Report of the 46th inter-laboratory study on the detection of Shiga toxin-producing *E. coli* (STEC) in leafy vegetables (PT46) - 2026

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1. OBJECTIVES OF THE STUDY

The study consisted in the detection and isolation of STEC in leafy vegetables samples and the **objectives** were:

- to improve the preparedness of the NRLs towards the detection and isolation of STEC strains in vegetables samples;
- to give further support to the NRLs for the accreditation of the ISO TS 13136:2012 and the EURL-VTEC method 04 for the detection of O104:H4.

This document represents the full evaluation report of the study.

2. PARTICIPANTS

Thirty-seven NRLs from 27 EU Member States, one candidate country and two EFTA Countries participated in the study. In particular, the participants were:

- 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
- Czech Republic,State Veterinary Institute Prague
- Denmark, Danish Veterinary, Food, Agriculture and Fisheries Agency
- Estonia, National Centre for Laboratory Research and Risk Assessment (LABRIS)
- Finland, Finnish Food Safety Authority Evira, Research and Laboratory Department, Food and Feed Microbiology Research Unit
- France, Vetagro Sup
- Germany, Federal Institute for Risk Assessment,
- Greece, Ministry of Rural Development and Food
- Hungary, National Food Chain Safety Office, Food Chain Safety Directorate
- Iceland, Matís ohf./Icelandic Food and Biotech R&D
- Ireland, Department of Agriculture, Food and the Marine
- Italy, Istituto Superiore di Sanità
- Latvia, Institute of Food Safety, Animal Health and Environment (BIOR)
- Lithuania, National Food and Veterinary Risk Assessment Institute
- Luxembourg, Laboratoire Vétérinaire et Alimentaire (ALVA)
- The Netherlands, National Institute for Public Health and the Environment – RIVM

- The Netherlands, Wageningen Food Safety Research WFSR
- Norway, Norwegian Veterinary Institute
- Poland, National Institute of Public Health (NIH)
- Poland, National Veterinary Research Institute (NVRI)
- Portugal, Instituto Nacional de Investigação Agrária e Veterinária
- Romania, Institute for Hygiene and Veterinary Public Health
- Serbia, Institute of Veterinary Medicine
- Slovakia, State Veterinary and Food Institute
- Slovakia, Public Health Authority of the Slovak Republic
- Slovenia, University of Ljubljana Veterinary Faculty, National Veterinary Institute
- Spain, National Plant Health and Hygiene Laboratory
- Spain, Laboratorio Central de Veterinaria de Algete (MAPA)
- Spain, Centro Nacional de Alimentación-AESAN
- Sweden, Swedish Food Agency
- Sweden, National Veterinary Institute
- Switzerland, Agroscope
- Switzerland, University of Zurich

Each Laboratory received its own individual participation report indicating the expected and the reported results after the deadline for submitting the results.

3. MATERIALS AND METHODS

3.1. Sample preparation

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

The salad used has been acquired at retail (ready-to-eat) and contained a natural background microflora of $4x10^4$ bacterial CFU per gram of lettuce ($2x10^4$ CFU of Enterobacteria per gram). The salad was 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 lettuce of the same batch were initially assayed 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 22nd of May 2026, using appropriate dilutions of an exponential liquid culture (0.5 OD read at 600 nm) of the STEC

strain ED0963 (O104:H7) possessing the *stx1* 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 0.125 log CFU/ml was associated with the standardized inoculum, using the procedure described in the ISO TS 19036. A proper dilution of the inoculum suspensions of strain ED0963 was added to the test portions corresponding to sample 2, to obtain the desired spiking concentration (Table 1). The inoculum used for spiking was also plated onto MacConkey agar plates to confirm the titer.

The sets of the test samples were labelled with randomly generated numerical codes different for each participant laboratory and stored at +4°C until shipped refrigerated on 25th of May 2026 by courier. The NRLs were requested to record the date of receipt and sample temperature and to start the analyses immediately upon receipt.

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

Contaminant (*Genotype*)	**Contamination level in:**	
	Sample 1	**Sample 2**
Strain ED0963, STEC O104:H7 *(stx1+, stx2-, eae- aggR-, aaiC-)*	-	50 CFU/25 g

3.2. 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.

The stability was assessed using samples spiked on the 19th of April 2026 and tested by ISO TS 13136:2012 after four, six and seven days since the initial contamination. The Real Time PCR screening was positive for the STEC target genes after 7 days from the spiking. Similarly, isolation of the STEC contaminating strain was successful up to 7 days from spiking.

When the test samples were prepared, six bags for each of the two levels of contamination 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 26th of May 2026. The Real Time PCR screening carried out for the homogeneity tests returned positive results for the STEC target genes in all the contaminated samples only, as expected.

3.3. 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 (EURL VTEC_Method_04_Rev 1: "*Detection and identification of Verocytotoxin-producing Escherichia coli (VTEC) O104:H4 in food by Real Time PCR*").

3.4. Collection and elaboration of the NRL results

The results were submitted through a dedicated website developed by the EURL for *E. coli* and the deadline was set on 15 June 2026.

3.5. Analysis of the NRL results

3.5.1. 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 or missing result for the identification of the O104 serogroup.

3.5.2. 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 isolated STEC strain was also assessed. In detail, 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 gene. Two penalties were assigned to the laboratories for not reporting the information on the serogroup of the STEC strain isolated. Two penalty points were assigned to the labs reporting the H4 flagellar antigen.

Finally, two penalty points were assigned in case of reporting STEC strain's isolation from sample 1.

3.5.3. 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 unsatisfactory (figure 4).

3.6. Evaluation of the performance of the method

Sensitivity (*Se*) and Specificity (*Sp*) were calculated for each of the STEC characteristics considered in the study and for the different steps of the ISO TS 13136:2012. 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

4. RESULTS

Almost all 37 participating Laboratories received the samples in good condition. Two reported to have received them in fair condition and one in bad, as the recorded temperature of the samples was 24°C. Nevertheless, this Laboratory successfully identified the STEC positive sample and could also isolate the contaminating STEC strain.

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

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

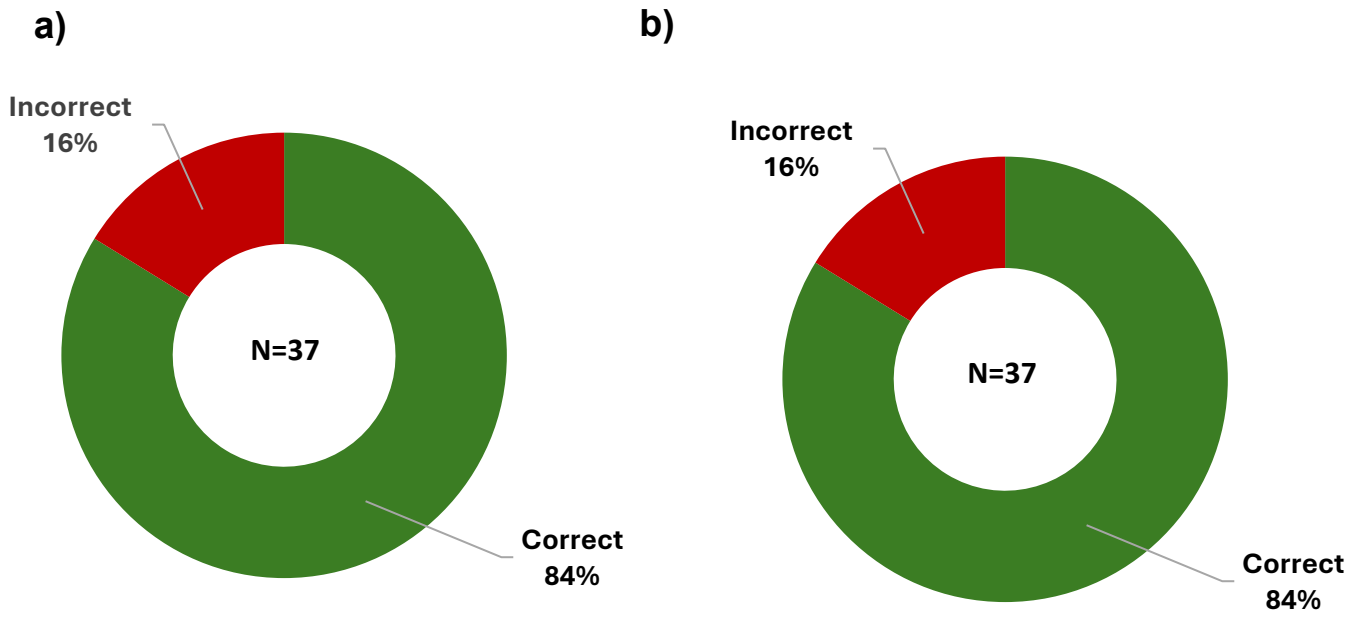


Figure 2. Real-time PCR detection of virulence and serogroup-associated genes in the enrichment cultures (yellow boxes represent the gold standards; green boxes: correct results and red boxes: incorrect results).

	Sample 1		Sample 2
Gold standard	Gold Standard - Negative	Gold standard	Gold Standard - *stx1*, O104
L000		L000	
L001		L001	
L002		L002	
L003		L003	
L004		L004	*stx1*, -
L006		L006	
L014		L014	
L016		L016	
L017		L017	
L018		L018	
L025		L025	
L144		L144	
L209		L209	
L222		L222	
L230		L230	
L256		L256	*stx1*, *eae*, -
L258		L258	
L308		L308	
L327		L327	*stx1*, *eae*, O104
L337		L337	
L370		L370	
L403		L403	negative
L462		L462	
L522		L522	
L615		L615	
L674		L674	negative
L685		L685	
L697		L697	*stx1*, -
L705		L705	
L708		L708	
L758		L758	
L846		L846	
L896		L896	
L972		L972	negative
L976		L976	
L986		L986	
L993		L993	

Figure 3. Isolation and genotyping of STEC strains from the salad samples (Yellow boxes represent the gold standards; green boxes: correct results and red boxes: incorrect results). L674, L972 and L403 had obtained negative results in the screening step. The *fliC* H7 is not covered by the method indicated to be applied by the laboratories, and the results H- or H? as well as blank were not considered as incorrect.

	Sample 1
Gold standard	Gold Standard - Not done
L000	
L001	
L002	
L003	
L004	
L006	
L014	
L016	
L017	
L018	
L025	
L144	
L209	
L222	
L230	
L256	
L258	
L308	
L327	
L337	
L370	
L403	
L462	
L522	
L615	
L674	
L685	
L697	
L705	
L708	
L758	
L846	
L896	
L972	
L976	
L986	
L993	

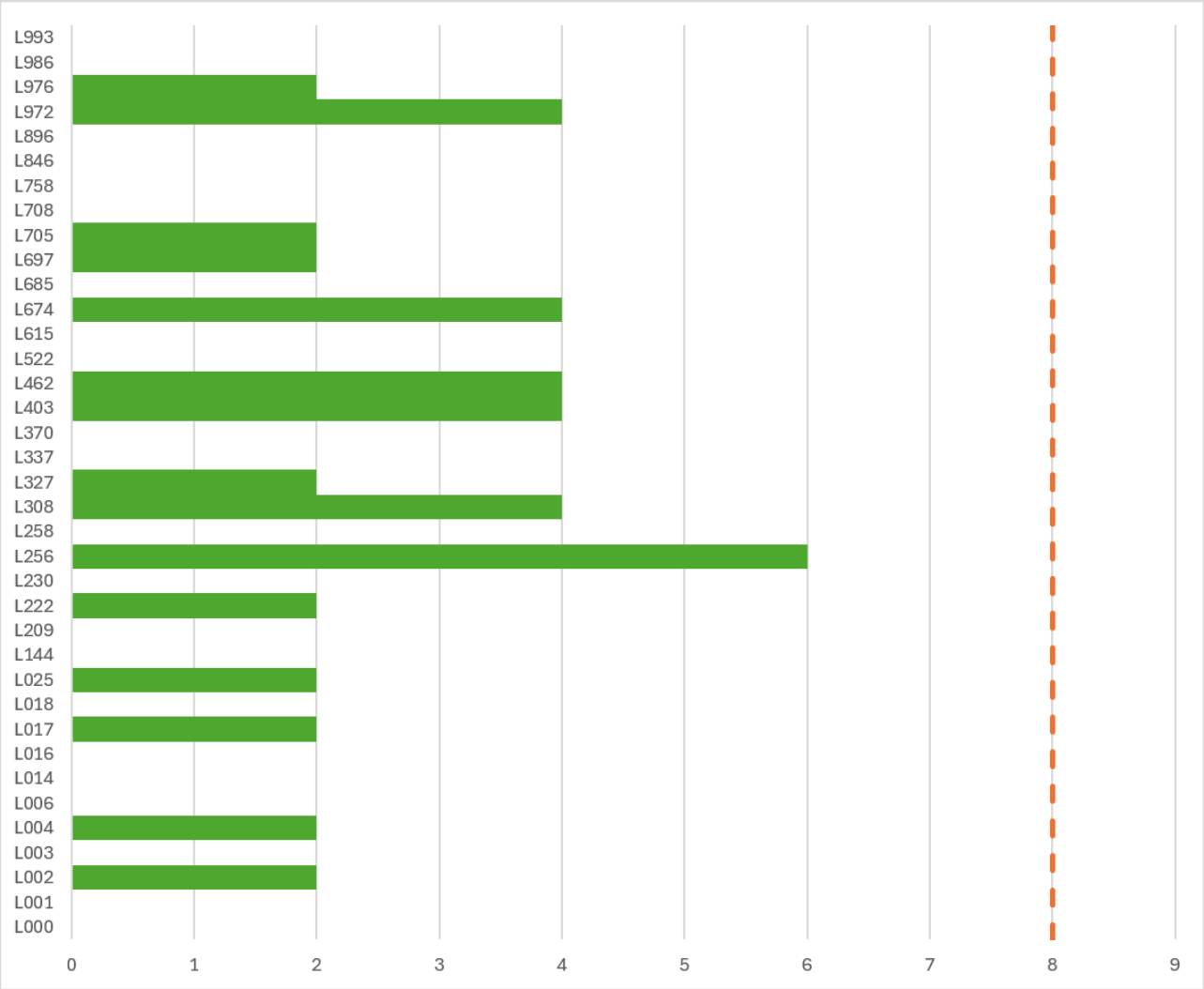
	Sample 2
Gold standard	Gold Standard - *stx1*, O104:H7
L000	
L001	
L002	
L003	
L004	
L006	
L014	
L016	
L017	Isolation not achieved
L018	
L025	*stx1*, O104:H4
L144	
L209	
L222	Isolation not achieved
L230	
L256	*stx1*, *eae*, O104:H7
L258	
L308	*stx1*, O104:H4
L327	
L337	
L370	
L403	Isolation not achieved
L462	-, O104
L522	
L615	
L674	Isolation not achieved
L685	
L697	
L705	*stx1*, O104:H4
L708	
L758	
L846	
L896	
L972	Isolation not achieved
L976	Isolation not achieved
L986	
L993	

All the participants correctly reported sample 1 as negative, and all but three laboratories correctly detected sample 2 as contaminated by STEC.

Concerning the isolation, 31 out of the 34 participants detecting *stx1* gene have been able to isolate the STEC contaminating strain from sample 2. Three laboratories reported the strain as O104:H4, whereas it was of serotype O104:H7. As a matter of fact, a few participants reported the detection of *stx*-negative *E. coli* positive for *fliC* H4 in sample 2, which could be the reason for the detection of H4 from the three laboratories, reporting the detection and isolation of STEC O104:H4 strain. It must be noted, however, that the EURL-VTEC Method 04 describes the determination of H4 only in isolated strains and should not be applied in the screening step, and requires the isolation of a pure bacterial culture for the determination of the *fliC* gene.

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. The three laboratories identifying both samples as negative for STEC will be contacted for follow-up. As shown in figure 4, all the laboratories complied with the definition of satisfactory proficiency. Laboratory L327 has been assigned 2 penalty points for the detection of *eae* in the screening step, according to the gold standard results obtained at the EURL for *E. coli*. Nevertheless, L327 reported to have isolated an *E. coli* positive for *eae* gene, in addition to the *stx1* O104 isolate. Considering that the salad used for PT46 contained a natural background flora and that the contamination may not be homogeneous, the presence of an EPEC strain cannot be excluded. Laboratory L308 reported “Yes” for the isolation from sample 1, accumulating two additional penalty points. However, this Laboratory had correctly reported negative results at the screening step and did not report any genotypic characteristics for the isolated strains, suggesting a mistake in reporting.

Figure 4. Evaluation of the NRLs performance in the PT procedures (screening and isolation steps). A total of 8 penalty points is the threshold set for unsatisfactory results.



The calculation of ***Se* and *Sp* in the screening step** was performed based on the results provided by all 34 participating NRLs which detected *stx1* in sample 2 and is reported in Table 1.

	Se	***Sp***
stx1	100%	NA
stx2	NA	100%
eae	NA	94.1%
O104	94.1%	NA

Table 1. Sensitivity and Specificity of the screening step.

The ***Se* in the isolation step** was 91.9% in sample 2. The Limit of detection (LOD_{50}) of the isolation step was not evaluated.

5. CONCLUDING REMARKS

The analysis of the results provided by 37 Laboratories participating in PT46 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 high sensitivity in the spiked sample.
3. Almost all the laboratories could isolate the STEC from sample 2
4. None of the participating Laboratory presented a non-satisfactory proficiency according to the parameters specified in the EURL procedure. However the three laboraotries that did not identify the contamination in both the samples shipped, will be contacted for a follow-up.
5. The performance parameters calculated in PT46 will be added to those already determined for other couples matrix/STEC strains 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.