Report of the 21st inter-laboratory study on the detection of Shiga toxin-producing *E. coli* (STEC) in sprouts (PT21)

Edited by:

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1. INTRODUCTION

The duties of the EU Reference Laboratory for *E. coli* (EURL-VTEC) include the organization of proficiency tests (PT) to assess the performance of the designated National Reference Laboratories (NRLs) for *E. coli* in the EU and EFTA Member States, EU Candidate Countries and certain third countries in using the methods for the detection of STEC in food and for the characterization of the isolated STEC strains.

Regulation (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 STEC O157, O26, O111, O103, O145 and O104:H4 in 25 g of product. This assessment shall be done by the application of the ISO TS 13136:2012 method, taking into account the adaptation provided by the EU Reference Laboratory for *E. coli* (EURL-VTEC) for the specific detection of STEC 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*").

This study, PT21, was carried out on sprout samples for the benefit of the network of NRLs, which must be prepared to test sprouts for the presence of STEC.

PT21 gives continuity to the previous studies conducted within the network of Reference Laboratories for *E. coli* on food matrices and this document represents the full evaluation report of the study.

2. DESIGN AND OBJECTIVES OF THE STUDY

The study consisted in the detection and isolation of STEC O26 present in sprout samples in different amounts.

The **objectives** of the study were:

- to improve the preparedness of the NRLs towards testing sprouts in compliance with Regulation (EU) No 209/2013;
- to improve the preparedness of the NRLs towards the detection and isolation of STEC strains not belonging to the O157 serogroup;
- to give further support to the NRLs and the Official Laboratories for the accreditation of the ISO TS 13136:2012.

3. PARTICIPANTS

A total of 39 NRLs representing 27 EU Member States, Egypt, Iceland, Norway, Russia and Switzerland participated in the study. Each NRL received its own individual Laboratory code, reported in the result tables and in the individual reports.

The NRLs participating in the study were:

- Austria, *Institut für Medizinische Mikrobiologie und Hygiene*, AGES
- Belgium, Scientific Institute of Public Health, *Direction Opérationnelle Maladies Transmissibles et Infectieuses*
- Bulgaria, National Diagnostic and Research Veterinary Institute
- Chile, *Sección Microbiología de Alimentos y Aguas, Departamento de Salud Ambiental, Instituto de Salud Pública de Chile*
- Croatia, Laboratory for food microbiology, Croatian Veterinary Institute
- Cyprus, Laboratory for the Control of Food of Animal Origin (LCFAO), Cyprus Veterinary Services
- Czech Republic, Veterinary Research Institute
- Denmark, FVST, *Mikrobiologisk Laboratorium*
- Egypt, Central Lab of Residue Analysis of Pesticides and Heavy Metals in Foods
- Estonia, Veterinary and Food Laboratory
- Finland, Finnish Food Safety Authority Evira, Research and Laboratory Department, Food and Feed Microbiology Research Unit
- France, *VetAgro Sup Campus Vétérinaire de Lyon*
- Germany, Federal Institute for Risk Assessment, Unit Food Technologies, Supply Chains and Food Defense, Department Biological safety
- Greece, National School of Public Health & Central Laboratory of Public Health, Department of Microbiology, National Reference Centre for Salmonella, Shigella, VTEC
- Hungary, National Food Safety Office, Food and Feed Safety Directorate, National Food Microbiological Reference Laboratory
- Iceland, Matís ohf. / Icelandic Food and Biotech R&D
- Ireland, Veterinary Public Health Regulatory Laboratory, Department of Agriculture, Food and the Marine
- Italy, *Istituto Superiore di Sanità*
- Latvia, Microbiological Division, Laboratory of Food and Environmental Investigations, Institute of Food Safety, Animal Health and Environment (BIOR)

- Lithuania, National Food and Veterinary Risk Assessment Institute, Molecular biology and GMO Section
- Luxembourg, *Ministère de l'Agriculture, de la Viticulture et de la Protection des consommateurs, Administration des services vétérinaires*, LMVE
- Norway, Norwegian Veterinary Institute, Section for microbiology
- Poland, National Institute of Public Health-National Institute of Hygiene, Department of Food Safety, Laboratory of Food Microbiology
- Poland, National Veterinary Research Institute, Department of Hygiene of food of animal origin
- Portugal, *Laboratório de Microbiologia dos Alimentos, Instituto Nacional de Investigação Agrária e Veterinária, I.P, Unidade Estratégica de Investigação e Serviços de Tecnologia e Segurança Alimentar* (LNIV)
- Romania, Department of Microbiology - Molecular Biology Unit, Institute for Hygiene and Veterinary Public Health
- Russia, International Department State Research Center for Microbiology and Biotechnology, Obolensk
- Slovakia, NRC of Environmental Microbiology, Public Health Authority of SR
- Slovenia, Veterinary Faculty UL, Nacional Veterinary Institute – Unit for Food of Animal Origin
- Spain, Microbiology Food Department, *Agencia Española de Consumo, Seguridad Alimentaria y Nutrición,* Spanish Agency for Consumers Affairs, Food Safety and Nutrition, National Center for Food -*Centro Nacional de Alimentación* (CNA)
- Sweden, *Livsmedelsverket*/The National Food Agency
- Sweden, National Veterinary Institute (SVA), Dept of Bacteriology
- Switzerland, Agroscope
- Switzerland, Institute for food safety and hygiene, University of Zurich
- The Netherlands, RIVM, Centre for Zoonoses and Environmental Microbiology
- The Netherlands, Food and Consumer Product Safety Authority (NVWA)
- UK, FW&E Laboratory – London, Public Health England
- UK, FW&E Laboratory – Porton, Public Health England
- UK, FW&E Laboratory – York, The Food and Environmental Research Agency (FERA), Public Health England

4. MATERIALS AND METHODS

4.1. Sample preparation

Three test samples (1, 2 and 3), each consisting of 25 g of red radish sprouts potentially contaminated with STEC, were sent in the blind to the NRLs.

The radish sprouts used have been acquired as retail packages and contained a natural background microflora (about 6 $x10^7$ CFU/g). The sprouts were portioned in 25 g samples in sterile stomacher bags and placed at + 4 °C until the preparation of the PT samples. Two 25 g portions of rocket were initially tested for the presence of STEC by applying the ISO TS 13136:2012 method. Both samples were negative for all the target genes at the screening.

The artificial contamination of the samples was carried out on the 13th of April 2018, using appropriate dilutions of an exponential liquid culture (0.5 OD read at 600 nm) of the STEC O26 strain C1188-02 positive for *stx1, stx2* and *eae* genes. The characteristics of the samples are reported in Table 1. An uncertainty of measurement of 0.209 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, low and high level. In detail, the set of samples sent to the NRLs contained 0, 2 and 20 estimated CFU per gram, respectively (Table 1). Serial dilutions of the inoculum suspensions of strain C1188-02 added to the samples were plated onto MacConkey agar plates to check their actual titer.

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 16 April 2018 by courier. The NRLs were requested to record date of delivery and sample temperature upon reception and to start the analyses immediately upon receipt.

Table 1: Characteristics of the red radish sprout samples assessed in the study

Contaminant (*Genotype*)	Contamination level in:		
	Sample 1	**Sample 2**	**Sample 3**
Strain C1188-02, STEC O26 *(stx1+, stx2+, eae+)*	-	Low: 2 CFU/g	High: 20 CFU/g

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.

The stability was assessed using samples spiked on the 26th of January 2018 and tested by ISO TS 13136:2012 after 3, 5 and 10 days since the initial contamination. The Real Time PCR screening was positive for the STEC target genes even after 10 days from the spiking. Isolation was successful for all the samples spiked with the high level of contamination at all the time points, whereas it was never achieved for the low level of contamination, even after 3 days form spiking.

When the PT21 test samples were prepared, ten bags for each of the two levels of contamination and two non-contaminated samples were randomly selected for homogeneity testing, enriched at 37 °C and analyzed by Real Time PCR for the presence of STEC O26 on the 16th and 17th of April 2018. The Real Time PCR screening carried out for the homogeneity tests were positive for the STEC target genes but showed very high threshold cycles for the low level of contamination. Moreover, for two samples with low-level contamination it was not possible to detect all the expected genes. In particular, in one sample it was not possible to detect *stx1* and in the other the presence of the genes *stx1* and wzx_{O26} was not identified.

4.3. Laboratory methods

Laboratories were requested to identify the presence of STEC using the method ISO TS 13136:2012 using Buffered Peptone Water as enrichment broth.

4.4. Collection and elaboration of the NRL results

The results were submitted through an online system, using a dedicated page in the “Restricted Area” of the EURL-VTEC website.

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 EURL-VTEC website.

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 STEC target genes in the enrichment cultures was evaluated by assigning four penalty points to each incorrect or missing result concerning the identification of virulence genes, *stx1* and *stx2*. Two penalty points have been assigned to the laboratories not identifying the presence of *eae* gene and the O26 serogroup-associated gene in the screening. Based on the results of the homogeneity testing, no penalties were assigned to the negative results in the PCR for the *stx1* and wzx_{O26} in the samples with a low level of contamination (Sample 2).

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

The performance of each NRL in the isolation and characterization of the STEC strains from the enrichment cultures of the positive samples was evaluated by assigning two penalty points to the lack of isolation from the sample 3. No penalty points were instead assigned to the lack of isolation from sample 2 (low level of contamination), as the contamination level was determined as being close to the limit of detection of the procedure.

As for strain characterization, four penalties have been assigned to incorrect detection of Stx-coding genes and two penalty points have been assigned to the laboratories not identifying the presence of *eae* gene and the O26 serogroup-associated gene in the STEC isolated strain.

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 eight was considered as unsatisfactory.

4.6. Evaluation of the performance of the method

Sensitivity (Se) and Specificity (Sp) were calculated for the various STEC characters considered in the study and for the different steps of the ISO TS 13136:2012. Therefore, Se and Sp were calculated for the PCR screening for *stx1*, *stx2* and *eae* genes, and for the isolation of the STEC O26 strain. The sensitivity and specificity were calculated according to the following formulas:

Sensitivity: Se = [true positives / (true positives + false negatives)] x 100

Specificity: Sp = [True negatives / (true negatives + false positives)] x 100

The limit of detection (LOD) has been calculated for the isolation step using the procedure described by Wilrich and Wilrich (Journal of AOAC international, Vol. 92 No. 6, 2009, 1763-1772).

5. RESULTS

All the 39 Laboratories receiving the samples returned results via the web platform.

As for the delivery conditions, 26 NRLs received the samples within 24 hours, 5 within 48 hours, 2 within 72 hours, L906 in 4 days and L400 in 10 days due to custom clearance procedures. The reported temperature at delivery was ≤ 4°C for 26 NRLs and between 5 °C and 8 °C for 10 laboratories. Two NRLs reported a temperature of 10 °C for the test samples and the L400 samples arrived in the laboratory at 25 °C.

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

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

As for the negative sample, two Laboratories incorrectly reported the detection of *stx1, stx2, eae* and wzx_{O26} genes. In one case, L391, this was likely due to exchange of two samples, whereas in the other case, L649, cross-contamination may have occurred during the analysis.

The detection of all the virulence genes and the O26 serogroup-associated gene in sample 2 (low level of contamination) was correctly identified by 31 laboratories (79.5 %). The presence of *stx1* and *stx2* genes was detected by 34 Laboratories (91.9 %), two failed in detecting *stx1* gene and three laboratories couldn't detect the presence of any *stx* genes. These were L391, probably due to the mentioned exchange of samples, L446 and L400. Moreover, one NRL didn't perform the screening for the presence of *eae* and wzx_{O26} genes, and two NRLs failed in detecting only wzx_{O26} gene.

As for sample 3 (high level of contamination), 36 NRLs (92.3 %) correctly identified the presence of *stx1* and *stx2* genes, and 31 NRLs (79.5 %) correctly reported the identification of the presence of all the target genes. Two laboratories reported sample 3 as negative for STEC. The detection of the wzx_{O26} gene was not achieved by two Laboratories, and one didn't carry out the detection of *eae* and wzx_{O26} gene. Finally, one NRL didn't detect the presence of *stx2* gene.

5.2. Isolation of the STEC O26 strain from PCR-positive samples.

The isolation of the contaminating STEC strain was generally successful for sample 3 (high level of contamination), as 32 (86.4 %) Laboratories out of the 37 detecting the presence of STEC could isolate the contaminating strain, but in one case (L935) the serogroup determination of the isolated strain was not achieved. As for the low-level of contamination sample, 23 (63.9 %) out of 36 NRLs detecting STEC in the screening step isolated the contaminating STEC strain. One Laboratory (L935) couldn't identify the serogroup of the isolated STEC strain, L350 isolated an EPEC O26 strain and one failed in detecting *stx1* gene. L521 reported not to have attempted the isolation from the low level of contamination, as the threshold cycles observed for the STEC target genes were quite high.

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 values not-matching with the expected results and the orange boxes correspond to 'test not done'.

NRL	Detection of virulence and serogroup-associated genes in:											
	Sample 1				Sample 2 Low level contamination				Sample 3 High level contamination			
	stx1	*stx2*	*eae*	*wzx$_{O26}$*	*stx1*	*stx2*	*eae*	*wzx$_{O26}$*	*stx1*	*stx2*	*eae*	*wzx$_{O26}$*
True value	-	-	-	-	+	+	+	+	+	+	+	+
L109												
L257												
L266												
L269												
L295					-							
L296												
L300												
L307												
L319												
L323												
L341												
L350												
L351												
L355										-		
L391	+	+	+	+	-	-						
L400*					-	-			-	-		
L429												
L446					-	-			-	-		
L494								ONT				
L521												
L542												
L576												
L598												
L599												
L609												
L649	+	+	+	+								
L662												
L689												
L789												
L802												
L803												
L813												
L906												
L920												
L935								ONT				ONT
L940					-							
L954												
L970												
L997												

***L400** received the test samples after 10 days from shipment and the temperature recorded was 25 °C.

Table 3. Isolation and genotyping of STEC strains from the radish sprout samples.

The green boxes indicate the correct results, in agreement with the true value reported on the top of each column. The red boxes indicate results non concordant with the gold standard. Orange boxes indicates that the test was not done even in the presence of *stx*-positive signals in the screening.

STEC strain isolation and genotyping from:									
NRL	Sample 1	Sample 2				Sample 3			
	-	STEC O26 Isolation	Genotype			STEC O26 Isolation	Genotype		
			stx1	*stx2*	*eae*		*stx1*	*stx2*	*eae*
True value	None	+	+	+	+	+	+	+	+
L109									
L257									
L266									
L269									
L295		-							
L296									
L300									
L307		-							
L319									
L323									
L341									
L350			-	-					
L351									
L355									
L391	O26 *vtx1 vtx2 eae*								
L400*									
L429		-							
L446*									
L494		-							
L521									
L542		-				-			
L576									
L598									
L599									
L609		-				-			
L649		-				-			
L662		-							
L689		-				-			
L789		-							
L802									
L803									
L813									
L906		-							
L920									
L935		ONT				ONT			
L940			-						
L954									
L970									
L997									

***** No *stx*-positive results were detected during the screening for any of the three samples.

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 and Figure 2 shows the number of NRLs grouped according to their score.

L400 was excluded from this analysis, as this NRL obtained the samples after 10 days and couldn't detect STEC virulence genes in any samples.

Four NRLs obtained a score equal or higher than 8 and their performance was not considered as satisfactory. Of these, one laboratory, L391, obtained a score of 16, and this was likely the result of samples exchange. L649 detected the presence of STEC in the screening step in all three samples, probably due to cross contamination when the analyses were carried out.

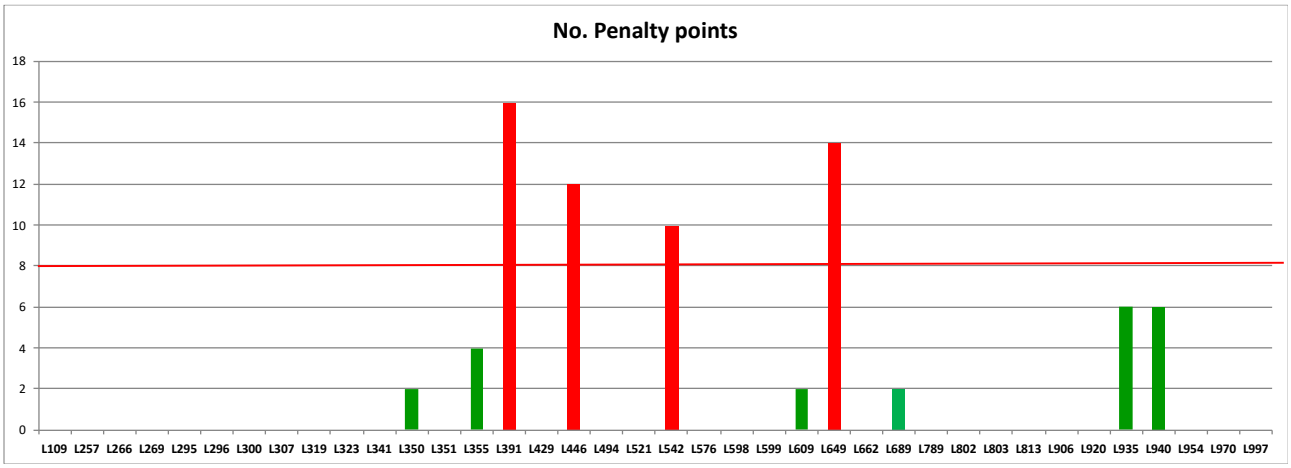

Figure 1. Evaluation of the NRL performance in the PT procedures (screening and isolation steps). The score was calculated according to the criteria described in section 4.5. The performance of the NRLs that obtained a score higher than 8 was not considered as satisfactory (red bars).

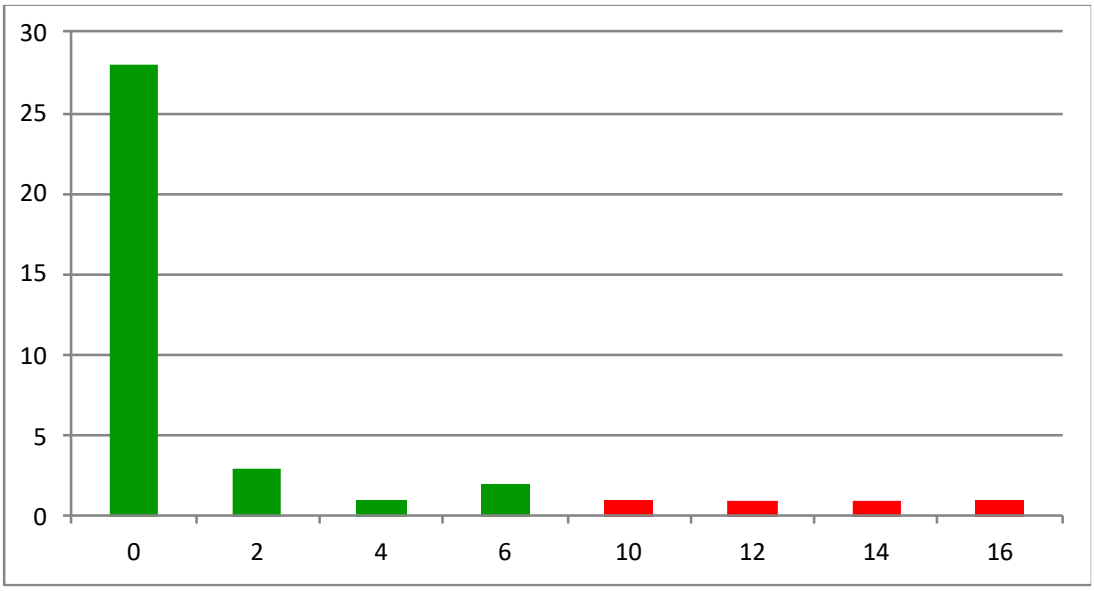

Figure 2. Number of NRLs within each penalty score. The score was calculated according to the criteria described in section 4.5. The red bars indicate the NRLs that obtained a score equal or higher than 8 and whose performance was not considered as satisfactory.

5.4. Evaluation of the performance of the methods

5.4.1. PCR screening step

The Sensitivity (Se) and Specificity (Sp) of the method was calculated for the detection of the *stx1, stx2, eae* and *wzx*$_{O26}$ genes in the screening step. The results provided by 37 out of the 39 NRLs submitting the results were considered for the determination of the sensitivity of the method at the *stx1* gene PCR applied to the low-level of contamination. The results from 38 labs were used to calculate the sensitivity of the method for the same target when detected in the high-level of contamination. As far as the *stx2*-specific PCR, the determination of the sensitivity was derived from the results of 38 NRLs either in the low or high level of contamination samples. As for the *eae* and *wzx*$_{O26}$ PCR, the results from 35 and 36 NRLs were used to infer the sensitivity in the low and high-level of contamination samples, respectively.

The results of L400, which clearly reflected a problem with the stability of the samples received, have been excluded from all the analyses.

As for the specificity, the results submitted by L391 and L649 were not considered.

The analysis of the results returned the following values:

- *stx1* PCR Se: 88.1 % (low level) and 97.4 % (high level).
- *stx2* PCR Se: 95.0 % (low level) and 95.0 % (high level).

- *eae* PCR Se: 100 % (low level) and 100 % (high level).
- *wzx*$_{O26}$ PCR Se: 94.6 % (low level) and 97.3 % (high level).
- *stx1* PCR Sp: 100 %.
- *stx2* PCR Sp: 100 %.

5.4.2. STEC O26 isolation

The Sensitivity of the isolation procedure was calculated from the results obtained from 35 and 37 NRLs for the low and high-level of contamination samples, respectively.

- Se: 76.1 % for the low contamination level.
- Se: 88.1 % for the high contamination level.

The Limit of detection (LOD) of the isolation step returned the following results when combining the data from the two levels of contamination:

No. of matrix	Name of matrix	Matrix effect	SD of log matrix effect	$LOD_{50\%}$ [1]			$LOD_{95\%}$ [2]			Test statistic matrix effect
				Detection limit	Lower conf. limit	Upper conf. limit	Detection limit	Lower conf. limit	Upper conf. limit	
i	$matrix_i$	F_i	s_{fi}	$d_{0.5,i}$	$d_{0.5,i,L}$	$d_{0.5,i,U}$	$d_{0.95,i}$	$d_{0.95,i,L}$	$d_{0.95,i,U}$	$\lvert z_i \rvert$
1		0,007	0,204	3,848	2,557	5,792	16,631	11,050	25,032	0,000
Combined data		0,007	0,204	3,848	2,557	5,792	16,631	11,050	25,032	0,000

6. CONCLUDING REMARKS

The consolidation of the use of the ISO TS 13136:2012 standard is crucial to ensure a harmonized approach to food testing for STEC throughout the EU.

PT21 concerned the application of the ISO TS 13136:2012 on sprout samples for the benefit of the network of NRLs, which must be prepared to test such a matrix for the presence of STEC, according to Regulation (EU) No 209/2013.

The analysis of the results provided by the Laboratories participating in the PT21 induces the following conclusions:

1. a high participation rate was observed: 39 laboratories covering 27 EU Member States, Egypt, Iceland, Norway, Russia and Switzerland participated in the study, confirming the consolidation of the network of National Reference Laboratories for *E. coli*;
2. the low level contamination used in this study (2 CFU/g) was very close to the LOD_{50}, therefore no penalty points were assigned for the lack of isolation in sample 2;

3. the contaminating STEC O26 strain was isolated by 32 Laboratories out of the 37 that had detected the presence of the STEC genes in sample 3 (high-level) and by 23 out of 36 NRLs that had detected the same genes in sample 2 (low level);
4. to evaluate the NRL proficiency, penalty points were assigned for all the incorrect results provided. As a whole, four NRLs showed an unsatisfactory performance; for one of these the underperformance was likely due to the exchange of two samples and another seemed to have cross-contaminated the samples;
5. the results of this PT allowed to determine the performance parameters of the method for a sprout type not considered before in the EURL EQA program. As in the other PT rounds, these performance parmaters will be added to those already determined for other couples matrix/STEC strain and made available through publication in the EURL-VTEC website with the aim to support the NRLs and Official Laboratories, which can refer to such parameters to have the method correctly accredited.