Report of the 20th inter-laboratory study on the detection of Shiga toxin-producing *E. coli* (STEC) in food (PT20)

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.

In November 2017 the EURL-VTEC organized a PT on the detection of Shiga toxin-producing *E. coli* (STEC) in vegetables, based on the application of the standard method for the detection of STEC in foodstuffs 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 study, PT20, represented the 13th PT on the detection of STEC in complex matrices based on the ISO TS 13136:2012 and was carried out on rocket salad samples.

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

- during the last years, a growing number of foodborne illnesses have been traced back to fruits and produce, increasing the concern that vegetables might be more important as a vehicle for human enteric pathogens, including STEC, than previously thought;
- rocket salad has been recently implicated in an outbreak of STEC O157 infection;
- this matrix had never been proposed in previous PTs organized by the EURL-VTEC.

PT20 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 detection and isolation of STEC from rocket salad samples spiked with different amounts of a STEC O111 strain.

The **objectives** of the study were:

- to improve the preparedness of the NRLs towards testing food commodities for the presence of STEC, by applying to the ISO TS 13136:2012;

- 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 40 NRLs representing 27 EU Member States, Egypt, Iceland, Norway, Russia and Switzerland participated in the study. Each NRL received its own individual Laboratory code, which is 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
- 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
- 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
- Malta, Public Health Laboratory
- 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, Department of Food Hygiene, State veterinary and food institute
- Slovakia, NRC of Environmental Microbiology, Public Health Authority of SR
- Slovenia, Veterinary Faculty UL, Nacional Veterinary Institute – Unit for Food of Animal Origin
- Spain, *Unidad Microbiología - Centro Tecnológico Agroalimentario de Lugo* (LSA-CETAL)
- Spain, Microbiology Food Department, *Agencia Española de Consumo, Seguridad Alimentaria y Nutrición, 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 samples (1, 2 and 3), each consisting of 25 g of rocket salad potentially contaminated with STEC, were sent in the blind to the NRLs.

The rocket salad used was purchased at retail on November the 7th, 2017 and contained a natural background microflora (about 7 x10^{4} CFU/g). The rocket was 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 10th of November 2017, using dilutions of an exponential liquid culture (0.5 OD read at 600 nm) of the STEC O111 strain ED476 positive for *stx1, stx2* and *eae* genes. The characteristics of the samples are reported 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, low and high level. In detail, the set of samples sent to the NRLs contained 0, 4 and 40 estimated CFU per gram, respectively (Table 1). Serial dilutions of the inoculum suspensions of strain ED476 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 13 November 2017 by courier. The NRLs were requested to start the analyses immediately upon receipt and to record date of delivery and sample temperature upon reception.

Table 1: Characteristics of the rocket salad samples assessed in the study

Contaminant (*Genotype*)	Contamination level in:		
	Sample 1	**Sample 2**	**Sample 3**
Strain ED476, STEC O111 *(stx1+, stx2+, eae+)*	-	Low: 4 CFU/g	High: 40 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 evaluated for samples spiked on the 1st of September 2017 and tested by ISO TS 13136:2012 after 3, 6, 10 and 13 days since the initial contamination. The Real Time PCR screening was positive for all the target genes even after 13 days from the spiking whereas isolation was successful up to 10 days form spiking.

When the test samples were prepared, eight bags for each of the two levels of contamination and two non-contaminated samples were randomly selected for homogeneity testing and analyzed according to the PT laboratory procedures on the 13th and 14th of November 2017. All the homogeneity tests gave the expected results.

4.3. Laboratory methods

Laboratories were requested to identify the presence of STEC using the method ISO TS 13136:2012.

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 4 penalty points to each incorrect or missing result concerning the identification of virulence genes, *stx1* and *stx2*, in the three samples. Two penalty points have been assigned to the laboratories not identifying the presence of *eae* gene and the O111 serogroup-associated gene in the screening.

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 responsible for positive PCR screening reactions in the enrichment cultures was evaluated by assigning 2 penalty points to the lack of isolation from the positive samples or when the isolation was not performed.

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 O111 serogroup-associated gene in the STEC 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 8 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 O111 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) was not calculated for the isolation step as the results obtained showed that only a few laboratories didn't succeed in isolating the STEC contaminating strain in both the spiking levels used.

5. RESULTS

The samples were sent to 40 NRLs and 37 of them returned results via the web platform. One Laboratory didn't receive the parcel containing the samples due to custom clearance procedures issues, whereas another participant couldn't submit the results since the Laboratory missed the correspondence between the samples and the blind codes. This NRL sent the results to the EURL-VTEC as an excel file and an O111 STEC possessing the genes *stx1*, *stx2* and *eae* was detected in two samples, whereas the third was negative, as expected. These results have been recorded by the EURL-VTEC but couldn't be used for the general analysis presented in this report. The last laboratory which didn't submit the results contacted the EURL-VTEC during the PT informing that, following a technical problem, it was not possible to submit the results.

As for the delivery conditions, 28 NRLs received the samples within 24 hours, 8 within 48 hours and L621 in 10 days due to custom clearance procedures.

The reported temperature at delivery was ≤ 4 °C for 16 NRLs and between 5 °C and 8 °C for the rest of the laboratories.

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

All the 37 NRLs returning the results carried out the detection of the virulence and serogroup-associated genes and the results are reported in Table 2.

As for the negative sample, one lab incorrectly reported the detection of *stx1* and *stx2* genes.

The presence of all the virulence genes and the O111 serogroup-associated gene in sample 2 (low level of contamination) was correctly identified by 34 laboratories (91.9 %). Three laboratories provided a total of five non-concordant results. No incorrect results were reported for the identification of *stx2* gene in this sample, whereas two laboratories didn't detect the presence of *stx1* in the screening step, with one of them failing in detecting *eae* gene too. In addition, one NRL failed to detect the presence of *eae* and *wbdl*$_{O111}$ genes.

As for sample 3 (high level of contamination), all labs but one correctly reported the identification of the presence of all the target genes. The NRL who reported the incorrect result was not able to detect *stx1*.

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

The isolation of the contaminating STEC strain was generally successful. One laboratory didn't participate in this part of the PT and performed the screening step only. A total of 34 laboratories isolated the STEC O111 strain from sample 2 (low level of contamination) and 35 from Sample 3 (high level of contamination). As for the isolated STEC strain characterization, all but two laboratories correctly identified the virulence gene set and the serogroup of the strain, whereas one laboratory failed to detect the presence of eae gene and another one couldn't detect the presence of *stx1*.

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.

NRL	Detection of virulence and serogroup-associated genes in:											
	Sample 1				Sample 2 Low level contamination				Sample 3 High level contamination			
	stx1	*stx2*	*eae*	*wbdl$_{O111}$*	*stx1*	*stx2*	*eae*	*wbdl$_{O111}$*	*stx1*	*stx2*	*eae*	*wbdl$_{O111}$*
True value	-	-	-	-	+	+	+	+	+	+	+	+
L128												
L136												
L163												
L174												
L178												
L226												
L318												
L404												
L446												
L527	+	+		ONT			-	ONT				
L546												
L552												
L562					-				-			
L600												
L607					-		-					
L621												
L653												
L659												
L700												
L703												
L712												
L723												
L724												
L738												
L744												
L783												
L792												
L825												
L827												
L843												
L862												
L894												
L925												
L944												
L950												
L979												
L987												

Table 3. Isolation and genotyping of STEC strains from the rocket salad 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.

STEC strain isolation and genotyping from:									
NRL	Sample 1	Sample 2				Sample 3			
	-	STEC O111 Isolation	Genotype			STEC O111 Isolation	Genotype		
			stx1	*stx2*	*eae*		*stx1*	*stx2*	*eae*
True value	None	+	+	+	+	+	+	+	+
L128						-			
L136									
L163									
L174									
L178									
L226									
L318									
L404									
L446									
L527					-				
L546									
L552									
L562		-					-		
L600									
L607		-							
L621									
L653									
L659									
L700									
L703									
L712									
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L783									
L792									
L825									
L827									
L843									
L862									
L894									
L925									
L944									
L950									
L979									
L987									

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.

Three NRLs obtained a score equal or higher than 8 and their performance was not considered as satisfactory. In particular, one laboratory (L562) failed to detect the *stx1* gene either in the enrichment culture or in the isolated STEC strain and the majority of the penalty points obtained refer to this problem with *stx1* identification.

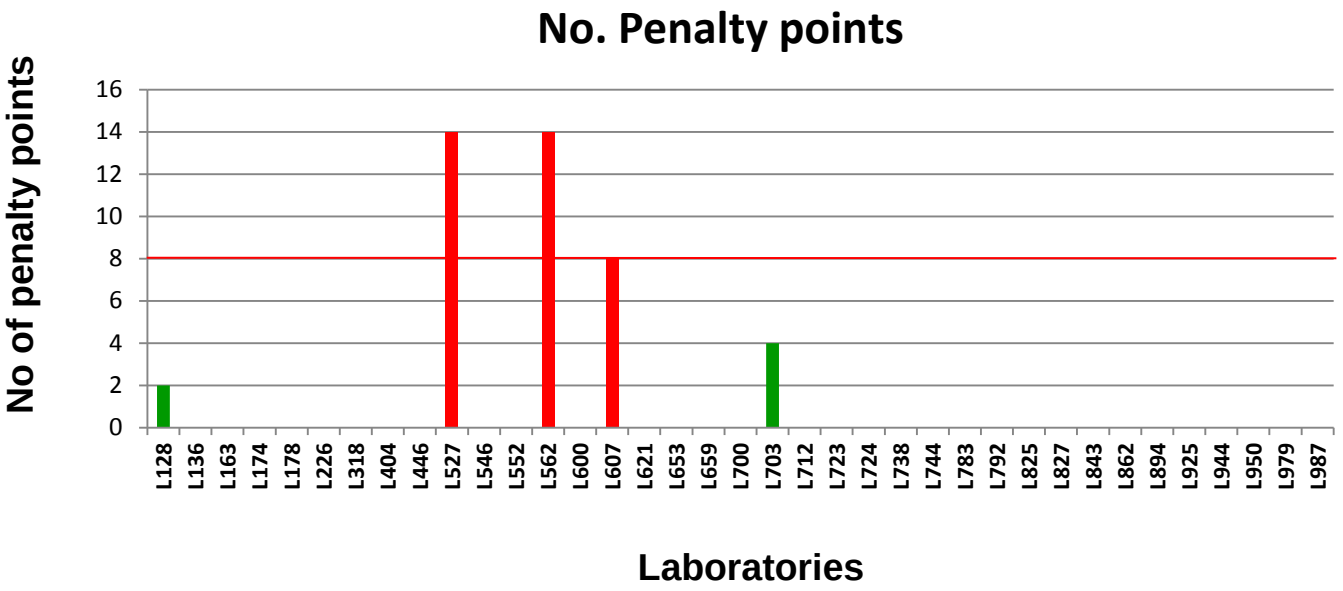

Figure 1. Evaluation of the NRL performance in the PT procedures (screening and isolation steps). The score was calculated according to the criteria described in section 4.5. The performance of the NRLs that obtained a score higher than 8 was not considered as satisfactory (red bars).

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 *wbdl*$_{O111}$ gene in the screening step. The results provided by 36 out of the 37 NRLs submitting the results were considered for the performances of the *stx1*-PCR, excluding those of L562, which clearly showed a general problem with the *stx1*-PCR. The results provided by all the 37 NRLs were considered for the performances of the *stx2* and *eae* PCR.

The analysis of the results returned the following values:

- *stx1* PCR Se: 97.2 % (low level) and 100 % (high level).
- *stx2* PCR Se: 100 % (low level) and 100 % (high level).
- *eae* PCR Se: 94.6 % (low level) and 100 % (high level).
- *wbdl*$_{O111}$ PCR Se: 97.3 % (low level) and 100 % (high level).
- *stx1* PCR Sp: 97.3 %.
- *stx2* PCR Sp: 97.3 %.

5.4.2. STEC O111 isolation step

The Sensitivity of the isolation procedure was the following:

- Se: 94.4 % for the low contamination level.
- Se: 97.2 % for the high contamination level.

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. At the same time, the definition of the performance parameters of the standard itself is necessary to grant the NRLs and Official Laboratories an easier access to accreditation, which in turn ensures the comparability of the results produced by different laboratories in the different Member States. The EURLs play a central role in developing methods, in producing data on their performances as well as in assessing the proficiency of the NRLs in using them through the delivery of PT schemes.

This PT aimed at extending the scopes of the EURL-VTEC PT schemes concerning the couples food matrix/contaminating STEC with respect to both assessing the NRLs network proficiency and the method's performances.

The PT20 was based on the analysis of rocket salad contaminated with a STEC O111, bringing to nine the number of matrices analyzed so far. The spectrum of food matrices included in the EURL-VTEC PT scheme covers most of the epidemiologically relevant food commodities such as milk, vegetables including sprouts and rocket, water and beef meat.

The analysis of the results provided by the laboratories participating in the PT20 induces the following remarks:

1. a high participation rate was observed: 40 laboratories covering 27 EU Member States, Egypt, Iceland, Norway, Russia and Switzerland participated in the study, confirming the consolidation of the network of national laboratories for *E. coli*;
2. the results of the study confirmed that the ISO TS 13136:2012 method represents a suitable tool for the detection of STEC in the food commodity most regarded as vehicles of human infections;
3. the presence of the STEC O111 virulence genes was identified correctly by 34 out of 37 NRLs (91.9 %) in samples 2 (low level of contamination) and by 36 out of 37 NRLs (97.3 %) in sample 3 (high level of contamination). The contaminating STEC O111 strain was isolated by 34 (94.4 %) and 35 (97.2 %) NRLs out of the 36 laboratories performing isolation, in samples 2 and 3 respectively;
4. to evaluate the NRL proficiency, penalty points were assigned for all the incorrect results provided. As a whole, three out of the 37 NRLs that contributed results showed an unsatisfactory performance;
5. the results of this PT allowed to determine the performance parameters of the method for the concerned couple matrix/STEC type, which sum to the other already determined

in the previous PT rounds and will be made available through publication in the EURL-VTEC website in order to support the NRLs and Official Laboratories, which can refer to such parameters to have the method correctly accredited.