EU Reference Laboratory for *E. coli*
Department of Veterinary Public Health and Food Safety
Unit of Foodborne Zoonoses
Istituto Superiore di Sanità

Report of the 16th inter-laboratory study on the detection of Verocytotoxin-producing *E. coli* (VTEC) in sprout spent irrigation water (PT16) - 2015

Edited by:

Silvia Arancia, Susan Babsa, Alfredo Caprioli, Paola Chiani, Clarissa Ferreri, Fabio Galati, Antonella Maugliani, Valeria Michelacci, Fabio Minelli, Stefano Morabito, Concetta Scalfaro, Rosangela Tozzoli

1. INTRODUCTION

The duties of the EU Reference Laboratory for *E. coli* (EU-RL VTEC) include the development of methods for the detection of VTEC in food and animal samples. In this respect, the EU-RL VTEC has coordinated the development of the ISO Technical Specification ISO/TS 13136:2012 "*Microbiology of food and animal feed -- Real-time polymerase chain reaction (PCR)-based method for the detection of food-borne pathogens -- Horizontal method for the detection of Shiga toxin-producing Escherichia coli (STEC) and the determination of O157, O111, O26, O103 and O145 serogroups*", published in November 2012. Later on, Regulation (EU) 209/2013, laying down microbiological criteria for sprouts, introduced for the first time microbiological criteria for VTEC in the EU legislation and prescribed for their detection the use of the ISO/TS 13136:2012, taking also into account the adaptation provided by the EU-RL VTEC for the specific detection of VTEC O104:H4 (EU-RL VTEC_Method_04_Rev 1: "*Detection and identification of Verocytotoxin-producing* Escherichia coli *(VTEC) O104:H4 in food by Real Time PCR* ").

Reg. (EU) 209/2013 also gives the food business operators producing sprouts the possibility to replace the sampling and testing of sprouts with the analysis of five samples of 200 ml of the water that has been used for their irrigation. However, testing spent irrigation water for the presence of VTEC or other enteric pathogens may pose technical problems, due to some characteristics of this particular matrix. For instance, if concentration of VTEC is pursued by a filtration step, the high density of the irrigation water, due to substances released by some species of sprouts, can make such a filtration difficult. Therefore, the EU-RL VTEC organized an inter-laboratory study (PT16) to evaluate the application of the ISO/TS 13136:2012 method to test sprout spent irrigation water. This document represents the report of the study.

2. OBJECTIVE AND DESIGN OF THE STUDY

The objective of the study was the evaluation of a procedure for the pre-treatment of sprout spent irrigation water samples to be entered in the analytical flow of the ISO/TS 13136:2012. Such a procedure makes use of centrifugation to concentrate the contaminating VTEC bacterial cells possibly present in the sample.

The study consisted in the assessment of different levels of contamination of sprout spent irrigation water samples with a VTEC strain belonging to one of the serogroups included in the microbiologic criterion laid down by Reg. (EU) 209/2013, following the prescriptions of the same Regulation. Three spent water samples were sent to the laboratories that

accepted to participate. Two of them were artificially contaminated with a VTEC strain and for one of them the contamination level was close to the expected limit of detection of the procedure.

3. PARTICIPANTS

The invitation to take part in the study was sent to the National Reference Laboratories (NRLs) for *E. coli* of EU Member States and other countries, as well as to a number of Italian laboratories involved in the official control of food and with experience in the application of the ISO/TS 13136:2012 method.

A total of 51 laboratories, 30 NRLs of EU Member States, 4 NRLs of non-EU countries and 17 Italian Official Laboratories (OLs) accepted to participate in the study.

Each laboratory received its own individual laboratory numerical code, which is reported in the result tables.

The NRLs participating in the study were:

- Austria, *Österreichische Agentur für Gesundheit und Ernährungssicherheit* GmbH
- Belgium, Scientific Institute of Public Health
- Belgium, Veterinary & Agrochemical Research Centre (VAR - CODA – CERVA)
- Bulgaria, National Diagnostic and Research Veterinary Institute
- Cyprus, Laboratory for the Control of Food of Animal Origin (LCFAO), Cyprus Veterinary Services
- Czech Republic, Veterinary Research Institute
- Denmark, National Food Institute, Technical University of Denmark
- Egypt, Central Laboratory of Residue Analysis of Pesticides and Heavy Metals in Foods
- Estonia, Veterinary and Food Laboratory
- Finland, Finnish Food Safety Authority Evira
- France, *VetAgro Sup Campus Vétérinaire de Lyon*
- Germany, Federal Institute for Risk Assessment (BfR)
- Greece, National School of Public Health & Central Laboratory of Public Health
- Hungary, National Food Microbiological Reference Laboratory
- Ireland, Central Veterinary Research Laboratory, Department of Agriculture, Food and Marine
- Italy, *Istituto Superiore di Sanità*
- Latvia, Institute of Food Safety, Animal Health and Environment (BIOR)
- Lithuania, National Food and Veterinary Risk Assessment Institute

- Luxembourg, *Ministère de l'Agriculture, de la Viticulture et de la Protection des consommateurs, Administration des services vétérinaires* (LMVE)
- Netherlands, National Institute for Public Health and the Environment (RIVM)
- Netherlands, Food and Consumer Product Safety Authority (NVWA)
- Norway, Norwegian Veterinary Institute
- Poland, National Institute of Public Health-National Institute of Hygiene, Warsaw
- Portugal, *Instituto Nacional de Investigação Agrária e Veterinária*, Vairão
- Romania, Institute for Hygiene and Veterinary Public Health
- Russia, State Research Center for Microbiology and Biotechnology, Obolensk
- Slovakia, Department of Food Hygiene, State veterinary and food institute, Dolný Kubín
- Slovakia, NRC of Environmental Microbiology, Public Health Authority, Bratislava
- Slovenia, Veterinary Faculty/ National Veterinary Institute
- Spain, AESAN, *Centro Nacional de Alimentación, Servicio de Microbiología Alimentaria*
- Spain, *Laboratorio Central de Veterinaria* (LCV)- *Sanidad Animal* (MAGRAMA), Algete
- Sweden, National Veterinary Institute (SVA)
- Switzerland, Institute for food safety and hygiene, University of Zurich
- UK, Public Health England, FWEM Laboratory, Porton

The Italian OLs participating in the study were:

- *Laboratorio di Prevenzione, Agenzia di Tutela della Salute (ATS) della Brianza, Oggiono (LC)*
- *IZS Abruzzo e Molise "G. Caporale", Igiene delle tecnologie alimentari e dell'alimentazione animale, Teramo*
- *IZS Puglia e Basilicata, UO Ricerca e Sviluppo Scientifico, Foggia*
- *IZS Puglia e Basilicata, Sezione di Putignano (BA)*
- *IZS Lombardia ed Emilia Romagna, Reparto Microbiologia, Brescia*
- *IZS Lombardia ed Emilia Romagna, Sezione di Bologna*
- *IZS Lazio e Toscana, Dir. Op. Controllo degli Alimenti, Roma*
- *IZS del Mezzogiorno, UO Microbiologia Alimentare, Sezione di Salerno, Fuorni (SA)*
- *IZS del Mezzogiorno, UO Diagnostica, Sezione di Salerno, Fuorni (SA)*
- *IZS della Sicilia, Area Microbiologia degli Alimenti, Palermo*
- *IZS Sardegna, Laboratorio di Microbiologia e Terreni Colturali, Sassari*
- *IZS Piemonte, Liguria e Valle D'Aosta, S.C. Controllo Alimenti e Igiene delle Produzioni, Torino*
- *IZS Piemonte, Liguria e Valle D'Aosta, S.C. Biotecnologie, Torino*

- *IZS Piemonte Liguria e Valle D'Aosta, Sezione di Genova*
- *IZS Umbria e Marche, Laboratorio Contaminanti Biologici, Perugia*
- *IZS delle Venezie, Sezione di Pordenone, Cordenons (PN)*
- *IZS delle Venezie, SC Analisi del Rischio e Sorveglianza in Sanità Pubblica, Legnaro (PD)*

4. MATERIALS AND METHODS

4.1. Sample preparation

The spent irrigation water used in the study was obtained from a local sprout producer who collected the water flowing from the production of red radish sprouts. The water was collected starting from 48 h after the beginning of the sprout production process, according to the prescriptions of Reg. (EU) 209/2013, and displayed a semi-colloid appearance due to the substances released by seeds during the sprouting process. The water specimens contained a natural background microflora (about 2,5 x 10^7 CFU/g) and were negative at the PCR screening for the genes that were the target of the method employed in the study. Three specimens (labeled A, B and C, each consisting of 200 ml of water in sterile plastic bottles) potentially contaminated with VTEC were sent in the blind to the laboratories.

The artificial contamination of the samples was carried out on 27 November 2015, using dilutions of an exponential liquid culture (0.5 OD read at 600 nm) of the VTEC O157 strain C210-03, described in Table 1. An uncertainty of measurement of 0.27 log CFU/ml was associated to the standardized inoculum, using the procedure described in the ISO/TS 19036:2006. The samples were spiked with three different levels of contamination: zero, high and low level. In detail, the set of samples sent to the laboratories contained 0, 200 and 500 estimated CFU per ml of VTEC O157 for the non-contaminated, low and high level samples, respectively. Serial dilutions of the inoculum suspensions added to the samples were plated onto MacConkey agar plates to check their titer. The stability tests showed that all the samples were positive at the PCR screening after nine days from the spiking. Strain isolation was still achieved from the samples with 500 CFU/g after nine days from contamination.

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

Table 1: Characteristics of the sprout spent irrigation water samples included in the study

Contaminant (*Genotype*)	Contamination level in:		
	Sample A	**Sample B**	**Sample C**
VTEC O157 *(vtx1+, vtx2+, eae+)*	High: 500 CFU/ml	Low: 200 CFU/ml	-

The test samples were labeled with randomly generated numerical codes different for each NRL, immediately refrigerated and transferred into refrigerated safety packages that were shipped on 30 November 2015 by courier. The NRLs were requested to start the analyses immediately upon receipt and to record the date of delivery and sample temperature upon reception.

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* artificially contaminated samples were prepared as described in the sample preparation section and tested along a period of time of nine days. Samples were spiked on 17 November 2015 and tested by Real Time PCR at 0, 3, 7 and 9 days since the initial contamination. The presence of the contaminating VTEC strain was detected in all the tests as expected.

When the test samples were prepared, 10 bottles for each of the three contamination levels were randomly selected for homogeneity testing and analyzed according to the PT laboratory procedure. All the homogeneity tests gave the expected results.

4.3. Laboratory methods

The technical procedure provided to the participating laboratories comprised the following sequential steps:

- Centrifugation of the water samples.
- Transfer of the resulting pellet presumptively containing VTEC into the enrichment medium. Assuming that contaminant bacteria present in sprouts may have undergone stressing conditions, the laboratories were requested to use buffered peptone water (BPW) as enrichment medium in the proportions 1/10 $vol_{pellet}/vol_{enrichment}$.
- Application of the ISO/TS 13136:2012 standard, according to Reg. (EU) 209/2013, to identify the presence of VTEC O157, O111, O26, O103, O145 and O104:H4, taking into account the adaptation provided by the EU-RL VTEC for the detection of VTEC

O104:H4 (available in the EU-RL web site, http://www.iss.it/vtec, Laboratory Methods Section).

- The isolation and characterization of the VTEC strains responsible for positive PCR screening reactions was accomplished according to the procedure described in the ISO/TS 13136:2012, taking into account the adaptation provided by the EU-RL VTEC for the detection of VTEC O104:H4.

4.4. Collection and elaboration of the results

The results were submitted directly through an on-line system, using a dedicated page in the “Restricted Area” of the EU-RL VTEC website.

The laboratories 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 a dedicated section for submitting the test results. This section also contained a *Shipment form* with the list of the samples to be analyzed and the fields to collect the 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 instant generated individual reports, containing the submitted and expected results, directly from the secure page of the EU-RL website.

4.5. Analysis of the results and evaluation of the performance of the method

Sensitivity (Se) and Specificity (Sp) were calculated for the various VTEC characters considered in the study and for the different steps of the ISO/TS 13136:2012. Therefore, Se was calculated for the PCR screening for *vtx1*, *vtx2*, *eae* and $rfbE_{O157}$ genes. Sp was calculated for the PCR screening for *vtx1* and *vtx2* genes.

The PT results were also used to calculate the Se and the LOD of the isolation step of the method. The latter parameter was calculated using the procedure described by Wilrich and Wilrich (*Journal of AOAC international, Vol. 92 No. 6, 2009, 1763-1772*).

5. RESULTS

The samples were sent to 51 laboratories and 50 of them returned results.

As far as the delivery conditions were concerned, 41 laboratories received the samples within 24 hours, 5 within 48 hours, 2 within 72 hours. The remaining three received the samples after 4 (L997) and 8 (L936) days, respectively, due to problems related with either the shipment courier or custom clearance procedures.

The temperature at delivery ranged between 2 °C and 8 °C for 42 laboratories, between 9 °C and 11 °C for 7 laboratories. For the remaining NRL, L936, that received the samples after 8 days, the temperature was 15°C.

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

For sample A (high level of contamination), 48 laboratories (96 %) identified correctly the presence of the virulence genes *vtx1*, *vtx2* and *eae*, while 2 laboratories provided a total of 3 incorrect results for these genes.

The detection of the serogroup-associated gene was performed correctly by 48 laboratories (96 %), which identified correctly the presence of the $rfbE_{O157}$ gene. One laboratory did not identify any serogroup and another one wrongly indicated the presence of serogroup O104. As a whole, 3 laboratories (6 %) provided a total of 5 incorrect results for sample A.

For sample B (low level of contamination), the presence of virulence genes was identified correctly by 47 laboratories (94 %) and the presence of the $rfbE_{O157}$ gene by 48 laboratories (96 %). As a whole, 4 laboratories (8 %) provided a total of 6 incorrect results for sample B.

Table 2. Real-time PCR detection of virulence and serogroup-associated genes in the enrichment cultures. The green boxes indicate the correct results, in agreement with the true value reported on the top of each column. The red boxes indicate the wrong results. A white box indicates that the test was not done.

NRL	Detection of virulence and serogroup-associated genes in:											
	Sample A High level contamination				Sample B Low level contamination				Sample C			
	vtx1	*vtx2*	*eae*	*rfbE$_{O157}$*	*vtx1*	*vtx2*	*eae*	*rfbEO$_{157}$*	*vtx1*	*vtx2*	*eae*	*rfbE$_{O157}$*
True value	+	+	+	+	+	+	+	+	-	-	-	-
L130												
L140						-						
L180												
L186												
L190												
L243												
L244												
L257												
L261												
L271												
L285												
L303												
L320												
L324												
L327												
L415												
L469				-				-				
L470												
L476	-				-							
L528												
L545												
L546												
L547												
L551												
L559												
L568												
L574												
L583												
L615												
L617												
L627												
L656												
L658												
L660												
L714												
L725												
L733												
L756												
L761												
L782												
L813												
L831												
L836												
L849												

Table 2 (continued). Real-time PCR detection of virulence and serogroup-associated genes in the enrichment cultures. The green boxes indicate the correct results, in agreement with the true value reported on the top of each column. The red boxes indicate the wrong results. A white box indicates that the test was not done.

NRL	Detection of virulence and serogroup-associated genes in:											
	Sample A High level contamination				Sample B Low level contamination				Sample C			
	vtx1	*vtx2*	*eae*	*rfbE*$_{O157}$	*vtx1*	*vtx2*	*eae*	*rfbE*$_{O157}$	*vtx1*	*vtx2*	*eae*	*rfbE*$_{O157}$
True value	+	+	+	+	+	+	+	+	-	-	-	-
L885												
L887												
L936	-		-	O104	-		-	O104				
L952												
L968												
L997												

5.2. Isolation of the VTEC O157 strain from PCR-positive samples.

Forty-four (88 %) of the 50 laboratories obtained the isolation of the VTEC O157 contaminating strain from the enrichment culture of sample A (Table 3).

The VTEC O157 contaminating strain was isolated from the enrichment cultures of sample B (low level of contamination) by 42 (84 %) laboratories.

Table 3. Isolation and genotyping of VTEC strains from the PCR-positive sprout spent irrigation water enrichment cultures. The green boxes indicate the correct results, in agreement with the true value reported on the top of each column. The red boxes indicate the wrong or missing results. A white box indicates that the test was not done.

VTEC strain isolation and genotyping from:									
NRL	Sample A				Sample B				Sample C
	VTEC O157 Isolation	Genotype			VTEC O157 Isolation	Genotype			-
		vtx1	*vtx2*	*eae*		*vtx1*	*vtx2*	*eae*	
True value	+	+	+	+	+	+	+	+	None
L130									
L140									
L180									
L186									
L190									
L243									
L244									
L257									
L261									
L271									
L285									
L303									
L320									
L324									
L327									
L415									
L469									
L470									
L476		-				-			
L528									
L545									
L546									
L547									
L551									
L559									
L568									
L574									
L583									
L615									
L617									
L627									
L656									
L658									
L660									
L714									

Table 3 (continued). Isolation and genotyping of VTEC strains from the PCR-positive sprout spent irrigation water enrichment cultures.

The green boxes indicate the correct results, in agreement with the true value reported on the top of each column. The red boxes indicate the wrong or missing results. A white box indicates that the test was not done.

VTEC strain isolation and genotyping from:									
NRL	Sample A				Sample B				Sample C
	VTEC O157 Isolation	Genotype			VTEC O157 Isolation	Genotype			-
		vtx1	*vtx2*	*eae*		*vtx1*	*vtx2*	*eae*	
True value	+	+	+	+	+	+	+	+	None
L725									
L733									
L756									
L761									
L782									
L813									
L831									
L836									
L849									
L885									
L887									
L936									
L952									
L968									
L997									

5.4. Evaluation of the performance of the methods

5.4.1. PCR screening step

The Sensitivity (Se) was calculated for the detection of the *vtx1*, *vtx2*, *eae* and $rfbE_{O157}$ genes, and the Specificity (Sp) for the *vtx1* and *vtx2* genes. The results provided by 49 of the 50 laboratories were considered, excluding L936, which was considered as outlier. The analysis of the results are reported in the table below:

***Table 4*. Performances of the PCR screening step**. Specificity (Sp) and Accuracy (Ac) were calculated only for the detection of *vtx* genes, since the ISO TS 13136:2012 method is sequential and does not require testing for *eae* and serogroup-associated genes in case of samples negative for the *vtx* genes. *Se= Sensitivity; Sp=Specificity; Ac= Accuracy.*

	Se (High)	Se (low)	Sp	Ac (High)	Ac (Low)
vtx1	97,9 %	97,9 %	100 %	98,97 %	98,97 %
vtx2	100 %	97,9 %	100 %	100 %	98,97 %
eae	97,9 %	97,9 %	N.A.	N.A.	N.A.
rfbE$_{O157}$	97,9 %	97,9 %	N.A.	N.A.	N.A.

5.4.2. VTEC O157 isolation step

The sensitivity and the limit of detection (LOD) were calculated for the isolation step, considering the results provided by 49 laboratories and excluding L936, which was considered as outlier. The analysis returned the following values:

- Se: 89.8 % (high level) and 85.7 % (low level).
- $LOD_{50\%}$: 104,9 CFU per gram (c.i. 79,4-138,7)
- $LOD_{95\%}$: 453,6 CFU per gram (c.i. 343,3-599,3).

6. CONCLUDING REMARKS

Reg. (EU) 209/2013 prescribes the absence of VTEC O157, O26, O157, O103, O145 and O104:H4 in sprouts to be consumed as raw, and allows the producers and the testing laboratories to analyze the spent irrigation water from the production process to assess the conformity to the microbiological criterion of the end product. Hence, the NRLs and the other laboratories involved in the official control of food must be prepared to test this matrix. On the other hand, spent irrigation water may be a problematic matrix for the verification of the VTEC presence and there are no established procedures for the treatment of such samples that ensure the quality of the results obtained with the official method ISO/TS 13136:2012. The EU-RL VTEC has therefore developed a simple procedure for the treatment of this peculiar matrix that would be suitable for the introduction in the analytical flux of the routine testing for STEC and took the opportunity of PT16 to evaluate the performances of the ISO/TS 13136:2012 applied to spent irrigation water samples treated with the mentioned procedure.

The PT16 specimens were contaminated with a VTEC O157 strain positive for all the virulence genes of VTEC. Two levels of contamination were included, the lowest being calculated experimentally as the one providing positive results in the isolation step in at least half of the samples, in order to determine the limit of detection of the procedure.

Since the aim of this PT was to evaluate the performances of a method rather than the proficiency of the participating laboratories, the invitation to participate was extended to the network of the Italian Official Laboratories, in order to maximize the number of results returned and to refine the calculations of the performance parameters.

The analysis of the results provided by the participating laboratories induces the following remarks:

1. A total of 51 laboratories, 30 NRLs of EU member States, 4 NRLs of non EU countries, and 17 Italian Official Laboratories accepted to participate in the study.
2. The analytical results, provided by 50 laboratories, confirmed the suitability and fit for purpose of the the developed treatment procedure for spent irrigation water, based on a simple centrifugation step. The presence of the VTEC O157 genes was identified correctly by 48 laboratories (96 %) in sample A (high level of contamination) and by 47 laboratories (94 %) in sample B (low level of contamination).
3. The contaminating VTEC O157 strain was isolated from both samples by the majority of the laboratories (88 % for sample A and 84 % for sample B).
4. The procedure for the treatment of spent irrigation water samples to be tested with the ISO/TS 13136:2012 method proved to be an effective tool for the verification of the conformity of the end product (sprouts) to the microbiological criterion laid down in the Reg. (EU) 209/2013.
5. The design of the whole study was done in the framework of the support provided to the NRLs and Official Laboratories, which can refer to the laboratory procedure provided for this PT scheme when testing spent irrigation water in compliance with regulatory requests.