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Report of the 14th inter-laboratory study on the detection of Verocytotoxin-producing *E. coli* (VTEC) in sprouts (PT14) - 2014

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 "*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 six rounds of the PT scheme of the EU-RL 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, carried out on sprouts. The reports of these PTs are available in the EU-RL web site (www.iss.it/vtec).

The 7th PT on the detection of VTEC in food (PT14) was carried out again 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 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.

PT14 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 VTEC, following the prescriptions of Reg. (EU) 209/2013.
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 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 new Reg. (EU) 209/2013.
- To determine the analytical performance parameters of the detection method, to give support to the NRLs and the Official Laboratories for its accreditation.

3. PARTICIPANTS

Forty-one NRLs representing the 28 EU Member States, Norway, Switzerland, Turkey, 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
- Egypt, Central Public Health Laboratories of Ministry of Health and Population
- 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, Feed Investigation National Reference Laboratory
- 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
- Turkey, Public Health Institution of Turkey (former Refik Saydam National Public Health Agency)
- Turkey, National Food Institute
- UK, Microbiology Services, Food Water and Environmental Microbiology Laboratory, Porton
- 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 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 21 November, using dilutions of an exponential liquid culture (0.5 OD read at 600 nm) of the VTEC 104 strain described in Table 1. An uncertainty of measurement of 0.125 log 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 (75-133) and 1000 (750-1,335) estimated CFU per gram for the non-contaminated, low and high level aliquots, respectively. The titer of the inoculum suspensions added to the samples was checked by plating serial dilutions on MacConkey agar plates. The initial limit of detection (LOD) of the method was estimated by running the tests (both PCR screening and isolation steps) on 10 blind replicates of each contamination level. The PCR tests were positive for all samples while the strain isolation was obtained from all the high titer samples and from 5

five of the 10 replicates of the low titer, identifying an estimated LOD of 100 CFU per gram for the isolation step of the ISO/TS 13136:2012, when applied to test the sprout mixture used in this study.

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 O104 *(vtx1+, vtx2-, eae-, fliC$_{H4}$-, aggR-, aaiC-)*	High: 1,000 (750-1,333) CFU/g	Low: 100 (75-133) 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 24 November 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 11 days since their preparation. Samples were spiked on 9 October 2014 and tested by Real Time PCR at 4, 6 and 11 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 performances 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* and wzx_{O104} genes, and Sp for the PCR screening for *vtx1*, *vtx2* and *eae* 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 41 NRLs, but one of them did not submit results for difficulties in reagents supply.

As far as the delivery conditions were concerned, 30 NRLs received the samples within 24 hours, five within 48 hours and two within 72 hours. The remaining three received the samples after 4, 11 and 14 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 30 NRLs, between 9 °C and 11 °C for five NRLs, between 12 °C and 17 °C for three NRLs. It was 22 °C and 28 °C, respectively, for two NRLs.

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

All the 40 NRLs carried out the detection of the virulence genes *vtx1*, *vtx2* and *eae*. The results are reported in Table 2.

For sample A (high level of contamination), 37 NRLs (92 %) identified correctly the presence of these genes, while 3 NRLs provided a total of 5 incorrect results.

For sample B (low level of contamination), the presence of the genes was identified correctly by 35 NRLs (87 %), while 5 NRLs provided a total of 7 incorrect results.

The detection of serogroup-associated genes was not performed by 2 NRLs. The other 38 NRLs (95 %) identified correctly the presence of the O104-associated gene in sample A (high level of contamination), while the gene was detected by 33 NRLs (82 %) in sample B (low level of contamination).

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

5.2. Isolation of the VTEC O104 strain from the PCR-positive samples.

Thirty-five (87 %) of the 40 NRLs obtained the isolation of the VTEC O104 contaminating strain from the enrichment culture of sample A (Table 3). However, 15 of them reported incorrect or missing results for the characterization of the isolated strain. In particular, two NRLs provided incorrect results for *vtx* genes and 14 for the H4-associated gene ($fliC_{H4}$). For the latter, two laboratories provided false positive results, and 12 laboratories did not perform the test, which is instead prescribed by Reg. (EU) 209/2013.

Only 6 NRLs tested the isolated VTEC O104 for the marker genes of entero-aggregative adhesion (*aaiC* and *aggR*). Testing the presence of these genes is not mentioned in Reg. (EU) 209/2013 and was not explicitly requested in the PT design. However, laboratories should consider that any O104 strains isolated from food should be submitted to this test.

The VTEC O104 contaminating strain was isolated from the enrichment culture of sample B (low level of contamination) by 27 (67 %) NRLs. Again, 11 NRLs provided incorrect or missing results for the characterization of the isolated strain.

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. ND 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*	wzx_{O104}	*vtx1*	*vtx2*	*eae*	wzx_{O104}	*vtx1*	*vtx2*	*eae*	wzx_{O104}
True value	+	-	-	+	+	-	-	+	-	-	-	-
L106												
L118		+	+			+	+	O157 O26 O111	+	+	+	O157
L157		+				+						
L206												
L240												
L258												
L273												
L307								-				
L313												
L354												
L367												
L401					-							
L402												
L433												
L463												
L519												
L534												
L544												
L551												
L566												
L605					-							
L608												
L633												
L674	-	+			-	+						
L679												
L687												
L691												
L692												
L699												
L712												
L718												
L737												
L775												
L776												
L804												
L905												
L949												
L969				ND				ND				
L986												
L987								-				

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. ND indicates that the test was not done (ND).

NRL	VTEC strain isolation and genotyping from:										
	Sample A					Sample B					Sample C
	VTEC O104 Isolation	Genotype				VTEC O104 Isolation	Genotype				-
		vtx1	*vtx2*	*fliCH4* (H4)	*aaiC* and/or *aggR*		*vtx1*	*vtx2*	*fliCH4* (H4)	*aaiC* and/or *aggR*	
True value	+	+	-	-	-	+	+	-	-	-	None
L106											
L118											
L157			+	ND				+	ND		
L206											
L240						-					
L258				ND					ND		
L273				ND					ND		
L307				+		-					
L313											
L354				ND					ND		
L367				ND					ND		
L401											
L402				ND					ND		
L433				ND					ND		
L463											
L519				ND					ND		
L534						-					
L544											
L551	-					-					
L566											
L605	ND										
L608											
L633											
L674		-	+				-	+			
L679											
L687						-					
L691				+							
L692						-					
L699				ND					ND		
L712											
L718				ND		-					
L737	-					-					
L775											
L776											
L804				ND					ND		
L905											
L949											
L969											
L986											
L987				ND							

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.

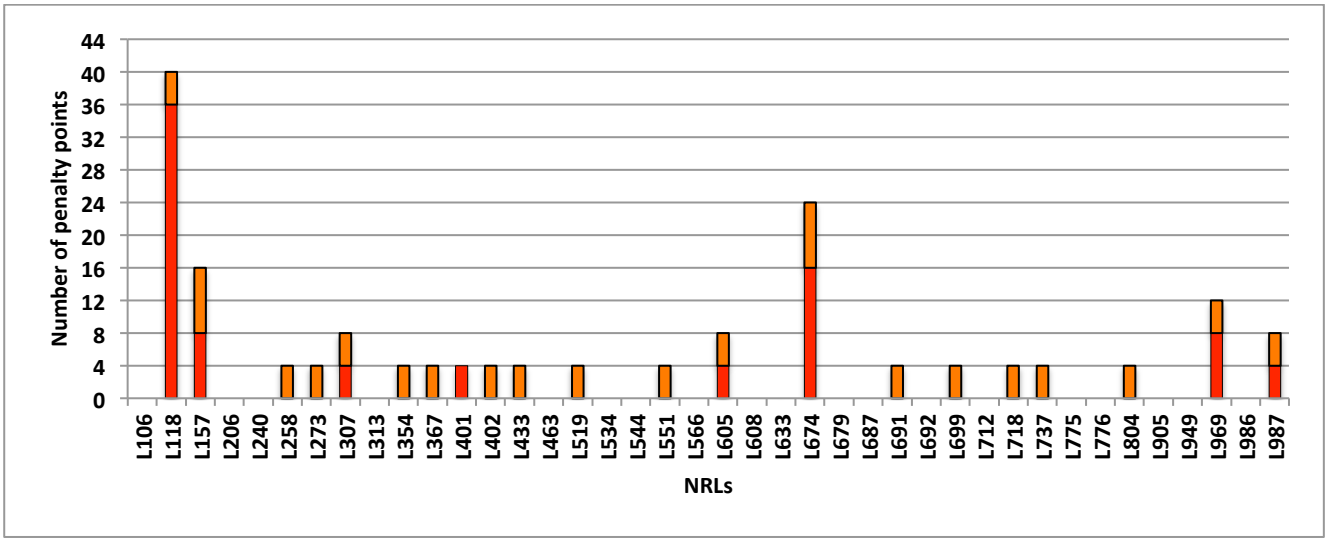

Figure 1. Evaluation of the NRL performance in the PT procedures. The score was calculated according to the criteria described in section 4.5. The points assigned for incorrect results in the Real Time PCR screening and isolation steps are marked in red and yellow, respectively. The performance of the 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.

Four NRLs obtained a score higher than 8, and their performance was not considered as satisfactory.

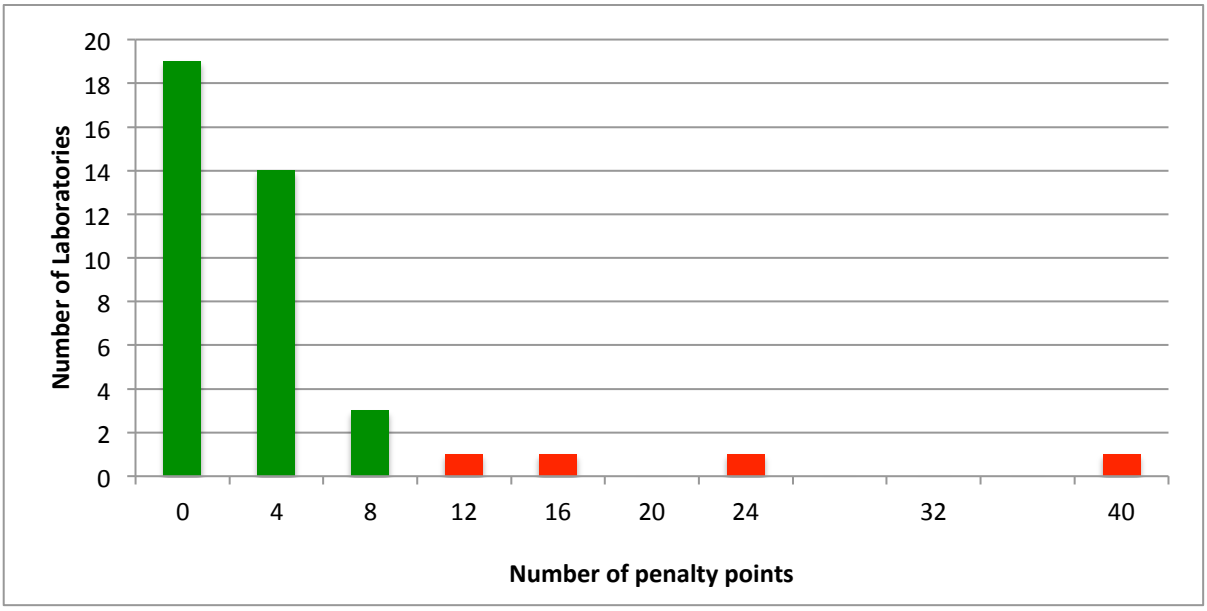

Figure 2. Number of NRLs within each penalty score. The score was calculated according to the criteria described in section 4.5. The red bars indicate the NRLs whose performance was not considered as satisfactory.

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* and wzx_{O104} genes, and the Specificity (Sp) for the *vtx1*, *vtx2,* and *eae* genes.

The results provided by 39 of the 40 NRLs were considered, excluding those of L118, which was considered as an outlier. The analysis of the results returned the following values:

- *vtx1* PCR Se: 97.4% (high level) and 92.3 % (low level).
- *vtx1* PCR Sp: 100 %.
- *vtx2* PCR Sp: 94.8 %.
- *eae* PCR Sp: 100 %.
- wzx_{O104} (O104) PCR Se: 100 % (high level) and 94.4 % (low level).

5.4.2. VTEC O104 isolation step

The sensitivity and the limit of detection (LOD) were calculated for this step, considering the results provided by the laboratories that carried out the test for the high (37 NRLs) and low level (35 NRLs) samples. These laboratories reported 35 and 27 correct results, respectively and the analysis returned the following values:

- Se: 94.6 % (high level) and 77.1 % (low level).

- $LOD_{50\%}$: 182.58 CFU per gram (c.i. 120.4-276.7)
- $LOD_{95\%}$: 789.1 CFU per gram (c.i. 520.6-1195.9).

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 in 2013, this study was conducted 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, taking into account the adaptation provided by the EU-RL VTEC for the detection of VTEC O104:H4. The sprout samples were contaminated with a VTEC O104 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. Forty-one NRLs, representing the 28 EU Member States, Egypt, Norway, Russia, Switzerland and Turkey joined the study, setting forth the growth and 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. As a matter of fact, the presence of the VTEC O104 genes was identified correctly by 36 NRLs (90 %) in sample A (high level of contamination) and by 32 NRLs (80 %) in sample B (low level of contamination, limit of detection).
3. The contaminating VTEC O104 strain was isolated from sample A by 35 NRLs (87 %) and from sample B by 27 NRLs (67 %).
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, only 4 laboratories out of the 40 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. Many NRLs did not complete the characterization of the isolated VTEC O104 strain by testing the presence of the H4 associated gene (*fliCH4*) and the marker genes of enteroaggregative adhesion (*aaic* and *aggR*). Testing for H4 is prescribed by Reg. (EU) 209/2013, and therefore penalty points were assigned to missing result. Since enteroaggregative adhesion is not mentioned in Reg. (EU) 209/2013 and was not esplicitly requested in the PT design, penalty points were not assigned to missing results. However, laboratories should consider that any O104 strains isolated from food should be submitted to this test.
6. The results of the study also confirmed that the addendum to the ISO/TS 13136:2012 provided by the EU-RL for the detection of VTEC O104:H4 is as robust as the ISO/TS 13136:2012 itself and represents a suitable tool for the detection of this VTEC serogroup in the food commodity regarded by the regulation (EU) 209/2013.
7. 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 with the specific food matrix for which the method is intended to be applied. The design of the study and the determination of these parameters were done to support the NRLs and Official Laboratories, which can refer to them in order to have the method correctly accredited.