Report of the 37th inter-laboratory study on the detection of Shiga toxin-producing *E. coli* (STEC) in sprouts (PT37) - 2023

Edited by:

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1. INTRODUCTION

PT37 was carried out to assess the presence of STEC in sprout samples.

The study was organized to consolidate the preparedness of the NRLs in verifying the compliance of this food commodity to Regulation (EU) No 209/2013, which amended Regulation (EC) No 2073/2005, and introduced the following microbiological criteria for sprouts: Absence of STEC O157, O26, O111, O103, O145 and O104:H4 in 25 g of end product.

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 in sprout samples and the **objectives** 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 for the accreditation of the ISO TS 13136:2012.

3. PARTICIPANTS

Thirty-three NRLs from the following 24 EU Member States and three EFTA Countries participated in the study.

- Austria, Institut für Medizinische Mikrobiologie und Hygiene, AGES
- Belgium, SCIENSANO – Foodborne Pathogens
- 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
- Denmark, Microbiological laboratory Ringsted, Danish Veterinary and Food Administration
- Estonia, Estonian 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 Chain Safety Office, Food Chain Safety Directorate, Microbiological NRL
- Iceland, Matís ohf. / Icelandic Food and Biotech R&D
- Ireland, 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, Laboratoire National de Santé
- Norway, Norwegian Veterinary Institute
- Poland, National Institute of Public Health (NIH) – National Research Institute
- Poland, National Veterinary Research Institute (NVRI), Department of Hygiene of food of animal origin
- Portugal, Laboratório de Microbiologia dos Alimentos, Instituto Nacional de Investigação Agrária e Veterinária, I.P.
- Romania, Department of Microbiology - Molecular Biology Unit, Institute for Hygiene and Veterinary Public Health
- Slovakia, Public Health Authority of the Slovak republic
- Slovakia, Veterrinary and Food Institute SVFI Dolny Kubin
- Slovenia, University of Ljubljana Veterinary Faculty, National Veterinary Institute
- Spain, National Plant Health Laboratory
- Spain, Laboratorio Central de Veterinaria de Algete (MAPA)
- Spain, Centro Nacional de Alimentación-AESAN
- Sweden, Swedish Food Agency
- Sweden, National Veterinary Institute (SVA), Dept of Bacteriology
- Switzerland, AGROSCOPE
- The Netherlands, National Institute for Public Health and the Environment - RIVM
- The Netherlands, Wageningen Food Safety Research WFSR

After reporting the results, each NRL received its own individual report of participation indicating the expected and the reported results.

4. MATERIALS AND METHODS

4.1. Sample preparation

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

The sprouts used have been acquired as a single batch from a local producer and contained a natural background microflora of $9.3X10^4$ bacterial CFU per gram of sprouts ($1X10^4$ CFU of enterobacteria per gram of sprouts). The sprouts were portioned in 25 g samples in sterile stomacher bags and placed at + 4 °C until the artificial contamination was carried out. Two 25 g portions of sprouts of the same batch 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 20th of October 2023, using appropriate dilutions of an exponential liquid culture (0.5 OD read at 600 nm) of the STEC strain ED0773 (O187:H28) possessing the *stx2* gene and negative for the presence of the *eae* gene. The characteristics of the samples are reported in Table 1. An uncertainty of measurement of 0.19 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 (Table 1). Serial dilutions of the inoculum suspensions of strain ED0773 added to the samples were plated onto MacConkey agar plates to confirm the titer.

The test samples were labeled with randomly generated numerical codes different for each participant laboratory and stored at 4°C until shipped refrigerated on 23rd of October 2023 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 ED0773, STEC O187:H28 *(stx1-, stx2+, eae-*)	-	Low: 5 CFU/g	High: 50 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 13rd of September 2023 and tested by ISO TS 13136:2012 after 0, 5, 7, and 12 days since the initial contamination. The Real Time PCR screening was positive for the STEC target genes even after 12 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 not achieved for the low level of contamination, after 5 days from spiking.

When the test samples were prepared, ten bags for each of the two levels of contamination and two non-contaminated test samples were randomly selected for homogeneity testing, enriched at 37°C and analyzed by Real Time PCR for the presence of the contaminating STEC on the 24th of October 2023. The Real Time PCR screening carried out for the homogeneity tests were positive for the STEC target genes.

4.3. Laboratory methods

Laboratories were requested to identify the presence of STEC using 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*")..

4.4. Collection and elaboration of the NRL results

The results were submitted through a dedicated Microsoft Form. The participating laboratories had to indicate in the Form their Lab code, provide the information on the arrival date, temperature, and quality of the samples, as well as the results obtained for each test of the blind samples.

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 *stx1* and *stx2*, and two penalty points for the incorrect identification of *eae* gene as well as the top-5 and O104 serogroups.

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 strain responsible for positive PCR screening reactions in the enrichment cultures was assessed. In detail, two penalty points were assigned in case of lack of isolation of STEC from sample 3. As for strain characterization, four penalties have been assigned to incorrect detection of Stx-coding genes. No penalties were assigned to the laboratories reporting the serogrouop of the STEC strain isolated as OND.

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 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

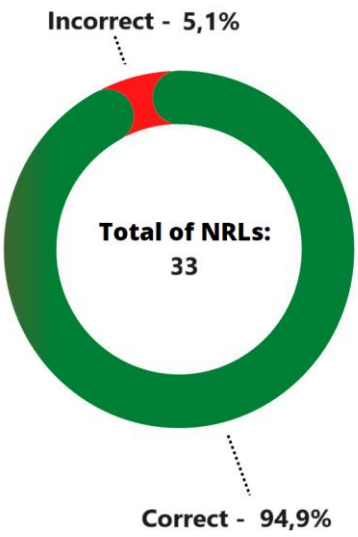
All the 33 Laboratories receiving the samples returned results via the web platform.

As for the delivery conditions, all the NRLs received the samples within 24 hours.

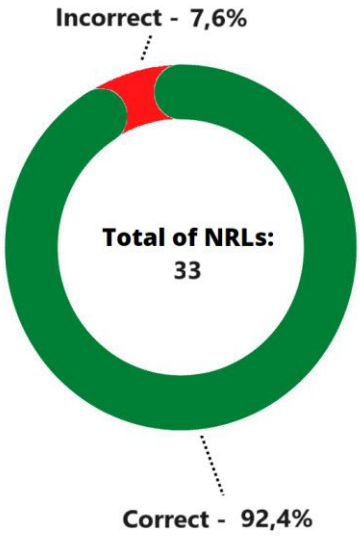
The reported temperature at delivery was ≤ 5 °C for 20 NRLs, between 5 °C and ≤ 8 °C for seven laboratories and between 8 °C and 13 °C for five NRLs. The L025 samples arrived in the laboratory at 20.6 °C.

The results submitted by the participating laboratories are summarized in **Figures 1 – 3**.

Figure 1. Percentage of Laboratories reporting the correct screening results (a) and isolating (b) the STEC strain (green: correct result; red: incorrect result).



a) Screening Step: % of Laboratories correctly detecting STEC in the spiked sample (green: correct results; red: incorrect results).



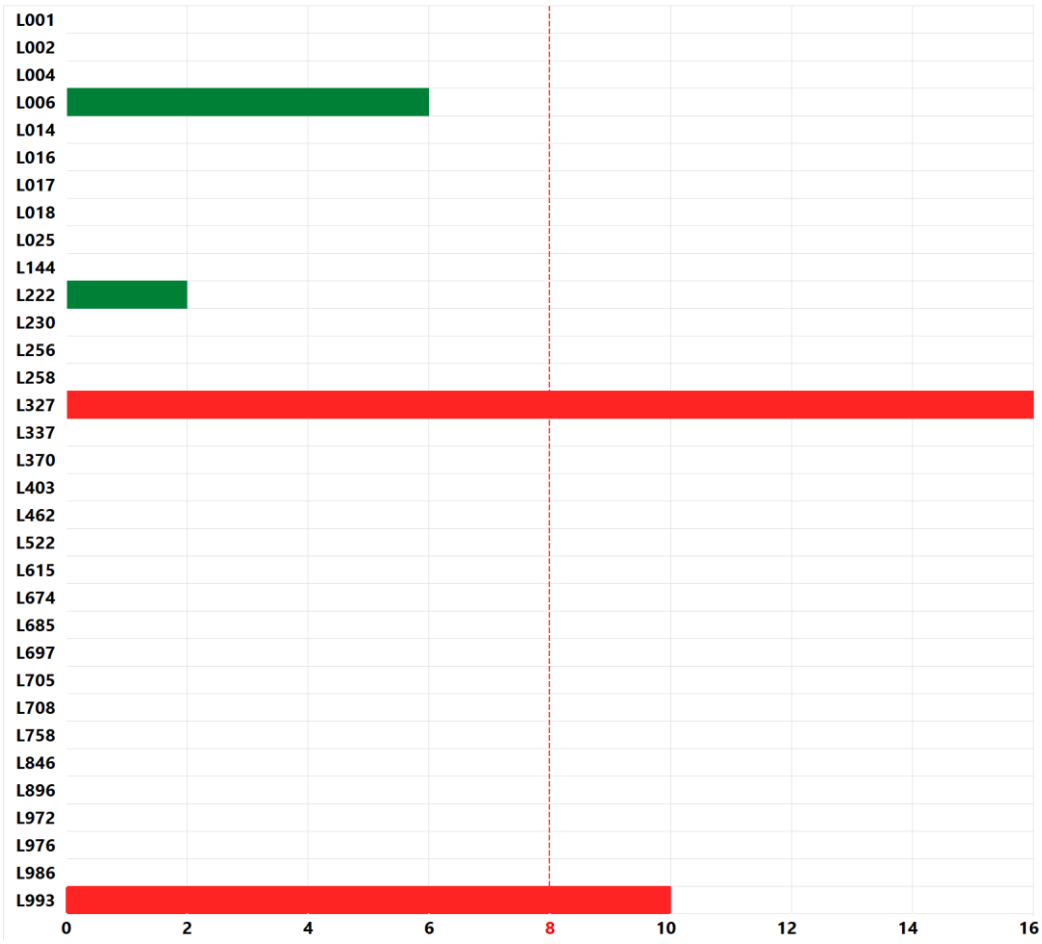
b) Isolation Step: % of Laboratories that successfully isolated the STEC strain detected in the screening step (green: correct results; red: incorrect results).

Figure 2. Real-time PCR detection of virulence and serogroup-associated genes in the enrichment cultures (yellow boxes represent the gold standards; green boxes: correct results and red boxes: incorrect results).

Figure 3. Isolation and genotyping of STEC strains from the sprouts samples (Yellow boxes represent the gold standards; green boxes: correct results and red boxes: incorrect results; *: correct identification of O187 serogroup).

For each NRL, the number of penalty points was determined using the criteria described in section 4.5. **Figure 4** shows the score achieved by each NRL. Only two laboratories did not comply the definition of satisfactory proficiency.

Figure 4. Evaluation of the NRLs performance in the PT procedures (screening and isolation steps).



The calculation of ***Se* in the screening step** was performed based on the results provided by 33 and 32 participating NRLs, respectively. The results reported for sample 3 by L993 were excluded, as an inversion of samples 1 and 3 was suspected.

The ***Sp* in the screening step** was calculated on the results provided by 32 participating NRLs. The results from L993 were excluded.

Table 1. Sensitivity and Specificity of the method.

	Se ***(low level)***	***Se*** ***(high level)***	***Sp***
stx1	NA	NA	98.0%
stx2	97.0%	100%	100%
eae	NA	NA	100%

The calculation of ***Se* in the isolation step** was based on the results provided by 33 and 32 participating NRLs, respectively. The results reported for sample 3 by L993 were excluded.

- Se: 93.9% for the low contamination level
- Se: 96.9% 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.

Table 2. Limit Of Detection (LOD). [1] $LOD_{50\%}$ = 50% Limit of Detection; [2] $LOD_{95\%}$ = 95% Limit of Detection.

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	Sprouts	0,022	0,244	1,236	0,758	2,016	5,343	3,277	8,712	0,000
Combined data		0,022	0,244	1,236	0,758	2,016	5,343	3,277	8,712	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.

PT37 concerned the application of the ISO TS 13136:2012 on sprout samples for the benefit of the network of NRLs, which are rquested to test such a matrix for the presence of STEC, according to Regulation (EU) No 209/2013.

The analysis of the results provided by 33 Laboratories participating in PT37 induces the following conclusions:

1. A high participation rate was observed, confirming the consolidation of the network of National Reference Laboratories for *E. coli*;
2. The virulence genes of the contaminating STEC strain were identified with satisfactory sensitivity in the spiked samples.
3. The majority of the laboratories could isolate the STEC from both the samples with low level and high level of contamination.
4. Four laboratories were able to correctly characterize the isolated strain as belonging to O187 serogroup.

5. Two participating laboratories presented a non-satisfactory performance and will be contacted. For one of these (L993) the underperformance was likely due to the exchange of two samples.
6. The LOD50 of the isolation step could be calculated and consisted in about 1 CFU/g.
7. As in the other PT rounds, the performance parmaters calculated in PT37 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.