Report of the 40th inter-laboratory study on the detection of Shiga toxin-producing *E. coli* (STEC) in spent irrigation water (PT40) - 2024

Edited by:

Arnold Knijn, Valeria Michelacci, Rosangela Tozzoli, Paola Chiani, Guendalina Fornari Luswergh, Federica Gigliucci, Theodora Socheri, Federica Melone, Fabio Minelli, Margherita Montalbano Di Filippo, Stefano Morabito

1. OBJECTIVE OF THE STUDY

PT40 consisted in assessing the presence of STEC in sprouts spent irrigation water samples with the objectives of:

- improving the preparedness of the NRLs towards testing sprouts spent irrigation water in compliance with Regulation (EU) No 209/2013;
- improving the preparedness of the NRLs towards the detection and isolation of STEC strains not belonging to the O157 serogroup;
- providing support to the NRLs for the accreditation of the ISO TS 13136:2012.

This document represents the full evaluation report of the study.

2. PARTICIPANTS

Twenty-five NRLs from the following 22 EU Member States and three EFTA Countries participated in the study. These were:

- Austria, AGES - Agency for Health and Food Safety
- Belgium, Sciensano - Service Food Pathogens
- Bulgaria, National Diagnostic and research Veterinary Medical Institute/NDRVMI/, NRL"Listeria and Escherichia coli"
- Cyprus, Laboratory for the Control of Food of Animal Origin (LCFAO), Cyprus Veterinary Services
- Denmark, Danish Veterinary and Food Administration Microbiological laboratory Ringsted
- Estonia, National Centre for Laboratory Research and Risk Assessment (LABRIS)
- Finland, Finnish Food Authority Laboratory and Research Division Microbiology Unit Food and Feed Microbiology Laboratory
- France, VETAGROSUP LMAP/LNR
- Germany, German Federal Institute for Risk Assessment (BfR), NRL E.coli
- 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)

- Luxembourg, Laboratoire National de Santé
- Norway, Norwegian Veterinary Institute
- Poland, Food Safety Department, National Institute of Public Health NIH, National Research Institute
- 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, State Veterinary and Food Institute SVFI Dolny Kubin
- Spain, National Plant Health Laboratory
- Sweden, National Veterinary Institute (SVA), Dept of Bacteriology
- Switzerland, AGROSCOPE
- The Netherlands, National Institute for Public Health and the Environment - RIVM

Each participating NRL received its own individual report of participation after the closure of the deadline for reporting the results.

3. MATERIALS AND METHODS

3.1. Sample preparation

Two test samples, each consisting of 200 ml of Alfalfa sprouting spent irrigation water potentially contaminated with STEC, were sent in the blind to the participating laboratories (Table 1).

The sprout spent irrigation water used was obtained in a single batch from a local producer and contained a natural background microflora equal to 10^7 CFU/ml of water (1.8×10^6 CFU of enterobacteria per milliliter of water). The water was aliquoted in 200 ml samples in plastic bottles with screw caps and placed at +4°C until artificial contamination was carried out. Two portions consisting in 200 ml of water from the same batch were initially tested for the presence of STEC by applying the ISO TS 13136:2012 method after pretreatment described in the EURL-VTEC_Method_09, available at the EURL-VTEC website. Both samples assayed were negative for all the STEC target genes.

The study design included the assay of two test samples artificially contaminated with 0 (blank) and 50 CFU/ml of a a STEC strain (Table 1). Artificial contamination of the samples was carried out on October the 11th, 2024, by using appropriate dilutions of an exponential

liquid culture (0.5 OD_{600}) of the STEC strain ED0963 (O104:H7) which possessed the *stx1* gene and was negative for the presence of the *eae* gene. The standardized inoculum was associated with an uncertainty of measurement equal to 0.125 log CFU/ml, calculated according to the procedure described in the ISO TS 19036:2006. Serial dilutions of the inoculum suspensions used for spiking were plated on MacConkey agar plates to confirm the titer.

The samples sent to the laboratories were labeled with randomly generated numeric codes, different for each laboratory, and were stored at +4°C until shipped refrigerated on the 14th of October 2024 by courier. Laboratories were asked to record the delivery date and the temperature of the samples received, and to start analyses within 18 hours from receipt, at latest.

Table 1: Characteristics of the spent irrigation water samples assessed in the study

Contaminant (*Genotype*)	**Contamination level in:**	
	Sample 1	**Sample 2**
Strain ED0963, STEC O104:H7 *(stx1+, stx2-, eae-*)	-	50 CFU/ml

Stability and homogeneity were evaluated according to the requirements of ISO 17043:2010. Stability was previously assessed using samples contaminated on 16 July 2024 and tested according to ISO TS 13136:2012, after pre-treatment (centrifugation) as described in the procedure EURL-VTEC_Method_09 and enrichment in BPW at 41.5 °C, after 0, 3, 7 and 9 days from initial contamination. The tests carried out showed that the Real Time PCR screening was positive for *stx1* and wzx_{O104} genes even after 9 days, while it was possible to isolate the STEC strain within 7 days from the spiking.

Six bottles for each of the two contamination levels (0 and 50 CFU/ml) were randomly selected from the batch of test samples prepared for the shipment to the laboratories to evaluate their homogeneity. These samples were pre-treated as indicated, enriched in BPW by incubating at 41.5 °C and analyzed on 15 October 2024 by Real Time PCR to identify the presence of the STEC strain, obtaining the expected results.

3.2. Laboratory methods

Laboratories were requested to identify the presence of STEC using the pre-treatment procedure for the samples available at the EURL for *E. coli* website (EURL-VTEC_Method_09_Rev 1: "*Laboratory procedure for testing spent irrigation water for the presence of STEC*"), followed by the application of the ISO TS 13136:2012 method, by enriching the samples in BPW at 41.5 °C and 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 2: "*Detection and identification of Verocytotoxin-producing Escherichia coli (VTEC) O104:H4 in food by Real Time PCR*").

3.3. Collection and elaboration of the NRL results

The results were submitted through a dedicated website developed by the EURL for *E. coli*. The participating laboratories were requested to 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.

3.4. Analysis of the NRL results

3.4.1. Evaluation of the NRL performance in the Real Time PCR screening step

The performance of each NRL was evaluated by assigning four penalty points to each incorrect or missing result concerning the identification of *stx* genes and two penalty points for the incorrect identification of *eae* gene as well as O104 serogroup.

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

The performance of each NRL was assessed as follows. Two penalty points were assigned in case of lack of isolation of STEC from sample 2. As for strain characterization, four penalties have been assigned to incorrect detection of Stx-coding genes and two penalties points for the incorrect identification of O104 serogroup.

3.4.3. 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 total score higher than eight was considered unsatisfactory.

3.5. Evaluation of the performance of the method

Sensitivity (*Se*) and Specificity (*Sp*) were calculated for the PCR screening for *stx1*, *stx2* and *eae* genes, and the *Se* only was estimated 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).

4. RESULTS

Twenty-four laboratories receiving the samples returned results.

As for the delivery conditions, all the NRLs received the samples in good condition within 48 hours. Three laboratories reported that temperature at delivery was between +3°C and +5°C, five between +5°C and +8°C and two in the range +9°C to +12°C. The rest of the participating NRLs did not report information on the temperature.

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).

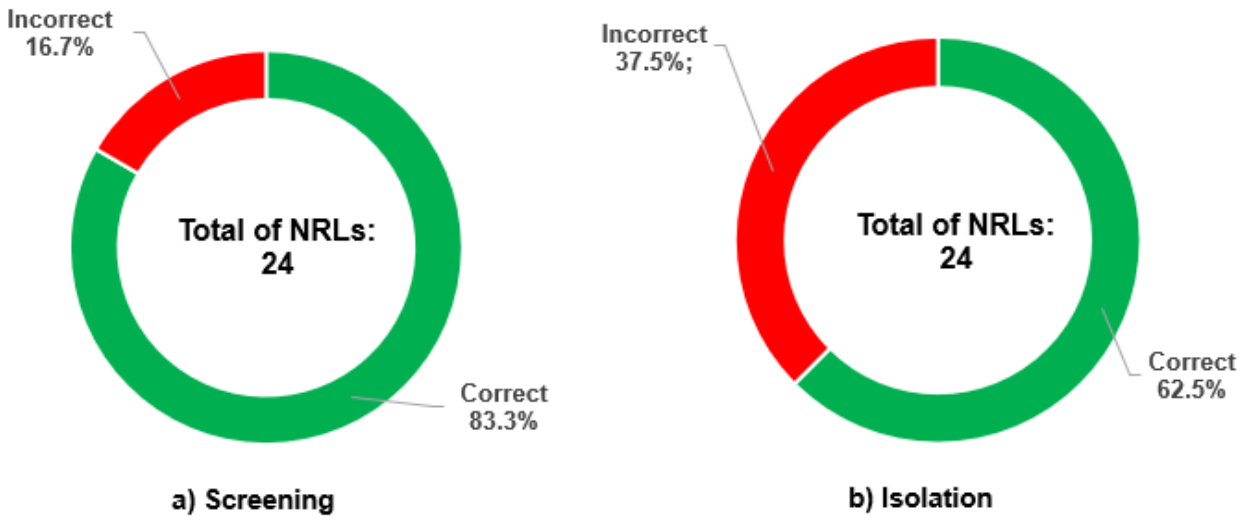
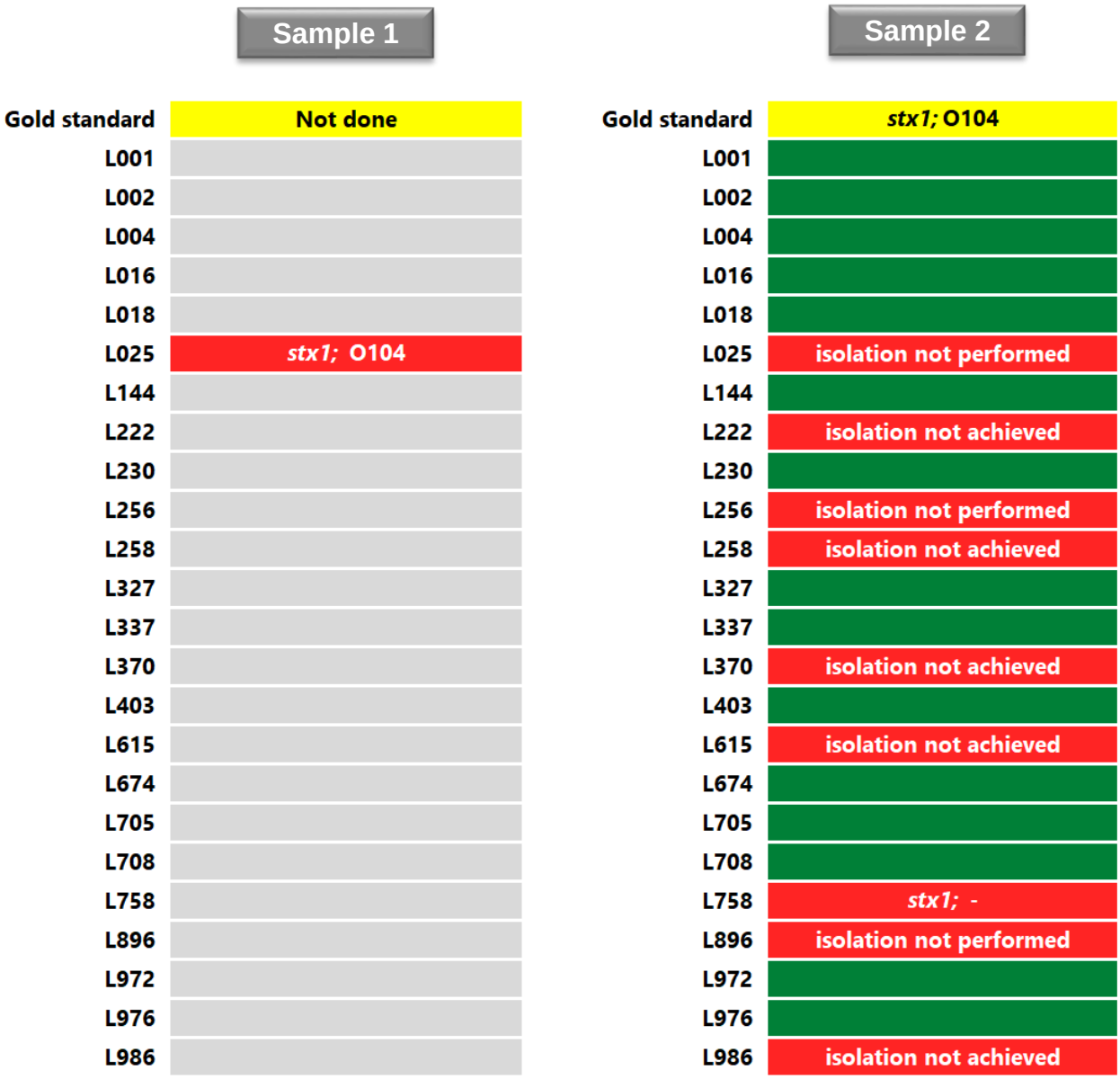


Figure 2. Real-time PCR detection of virulence and serogroup-associated genes in the enrichment cultures (yellow boxes represent the gold standards; green boxes: correct results and red boxes: incorrect results).

Sample 1	
Gold standard	Negative
L001	
L002	
L004	
L016	
L018	
L025	*stx1;* O104
L144	
L222	
L230	
L256	
L258	
L327	
L337	
L370	
L403	
L615	
L674	
L705	
L708	
L758	
L896	
L972	
L976	
L986	

Sample 2	
Gold standard	*stx1;* O104
L001	
L002	
L004	
L016	
L018	
L025	none
L144	*stx1;* -
L222	
L230	
L256	none
L258	
L327	
L337	
L370	*stx1;* -
L403	
L615	
L674	
L705	
L708	*stx1;* -
L758	*stx1;* -
L896	none
L972	
L976	
L986	

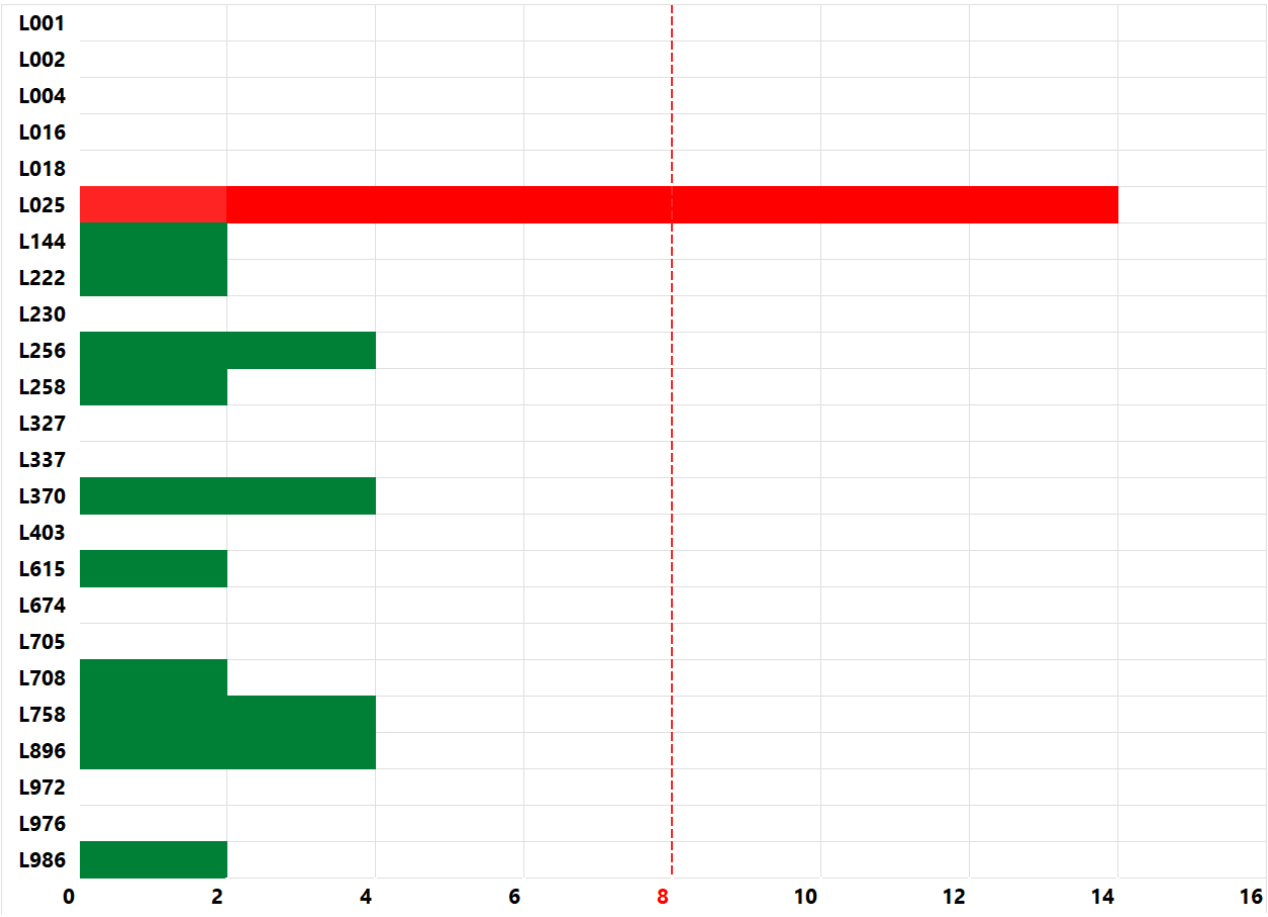
Figure 3. Isolation and genotyping of STEC strains from the sprouts samples (Yellow boxes represent the gold standards; green boxes: correct results and red boxes: incorrect results).



One laboratory reported to have achieved isolation of the STEC contaminating strain only after applying acid treatment.

For each NRL, the number of penalty points was determined using the criteria described in section 3.4. **Figure 4** shows the score achieved by each NRL. Only one laboratory did not comply with the definition of satisfactory proficiency.

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



The calculation of ***Se and Sp* in the screening step** was performed based on the results provided by 23 out of the 24 participating NRLs. The results reported by L025 were excluded, as an inversion of samples 1 and 2 was suspected.

Table 2. Sensitivity and Specificity of the method.

	Se	***Sp***
stx1	91.3%	100%
stx2	NA	100%
eae	NA	100%
***wzx*$_{O104}$**	84%	NA

The calculation of ***Se* in the isolation step** was based on the results provided by the 21 NRLs that detected the presence of STEC in the screening step but the L025, due to the samples swap. The sensitivity of the isolation step was 76.2%.

The Limit of detection (LOD) of the isolation step returned the results reported in Table 3.

Table 3. 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		0,000	0,272	24,150	14,017	41,609	104,375	60,580	179,830	0,000
Combined data		0,000	0,272	24,150	14,017	41,609	104,375	60,580	179,830	0,000

5. 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.

Reg. (EU) 209/2013 prescribes the absence of STEC O157, O26, O103, O145 and O104:H4 in sprouts to be consumed as raw and allows testing laboratories to analyze the spent irrigation water from the production process to assess the conformity to the microbiological criterion of the end product. This PT allowed the evaluation of the performances of the ISO TS 13136:2012 with the procedure developed by the EURL-VTEC for the treatment of spent irrigation water applied to samples of this matrix contaminated with STEC O104. The analytical results, provided by 24 laboratories, confirmed the suitability of the treatment procedure for spent irrigation water, based on a simple centrifugation step and increased enrichment temperature (41.5°C instead of 37°C), as the contaminating STEC strain was isolated from the spiked sample by 15 laboratories (76.2%) out of the 21 NRLs that could detect a positive signal in the screening step.

The analysis of the results provided by 24 Laboratories participating in PT40 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 sample.

3. Four laboratories didn't report the presence of the *wzx*$_{O104}$ gene in the screening: this might be the result of a failure in the detection of such gene or because of screening the samples for the virulence genes only. This represents a diversion from the procedure and generates penalty points, nevertheless two out of these four laboratories were able to characterize the isolated strain as belonging to O104 serogroup.
4. The majority of the laboratories could isolate the STEC from sample 2, while about 23.8% of them could not. This result relates to the limit of detection of the procedure (LOD_{50}) when used to this type of spent irrigation water, which has been estimated at 24 CFU/ml. One laboratory reported that isolation was only achieved through acid treatment.
5. Only one participating laboratory (L025) presented a non-satisfactory performance, probably due to the exchange of the two samples.
6. As in the other PT rounds, the performance parmaters calculated in PT40 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 for the purpose of the method accreditation.