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Report of the 34th inter-laboratory study on the detection of Shiga toxin-producing *E. coli* (STEC) in sprout spent irrigation water (PT34) results from non-EU NRLs - 2022

Edited by:

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1. OBJECTIVES AND DESIGN OF THE STUDY

PT34 regarded the detection and isolation of STEC in spent irrigation water. This document represents the full report on the analysis of the results reported by non-EU Countries participating in this study. The participants were requested to apply the same pre-treatment procedure of sprout irrigation water used in PT30 (EURL-VTEC_Method_09, available at the EURL-VTEC website), based on the centrifugation of the spent irrigation water and enrichment carried out at 41.5°C.

The objectives of the study were:

– to optimize the procedure for the pre-treatment of spent irrigation water for the detection of STEC;

– to improve the preparedness of the NRLs towards testing spent irrigation water for the presence of STEC, by applying to the ISO TS 13136:2012;

– to give further support to the NRLs for the accreditation of the ISO TS 13136:2012.

The study consisted in the analysis of sprout spent irrigation water samples containing a STEC strain belonging to one of the serogroups included in the microbiologic criterion laid down by Reg. (EU) 209/2013.

Two samples were sent to the laboratories that accepted to participate. One was spiked with a STEC O157 strain.

2. PARTICIPANTS

Five non-EU Member States participated in the study. Each participant received its own individual laboratory numerical code (Lab code), which is indicated in the result tables.

The laboratories participating in the study were:

- Chile, *Dep. Salud Ambiental, Instituto de Salud Pública de Chile*
- Egypt, Central Laboratory of Residue Analysis of Pesticides and Heavy Metals in Foods (QCAP Lab)
- UK, Health Security Agency, FWE Laboratory, York
- UK, Health Security Agency, FWE Laboratory, London
- UK, Health Security Agency, FWE Laboratory, Porton

3. MATERIALS AND METHODS

3.1. Sample preparation

The spent irrigation water used in the study was obtained from a local sprout producer who

collected the water flowing from the production of red radish sprouts. The water was collected starting at 48 h from the starting of the sprout production process, accordingto the prescriptions of Reg. (EU) 209/2013.

The water specimens contained natural background microflora (about 2 x 10^6 CFU/ml) and were negative at the Real Time PCR screening for the genes target of the method ISO TS 13136. Two samples, each consisting of 200 ml of water in sterile plasticbottles, potentially contaminated with STEC, were sent in the blind to the laboratories.

The artificial contamination of the samples was carried out using dilutions of an exponential liquid culture (0.5 OD at 600 nm) of the STEC O157 strain C210-03. An uncertainty of measurement of 0.24 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 added to the samples were plated onto MacConkey agar plates to check their titer. The set of two samples sent to the laboratories contained 0 and 100 estimated CFU per ml of STEC O157, respectively.

Table 1: Characteristics of the sprout spent irrigation water samples of the study

Contaminant (*Genotype*)	**Contamination level in:**	
	Sample 1	**Sample 2**
C210-03 STEC O157 *(stx1+; stx2+, eae+)*	-	100 CFU/ml

The test samples were spiked on the 17th October 2022, labeled with randomly generated numerical codes different for each NRL, immediately refrigerated and transferred into refrigerated safety packages and sent to the participants on the same day of preparation by courier.

The stability tests showed that all the samples were positive at the Real Time PCR screening after 5 days from the spiking. The characteristics of the samples are reported in **Table 1** and were considered as the gold standard.

The NRLs were requested to record the date of delivery and sample temperature upon reception and to start the analyses immediately.

3.2. Collection and elaboration of the results

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

3.3. Evaluation of the NRLs 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* genes, and two penalty points for the incorrect detection of *eae*. The performance of laboratories that obtained a score higher than eight was considered as unsatisfactory.

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

Two penalty points were assigned in case of lack of isolation of STEC from sample 2 and two penalty points were assigned to laboratories that reported the identification of a serogroup different from that of the STEC strain used to contaminate the samples (O157) .

3.5 Evaluation of the performance of the method

Sensitivity (*Se*) and Specificity (*Sp*) were calculated for the screening and isolation steps, respectively.

Sensitivity: *Se* = [true positives / (true positives + false negatives)] x 100

Specificity: *Sp* = [true negatives / (true negatives + false positives)] x 100

4. RESULTS

Test samples were sent to 5 laboratories and 4 reported the results.

The parcel containing the specimens were sent on the 17th October 2022 and were received by three participants on the 18th-19th of October, while the samples were delivered to the remaining two labs on the 21th-23th of October (L563 and L987, respectively). As far as the shipment conditions were concerned, the temperature at delivery ranged between 1.0 °C and 12.0 °C for most of the laboratories. Two participants recorded the temperature of the parcel as 18°C and 22°C (L563 and L987, respectively).

The results submitted by the participating laboratories are shown in **Figures 1 - 3**.

Figure 1. Percentage of Laboratories correctly detecting and isolating STEC strain in the spiked sample (green: correct result; red: incorrect result).

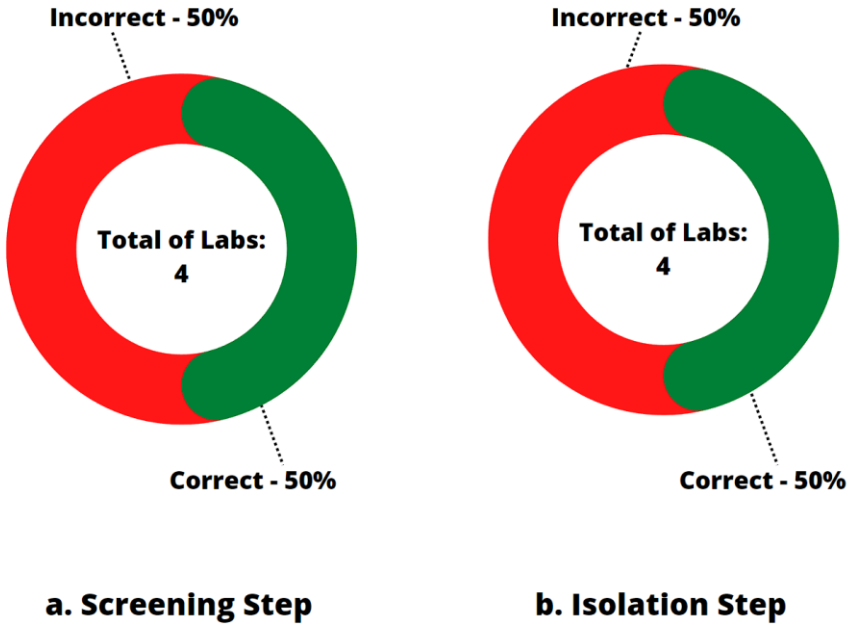


Figure 2. Real-time PCR detection of virulence and serogroup-associated genes in the enrichment cultures (yellow boxes represented gold standards; green boxes: correct results and red boxes: incorrect results).

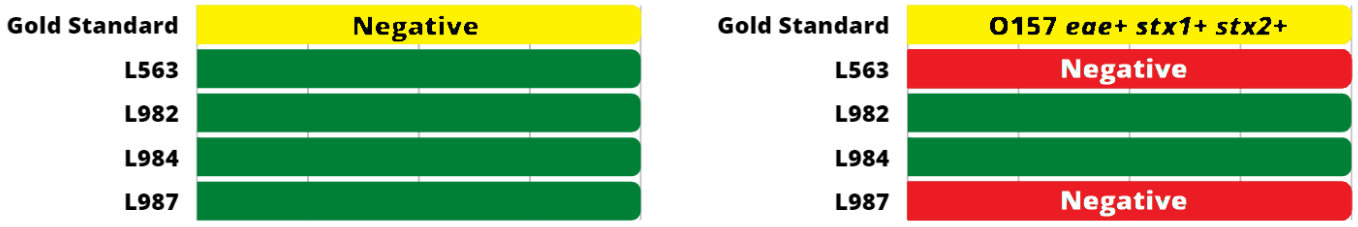
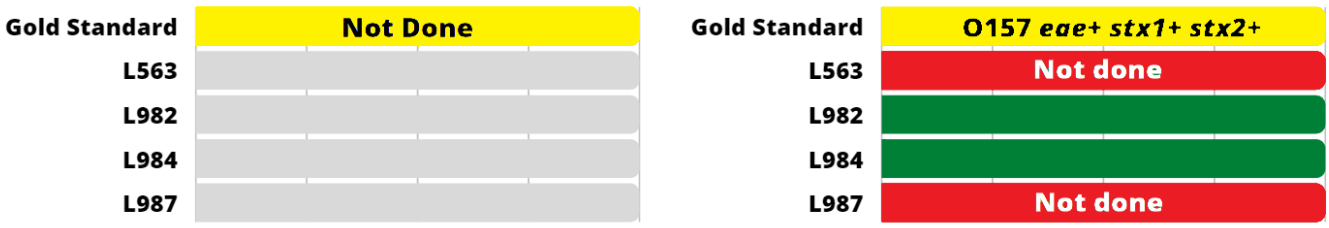
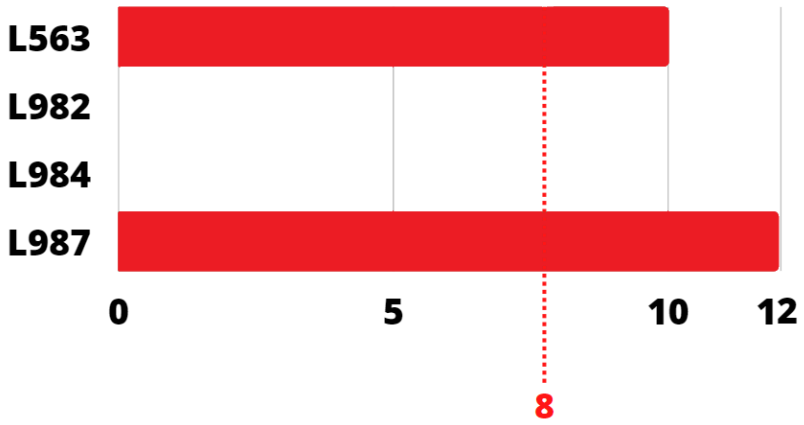


Figure 3. Isolation and genotyping of STEC strains from the spent irrigation water. (Yellow boxes represented gold standards; green boxes: correct results and red boxes: incorrect results).



For each NRL, the number of penalty points was determined using the criteria described in sections 3.3 - 3.4. **Figure 4** shows the score achieved by each NRL. 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 score was calculated according to the criteria described in sections 3.3 - 3.4. Two laboratories showed unsatisfactory performance (red bars).



The calculation of ***Se* and *Sp* in the screening step** returned the following results:

	Se	***Sp***
stx1	67.7%	100%
stx2	67.7%	100%
eae	67.7%	NA
***rfbE*$_{O157}$**	67.7%	NA

The ***Se* of the isolation step** has been calculated as **100%,** evaluated on the basis of the results provided by 2 laboratories detecting STEC in the screening.

5.CONCLUSIONS

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 the producers and the testing laboratories to analyze the spent irrigation water from the production process to assess the compliance to the microbiological criterion of the end product. Spent irrigation water is a problematic matrix

for the verification of the presence of STEC and there are no established procedures for the treatment of such samples that ensure the quality of the results obtained with the official method ISO TS 13136:2012. The EURL-VTEC developed a procedure for the treatment of this peculiar matrix and evaluated the performances of the ISO TS 13136:2012 applied to spent irrigation water samples contaminated with STEC O157.

The analytical results provided by four laboratories participating at the PT34, showed that the virulence genes of the contaminating STEC O157 strain were identified by two laboratories (50% of the participants). The other two participants obtained a score corresponding to a non-satisfactory performance in the detection of STEC virulence and serogroup-associated genes (L563 and L987). Anyway, it has to be considered that these two laboratories received the test samples after 5 and 6 days from the preparation and shipment, at the limit or out of the estimated range of stability explaining, together with the reception of the test samples at high temperatures (18°C and 22°C), the incorrect results reported. Therefore, the penalty points accumulated may not be due to a unsatisfactory performance of these laboratories.