EU Reference Laboratory for *E. coli*
Department of Food Safety, Nutrition and Veterinary Public Health
Unit of Microbiological Food Safety and Foodborne Diseases
Istituto Superiore di Sanità

Report of the 39th inter-laboratory study on the detection of Shiga toxin-producing *E. coli* (STEC) in cheese (PT39) - 2024

Edited by:

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

1. INTRODUCTION

PT39 consisted in the detection of STEC in cheese samples, with the aim of consolidating the preparedness of the NRLs in testing such matrix for the presence of STEC. The PT was organized according to the International Standard ISO/IEC 17043:2010 “Conformity assessment – General requirements for proficiency testing”, and this document represents the full evaluation report of PT39.

2. DESIGN AND OBJECTIVES OF THE STUDY

The study consisted in the examination of artificially contaminated cheese samples and the **objectives** were:

- to improve the preparedness of the NRLs towards testing cheese samples for the detection and isolation of STEC according 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 support to the NRLs for the accreditation of the ISO TS 13136:2012.

3. PARTICIPANTS

Thirty laboratories consisting in twenty-seven NRLs from 22 EU Member States and three NRLs from EFTA Countries, participated in the study. The participating laboratories are listed below:

- Austria, Institut für medizinische Mikrobiologie und Hygiene (AGES)
- Belgium, SCIENSANO – Foodborne Pathogens Service
- Bulgaria, National Diagnostic and Research Veterinary Medical Institute (NDRVMI)
- Cyprus, Laboratory for the Control of Food of Animal Origin (LCFAO), Cyprus Veterinary Services
- Czech Republic, State veterinary institute Prague
- 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 Section
- France, VetAgro Sup Campus Vétérinaire LMAP/LNR E. coli STEC
- Germany, Bundesinstitut für Risikobewertung
- Hungary, National Food Chain Safety Office, Food Chain Safety Laboratory 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 Food Safety
- Portugal, Laboratório de Microbiologia dos Alimentos, Instituto Nacional de Investigação Agrária e Veterinária, I.P.
- Slovakia, Public Health Authority of the Slovak Republic
- Slovakia, State veterinary and food institute, SVFI Dolny Kubin
- Slovenia, University of Ljubljana, Veterinary faculty, National Veterinary Institute
- Spain, Laboratorio Central de Veterinaria, Algete-Ministerio de Agricultura, Pesca y Alimentación - MAPA
- Spain, AESAN OA. Centro Nacional de Alimentciòn. Servicio de Microbiologìa Alimentaria I (MA)
- 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 per each sample.

4. MATERIALS AND METHODS

4.1. Sample preparation

The cheese was purchased from a local retailer. The presence of natural background microflora was evaluated by plating on TSA and MacConkey agar serial dilutions of 25 gr of cheese homogenized in 225 ml of Buffered Peptone Water (BPW). No growth was observed on both media. Two samples consisting of 25 g of cheese have been 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.

Two test samples (1 and 2), each consisting of 25 g of cheese potentially contaminated with STEC, were sent in the blind to the NRLs. The characteristics of the contaminated samples are reported in Table 1.

The artificial contamination of the samples was carried out on 17 May 2024, using appropriate dilutions of an exponential liquid culture (0.5 OD read at 600 nm) of the STEC strain C1188-02. An uncertainty of measurement of 0.209 log CFU/ml was associated to the standardized inoculum, calculated using the procedure described in the ISO TS 19036:2006. Serial dilutions of the inoculum suspensions of strain C1188-02 used to spike the samples were streaked onto MacConkey agar plates to confirm the titer.

The test samples were labeled with randomly generated numerical codes, different for each laboratory, and stored at 4°C until shipped refrigerated on 20 May 2024 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 cheese samples assessed in the study

Contaminant (*Genotype*)	**Contamination level in:**	
	Sample 1	**Sample 2**
Strain C1188-02, STEC O26:H11 *(stx1+, stx2+, eae+)*	-	2 CFU/25 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.

Stability was assessed using samples spiked on 13 February 2024 and tested by ISO TS 13136:2012 after 0, 2, 6, and 9 days since the initial contamination. The Real Time PCR screening for the STEC target genes and isolation were both successful for all the samples spiked at all the time points.

Six specimens were randomly selected for homogeneity testing among the spiked samples prepared for shipment. All were enriched at 37°C and analyzed by Real Time PCR for the presence of the contaminating STEC on 20 May 2024. The Real Time PCR screening for

the STEC target genes was positive, and the O26 STEC strain was isolated from all six replicates. Similarly, all the six replicates selected from non-spiked samples were negative for the presence of STEC.

4.3. Laboratory methods

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

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. Scoring of the Real Time PCR screening step

The results reported were categorized by assigning penalty points. Four penalty points have been assigned to each incorrect or missing result concerning the identification of *stx1* and *stx2* genes, and two penalty points for the incorrect identification of *eae* gene as well as the top-5 serogroups-associated genes.

4.5.2. Scoring of the isolation and characterization of the STEC strains isolated from the PCR-positive enrichment cultures

The results reported were categorized by assigning two penalty points 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, two penalties to lack of identification of *eae* gene, and two for the identification of a serogroup different from that of the STEC strain used to contaminate the sample (O26).

4.5.3. Evaluation of the NRL performance

The sum of the penalty points obtained in the different steps of the procedure originated a total score, used to evaluate the performance of the NRLs in the PT. Laboratories that obtained a score higher than eight were considered underperforming.

4.6. Evaluation of the performance of the method

Sensitivity (*Se*) and Specificity (*Sp*) were calculated for the PCR screening of *stx1*, *stx2* and *eae* genes, and for the isolation of the STEC strain 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).

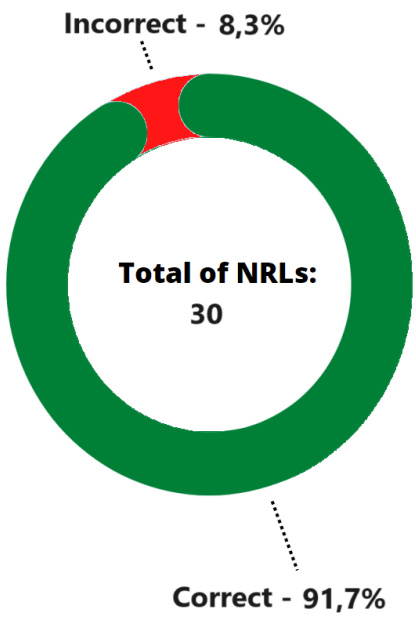
5. RESULTS

All the 30 Laboratories receiving the samples returned results via the web platform.

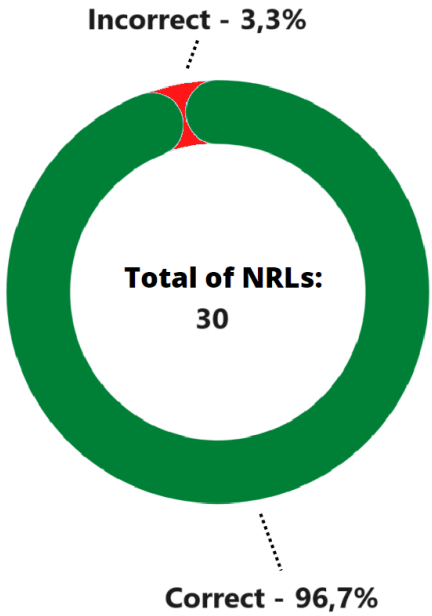
As for the delivery conditions, all the NRLs received the samples within 24 hours, except for one that received the sample in 48 hours.

The reported temperature at delivery was > 15 °C (17.5 °C) for nine NRLs (L025, L144, L327, L337, L370, L705, L896, L972 and L993). Nevertheless, this did not affect the results obtained by these NRLs on the test samples. The results submitted by the participating laboratories are summarized in **Figures 1 – 3**.

Figure 1. Percentage of Laboratories reporting the correct results for the screening of samples (a) and isolation of the STEC strain (b) (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 per laboratory (values in the yellow boxes are the gold standards; green boxes: correct results; red boxes: incorrect results).

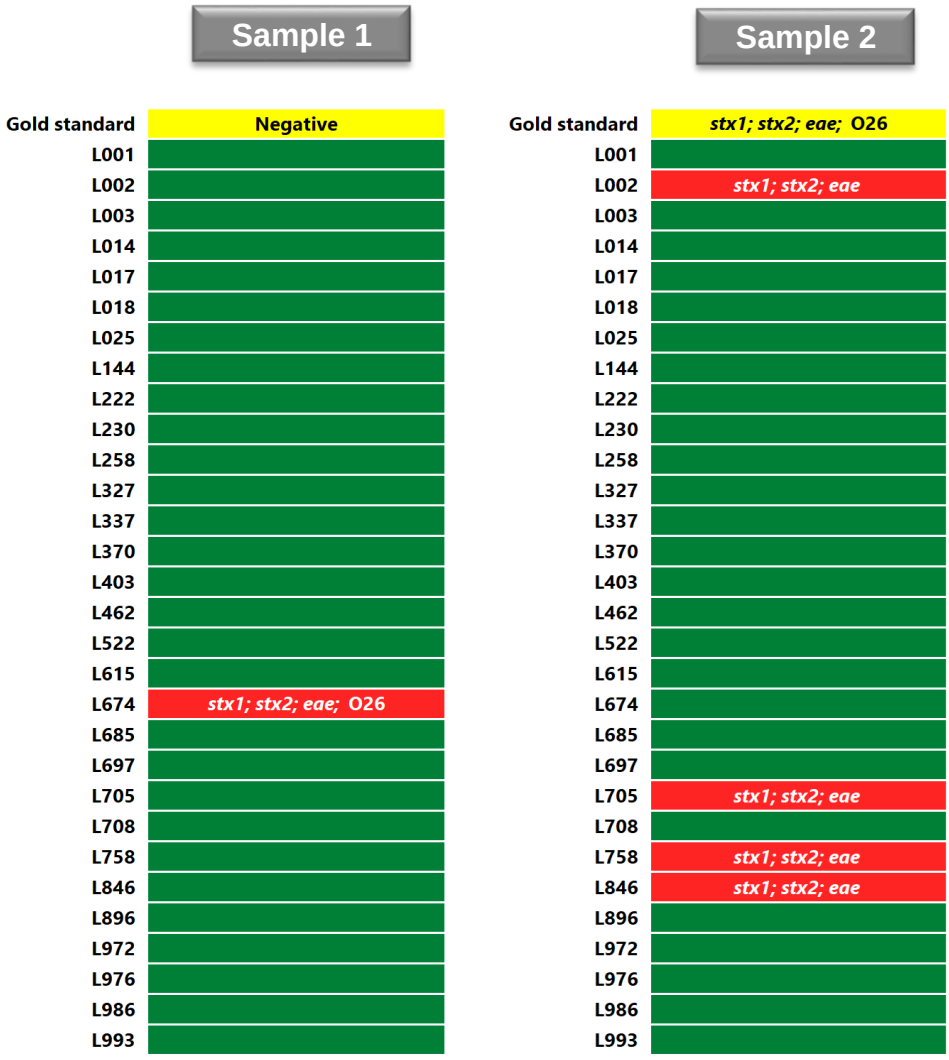
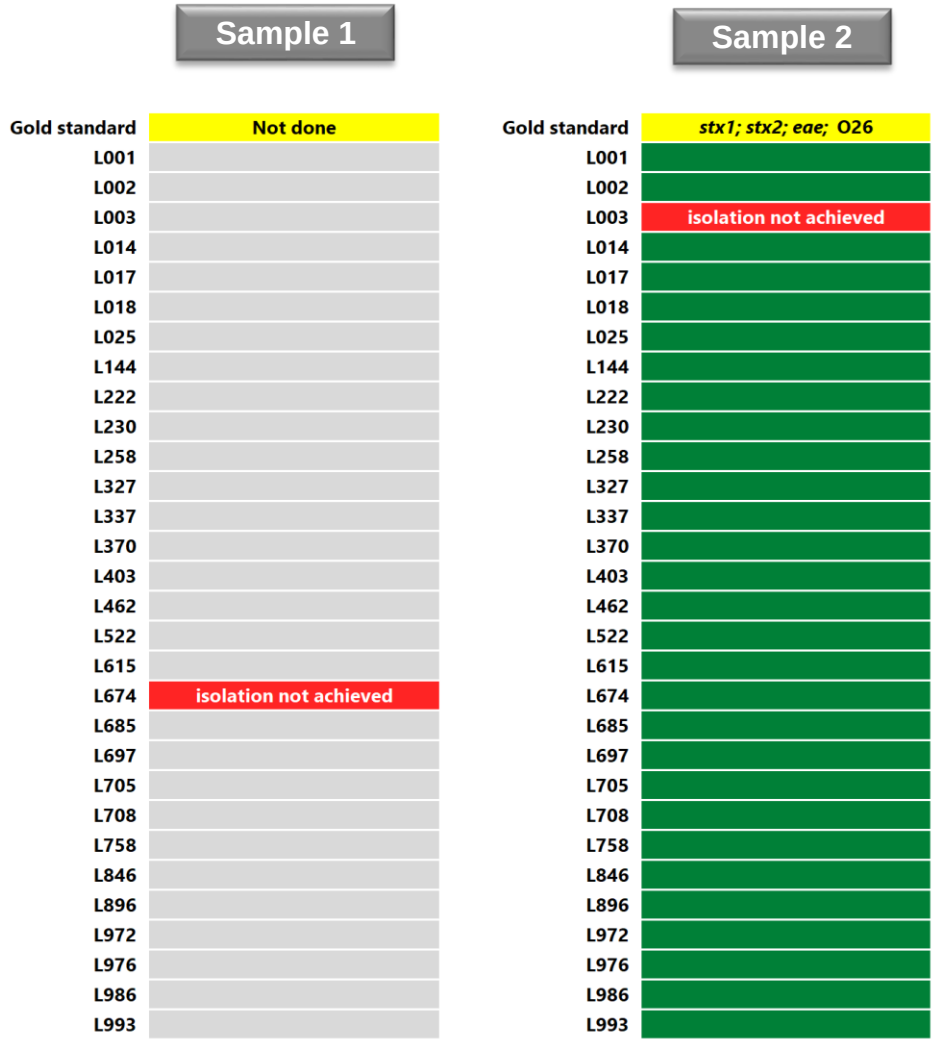
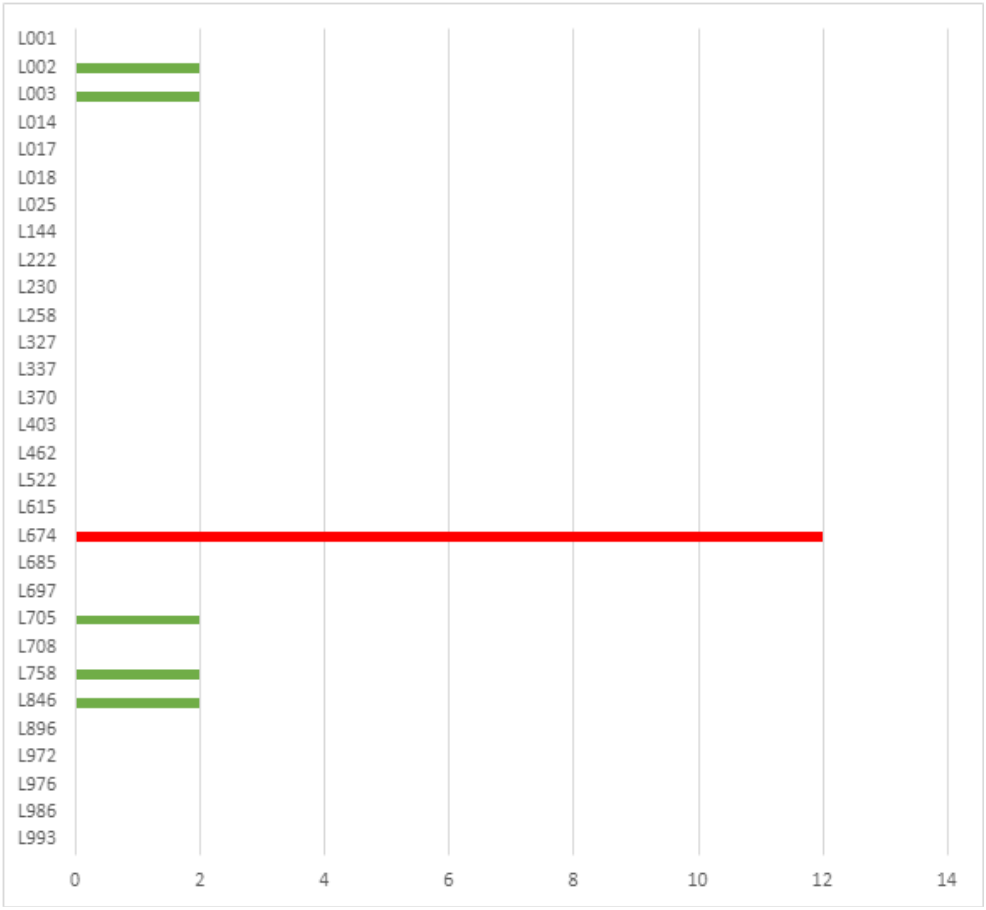


Figure 3. Isolation and genotyping of STEC strains from the cheese samples (values in the yellow boxes are the gold standards; green boxes: correct results; red boxes: incorrect results).



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 one laboratory did not comply with the definition of satisfactory proficiency.

Figure 4. Evaluation of the NRLs performance.



The calculation of ***Se* and *Sp* in the screening step** was based on the results provided by all the 30 participating NRLs.

Table 2. Sensitivity and Specificity of the method in the screening step.

	Se	***Sp***
stx1	100%	96.7%
stx2	100%	96.7%
eae	100%	96.7%

The calculation of ***Se* in the isolation step** was based on the results provided for Sample 2 by all the 30 participating NRLs. The Sensitivity was calculated as 96.7%

Table 3 reports the evaluation of the Limit of detection (LOD).

Table 3. Limit of Detection (LOD) calculated based on PT39 results.

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	Cheese	1,701	0,289	0,016	0,009	0,029	0,070	0,040	0,126	2,301

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.

PT39 concerned the application of the ISO TS 13136:2012 on cheese samples.

The analysis of the results provided by 30 Laboratories participating in PT39 induces the following conclusions:

1. A high participation rate was observed, confirming the consolidation of the network of National Reference Laboratories (NRLs) for *E. coli*;
2. The virulence genes of the contaminating STEC strain were identified with 100% sensitivity in the spiked sample. Four NRLs did not detect O26 serogroup for Sample 2 in the screening step.
3. The majority of the laboratories could isolate the STEC from the spiked sample.
4. Only one participating laboratory presented a non-satisfactory performance, possibly due to contamination of Sample 1 during the analysis, and will be contacted for a follow-up of this underperformance.
5. The results obtained in this PT showed a very low LOD_{50} (0.016 CFU/g), as 96.7% of the participating laboratories could isolate the contaminating STEC strain. However, the lack of contaminant microflora in the cheese probably contributed to this value.
6. As in the other PT rounds, the performance parmaters calculated in PT39 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.