Report of the 12th inter-laboratory study on the detection of VTEC and other pathogenic *E. coli* in sprout samples - 2013

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: CEN/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 had been recommended by EFSA for the detection of the main pathogenic serogroups in food and animal samples (Guidance on the technical specifications for the monitoring and reporting of VTEC on animals and food, *EFSA Journal* 2009; 7:1366). More recently, it was prescribed for the detection of VTEC by Regulation (EU) No 209/2013, which has introduced for the first time microbiological criteria for VTEC in the EU legislation. The method had already been adopted and evaluated in five rounds of the PT scheme of the EU-RL VTEC: 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, and PT9, carried out on seeds intended for sprouting. The reports of these PTs are available in the EU-RL web site (www.iss.it/vtec).

The sixth PT on the detection of VTEC in food (PT12) was carried out on sprouts. The

choice of this matrix was due to the following reasons:

- During the last years, a growing number of epidemic episodes have brought to the attention the role of sprouts intended for direct consumption (ready to eat) in the transmission of Salmonella and VTEC infections.
- Sprouts have been implicated as the source of the outbreak of VTEC O104:H4 infections occurring in Europe in 2011.
- 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 gr of product, as determined by the CEN/ISO/TS 13136 method.

Beside VTEC, the study included the detection of other groups of pathogenic *E. coli*: namely, Enteroaggregative *E. coli* (EAggEC), Enterotoxigenic *E. coli* (ETEC), and Enteroinvasive *E. coli* (EIEC), which are well known causes of traveler diarrhea, but have been also reported in sporadic cases and foodborne outbreaks in industrialized countries.

PT12 complemented 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 the contamination of sprout samples with VTEC and other groups of pathogenic *E. coli*: namely, EAggEC, ETEC, and EIEC. The methods employed were the CEN/ISO/TS 13136 for the detection of VTEC in foodstuffs, and specific standard laboratory procedures (SOP) developed by the EU-RL for the other pathogroups. Three artificially contaminated sprout samples were sent to the laboratories that accepted to participate in the PT.

The **objectives** of the study were: i) to further train the NRLs in the use of the international standard method CEN/ISO/TS 13136 for the detection of VTEC; ii) to introduce reliable methods for the detection of pathogenic *E. coli* other than VTEC in the diagnostic panel available in the *E. coli* NRL network.

3. PARTICIPANTS

Thirty-eight NRLs representing 28 EU Member States, Argentina, Egypt, Norway and Switzerland participated in the study. Each NRL received its own individual laboratory numerical code, which was reported in the result tables.

The NRLs participating in the study were:

- Argentina, Instituto de Genética Veterinaria "Ing. Fernando Noel Dulout" CONICET, Universidad Nacional de La Plata
- 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, 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
- 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, Centro Nacional de Alimentación, Servicio de Microbiología Alimentaria, AESAN
- Spain, Laboratorio Central de Veterinaria de Algete (MAGRAMA)
- Sweden, Livsmedelsverket/The National Food Agency
- Sweden, National Veterinary Institute (SVA)
- Switzerland, Institute for food safety and hygiene, University of Zurich
- UK, Health Protection Agency Microbiology Services, Food Water and Environmental Microbiology Laboratory, Preston
- UK, Public Health England, Food, Water and Environmental Microbiology, Porton

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 from a retailer. 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 or other *E. coli* patho-groups were sent in the blind to the NRLs. The artificial contamination of the samples was carried out on 18 November, using dilutions of exponential liquid cultures of the VTEC and EIEC strains described in Table 1. The titer of the inoculum suspensions in PBS was assessed by plating serial dilutions on MacConkey agar plates and the average contamination level recorded was 1.52×10^3 CFU per g of sprouts or the VTEC strain, and 1.84×10^2 CFU per g of sprouts for the EIEC strain.

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*)	**Sample A**	**Sample B**	**Sample B**
VTEC O157 *(vtx1, vtx2, eae)*	1.5 x 10^3 CFU/g	-	-
EIEC (*ipaH*)	-	1.8 x 10^2 CFU/g	-

The test samples were labeled with randomly generated numerical codes different for each NRL and immediately transferred into refrigerated safety packages that were shipped the same day of preparation by a courier. The NRLs were requested to start the analyses immediately upon receipt and to record date and time of sample delivery, date and time the analyses start, and the 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 8 days since their preparation. Samples were spiked on 31 October and five aliquots for each type of contaminated sprouts were tested on 31 October, 5 November, and 7 November. The contaminating VTEC and EIEC strains were detected in all the tests as expected.

When the test samples were prepared, 5 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

The laboratories were requested to identify the presence of VTEC belonging to the serogroups O157, O111, O26, O103, O145, and O104 using the method CEN/ISO/TS 13136, according to Reg. (EU) No 2019/2013, 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.

To detect the presence of the other *E. coli* patho-groups, the sample enrichment cultures negative for the presence of *vtx* genes had to be tested for the following target genes:

- *aaiC* and *aggR* for EAggEC
- *lt*, *sth,* and *stp* for ETEC
- *ipaH* for EIEC

The Real Time PCR SOPs for the detection of these target genes were available in the EU-RL web site (http://www.iss.it/vtec), Laboratory Methods section.

The isolation of the pathogenic *E. coli* strains responsible for the positive PCR screening reactions was accomplished by streaking the enrichment culture positive at the Real Time PCR screening step onto suitable solid media and testing up to 50 colonies with *E. coli* morphology for the presence the virulence genes detected in the enrichment cultures. For VTEC, the procedure was that described in the CEN/ISO/TS 13136. For the other pathogroups, the methods were described in the respective SOPs available in the EU-RL web site.

The VTEC strains isolated had to be characterized by determining the presence of *vtx1*, *vtx2*, and *eae* genes and the serogroup. For the strains belonging to the other patho-groups the determination of the presence of the target virulence gens was sufficient.

The laboratory procedure sent to the NRLs is reported as **Annex 1**.

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 the 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 identification of the target virulence genes of the different groups of pathogenic E. coli in the enrichment cultures (screening step)

The performance of each NRL in identifying the target virulence genes of the different patho-groups of *E. coli* in the enrichment cultures was evaluated by assigning penalty points for the virulence genes that were identified incorrectly. The penalty points were assigned as follows, according to the public health relevance of the different patho-groups:

- **4 penalty points** were assigned to each incorrect or missing result concerning the identification of the *vtx* genes, that represent the main virulence determinants of VTEC and the main target of the ISO/TS 13136:2012, the international standard aiming at the detection of these pathogenic *E. coli* in food. The detection of these genes was the object of several previous PTs. Moreover, there is a microbiological criterion for the presence of VTEC in a food commodity in a EU regulation (Reg. EU 209/2013).
- **2 penalty points** were assigned to each incorrect result concerning the identification of the other virulence genes considered in the PT (*eae, AggR, aaiC, lt, st, ipaH*).
- **1 penalty point** was assigned to each result reported as "Not Done".

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

The performance of each NRL in the isolation and identification of the pathogenic *E. coli* strains responsible for the positive PCR screening reactions in the enrichment cultures was evaluated by assigning penalty points to the lack of isolation, according to the following criteria:

- **4 penalty points** were assigned to the lack of isolation of VTEC strains.
- **2 penalty points** were assigned to the lack of isolation of the strains belonging to other patho-groups.

4.5.3. Evaluation of the NRL performance in the overall procedure

The sum of the penalty points obtained in the Real Time PCR screening and the isolation steps originated a total score, used to evaluate the overall performance of the NRLs in the PT. In particular, a threshold of 8 penalty points was set in order to identify the laboratories performing adequately. The NRLs that summed up a score higher than 8 without making errors in the identification of *vtx* genes or in the isolation of the VTEC strain represented an exception and their performance was still considered as satisfactory.

5. RESULTS

The samples were sent to 38 NRLs, 37 of which submitted their results.

As far as the delivery conditions were concerned, 26 NRLs provided the detailed information requested, while the remaining reported only that no problems were

encountered with the delivery. Among the 26 NRLs providing the information, 22 received the samples within 24 hours, and 4 within 48 hours. The reported temperatures ranged between 1°C and 7 °C.

5.1. Real-time PCR detection of the target virulence genes of the *E. coli* pathogroups in the enrichment cultures

All the 37 NRLs carried out the detection of the VTEC/EPEC-associated genes *vtx1*, *vtx2* and *eae*, while the detection of all or part of the target virulence genes of EAggEC, ETEC, and EIEC was not performed by 10 NRLs (Table 2).

Twenty-two NRLs identified correctly the presence/absence of all the target genes in the three test samples, while six NRLs provided a total of 14 incorrect results.

As for the detection of *vtx* genes, 34 NRLs (92%) identified correctly their presence/absence in the three test samples, while three NRLs provided a total of four incorrect results: one false negative for *vtx1*, one false positive for *vtx1*, and two false positive for *vtx2.*

As for the detection of the other virulence genes, the *ipaH* gene was correctly identified in sample B by 28 of the 31 NRLs (90%) that carried out the test. As a whole, five NRLs provided a total of 10 incorrect results for the genes other than *vtx*: three false negative and one false positive for *ipaH*, one false negative and two false positive for *eae,* one false positive each for the *aggR*, *aaic*, and *sth* genes. The NRL L227 reported the presence of the *ipaH* gene in the negative Sample C and missed its detection in the positive sample B, probably due to an exchange of samples.

For the VTEC-positive sample A, all the NRLs reported correctly the presence/absence of the serogroup-associated genes in the enrichment cultures, according to the ISO/TS 13136 method (Table 3).

Table 2. Real-time PCR detection of virulence and serogroup-associated genes in the enrichment cultures. The green boxes highlight the correct results, the red boxes the wrong results or tests that were not performed.

NRL	Table 2 - Detection of virulence genes in:																												
	Sample A									Sample B									Sample C										
	vtx1	*vtx2*	*eae*	*aggR*	*aaiC*	*lt*	*sth*	*stp*	*ipah*	*vtx1*	*vtx2*	*eae*	*aggR*	*aaiC*	*lt*	*sth*	*stp*	*ipah*	*vtx1*	*vtx2*	*eae*	*aggR*	*aaiC*	*lt*	*sth*	*stp*	*ipah*
True value	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
L105																											
L151																											
L162													ND	ND	ND	ND	ND	ND				ND	ND	ND	ND	ND	ND
L163																											
L166													ND	ND	ND	ND	ND										
L172																											
L187																											
L209														ND		ND	ND	-					ND		ND	ND	
L227																		-									+
L232													ND	ND	ND	ND	ND	ND				ND	ND	ND	ND	ND	ND
L268																											
L318					+		+				+	+	+					-									
L376	-																										
L384															ND	ND	ND	ND						ND	ND	ND	ND
L416																											ND
L430																											
L460																											
L518																											
L542																											
L549																											
L566																											
L607																			+	+	+						
L614													ND	ND	ND	ND	ND	ND				ND	ND	ND	ND	ND	ND
L623																											
L677													ND	ND	ND	ND	ND					ND	ND	ND	ND	ND	
L694																											
L706															ND	ND	ND										
L728																											
L732																											
L751																											
L758																											
L920																											
L936													ND	ND	ND	ND	ND	ND				ND	ND	ND	ND	ND	ND
L950			-																								
L962																											
L975													ND	ND	ND	ND	ND	ND				ND	ND	ND	ND	ND	ND
L988																											

Table 3. Real-time PCR Detection of virulence and serogroup-associated genes in the enrichment culture of sample A, according to the method ISO/TS 13136. The green boxes highlight the correct results, the red boxes the wrong results.

NRL	Detection of virulence and serogroup-associated genes in sample A								
	vtx1	*vtx2*	*eae*	*O157*	*O26*	*O103*	*O111*	*O145*	*O104*
True value	+	+	+	+	-	-	-	-	-
L105									
L151									
L162									
L163									
L166									
L172									
L187									
L209									
L227									
L232									
L268									
L318									
L376	-								
L384									
L416									
L430									
L460									
L518									
L542									
L549									
L566									
L607									
L614									
L623									
L677									
L694									
L706									
L728									
L732									
L751									
L758									
L920									
L936									
L950			-						
L962									
L975									
L988									

5.2. Isolation of the pathogenic *E. coli* strains from the PCR-positive samples.

Thirty-three (89%) of the 37 NRLs obtained the isolation of the VTEC O157 contaminating strain from the enrichment culture of sample A (Table 4).

Table 4. Isolation and genotyping of pathogenic *E. coli* strains from the PCR-positive sprout enrichment cultures. The green boxes highlight the correct results, the red boxes the wrong results or tests that were not performed.

NRL	*E. coli* strain isolation and genotyping from:							
	Sample A					Sample B		Sample C
	Pathogroup	Genotype				Pathogroup	Genotype	Pathogroup
		Serogroup	*vtx1*	*vtx2*	*eae*		*ipah*	
True value	VTEC Isolation	O157	+	+	+	EIEC Isolation	+	None
L105								
L151								
L162						NO		
L163								
L166								
L172								
L187								
L209						NO		
L227						NO		EIEC
L232						NO		
L268								
L318						NO		
L376			-					
L384						NO		
L416								
L430								
L460	NO							
L518								
L542								
L549								
L566	NO							
L607								VTEC
L614						NO		
L623								
L677								
L694								
L706								
L728								
L732								
L751								
L758								
L920								
L936						NO		
L950	NO							
L962								
L975	NO					NO		
L988								

The NRL L607 reported the isolation of the VTEC O157 strain also from the negative Sample C, and this was probably due to a cross-contamination with the positive sample A. All but one (L376) the NRLs identified correctly the genotype of the isolated VTEC O157 strain.

The EIEC strain was correctly isolated from Sample B by 28 NRLs (76%). The NRL L227 reported the isolation of the EIEC strain from the negative Sample C and missed its detection in the positive sample B, probably due to an exchange of 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, marking with different colors the points assigned for the incorrect results in the Real Time PCR screening and isolation steps, and those assigned for tests not done. Figure 2 shows the number of NRLs grouped according to their score. Ten NRLs obtained a score higher than 8. However, for eight of them the high score was mainly due to the fact that they did not perform the detection of all or part of the test for the detection of the *E. coli* patho-groups other than VTEC.

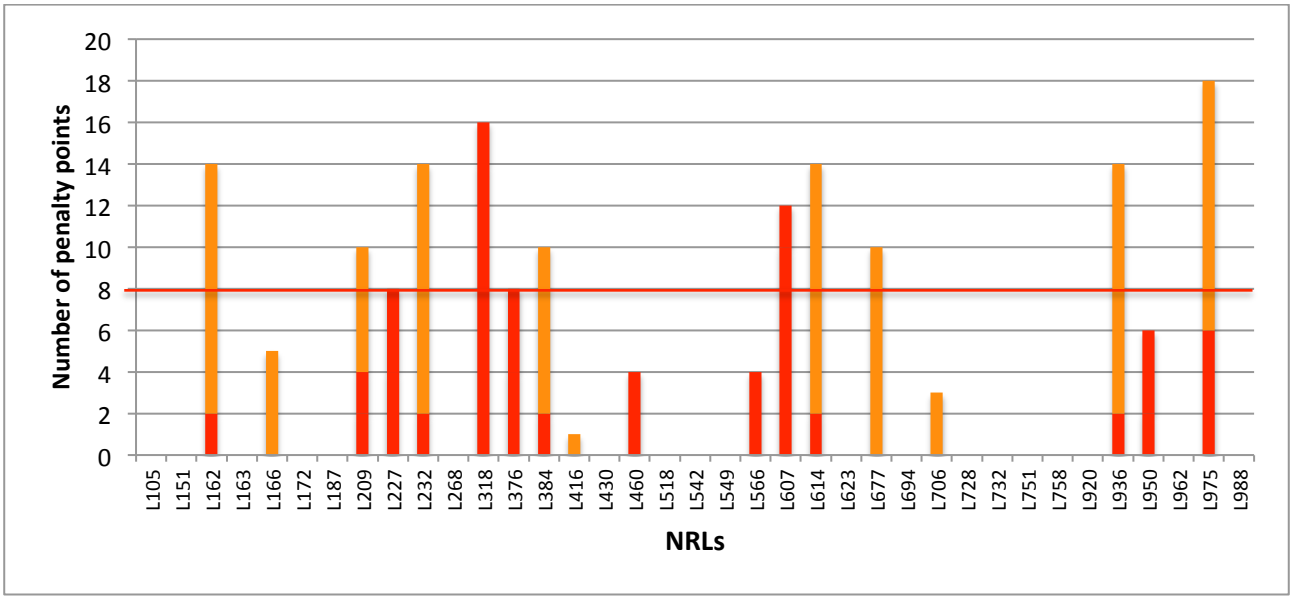

Figure 1. Evaluation of the NRL performance in the PT procedures. The score was calculated according to the criteria described in section 4.5. The points assigned for incorrect results in the Real Time PCR screening and isolation steps are marked in red, those assigned for the tests not done in yellow.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

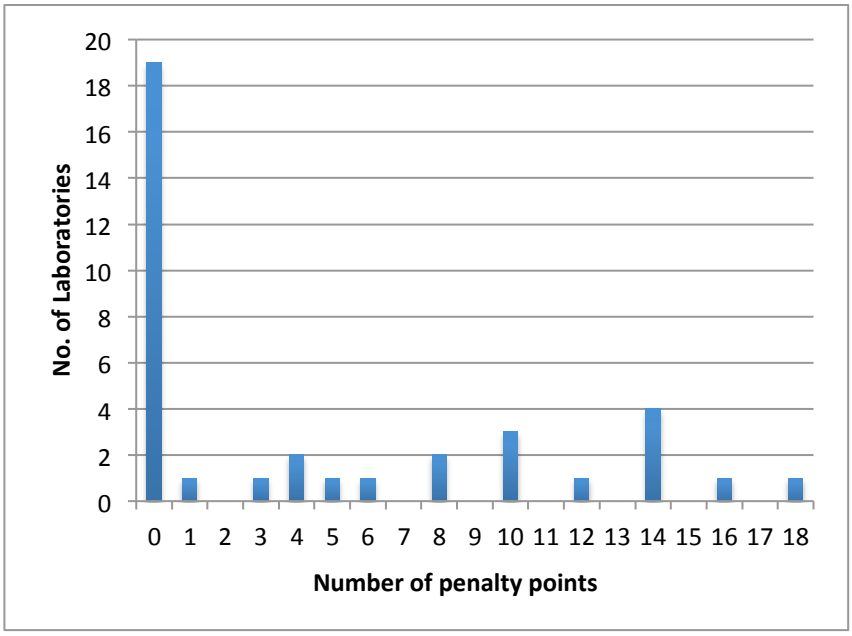

Figure 2. Number of NRLs within each penalty score. The score was calculated according to the criteria described in section 4.5.

6. REMARKS

1. This study was conducted on sprouts, since Reg. (EU) No 209/2013 has recently introduced for this matrix microbiological criteria that include the absence of VTEC O157, O26, O111, O103, O145 and O104:H4. As prescribed by the aforementioned Regulation, the method used for the detection of VTEC was the ISO/TS 13136:2012.
2. The PT scheme was extended in order to cover the detection of other groups of pathogenic *E. coli*, which have been increasingly reported in foodborne outbreaks in industrialized countries.
3. Thirty-eight NRLs representing all the 28 EU Member States, Norway, Switzerland Argentina, and Egypt joined the study. However, one of the laboratories did not perform the analyses for difficulties in acquiring the needed reagents due to budget constraints.
4. The large participation in the PT and the results concerning the detection of the VTEC strain in Sample A confirmed that nearly all the European NRLs are now able to perform the standard method ISO/TS 13136:2012 for the detection of VTEC in food.
5. Ten NRLs did not perform the detection of all or part of the target virulence genes of EAggEC, ETEC, and EIEC, due to either NRL specificity (activity limited to animal samples) or budget constraints. However, 28 of the 31 NRLs (90%) that carried out the specific test identified correctly the presence of the EIEC strain in sample B.

6. In the previous PT rounds on the detection of VTEC in food, the NRL performance was evaluated by determining the Cohen's Kappa value. In this PT, assumed that the majority of the NRLs has become accustomed with this new molecular detection approach, the performance was evaluated by a more precise, score-based system score-based system based on the assignment of penalty points to the incorrect results reported. Such a score was used to identify the aspects of the NRLs proficiency to be improved.
7. In conclusion, most NRLs performed satisfatorily in the detection of VTEC in sprouts, indicating that a very good level of preparedness has been established in the EU towards these foodborne pathogens. Moreover, most NRLs are now able to identify other groups of pathogenic E. coli in the case of foodborne outbreaks due to these pathogens.

Annex 1

12th inter-laboratory study on the detection of Verocytotoxin-producing *E. coli* (VTEC) and other pathogenic *E. coli* in sprouts (PT12)

Laboratory Guideline

The **12th inter-laboratory study** (PT12) among the National Reference Laboratories (NRLs) for *E. coli* in the EU Member States and other European countries is dedicated to the detection and isolation of VTEC, as well as other *E. coli* strains belonging to patho-groups other than VTEC, in sprout samples. The patho-groups other than VTEC object of this study are: Enteroaggregative *E. coli* (EAggEC), Enterotoxigenic *E. coli* (ETEC), and Enteroinvasive *E. coli* (EIEC).

1. Treatment of the samples

The samples are constituted by 25 g of sprouts placed in stomacher bags with filters.
Assuming that the bacteria in the sprout samples my have undergone stressing conditions, the enrichment medium will be buffered peptone water (BPW).
The samples are therefore added with 225 ml BPW directly in the stomacher bag, and homogenised in a stomacher peristaltic blender. The enrichment cultures are then incubated for 18-24 hrs at 37 °C (either static or in agitation).

2. Detection of the presence of pathogenic *E. coli* strains in the enrichment cultures by Real Time PCR amplification of their target virulence genes

One ml aliquot of the enrichment cultures is taken at this stage and used for DNA extraction and purification, according to the ISO/TS 13136:2012. The remaining culture shall be stored at +4 °C for the isolation steps that will follow a positive PCR result.

2.1. Detection of the presence of VTEC belonging to serogroups O157, O111, O26, O103, O145, and O104.

This part of the test will be performed according to the 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.

The detection of VTEC O104 will be carried out according to the adaptation provided by the EU-RL VTEC (available in the EU-RL web site, http://www.iss.it/vtec, Laboratory Methods Section - EU-RL_VTEC_Method_04_Rev1).

2.2. Detection of the presence of pathogenic E. coli strains other than VTEC

If the sample is negative for the presence of *vtx* genes, it will be tested for the following target genes:

- *aaiC* and *aggR* for **EAggEC**
- *lt*, *sth,* and *stp* for **ETEC**
- *ipaH* for **EIEC**

The Real Time PCR procedures for the detection of these target genes are available in the EU-RL web site (http://www.iss.it/vtec), Laboratory Methods Section.

In particular the procedure for:

aaiC and *aggR* genes detection is the EU-RL_VTEC_Method_05_Rev1;

lt, *sth,* and *stp* genes detection is the EU-RL_VTEC_Method_08_Rev0;

ipaH gene detection is the EU-RL_VTEC_Method_07_Rev0.

3. Isolation and identification of the pathogenic *E. coli* strains responsible for the positive PCR screening reactions

The enrichment cultures positive at the Real Time PCR screening step will be subjected to the isolation of the *E. coli* strains responsible for the positive PCR.

This will be accomplished by streaking the enrichment culture on suitable solid media (MacConkey agar plates or other media for *E. coli* isolation, such as TBX) and testing up to 50 colonies with typical *E. coli* morphology for the presence the virulence genes detected in the enrichment cultures. This can be achieved by testing pools consisting of 10 colonies each. Should a pool result positive in PCR tests, go back to test the single colonies of the pool to identify the one(s) containing the gene(s). If the Real Time PCR

screening step of the ISO/TS 13136:2012 indicated the presence of a given VTEC serogroup, the isolation can be facilitated by an immuno-magnetic separation step, specific for that serogroup.

The VTEC strains isolated will be characterized by determining the presence of *vtx1*, *vtx2*, and *eae* genes and the serogroup. For the strains belonging to the other patho-groups the determination of the presence of the target virulence genes will be sufficient.