroups by using the Real-time PCR-based Technical Specification;
3. Mandatory: identification and characterization of the VTEC strains isolated by serogrouping and identification of virulence genes.

3. PARTICIPANTS

Twenty-nine NRLs from 24 EU Member States and the NRL of Switzerland 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 - Institute of Public Health, National Reference Laboratory in food microbiology
- Belgium - Veterinary and Agrochemical Research Centre
- Cyprus - Laboratory for the Control of Food of Animal Origin (LCFAO)
- Czech Republic - Veterinary Research Institute
- Denmark - Department of Microbiology and Risk Assessment, National Food Institute, Technical University of Denmark
- Estonia - Veterinary and Food Laboratory
- Finland - Finnish Food Safety Authority, Evira, Helsinki *
- France - Ecole Nationale Vétérinaire de Lyon
- Germany - Federal Institute for Risk Assessment (BfR)
- Greece – Central Public Health Labratory
- 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
- Italy - Istituto Superiore di Sanità
- Latvia - National Diagnostic Centre
- Lithuania - National Veterinary Laboratory
- Malta - Public Health Laboratory, National Reference Laboratory
- Poland - National Veterinary Research Institute, Pulawy

- Poland - National Institute of Public Health,
- Portugal - Laboratório Nacional de Investigação Veterinária
- Romania - Institute for Hygiene and Veterinary Public Health
- Slovakia - State Veterinary and Food Institute
- Slovenia - National Veterinary Institute, Veterinary Faculty, University of Ljubljana
- Spain - Laboratorio Central de Veterinaria de Algete
- Spain Agencia Española de Seguridad Alimentaria y Nutrición
- Sweden - National Veterinary Institute, SVA
- Sweden – National Food Administration
- Switzerland - Institute for Food Safety & Hygiene, University of Zurich
- The Netherlands - Laboratory of the Food and Consumer Product Safety Authority, VWA

4. MATERIALS AND METHODS

A panel of samples constituted by 5 sponges (samples A-E) containing the VTEC strains at different concentration and a background flora was sent to the NRLs. The samples were sent in refrigerated packages and 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.

The NRLs were requested to identify the presence of VTEC O157 by using a modified ISO 16654:2001 procedure for *E. coli* O157 in food and feed. In particular, the enrichment step was performed by adding 90 mL of pre-warmed BPW to the sponge and by incubating at 41.5 ±1°C for 18 hours. The isolated *E.coli* O157 strains were confirmed as VTEC by testing for the presence of *eae* and *vtx* genes.

As a facultative section of the study, the NRLs were requested to examine the enrichment cultures for the presence of the serogroup-specific genes of the main pathogenic VTEC non-O157 (O26, O103, O111 and O145), as well as their virulence genes (*vtx*1 and *vtx*2, and *eae*) using the Real-Time PCR detection method. The laboratory procedure provided by the CRL is reported in **Annex 1**.

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

Table 1: Characteristics of the content of the sponges included in the study

Sample/Contaminant	Sample A	Sample B	Sample C	Sample D	Sample E
VTEC O157 *vtx1, vtx2, eae*	2 CFU/ml	$2x10^3$ CFU/ml	20 CFU/ml	none	none
VTEC O26 *vtx1, eae*	none	40 CFU/ml	$4x10^3$ CFU/ml	40 CFU/ml	none
E. coli	10^2 CFU/ml	10^2 CFU/ml	10^2 CFU/ml	10^2 CFU/ml	10^2 CFU/ml
K. pneumoniae	$2x10^2$ CFU/ml	$2x10^2$ CFU/ml	$2x10^2$ CFU/ml	$2x10^2$ CFU/ml	$2x10^2$ CFU/ml
S. faecalis	$5x10^2$ CFU/ml	$5x10^2$ CFU/ml	$5x10^2$ CFU/ml	$5x10^2$ CFU/ml	$5x10^2$ CFU/ml

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 samples correctly identified. For serogroup identification, a positive result was defined as the correct identification of the strain. Specificity was defined as the proportion of negative samples correctly identified.

95% confidence interval (95%CI) was calculated for all the above mentioned parameters.

5. RESULTS

The samples were received within 48h from the shipment by 29 NRLs, while the remaining NRL received the samples after 10 days and could not participate in the study. Therefore,

all the results refer to 29 NRLs. The temperature of the samples at delivery ranged between 1 and 12°C. It resulted higher than 8°C for 4 shipments.

5.1 Section 1 – Detection of E.coli O157 by a modified ISO16654:2001 procedure (mandatory)

All the 29 participating NRLs performed this section. Twenty-seven identified correctly the presence/absence of *E.coli* O157 (Table 2). One NRL failed to detect the presence of *E.coli* O157 in sample C while one NRL reported a false positive result for sample E.

Table 2. *Detection of E.coli O157 (modified ISO16654:2001 procedure)*

Test	Sample	True Value	Laboratories																													
			L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	L17	L18	L19	L20	L21	L22	L23	L24	L25	L26	L28	L29	L30
Isolation of E.coli O157	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	C	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	E	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

All the NRLs but one (L3), also perfomed the PCR assays for the molecular characterization of the isolated strains: *vtx1*, *vtx2*, *eae* genes detection. The results of the PCR assays are reported in table 3, according to the gold standard values.

Table 3. Detection of virulence genes in the *E.coli* O157 strains isolated from the samples

Test detection of:	Sample	True Value	Laboratories																													
			L1	L2	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	L17	L18	L19	L20	L21	L22	L23	L24	L25	L26	L28	L29	L30		
vtx1	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	C	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
vtx2	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
	C	+	+		+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
eae	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
	B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
	C	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+

24 NRLs identified correctly the presence of the virulence genes in the 3 *E.coli* O157 strains isolated. One NRL (L15) failed to identify the presence of *vtx1* in two strains and that of *vtx2* in one. Two NRLs (L15, ad L18) did not identify *vtx2* in one strain, and one NRL (L19) failed to identify *eae* in all the 3 VTEC O157 strains.

5.2 Section 2 - Detection of the presence of VTEC non-O157 by Real-time PCR (facultative)

This facultative part of the study was carried out by 16 NRLs. The results of the detection of the virulence genes (*vtx1*, *vtx2*, *eae*) in the swab enrichment cultures are reported in table 4.

Table 4. Detection of virulence genes in the enrichment cultures (Real-time PCR method)

Test detection of:	Sample	True Value	Laboratories														
			L1	L2	L4	L7	L8	L9	L11	L12	L14	L15	L17	L20	L21	L22	L25	L30
vtx1	A	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
	B	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
	C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	D	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
vtx2	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	C	+	+	-	-	+	+	-	-	-	-	-	-	-	+	-	-	+
	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
eae	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	D	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	E	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-

Fifteen of the 16 NRLs (94%) detected correctly the presence of *vtx1* and *eae* in all the positive samples, while one NRL (L15) missed 2 of the 4 *vtx1*-positive samples and reported the presence of *eae* in one negative sample. Conversely, only 5 NRLs (31%) detected correctly the presence of *vtx2* in all the positive samples. The other 11 NRLs made the same error (false negative result) and did not detect the presence of the *vtx2* gene in sample C. This was likely due to the presence, in this sample, of a much higher amount of the *vtx1* gene (possessed by both the VTEC strains present in the sample) with respect to the *vtx2* gene (present only in the VTEC O157 strain, see Table 1). In fact, the amplification of the two genes relies on the use of the same primer pair and the discrimination between *vtx1* and *vtx2* is achieved by using two different probes. In the condition of sample C, the competition of the two *vtx* genes for the same primers did probably favour the annealing of the oligonucleotides to the *vtx1* sequence present in excess, hindering the *vtx2* , present in a much lower concentration.

The detection of the serogrup-specific genes in the enrichment cultures was performed by 14 NRLs. The results are reported in table 5.

Table 5. Detection of serogrup-specific genes in the enrichment cultures (Real-time PCR method)

Test detection of serogroup associated genes:	Sample	True Value	Laboratories													
			L1	L2	L4	L7	L8	L9	L12	L14	L15	L17	L21	L22	L25	L30
O157	A	+	+	+	+	+	+	+	+	+	+	+		+	+	+
	B	+	+	+	+	+	+	+	+	+	+	+		+	+	+
	C	+	+	+	+	+	+	+	+	+	+	+		+	+	+
	D	-	-	-	-	-	-	-	-	-	-	-		-	-	-
	E	-	-	-	-	-	-	-	-	-	-	-		-	-	-
O26	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	D	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O111	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O103	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O145	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

All the NRLs identified correctly the presence/absence of the non-O157serogrup-specific genes in the enrichment cultures. One NRL (L22) didn't include in the assay the serogroup O157.

5.3 Isolation of VTEC O26 from the RT PCR-positive enrichment cultures

All the 14 NRLs that had performed the detection of both the virulence and serogrup-specific genes in the enrichment cultures by Real-time PCR also made the attempt to isolate the VTEC O26 responsible for the positive PCR results. The results are reported in table 6.

Table 6. Isolation of VTEC O26 from *the RT PCR-positive* enrichment cultures

Test	Sample	True Value	Laboratories													
			L1	L2	L4	L7	L8	L9	L12	L14	L15	L17	L21	L22	L25	L30
Isolation of O26	B	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
	C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	D	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Thirteen NRLs correctly isolated the VTEC O26 strain from the 3 positive samples, while one NRL (L9) did not isolate *E.coli* O26 from sample B, where it was present at a lower concentration with respect to the VTEC O157 strain also present in that sample. Finally, one NRL (L15) reported the isolation of *E.coli* O111 from samples D and E, despite *E.coli* O111 had not been included in the samples and despite the same NRL had obtained negative results for O111-associated genes in the previous screening by Real-Time PCR (see Table 5).

Five of the NRLs that didn't perform the preliminary Real-Time PCR screening for the "top 5" VTEC serogroups made the attempt to isolate the non-O157 VTEC from the enrichment cultures of all the samples. The results are reported in table 7.

Table 7. Isolation of VTEC non-O157 from the enrichment cultures

Test	Sample	True Value	Laboratories				
			L11	L13	L20	L24	L29
isolation of VTEC non-O157	A	-	-	-	-	-	-
	B	VTECO26	-	-	VTECO26	VTECO26	VTECO26
	C	VTECO26	VTECO26	VTECO26	VTECO26	VTECO26	VTECO26
	D	VTECO26	VTECO26	VTECO26	VTECO26	VTECO26	VTECO26
	E	-	-	-	-	-	-

Three NRLs isolated correctly VTEC O26 from the 3 positive samples. Two NRLs (L11 and L13) did not isolate VTEC O26 from sample B, where it was present at a lower concentration with respect to the VTEC O157 strain also present in that sample.

The molecular characterization of the *E.coli* O26 strains isolated from the samples was carried out by 12 of the 19 NRLs that had isolated these strains. As shown in table 8, all the NRLs identified correctly the presence/absence of the virulence genes in all the isolated strains.

Table 8. Detection of virulence genes in the *E.coli* O26 strains isolated from the samples

Test detection of:	Sample	True Value	Laboratories															
			L1	L2	L4	L7	L8	L11	L12	L14	L17	L20	L21	L22	L24	L25	L29	L30
vtx1	B	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+
	C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	D	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
vtx2	B	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-
	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
eae	B	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+
	C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	D	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

5.4 Overview of the analytical performances

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

The level of agreement between the results of each NRL and the true values were evaluated by the Cohen's Kappa values (Table 9). The overall agreement (K) was 0.94 (CI: 0.91 – 1). For the mandatory part of the study, an "excellent" agreement (K>0.75, herein represented in green color) was reported for most of the NRLs (27/29) while two NRLs had a lower analytical performance for the ISO method, even if considered "good" (0.40<K<0.75, herein represented in yellow). Similar results were reported for the facultative Real-Time PCR assays for the detection of the "top 5" VTEC serogroups and for the isolation and genotyping of the VTEC O26 strains, with almost all the NRLs that carried out these parts reporting excellent agreement. In none of the assays there were NRLs reporting results with Kappa values falling in the "critical" area (k<0.40).

Table 9. Agreement (Cohen's Kappa) between the results obtained by the NRLs in each analytical sections and the true values of the samples. Green and yellow colours refer to values of Kappa >0.75 (excellent agreement) and 0.45<K<0.75 (good agreement), respectively.

	Mandatory		Facultative			
Test	1		2		3	
NRLs	Isolation of E.coli O157	Identification of virulence genes of isolated O157 strains	Identification of vtx1, vtx2, eae (Real-Time PCR)	Identification of serogroup-associated genes (Real-Time PCR)	Isolation of VTEC O26 strains	Identification of virulence genes in isolated VTEC O26 strains
L1	1,00	1,00	1,00	1,00	1,00	1,00
L2	1,00	1,00	0,84	1,00	1,00	1,00
L3	1,00					
L4	0,62	0,62	0,84	1,00	0,90	0,91
L5	1,00	1,00				
L6	1,00	1,00				
L7	1,00	1,00	1,00	1,00	1,00	1,00
L8	1,00	1,00	1,00	1,00	1,00	1,00
L9	1,00	1,00	0,84	1,00	0,90	
L10	1,00	1,00				
L11	1,00	1,00	0,84		0,62	0,68
L12	1,00	1,00	0,84	1,00	1,00	1,00
L13	0,55	1,00			0,62	
L14	1,00	1,00	0,84	1,00	1,00	1,00
L15	1,00	0,67	0,41	1,00	0,50	na
L16	1,00	1,00				
L17	1,00	1,00	0,84	1,00	1,00	1,00
L18	1,00	0,87				
L19	1,00	0,67				
L20	1,00	1,00	0,84		1,00	1,00
L21	1,00	1,00	1,00	0,70	1,00	1,00
L22	1,00	1,00	0,84	1,00	0,90	1,00
L23	1,00	1,00				
L24	1,00	1,00			1,00	1,00
L25	1,00	1,00	0,84	1,00	1,00	1,00
L26	1,00	1,00				
L28	1,00	1,00			1,00	1,00
L29	1,00	1,00				
L30	1,00	1,00	1,00	1,00	1,00	1,00

The sensitivity and specificity values for each analytical assay are reported in tables 10 and 11.

Table 10. Sensitivity of the analytical assays for each NRL

	Mandatory		Facultative			
Analytical section	1		2		3	
NRLs	Isolation of E.coli O157	Identification of virulence genes of isolated O157 strains	Identification of vtx1, vtx2, eae (Real-Time PCR)	Identification of serogroup-associated genes (Real-Time PCR)	Isolation of VTEC O26 strains	Identification of virulence genes in isolated VTEC O26 strains
L1	100%	100%	100%	100%	100%	100%
L2	100%	100%	91%	100%	100%	100%
L3	100%	na				
L4	67%	100%	91%	100%	71%	100%
L5	100%	100%				
L6	100%	100%				
L7	100%	100%	100%	100%	100%	100%
L8	100%	100%	100%	100%	100%	100%
L9	100%	100%	91%	100%	71%	0%
L10	100%	100%				
L11	100%	100%	91%		67%	100%
L12	100%	100%	91%	100%	100%	100%
L13	100%	100%			67%	
L14	100%	100%	91%	100%	100%	100%
L15	100%	67%	73%	100%	14%	n.a.
L16	100%	100%				
L17	100%	100%	91%	100%	100%	100%
L18	100%	89%				
L19	100%	67%				
L20	100%	100%	91%		100%	100%
L21	100%	100%	100%	100%	100%	100%
L22	100%	100%	91%	100%	71%	100%
L23	100%	100%				
L24	100%	100%			100%	100%
L25	100%	100%	91%	100%	100%	100%
L26	100%	100%				
L28	100%	100%			100%	100%
L29	100%	100%				
L30	100%	100%	100%	100%	100%	100%

Table 11. Specificity of the analytical assays for each NRL

	Mandatory		Facultative			
Analytical section	1		2		3	
NRLs	Isolation of E.coli O157	Identification of virulence genes of isolated O157 strains	Identification of vtx1, vtx2, eae (Real-Time PCR)	Identification of serogroup-associated genes (Real-Time PCR)	Isolation of VTEC O26 strains	Identification of virulence genes in isolated VTEC O26 strains
L1	100%	na	100%	100%	100%	100%
L2	100%	na	100%	100%	100%	100%
L3	100%	na				
L4	100%	na	100%	100%	100%	100%
L5	100%	na				
L6	100%	na				
L7	100%	na	100%	100%	100%	100%
L8	100%	na	100%	100%	100%	100%
L9	100%	na	100%	100%	100%	n.a.
L10	100%	na				
L11	100%	na			100%	100%
L12	100%	na	100%	100%	100%	100%
L13	50%	na			100%	
L14	100%	na	100%	100%	100%	100%
L15	100%	na	75%	100%	100%	n.a.
L16	100%	na				
L17	100%	na	100%	100%	100%	100%
L18	100%	na				
L19	100%	na				
L20	100%	na			100%	100%
L21	100%	na	100%	100%	100%	100%
L22	100%	na	100%	100%	100%	100%
L23	100%	na				
L24	100%	na			100%	100%
L25	100%	na	100%	100%	100%	100%
L26	100%	na				
L28	100%	na			100%	100%
L29	100%	na				
L30	100%	na	100%	100%	100%	100%

6. REMARKS

- Twenty-nine of the 30 NRLs designated at the moment of the study by the EU Member States participated in the inter-laboratory study, together with the NRL of Switzerland. The remaining NRL, the National Referente Centre for Environmental Microbiology of Slovakia, did not participate because the analysis of animal samples falls into the competences of the other NRL of Slovakia, the State Veterinary and Food Institute, which indeed participated in the study. One of the NRLs (L27) received the samples after 10 days and could not participate in the study. This NRL received a new set of samples in November and could perform the test separately.
- All the NRLs carried out the detection of VTEC O157 using the modified ISO 16654:2001 procedure, and all but one the PCR detection of virulence genes in the isolated strains.
- For both the assays, most of the NRLs had excellent agreement (K value>0.75) and only two labs obtained a good agreement (K value >0.45 and <0.75). The overall agreement values were 0.97 and 0.95 for the isolation of *E.coli* O157 and for the genotyping, respectively.
- A good accuracy of these tests was obtained in terms of sensitivity and specificity.
- Fourteen NRLs performed the facultative section and applied the complete Real Time PCR-based Technical Specification for the detection of the main pathogenic VTEC non-O157 serogroups.
- All these NRLs detected correctly the presence of serogroup-associated genes; most of them identified correctly the presence of *vtx1* and *eae* genes in all the samples, while most (11NRLs) failed to identify the presence of *vtx2* in sample C (see 5.2 section 2). However, this false-negative result did not modify the final outcome of the assay, since the detection of the *vtx* gene, without discriminating the two *vtx* types, is sufficient to go through the next steps of the procedure: the detection of the *eae* and the serogroup-associated genes.
- The isolation of VTEC O26 was carried out successfully from the 3 positive samples by 13 of the 14 NRLs who participated in this part of the study.
- As a whole, the Real-time PCR-based horizontal Technical Specification for the detection of non-O157 VTEC indicated in the EFSA Guidance on the technical specifications for the monitoring and reporting of VTEC on animals and food, provided a satisfactory result, allowing in most NRLs the detection and isolation of VTEC O26 from samples where it was present at a concentration of 40 CFU/g.

Annex 1

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

Outline of the methods

Detection of VTEC O157

The ISO 16654:2001 standard method for the detection of *E. coli* O157 in food and feed, shall be used for cattle hide swabs, with the following modification, concerning the enrichment step:

- The sponge shall be added with 90 mL of pre-warmed BPW and incubated at 41.5 ±1°C for 18 hours.

The immunomagnetic separation and isolation steps will be performed at the end of the enrichment stage according to the protocol described in ISO 16654:2001, as well as the identification of suspected colonies. The *E. coli* O157 strains isolated must be confirmed as VTEC by testing for the presence of *eae* and *vtx* genes. The PCR method for detecting *eae* and *vtx* genes is provided in **Annex 1**. The procedure is summarized in Figure 1.

Detection of non-O157 VTEC

The recommended detection method is a Real-time PCR protocol submitted to ISO in the form of “Technical Specification” by Working Group 6 of the Technical Committee 275 of the European Normalisation Committee (CEN TC275/WG6). The method targets both virulence genes (*vtx*1 and *vtx*2, and *eae*) and serogroup-specific genes for O26, O103, O111 and O145. It shall be used for screening samples, but requires a confirmation step with the isolation of the VTEC strains responsible for the positive PCR reactions.

The method is applied to the same enrichment culture performed for the isolation of VTEC O157. One ml aliquot of such a culture is used for DNA extraction and purification. This step shall be accomplished according to the ISO 20837:2006 “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.

Real-time PCR is performed using the primers and probes described in the guideline included as **Annex 2**. Amplification conditions to be applied will depend on the system used, and will refer to the instructions supplied with the instrument and kit of choice.

The method is sequential:

Step 1: Enrichment of the sample.

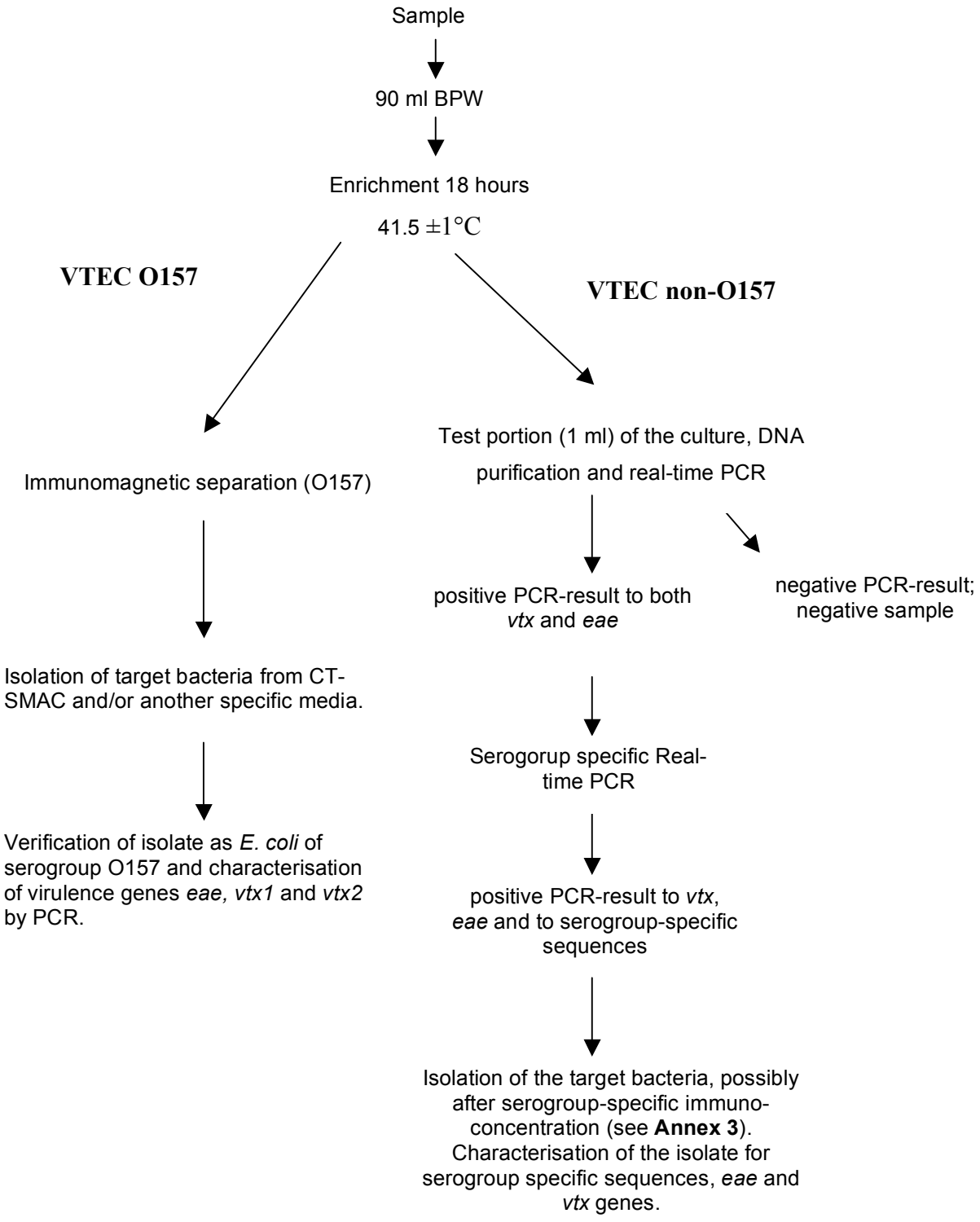
Step 2: Detection of the genes *vtx1*, *vtx2* and *eae.*

Step 3: Samples positive for both *vtx* and *eae* at the second step are tested for the serogroup-associated genes (molecular serogrouping).

Step 4: Isolation of the VTEC strain; samples positive at the same time for *vtx*, *eae*, and 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 included as **Annex 3**.

Step 5: Characterisation of the isolate i.e. identification, detection of *vtx* genes, the *eae* gene and the serogroup gene.

Figure 1. Flow-diagram for the detection of VTEC in cattle hide swabs



Annex 1

Multiplex PCR for the characterisation of VTEC virulence genes: *vtx1*, *vtx2* and *eae*

1. Principle of the method

The method is based on PCR amplification of specific DNA regions from a DNA template, with oligonucleotides triggering the start of the PCR reaction. Detection of *vtx1*, *vtx2* and *eae* is performed by a multiplex PCR reaction using specific primers (Table 1).
The primer pairs used, stx1F/stx1R and stx2F/stx2R (Paton & Paton, 1998), are able to detect the genes *vtx1* and *vtx2*, respectively. The latter recognise all the variants of *vtx2*, except *vtx2f*. The amplification of the *vtx2f* variant can be obtained by an individual primers set: 128-1/128-2 (Schmidt et al, 2000). The primers used for the detection of the intimin-coding gene *eae* (Paton & Paton, 1998) recognise all the reported polymorphic variants of this gene.
The method does not allow for the discrimination of the *vtx2* variant genes, with the exception of *vtx2f*, and is intended for use with bacterial cultures only.
The method is composed of the following steps:

- template preparation;
- setting-up of the PCR reaction;
- determination of the PCR results by agarose horizontal gel electrophoresis.

2. Template preparation

Cultures streaked onto solid media (e.g. TSA) are processed as follows:

- pick a single bacterial colony up with a sterile 1 µl loop;
- prepare the template by suspending the bacteria in 100 µl of 0.22 µm filter-sterilised MilliQ water and boil for 10 minutes.

3. Setting up the PCR reaction

For each sample, set up a 50 µl reaction (reaction buffer 1X, $MgCl_2$ 1.2 mM, dNTPs 0.2 mM each, 50 pmoles of each primer, 2 Uts of *Taq* polymerase and 10 µl of DNA template).

The volume of the reagents can be scaled according to the final volume of reaction. MilliQ water must be used for PCR reactions.
In each PCR assay, a positive and two negative controls must be included. The positive controls are DNA templates obtained from *E. coli* strains possessing the virulence genes tested, while a negative control is the DNA from a non-pathogenic *E. coli* isolate (no virulence genes harboured) and the other is constituted by a sample without template added.
The reactions are incubated in a thermal cycler programmed with the thermal profile described by Paton & Paton (1998): 35 PCR cycles, each consisting of 1 min of denaturation at 95°C; 2 min of annealing at 65°C for the first 10 cycles, decrementing to 60°C by cycle 15; and 1.5 min of elongation at 72°C, incrementing to 2.5 min from cycles 25 to 35.

4. Agarose gel electrophoresis

Prepare a 2.5% (w/v) agarose gel in 1X Tris/Borate/EDTA (TBE) or Trs/Acetate/EDTA (TAE). Each well of the gel is loaded with 15 µl of each reaction added with loading die at 1X final concentration. Run the samples in 1X running buffer (TBE or TAE) in constant voltage (100 V). Use a molecular weight marker suitable for the assignment of the correct molecular weights to the amplicons produced (refer to Table 1 in this appendix). Consider that a correct band assignment is a crucial point in the assessment of the presence of the virulence genes. Make sure that the bands produced by the reference strains match exactly the expected molecular weight. The use of 2.5% agarose gels is needed to get a satisfactory resolution between the bands referring to *eae* (384 bp) and *vtx2f* (428 bp) genes.
Ethidium bromide should be added to agarose gels to allow the visualisation of DNA. This reagent is a DNA intercalating agent commonly used as a nucleic acid stain in molecular biology laboratories. When exposed to ultraviolet light, it will fluoresce with a red-orange color. Ethidium bromide should be added to a final concentration of 0.5 µg/ml before pouring the agarose gel in the electrophoresis gel cast. Alternatively the agarose gel can be stained after electrophoresis in a 0.5 µg/ml ethidium bromide aqueous solution.

5. Reference strains

A VTEC strain harbouring *vtx1*, *vtx2* and *eae* genes should be used as positive control for all these genes. An example is the *E. coli* O157 EDL933 reference strain (ATCC no 43895).
A VTEC strain harbouring *vtx2f* gene should be used as positive control for this gene.
Any *E. coli* K12 strain such as LE392 can be used as negative control.
PCR controls are prepared as described in section 5.2 (Template preparation). The control templates can be prepared in advance and stored in 10 µl ready-to-use aliquots at -20°C for eight months.

6. Interpretation of the results

Samples showing amplification fragments of the expected size (see Table 1) are considered as positive for related target genes. Positive and negative controls must be included in each reaction and give positive and negative results, respectively.

Table 1. Primer sequences and amplicon sizes

Target gene	Primer Name (reference)	Primer Sequence	Amplicon Size (bp)
eae	*eae*AF (Paton & Paton, 1998)	GACCCGGCACAAGCATAAGC	384
	*eae*AR (Paton & Paton, 1998)	CCACCTGCAGCAACAAGAGG	
vtx1	stx1F (Paton & Paton, 1998)	ATAAATCGCCATTCGTTGACTAC	180
	stx1R (Paton & Paton, 1998)	AGAACGCCCACTGAGATCATC	
vtx2 (group)	stx2F (Paton & Paton, 1998)	GGCACTGTCTGAAACTGCTCC	255
	stx2R (Paton & Paton, 1998)	TCGCCAGTTATCTGACATTCTG	
vtx2f	128-1 (Schmidt et al. 2000)	AGA TTG GGC GTC ATT CAC TGG TTG	428
	128-2 (Schmidt et al. 2000)	TAC TTT AAT GGC CGC CCT GTC TCC	

References

Paton AW, Paton JC, 1998. Detection and characterisation of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for *stx1*, *stx2*, *eaeA*, enterohemorrhagic *E. coli* hlyA, rfbO111, and rfbO157. J Clin Microbiol 36, 598-602.

Schmidt H, Scheef J, Morabito S, Caprioli A, Wieler L, Karch H, 2000. A new Shiga toxin 2 variant (Stx2f) from *Escherichia coli* isolated from pigeons. Appl. Environment Microbiol 66, 1205-1208.

Annex 2

Real-time PCR for detection and identification of VTEC

1. Principle of the method

This Real-time PCR protocol aims at the detection and identification of the major virulence genes and serogroup-associated genes characterising the VTEC strains that are considered to be pathogenic to humans. These genes include:

1) *vtx* genes (*vtx1*, *vtx2* and its variants) encoding the Verocytotoxins (Shiga toxins), the main virulence factors of VTEC;
2) the *eae* gene, encoding a 90KDa protein, the intimin, which is the key factor for the induction of the "attaching and effacing" lesion on the enterocyte, a typical feature of the pathogenic VTEC strain;
3) genes associated with the VTEC serogroups that are mainly isolated from human cases of severe disease: O157, O26, O111, O103, and O145. The genes are either comprised in the operons encoding the different lipopolysaccharides (LPS) constituting the O antigens or are anyhow associated to each serogroup in a unique manner (Table 1).

2. Operating procedures

The protocol is based on the 5' nuclease PCR assay. Considering that Real-time PCR may use different instruments and probes labelling chemistry, the amplification conditions to be applied may vary depending on the system used. Refer to the instructions supplied with the instrument and kit of choice.
The primers and probes to be used are listed in the tables below. The chemistry of the reporter and quencher phluorophores are not indicated being largely dependent on the Real-time PCR systems available in each laboratory.

3. Controls

A VTEC strain harbouring *vtx1*, *vtx2* and *eae* genes should be used as positive control for the virulence genes. An example is the *E. coli* O157 EDL933 reference strain (ATCC no 43895). For serogroup-associated genes, reference strains belonging to each of the serogroup should be used.

The Real-time PCR procedure requires an **inhibition/extraction control**. Details on the possible systems to be used as inhibition/extraction control are given below. In particular, two different internal amplification controls (IACs) can alternatively be used:

- A commercially available TaqMan® Exogenous Internal Positive Control (Applied Biosystems, Foster City, CA, USA). The kit includes all reagents 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. (2007. Appl Environ Microbiol 73**,** 1892-1898). Approximately 100 copies of target DNA (pUC 19) should be used per PCR reaction. The size of the IAC is 119 bp.

The latter may be also used as an extraction control by adding 100 copies of the pUC 19 plasmid to the sample aliquot prior to the DNA purification step.

Table 1. Degenerate primers and TaqMan probes used in 5' nuclease PCR assays for the detection of virulence genes

Target gene (Ref.)	Forward primer, reverse primer and probe sequences (5'-3') [a]	Amplicon size (bp)	Location within sequence	GenBank accession number
vtx1 (1)	TTTGTYACTGTSACAGCWGAAGCYTTACG CCCCAGTTCARWGTRAGRTCMACRTC **Probe-** CTGGATGATCTCAGTGGGCGTTCTTATGTAA	131	878–906 983–1008 941–971	M16625
vtx2 (1)	TTTGTYACTGTSACAGCWGAAGCYTTACG CCCCAGTTCARWGTRAGRTCMACRTC **Probe-** TCGTCAGGCACTGTCTGAAACTGCTCC	128	785–813 785–813 838–864	X07865
Eae (2)	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)

(1) Perelle S. et al. Mol Cell Probes 2004 18: 185–192
(2) Møller Nielsen E. and Thorup Andersen M. J Clin. Microbiol. 2003 41: 2884-2893
(3) Perelle S. et al. J. Appl. Microbiol. 2005 98: 1162–1168

Table 2. Primers and probes used for the amplification of O antigen-specific genes in 5' nuclease PCR assays

Target gene (serogroup)	Forward primer, reverse primer and probe sequences (5'-3')	Amplicon size (bp) (Ref.)	Location within sequence	GenBank accession number
§*rfbE* (O157)	TTTCACACTTATTGGATGGTCTCAA CGATGAGTTTATCTGCAAGGTGAT **Probe-** AGGACCGCAGAGGAAAGAGAGGAATTAAGG	88 (1)	348–372 412–435 381–410	AF163329
§*wbdI* (O111)	CGAGGCAACACATTATATAGTGCTTT TTTTTGAATAGTTATGAACATCTTGTTTAGC **Probe-** TTGAATCTCCCAGATGATCAACATCGTGAA	146 (1)	3464–3489 3579–3609 3519–3548	AF078736
§*wzx* (O26)	CGCGACGGCAGAGAAAATT AGCAGGCTTTTATATTCTCCAACTTT **Probe-** CCCCGTTAAATCAATACTATTTCACGAGGTTGA	135 (1)	5648–5666 5757–5782 5692–5724	AF529080
§*ihp1* (O145)	CGATAATATTTACCCCACCAGTACAG GCCGCCGCAATGCTT **Probe-**CCGCCATTCAGAATGCACACAATATCG	132 (1)	1383–1408 1500–1514 1472–1498	AF531429
**wzx* (O103)	CAAGGTGATTACGAAAATGCATGT GAAAAAAGCACCCCCGTACTTAT **Probe-**CATAGCCTGTTGTTTTAT	99 (3)	4299–4323 4397–4375 4356–4373	AY532664

(1) Perelle S. et al. Mol Cell Probes 2004 18: 185–192

(2) Møller Nielsen E. and Thorup Andersen M. J Clin. Microbiol. 2003 41: 2884-2893

(3) Perelle S. et al. J. Appl. Microbiol. 2005 98: 1162–1168

Annex 3:

Isolation of VTEC O26, O103, O111 AND O145 from Real-time PCR positive samples

The Real-time PCR method proposed for the detection of VTEC belonging to the pathogenic serogroups O26, O103, O111 and O145 is based on screening samples for the presence of *vtx* (VT-coding genes), *eae* (intimin-coding gene) and specific serogroup-associated genes. Samples positive at the same time for *vtx*, *eae*, and a serogroup-associated gene must be submitted to a further step aimed at the isolation of the VTEC strain, to confirm the simultaneous presence of the genes in the same live bacterial cell. Flow of the operations may include:

1) Submit the enrichment culture maintained as a back-up to serogroup-specific enrichment (SSE), using immuno-capture systems such as immuno-magnetic separation (IMS) or equivalent following instructions supplied by the manufacturer.
2) Streak the SSE onto Trpytone-bile-glucuronic medium (TBX) or onto a specific selective medium where available (see Note 1) and incubate for 18 to 24 hours at 37°C.
3) Pick from 10 to 50 colonies with *E. coli* morphology or with characteristic aspect according to the medium used (see Note 1) and point-inoculate on nutrient agar (NA) (see Note 2) and H_2O (the colonies may be pooled in water up to a number of ten per pool).
4) Perform conventional PCR (Annex 13) or Real-Time PCR (Annex 2) on the H_2O pools to assess the presence of the *vtx* and the *eae* genes.
5) If a PCR 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 isolates as *E. coli* and confirm the serogroup the sample was positive for in the screening PCR assay (see Note 3).

NOTE 1: For VTEC O26 isolation, a differential solid media (MacConkey) containing Rhamnose instead of lactose is commercially available (RMAC). It is very effective in distinguishing VTEC O26 strains, which do not ferment Rhamnose, from other *E. coli*. Differential and confirmation plating media for VTEC serotypes O26, O103, O111, O145, and sorbitol-positive and -negative O157 have been recently proposed (1, 2). Enterohaemolysin Agar can also be used. It detects Enterohaemolysin production, which is a common feature of VTEC pathogenic to humans (3).

NOTE 2: There are several types of nutrient agar media available commercially either ready-to-use plates or prepared in house from dehydrated powders. Every type of non-selective nutrient agar media (e.g. TSA), including Enterohaemolysin Agar, is suitable for the purpose of maintaining the colonies for further characterisation.
NOTE 3: Colony confirmation as *E. coli* may be achieved by using commercial biochemical galleries or by assessing the indole production. Confirmation of the serogroup the sample was positive to in the screening PCR assay may be achieved either by PCR or by agglutination with commercial antisera.

References

1. Posse B, De Zutter L, Heyndrickx M, Herman L, 2008b. Quantitative isolation efficiency of O26, O103, O111, O145 and O157 STEC serotypes from artificially contaminated food and cattle faeces samples using a new isolation protocol. J Appl Microbiol, 227-235.
2. Possé B, De Zutter L, Heyndrickx M, Herman L, 2008. Novel differential and confirmation plating media for Shiga toxin-producing *Escherichia coli* serotypes O26, O103, O111, O145 and sorbitol-positive and -negative O157. FEMS Microbiol Lett 282,124-131.
3. Beutin L, Zimmermann S, Gleier K. Rapid detection and isolation of shiga-like toxin-producing Escherichia coli by direct testing of individual enterohemolytic colonies from washed sheep blood agar plates in the VTEC-RPLA assay. J Clin Microbiol. 1996; 34:2812-4.

Figure 1. Flow-diagram of the isolation procedure