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Report of the 4th inter-laboratory study on the detection of VTEC non-O157 in food samples - 2010

1. INTRODUCTION

The duties of the EU Reference Laboratory for VTEC (EU-RL VTEC) include the organisation of proficiency tests (PT) on the detection of VTEC belonging to the main pathogenic serogroups in food and animal samples 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 3rd PT, carried out in 2009 (report available here), concerned the detection of the "Top 5" VTEC pathogenic serogroups (O157, O26, O103, O111, O145) in animal carcass swabs, with the aim of preparing the NRLs to properly assist their competent authorities in carrying out the monitoring of VTEC foreseen by EFSA. The participation and the performance of the NRLs were very good as far as the detection of VTEC O157 by the cultural method was concerned. However, only half of the laboratories performed the facultative detection of VTEC non-O157 using the draft ISO Technical Specification "Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (STEC) belonging to O157, O111, O26, O103 and O145 serogroups - Qualitative Method" (ISO/WD TS 13136). This Real-time PCR-based method 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).

Therefore, the 4th PT was focused on the detection of VTEC non-O157 belonging to the main pathogenic serogroups in food samples containing the organisms of interest together with background microbial flora.

The results of the PT have been discussed during the 5th Annual Workshop of the EU reference laboratories for *E. coli* held in Rome on 8 October 2010 (agenda and

presentations available here). 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 (milk) samples with the main pathogenic VTEC non-O157 serogroups (O26, O103, O111, O145) by using the Real-time PCR-based method (ISO/WD TS 13136) recommended by EFSA.

Two milk samples potentially contaminated with VTEC non-O157 and containing a 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

Twenty-nine NRLs representing 23 EU Member States, Norway, and 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 (also representing Luxembourg)
- Belgium - Veterinary and Agrochemical Research Centre
- 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
- Finland - Finnish Food Safety Authority, Evira, Kuopio
- France - VetAgro Sup Campus Vétérinaire de Lyon
- Germany - Federal Institute for Risk Assessment (BfR)

- 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
- Lithuania - National Food and Veterinary Risk Assessment Institute
- Netherlands - Laboratory of the Food and Consumer Product Safety Authority, VWA
- Norway – National Veterinary Institute
- Poland - National Veterinary Research Institute, Pulawy
- Portugal - Laboratório Nacional de Investigação Veterinária
- Romania - Institute for Hygiene and Veterinary Public Health
- Slovakia - State Veterinary and Food Institute
- Slovakia - Public Health Authority
- Slovenia - National Veterinary Institute, Veterinary Faculty, University of Ljubljana
- Spain - Laboratorio Central de Sanidad Animal
- Spain Agencia Española de Seguridad Alimentaria y Nutrición
- Sweden - National Veterinary Institute, SVA
- Sweden – National Food Administration, SLV
- Switzerland - Institute for Food Safety & Hygiene, University of Zurich
- UK - Health Protection Agency, Food Water and Environmental Microbiology Network, Regional Microbiology Network (also representing Malta).

4. MATERIALS AND METHODS

Two milk samples (samples A and B) potentially contaminated with VTEC non-O157 and containing a background microflora were sent in the blind to the NRLs. The NRLs were requested to identify the presence of VTEC non-O157 belonging to the main pathogenic serogroups (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 milk samples included in the study

Contaminant	Sample A	Sample B
VTEC O103, *vtx2, eae*	40 CFU/ml	0
E. coli ATCC35218	10^2 CFU/ml	10^2 CFU/ml
E. faecium ATCCL565	10^2 CFU/ml	10^2 CFU/ml

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 O103 *vt2, eae*: 0.24 log cfu/ml

E. faecium ATCCL565: 0.38 log cfu/ml

E. coli ATCC35218: 0.22 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 27 April 2010, 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 27 and 29 April, and 3 , 5, 10, 12, and 14 May. All the tests yielded the expected results.

The test samples were prepared on 14 May and labelled with randomly generated numerical codes different for each NRL. The homogeneity of the test samples was assessed by testing three 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 17 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 17 May. Twenty-seven NRLs received the samples on 18 May and the remaining two on 19 May. For 24 NRLs, the temperature at delivery ranged between 2°C and 8°C; for the remaining NRLs, 4 received the samples at temperatures <2°C and 4 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 25 (86%) of the 29 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.

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

NRL	Detection of genes in:													
	Sample A							Sample B						
	vtx1	*vtx2*	*eae*	O26	O103	O111	O145	*vtx1*	*vtx2*	*eae*	O26	O103	O111	O145
True value	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L01	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L02	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L03	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L04	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L10	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L12	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L14	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L15	-	+	+	-	-	-	-	-	-	-	-	-	-	-
L19	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L21	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L22	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L23	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L24	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L25	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L27	-	+	+	-	+	-	-	-	+	+	-	+	-	-
L28	+	+	+	-	+	-	-	-	+	+	-	+	-	-
L29	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L31	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L34	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L36	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L37	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L41	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L42	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L44	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L45	-	+	+	-	-	-	-	-	-	-	-	-	-	-
L46	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L48	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L49	-	+	+	-	+	-	-	-	-	-	-	-	-	-
L50	-	+	+	-	+	-	-	-	-	-	-	-	-	-

Four NRLs made errors. L15 and L45 failed to identify the presence of the O103 serogroup-specific gene in Sample A. L27 and L28 wrongly reported the presence of the

VTEC O103 target genes in the negative Sample B, probably because of a cross contamination between the two samples occurred during the analytical procedure. The NRL L28 also made an error in Sample A, reporting the presence of *vtx1*.

5.2. Isolation of the VTEC O103 strain from the PCR-positive samples

Among the 25 NRLs that had detected correctly the presence/absence of the target genes in the enrichment cultures, 24 (96%) performed successfully the isolation step (Table 3).

Table 3. Isolation of VTEC O103 from the milk samples. The green boxes highlight the correct results, the red boxes the wrong results.

NRL	Isolation of VTEC O103 from:	
	Sample A	Sample B
True Value	+	-
L01	+	-
L02	+	-
L03	+	-
L04	+	-
L10	+	-
L12	+	-
L14	+	-
L15	-	-
L19	+	-
L21	+	-
L22	+	-
L23	+	-
L24	+	-
L25	+	-
L27	+	+
L28	+	+
L29	+	-
L31	+	-
L34	+	-
L36	+	-
L37	+	-
L41	+	-
L42	+	-
L44	+	-
L45	-	-
L46	+	-
L48	+	-
L49	+	-
L50	-	-

The two NRLs (L15 and L45) that had failed to identify the presence of the VTEC O103 target genes in sample A also failed to isolate the strain. Another NRL (L50) did not attempt the isolation of the VTEC strain despite it had correctly identified the O103 serogroup-associated gene. Finally, the NRLs L27 and L28 reported the isolation of VTEC O103 from the negative sample B, in agreement with the wrong positive results reported in the PCR screening step.

The NRL L28 also made an error in genotyping the isolated VTEC strain, reporting the presence of the *vtx1* gene.

5.3 Overview of the analytical performances

The analytical performances of the NRLs were evaluated in terms of agreement, sensitivity and specificity for the Real-time PCR step finalized to the detection of the virulence and serogroup-associated genes in the enrichment cultures.

The level of agreement between the results of each NRL and the true values were evaluated by the Cohen's Kappa values. The overall agreement (K) was 0.94 (CI: 0.84 – 1) and the values of each NRL are reported in Table 4.

Table 4. 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).

	Cohen's Kappa values for NRL:														
NRL	**L01**	**L02**	**L03**	**L04**	**L10**	**L12**	**L14**	**L15**	**L19**	**L21**	**L22**	**L23**	**L24**	**L25**	**L27**
K value	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**0.75**	**1**	**1**	**1**	**1**	**1**	**1**	**0.14**
NRL	**L28**	**L29**	**L31**	**L34**	**L36**	**L37**	**L41**	**L42**	**L44**	**L45**	**L46**	**L48**	**L49**	**L50**	
K value	**0.20**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**0.75**	**1**	**1**	**1**	**1**	

Most NRLs (25 of the 29) obtained an "excellent" agreement, while two NRLs (L15 and L45) had a lower analytical performance, even if considered "good". The two remaining NRLs (L27 and L28) had an analytical performance that was considered as "poor".

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 97.7% (95% CI: 94.5% - 100%) and the overall specificity was 97.8% (95% CI: 96.2% - 99.4%). The sensitivity and specificity values for each NRL are reported in Table 5.

Table 5. 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**	**L02**	**L03**	**L04**	**L10**	**L12**	**L14**	**L15**	**L19**	**L21**
Se	100%	100%	100%	100%	100%	100%	100%	67%	100%	100%
Sp	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
NRL	**L22**	**L23**	**L24**	**L25**	**L27**	**L28**	**L29**	**L31**	**L34**	**L36**
Se	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Sp	100%	100%	100%	100%	73%	64%	100%	100%	100%	100%
NRL	**L37**	**L41**	**L42**	**L44**	**L45**	**L46**	**L48**	**L49**	**L50**	
Se	100%	100%	100%	100%	67%	100%	100%	100%	100%	
Sp	100%	100%	100%	100%	100%	100%	100%	100%	100%	

The four NRLs (L15, L45, L27, and L28) that made errors in the first step of Real time PCR screening of the pre-enrichment cultures had a poor performance also in the step of isolation of the VTEC strain. A poor performance in the isolation step was also recorded for L50 that failed to isolate the VTEC O103 strain despite the correct identification in the molecular screening step.

6. REMARKS

1. Twenty-nine NRLs representing 23 EU Member States participated in the study, together with the NRLs of Norway and Switzerland.
2. This represented a substantial increase if compared with the 16 NRLs that performed the Real Time PCR-based Technical Specification for the detection of the main pathogenic VTEC non-O157 serogroups in carcass swabs in the 3rd Inter-laboratory Study carried out in 2009.
3. Twenty-four NRLs (82%) reported correctly the isolation of VTEC O103 from sample A and a negative result for sample B.

4. Five NRLs provided incorrect results: two failed to identify the presence of VTEC O103 in the positive samples and two wrongly reported the presence of the strain also in the negative Sample B. The remaining NRL failed to isolate the strain after a correct detection of virulence and serogroup-associated genes by Real-Time PCR.
5. The false positive results were probably due to a cross contamination between the two samples during the analytical procedure. The errors involving the first step of Real time PCR screening of the pre-enrichment cultures affected the following step of isolation of the VTEC strain.
6. Most NRLs had an excellent or good or 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.
7. The web site section implemented for the submission of the results of the inter-laboratory studies proved to be a valuable tool for gathering and managing the results. It will be further improved, based on the comments collected from the users.
8. This PT further confirmed that the Real-time PCR-based horizontal Technical Specification (ISO/WD TS 13136) represents a robust tool for the detection of non-O157 VTEC in food samples, allowing most NRLs to isolate the VTEC O103 strain from samples where it was present at a concentration of 40 CFU/g.

Annex 1

4th inter-laboratory study on Verocytotoxin-producing *E. coli* (VTEC): detection and identification in milk samples

Laboratory procedure

The method to be used is the ISO-CEN draft Technical Specification "Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (STEC) belonging to O157, O111, O26, O103 and O145 serogroups - Qualitative Real-time polymerase chain reaction (PCR)-based Method". This method has been developed by the "STEC *ad hoc* Group" within the WG 6 of 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, followed by the isolation of the VTEC strains responsible for the positive PCR reactions.

The method is applied to an enrichment culture performed by adding a test portion of 25 ml of the milk sample to 225 ml of mTSB containing 12 mg/lt Acriflavin, and incubating for 18 -24 h at 37°C ± 1°C (see **Flow diagram** below). 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 included as **Annex 1**. 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.

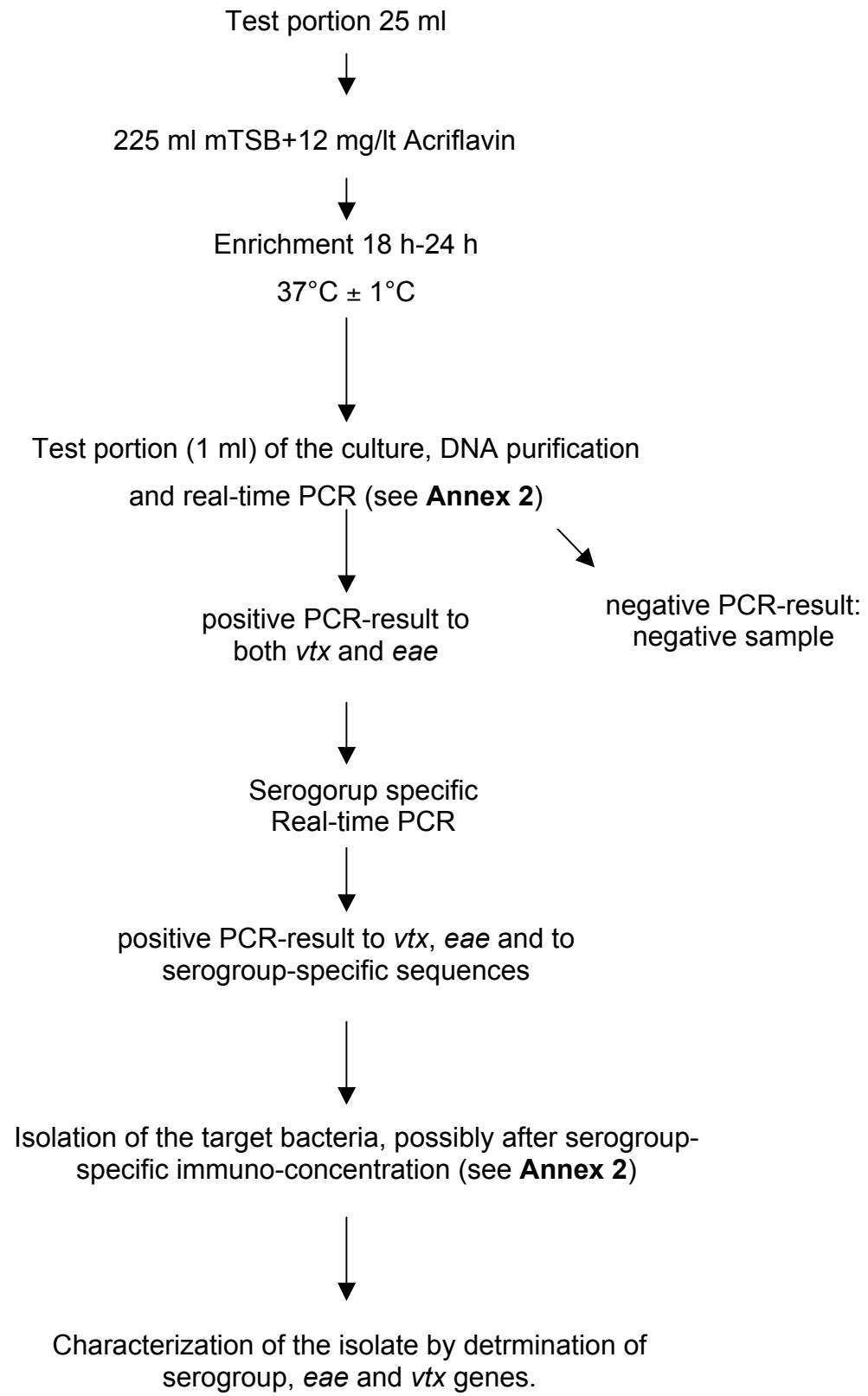
Step 2: Detection of the genes *vtx1*, *vtx2* and *eae.*

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 2**.

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

Flow-diagram for the detection of VTEC non-O157 in milk samples



Annex 1

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:

1. 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 2:

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 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 or Real-Time PCR 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

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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