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

Edited by:

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

1. INTRODUCTION

PT37 consisted in the assessment of 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

Three non-EU NRLs participated in the study:

- Egypt, The Central Laboratory of residues analysis of pesticides and heavy metals in food
- UK, United Kingdom Health Security Agency (UKHSA), Food, Water & Environmental Microbiology - London
- UK, United Kingdom Health Security Agency (UKHSA), Food, Water & Environmental Laboratory - Porton

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

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 participating laboratories 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 in the contaminated samples, whereas the blank samples tested negative for STEC.

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 samples 2 and 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.

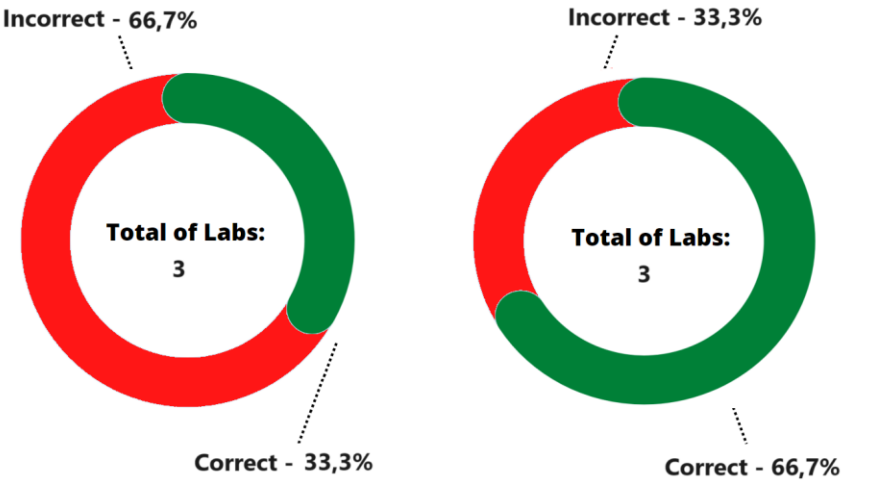
5. RESULTS

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

As for the delivery conditions, L027 received the parcel in 24 hours, L984 in 48 hours and L563 in five days. The reported temperature at delivery for each NRL was 11.4 °C for L027, 32 °C for L563 and 3.9 °C for L984.

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).



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

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).

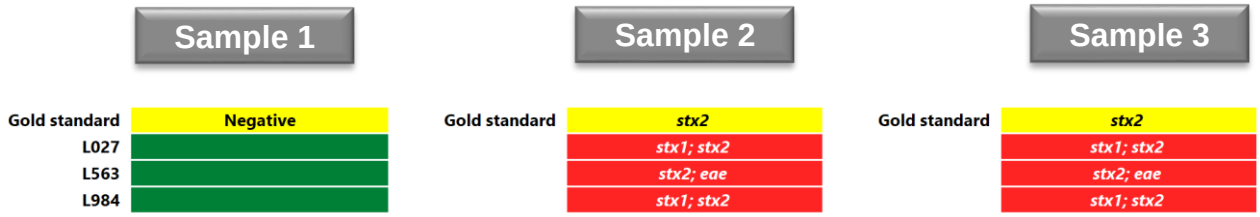
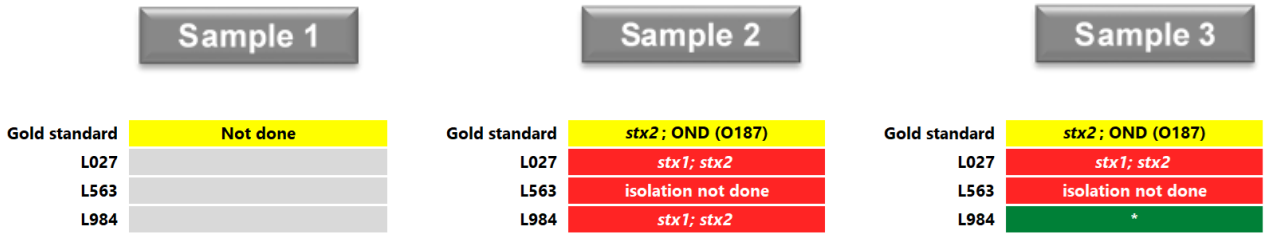


Figure 3. Isolation and genotyping of STEC strains from the sprouts samples (Yellow boxes represent the gold standards; green boxes: correct results and red boxes: incorrect results; *: correct identification of O187 serogroup).



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.

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

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.

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

The analytical results provided by three non-EU laboratories participating in PT37 showed that the presence of a contaminating STEC in samples 2 and 3 was correctly identified by all of them at the screening step. Nevertheless, one laboratory (L563) wrongly detected the presence of *eae* gene, while the other two laboratories reported the presence of both *stx1* and *stx2* genes, but explained that the Real Time PCR method they use is not able to discriminate between the two types. One laboratory (L563) did not attempt isolation of the contaminating strain from samples 2 and 3. The other two laboratories could isolate a contaminating STEC strain from both the samples, but were not able to discriminate between *stx1* and *stx2*, declaring that the discrimination is routinely performed through whole genome sequencing (WGS) of the isolated strains at a later time. L984 could perform WGS on the strain isolated from sample 3, correctly identifying both the presence of *stx2* virulence gene and the O187:H28 serotype. Therefore, despite obtaining several penalty points, L027 and L984 proved able to identify and isolate a contaminating STEC strain from both the spiked samples.