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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) - 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. 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, was applied.

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 assessment of two sprout spent irrigation water samples, with one of them being spiked with a STEC strain belonging to one of the serogroups included in the microbiological criterion laid down by Reg. (EU) 209/2013, following the prescriptions of the same Regulation.

2. PARTICIPANTS

Twenty-five NRLs EU Member States, plus two EFTA countries, participated in the study. Each NRL received its own individual laboratory numerical code (Lab code), which is reported in the result tables.

The NRLs participating in the study were:

- Austria, *Österreichische Agentur für Gesundheit und Ernährungssicherheit* GmbH (AGES)
- Belgium, SCIENSANO - Foodborne Pathogens
- Bulgaria, National Diagnostic and Research Veterinary Institute (NDRVMI)
- Czeck Republic, Hygiene of food and feed
- Denmark, Microbiological laboratory Ringsted
- Estonia, Veterinary and Food Laboratory
- Finland, Finnish Food Safety Authority
- France, *VetAgro Sup*
- Germany, Federal Institute for Risk Assessment
- Hungary, National Food Chain Safety, Microbiological Reference Laboratory
- Iceland, Matís ohf. / Icelandic Food and Biotech R&D
- Ireland, Central Veterinary Research Laboratory, Department of Agriculture, Food and Marine
- Italy, *Istituto Superiore di Sanità*

- Latvia, Institute of Food Safety, Animal Health and Environment (BIOR)
- Lithuania, National Food and Veterinary Risk Assessment Institute (NFVRAI)
- Luxembourg, Laboratoire Nationale de Santé, Departement Protection de la Santé, service surveillance alimentaire
- Netherlands, National Institute for Public Health and the Environment (RIVM)
- Netherlands, Wageningen Food Safety Research (WFSR)
- Norway, Norwegian Veterinary Institute
- Poland, National Institute of Public Health-National Institute of Hygiene, Warsaw
- Portugal, *Instituto Nacional de Investigação Agrária e Veterinária*, Vairão
- Slovakia, Department of Food Hygiene, State veterinary and food institute, Dolný Kubín
- Slovakia, NRC of Environmental Microbiology, Public Health Authority, Bratislava
- Slovenia, Veterinary Faculty/ National Veterinary Institute
- Spain, National Plant Health Laboratory
- Sweden, Swedish Food Agency
- Sweden, National Veterinary Institute (SVA)

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 from 48 h after the beginning of the sprout production process, according to the prescriptions of Reg. (EU) 209/2013.

The water specimens contained a natural background microflora (about 2 x 10^6 CFU/ml) and were negative at the PCR screening for the genes targeted by ISO TS 13136:2012. Two specimens, each consisting of 200 ml of water in sterile plastic bottles, potentially contaminated with STEC, were sent in the blind to the participating laboratories.

The artificial contamination of the samples was carried out on early morning of the 17th October 2022, using dilutions of an exponential liquid culture (0.5 OD read 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, using the procedure described in the ISO/TS 19036:2006. In detail, the set of samples sent to the laboratories contained 0 and 100 estimated CFU/ml of STEC O157, respectively. Serial dilutions of the inoculum suspensions added to the samples were plated onto MacConkey agar plates to check their titer. The stability tests carried out in early October 2022, 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.

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

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

3.2. Collection and elaboration of the results

The results were collected 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.

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* as well as the top-5 and O104 serogroups. 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

The proficiency 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 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 as follows:

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

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

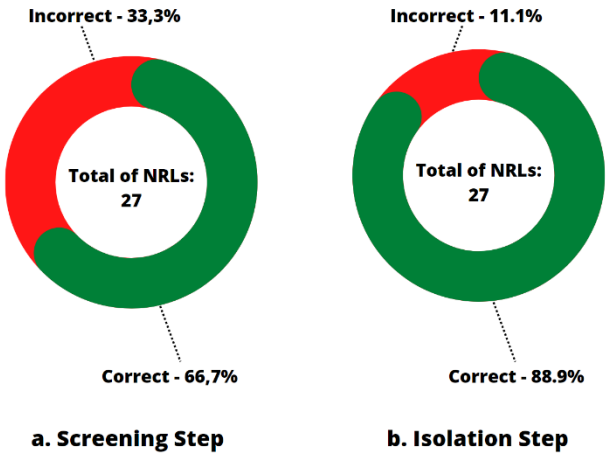
4. RESULTS

Test samples were sent to 27 laboratories and all reported the results.

The parcel containing the specimens were sent on the 17th October 2022 and were received by the majority of participants on the 18th-19th of October at latest. 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.

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

Figure 1. Percentage of Laboratories correctly detecting (a) and isolating (b) the STEC strain contaminating sample 2 (green: correct result; red: incorrect result).



a. Screening Step **b. Isolation Step**

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).

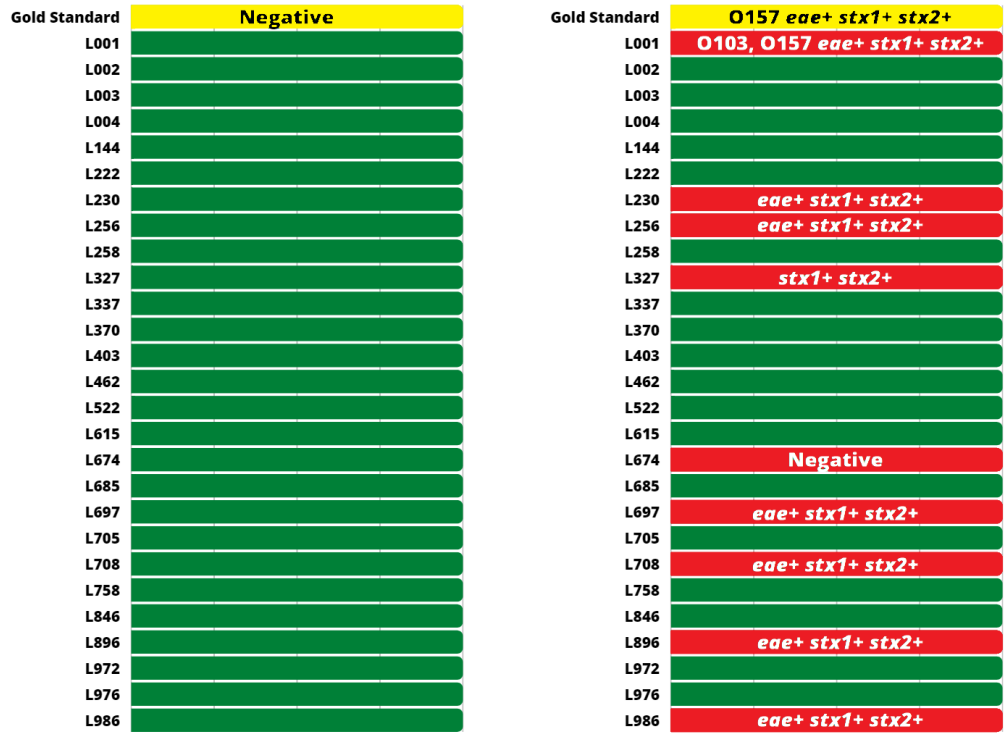
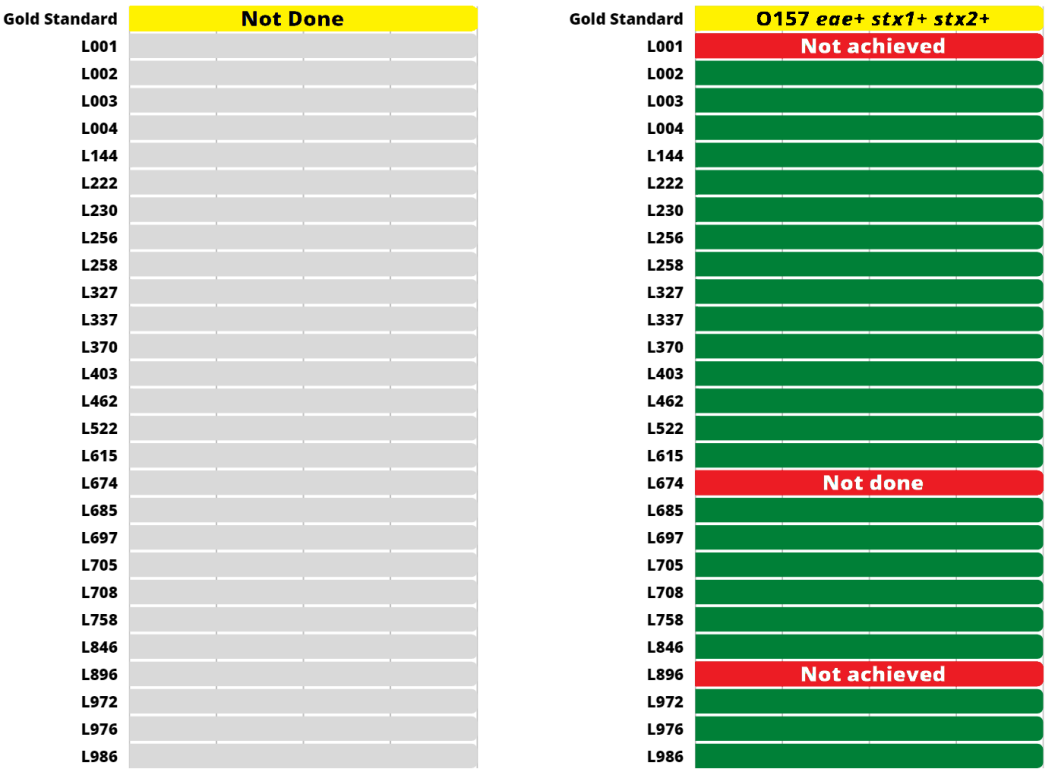
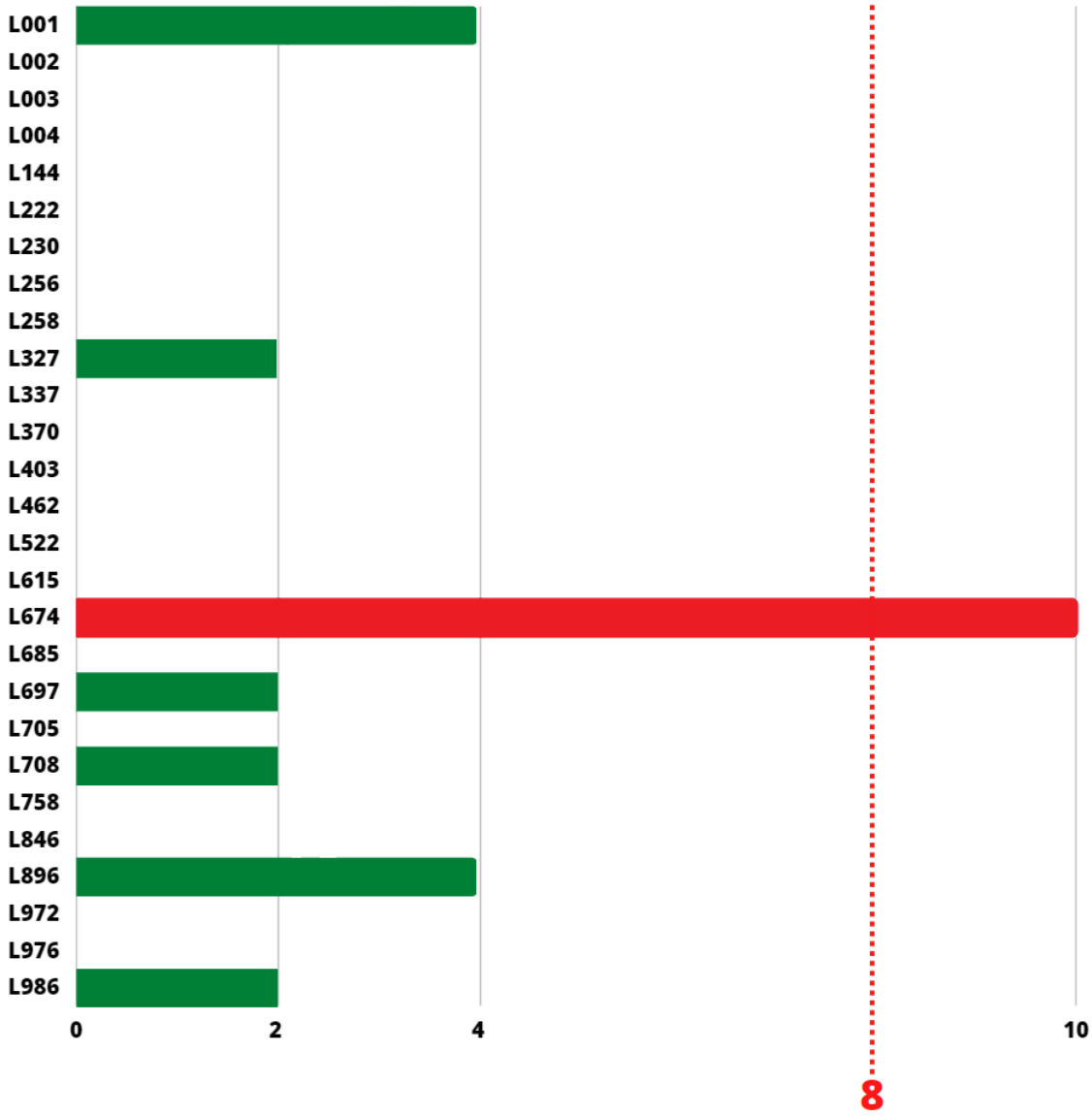


Figure 3. Isolation and genotyping of STEC strains from the spent irrigation water. (Yellow boxes represent the gold standards; green boxes: correct results and red boxes: incorrect results).



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. Only one laboratory did not comply 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** returned the following results:

	Se	***Sp***
stx1	96.4%	100%
stx2	96.4%	100%
eae	96.3%	NA
***rfbE*$_{O157}$**	83.3%	NA

The ***Se* of the isolation step** has been calculated as **92.9%,** evaluated on the basis of the results provided by 26 laboratories.

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 conformity 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 27 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), at least for STEC O157, as the the contaminating STEC strain virulence genes was isolated by 24 laboratories (89%) from the spiked sample.

The overall considerations from PT34 are the following:

1. The virulence genes of the contaminating STEC O157 strain were identified with satisfactory sensitivity in the spiked sample.
2. Many laboratories didn't report the presence of the *rfbE*$_{O157}$ 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. The latter represents a diversion from the procedure and generates penalty points, but the result does not hinder the correct identification of the contaminated sample.
3. One participating laboratory presented a non-satisfactory performance and will be

contacted.

4. STEC O157 was isolated by the majority of laboratories, representing 92.3 % of the participating laboratories detecting STEC.