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Report of the 15th inter-laboratory study on the detection of Verocytotoxin-producing *E. coli* (VTEC) in sprouts (PT15) - 2015

Edited by:

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1. INTRODUCTION

The duties of the EU Reference Laboratory for *E. coli* (EU-RL VTEC) include the development of methods for the detection of VTEC in food and animal samples and the organization of proficiency tests (PT) on those methods for the designated National Reference Laboratories (NRLs) for *E. coli* in the EU and EFTA Member States, EU Candidate Countries and certain third countries.

A method for the detection of VTEC in foodstuffs was published on November 2012 as an 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*". This method, together with the EU-RL procedure "*Detection and identification of Verocytotoxin-producing Escherichia coli (VTEC) O104:H4 in food by Real Time PCR* (EU-RL VTEC_Method_04_Rev 1)", had been prescribed for the detection of VTEC in sprouts by Regulation (EU) No 209/2013, which has introduced for the first time microbiological criteria for VTEC in the EU legislation.

The method was already adopted and evaluated in seven rounds of the EU-RL PT scheme on the detection of VTEC in food matrices: PT3, carried out on bovine carcass swabs; PT4, carried out on milk samples; PT7, carried out on vegetable samples; PT8, carried out on water samples; PT9, carried out on seeds intended for sprouting; and PT12 and PT14, carried out on sprouts. The reports of these PTs are available in the EU-RL web site (www.iss.it/vtec).

The 8th PT on the detection of VTEC in food (PT15) was again carried out on sprouts, according to Reg. (EU) 209/2013.

The choice of this matrix was due to the following reasons:

- After the outbreak of VTEC O104:H4 infections occurred in Europe in 2011, sprouts continue to be implicated in epidemic episodes of Salmonella and VTEC infections.
- Reg. (EU) No 209/2013, which is in place by 1 July 2013, amended Regulation (EC) No 2073/2005 and introduced microbiological criteria for sprouts, including the absence of VTEC O157, O26, O111, O103, O145 and O104:H4 in 25 g of product, as determined by the ISO/TS 13136:2012 method.
- According to the new EU rules, laboratories involved in the official control of food must be prepared to test sprouts for the presence of VTEC.

PT15 gives continuity to the previous studies conducted within the network of Reference Laboratories for *E. coli* on matrices possibly involved in the control of sprout production and this document represents the full evaluation report of the study.

2. DESIGN AND OBJECTIVES OF THE STUDY

The study consisted in the assessment of different levels of contamination of sprout 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 sprout samples were sent to the laboratories that accepted to participate in the PT. 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.

The **objectives** of the study were:

- To improve the preparedness of the NRLs towards testing sprouts for the presence of VTEC, according to the Reg. (EU) 209/2013.
- To expand the range of serogroups for which the analytical performance parameters of the detection method have been determined.
- To give further support to the NRLs and the Official Laboratories for the accreditation of the ISO/TS 13136:2012.

3. PARTICIPANTS

Thirty-six NRLs representing 26 EU Member States, Norway, Switzerland, Russia, and Egypt participated in the study.

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

The NRLs participating in the study were:

- Austria, *Österreichische Agentur für Gesundheit und Ernährungssicherheit* GmbH
- Belgium, Scientific Institute of Public Health
- Belgium, Veterinary & Agrochemical Research Centre (VAR - CODA – CERVA)
- Bulgaria, National Diagnostic and Research Veterinary Institute
- Croatia, Croatian 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
- Netherlands, Centre for Zoonoses and Environmental Microbiology (RIVM)
- Netherlands, Food and Consumer Product Safety Authority (NVWA)
- Norway, Norwegian Veterinary Institute
- Poland, National Institute of Public Health-National Institute of Hygiene, Warsaw
- Poland, National Veterinary Research Institute, Department of Hygiene of food of animal origin, Pulawy
- 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, *Livsmedelsverket*/The National Food Agency
- Sweden, National Veterinary Institute (SVA)
- Switzerland, Institute for food safety and hygiene, University of Zurich
- UK, Public Health England, Food, Water and Environmental Microbiology Laboratory, Preston

4. MATERIALS AND METHODS

4.1. Sample preparation

The sprouts used in the study consisted of a commercial mixture of alpha-alpha (90 %) and watercress (10 %) sprouts acquired as retail packages. They contained a natural background microflora (about $9x10^7$ CFU/g) and were negative at a PCR screening for the genes that were the target of the PT. Three samples (samples A, B and C, each consisting of 25 g of sprouts) potentially contaminated with VTEC were sent in the blind to the NRLs.

The artificial contamination of the samples was carried out on 17 April 2015, using dilutions of an exponential liquid culture (0.5 OD read at 600 nm) of the VTEC O111 strain ED 476, described in Table 1. An uncertainty of measurement of 0.138 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 NRLs contained 0, 100 (73-137) and 1000 (727-1,374) estimated CFU per gram for the non-contaminated, low and high level aliquots, respectively. Serial dilutions of the inoculum suspensions added to the samples were plated onto MacConkey agar plates to check their titer. The concentration of the VTEC O111 strain was established on the basis of the stability tests (both PCR screening and isolation steps) run on replicates of each contamination level in a time period spanning 12 days from samples spiking. The PCR tests were positive for all samples with 100 and 1000 CFU/g of the contaminant VTEC, even after 12 days from the spiking. Conversely, strain isolation was not obtained for the sample with 100 CFU/g after 10 days from contamination. The possibility of spiking with a lower (10 CFU/g) VTEC O111 titer was also evaluated but the PCR tests gave negative results after 10 days from spiking.

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

Table 1: Characteristics of the sprout samples included in the study

Contaminant (*Genotype*)	Contamination level in:		
	Sample A	**Sample B**	**Sample C**
VTEC O111 (*vtx1+, vtx2+, eae+*)	High: 1,000 (727-1,374) CFU/g	Low: 100 (73-137) CFU/g	-

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 20 April by courier. The NRLs were requested to start the analyses immediately upon receipt and to record 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 13 days since their preparation. Samples were spiked on 6 March 2015 and tested by Real Time PCR at 3, 6 and 13 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 bags for each of the three samples were randomly selected for homogeneity testing and analyzed according to the PT laboratory procedures. All the homogeneity tests gave the expected results.

4.3. Laboratory methods

According to Reg. (EU) 209/2013, laboratories were requested to identify the presence of VTEC O157, O111, O26, O103, O145 and O104:H4 using the method ISO/TS 13136:2012, 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). Assuming that contaminant bacteria present in sprouts may have undergone stressing conditions, the NRLs were requested to use buffered peptone water (BPW) as enrichment medium.

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 NRL results

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

4.5. Analysis of the NRL results

4.5.1. Evaluation of the NRL performance in the real time PCR screening step

The performance of each NRL in identifying VTEC target genes in the enrichment cultures was evaluated by assigning 4 penalty points to each incorrect or missing result concerning the identification of virulence and serogroup-associated genes in the three samples.

4.5.2. Evaluation of the NRL performance in the isolation of VTEC strains from the PCR-positive enrichment cultures

The performance of each NRL in the isolation and characterization of the VTEC strains responsible for positive PCR screening reactions in the enrichment cultures was evaluated by assigning 4 penalty points to the lack of isolation from sample A (high level of contamination) and to incorrect or missing results in strains characterization. No penalty points were assigned to the lack of isolation from sample B (low level of contamination), because the contamination level was close to the limit of detection of the procedure.

4.5.3. Evaluation of the NRL performance in the overall procedure

The sum of the penalty points obtained in the different steps of the procedure originated a total score, used to evaluate the overall performance of the NRLs in the PT. The performance of laboratories that obtained a score higher than 8 was considered as unsatisfactory.

4.6. Evaluation of the performance of the method

Sensitivity (Se) and Specificity (Sp) were differentially 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 wzx_{O111} genes, and for the isolation of the VTEC O111 strain. Sp was calculated for the PCR screening for *vtx1* and *vtx2* genes.

The PT results were also used to calculate the LOD of the isolation step of the method, 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 36 NRLs and all of them returned results.

As far as the delivery conditions were concerned, 30 NRLs received the samples within 24 hours, three within 48 hours and one within 72 hours. The remaining two received the samples after 5 (L568) and 8 (L936) days, respectively, due to problems related with either the shipment courier or custom clearance procedures.

The temperature at delivery ranged between 4 °C and 8 °C for 31 NRLs, between 8 °C and 9 °C for two NRLs, and was 10 °C and 13 °C, respectively, for two NRLs. For the remaining NRL, L936, that received the samples after 8 days, the temperature was over 30 °C.

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

All the 36 NRLs carried out the detection of the virulence and serogroup-associated genes, and the results are reported in Table 2.

For sample A (high level of contamination), 32 NRLs (89 %) identified correctly the presence of the virulence genes *vtx1*, *vtx2* and *eae*, while 4 NRLs provided a total of 8 incorrect or missing results for these genes. The detection of serogroup-associated genes was performed correctly by 32 NRLs (89 %), which identified correctly the presence of the wzx_{O111} gene.

For sample B (low level of contamination), the presence of virulence genes was identified correctly by 30 NRLs (83 %), and the presence of the wzx_{O111} gene by 32 NRLs (89 %). Eight NRLS provided a total of 14 incorrect results.

A laboratory (L469) provided false positive PCR results for the uncontaminated sample C.

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. The white boxes indicates that the test was not done. ONT: O untypable.

NRL	Detection of virulence and serogroup-associated genes in:											
	Sample A High level contamination				Sample B Low level contamination				Sample C			
	vtx1	*vtx2*	*eae*	wzx_{O111}	*vtx1*	*vtx2*	*eae*	wzx_{O111}	*vtx1*	*vtx2*	*eae*	wzx_{O111}
True value	+	+	+	+	+	+	+	+	-	-	-	-
L130												
L140						-						
L180												
L190												
L213												
L244												
L257												
L261		-				-						
L285												
L317	-	-			-	-						
L324					-							
L327												
L400												
L415												
L469				O157	-	-			+	+	+	
L470												
L498												
L528												
L545												
L546												
L547								O157/O111				
L568												
L574												
L583												
L615												
L627												
L658												
L714												
L782				ONT				ONT				
L831	-				-							
L849												
L887												
L936												
L957												
L968												
L997												

5.2. Isolation of the VTEC O111 strain from PCR-positive samples.

Twenty-three (64 %) of the 36 NRLs obtained the isolation of the VTEC O111 contaminating strain from the enrichment culture of sample A (Table 3).

Table 3. Isolation and genotyping of VTEC strains from the PCR-positive sprout 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. The white boxes indicates that the test was not done.

VTEC strain isolation and genotyping from:									
NRL	Sample A				Sample B				Sample C
	VTEC O111 Isolation	Genotype			VTEC O111 Isolation	Genotype			-
		vtx1	*vtx2*	*eae*		*vtx1*	*vtx2*	*eae*	
True value	+	+	+	+	+	+	+	+	None
L130									
L140									
L180									
L190									
L213									
L244									
L257									
L261			-				-		
L285									
L317									
L324						-			
L327									
L400									
L415									
L469									
L470									
L498									
L528									
L545									
L546									
L547									
L568				-				-	
L574									
L583									
L615									
L627									
L658									
L714		-	-	-		-	-	-	
L782									
L831									
L849									
L887									
L936									
L957									
L968									
L997									

Three of the laboratories that isolated the VTEC strain reported incorrect or missing results for its characterization. In particular, 2 NRLs provided incorrect results for *vtx* genes and 2 for the *eae* gene.

The VTEC O111 contaminating strain was isolated from the enrichment cultures of sample B (low level of contamination) by 16 (44 %) NRLs. Again, 4 NRLs provided incorrect or missing results for the characterization of the isolated strain.

5.3. Evaluation of the NRL performance in the PT procedures

For each NRL, the number of penalty points was determined using the criteria described in section 4.5.

Figure 1 shows the score achieved by each NRL, marking with different colors the points assigned for the incorrect results in the Real Time PCR screening and isolation steps.

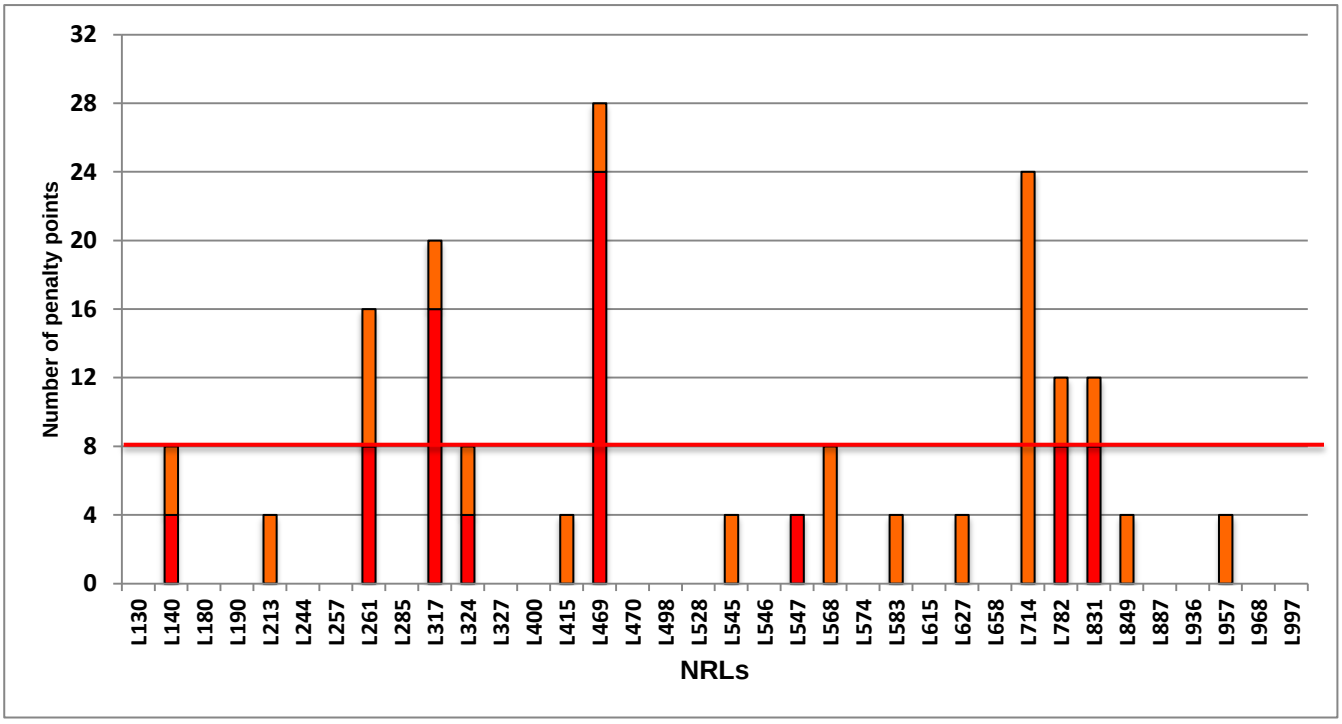

Figure 1. Evaluation of the NRL performance in the PT procedures. The score was calculated according to the criteria described in section 4.5. The points assigned for incorrect results in the Real Time PCR screening and isolation steps are marked in red and yellow, respectively. The performance of the NRLs that obtained a score higher than 8 was not considered as satisfactory.

Figure 2 shows the number of NRLs grouped according to their score. Six NRLs obtained a score higher than 8, and their performance was not considered as satisfactory.

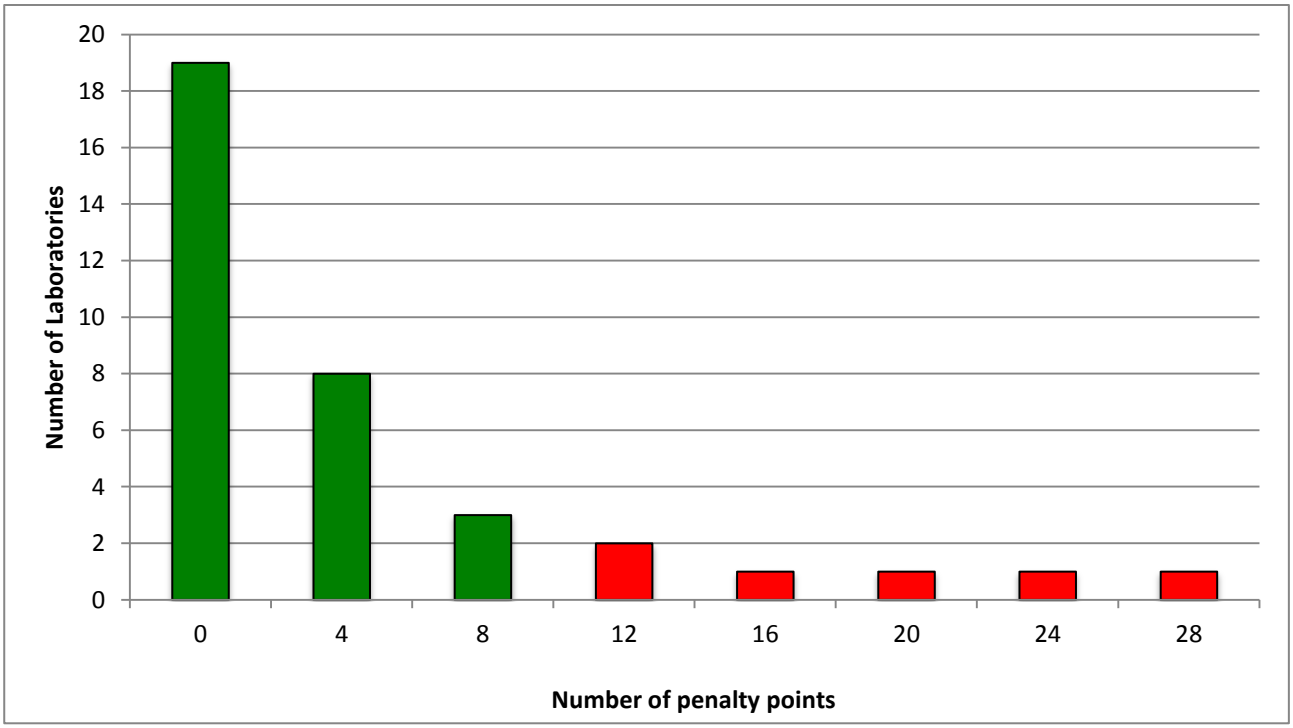

Figure 2. Number of NRLs within each penalty score. The score was calculated according to the criteria described in section 4.5. The red bars indicate the NRLs that obtained a score higher than 8 and whose performance was not considered as satisfactory.

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 wzx_{O111} genes, and the Specificity (Sp) for the *vtx1* and *vtx2* genes. The results provided by 33 of the 36 NRLs were considered, excluding those of L317, L469 and L936, which were considered as outliers. The analysis of the results returned the following values:

- *vtx1* PCR Se: 97.0 % (high level) and 94.0 % (low level).
- *vtx2* PCR Se: 97.0 % (high level) and 94.0 % (low level).
- *eae* PCR Se: 100 % (high level) and 100 % (low level).
- wzx_{O111} (O111) PCR Se: 97.0 % (high level) and 94.0 % (low level).
- *vtx1* PCR Sp: 100 %.
- *vtx2* PCR Sp: 100 %.

5.4.2. VTEC O111 isolation step

The sensitivity and the limit of detection (LOD) were calculated for the isolation step, considering the results provided by 33 NRLs and excluding those provided by L317, L469, and L936, which were considered as outliers. These laboratories reported 23 and 15

correct results for the high and low level samples, respectively. The analysis returned the following values:

- Se: 69.7 % (high level) and 45.5 % (low level).
- $LOD_{50\%}$: 377.4 CFU per gram (c.i. 256.5-556)
- $LOD_{95\%}$: 1,631.1 CFU per gram (c.i. 1,106.9-2,403.3).

6. CONCLUDING REMARKS

Reg. (EU) 209/2013 prescribes the absence of VTEC O157, O26, O111, O103, O145 and O104:H4 in sprouts to be consumed as raw, and the NRLs and the other laboratories involved in the official control of food must be prepared to test this matrix. Therefore, similarly to PT12 and PT14, conducted in 2013 and 2014, respectively, this study was carried out using this food commodity as the test matrix.

As specified in the aforementioned regulation, the method used in this PT for the detection of VTEC was the ISO/TS 13136:2012. The sprout samples were contaminated with a VTEC O111 strain, a serogroup that had never been included before in the PTs on the detection of VTEC in food matrices. Two levels of contamination were included, the lowest being around the limit of detection of the analytical method estimated for this food matrix. The analysis of the results provided by the participating laboratories induces the following remarks:

1. Thirty-six NRLs representing 26 EU Member States, Norway, Switzerland, Russia and Egypt joined the study, confirming the consolidation of the network of national laboratories for *E. coli*.
2. The PCR screening of the samples confirmed the suitability and fit for purpose of the method ISO/TS 13136:2012. The presence of the VTEC O111 genes was identified correctly by 31 NRLs (86 %) in sample A (high level of contamination) and by 28 NRLs (78 %) in sample B (low level of contamination, limit of detection).
3. The contaminating VTEC O111 strain was isolated from sample A by 23 NRLs (64 %) and from sample B by 17 NRLs (47 %).
4. To evaluate the NRL performance, penalty points were assigned for all the incorrect results provided, with the exception of those related with the isolation step for sample B, because the contamination level was set at the limit of detection of the isolation procedure. As a whole, using these metrics, 6 out of the 36 NRLs that contributed results had an unsatisfactory performance, confirming that nearly all the European

NRLs are able to detect VTEC contamination in sprouts, according to the prescriptions of Reg. (EU) 209/2013.

5. The results of the study confirmed that the ISO/TS 13136:2012 method represents a suitable tool for the detection of VTEC O111 in the food commodity regarded by the regulation (EU) 209/2013. However, the isolation of the VTEC O111 strains was not achieved from a relevant proportion of PCR-positive samples. This was likely due to the lack of an efficient selective/differential isolation medium, such as those vailable for VTEC O157 and, in a lesser extent VTEC O26, to be employed after the enrichment step.
6. The results of the PT allowed to determine the performance parameters of the method indicated in the Reg. (EU) 209/2013 for the detection of VTEC in sprouts, including sensitivity, specificity and the limit of detection for VTEC O111 in the specific food matrix for which the method is intended to be applied. The design of the whole study, planned to come to the determination of these parameters, was done to support the NRLs and Official Laboratories, which can refer to such parameters to have the method correctly accredited.