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Report of the 30th inter-laboratory study on the detection of Shiga toxin-producing *Escherichia coli* (STEC) in sprout spent irrigation water (PT30) - 2021

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1. OBJECTIVES AND DESIGN OF THE STUDY

PT30 was organized with the objective of expanding the observations related with the testing of sprout spent irrigation water, and to check whether the procedure developed in the previous years for testing this matrix was still applicable or if modification must deployed in order to ensure that the compliance with the STEC microbiological criterion set in EU Regulation 2073 can be assessed. The participating Laboratories were requested to carry out the pre-treatment procedure developed by the EURL for *E. coli* and then apply ISO/TS 13136:2012 for detecting the presence of STEC in sprout irrigation water, carrying out the enrichment at 41.5°C. This report presents the analysis of the results reported by the participating Laboratories, including NRLs for *E. coli* in the EU and the Italian Official Laboratories (OLs).

2. PARTICIPANTS

NRLs and Italian Official Laboratories (OLs) were invited to take part to the voluntary inter-laboratory study to expand the number of determinations. A total of 46 Laboratories agreed to participate, and are listed below:

EU-NRLs

1. Austria, *Institut für Medizinische Mikrobiologie und Hygiene Geschäftsfeld Öffentliche Gesundheit* (AGES), Graz
2. Austria, *Institut für Lebensmittelsicherheit* (AGES), Wien
3. Belgium, Foodborne Pathogens Service, Scientific Directorate Infectious Diseases in Humans (Sciensano)
4. Bulgaria, National Diagnostic and Research Veterinary Institute (NDRVMI, BFSA)
5. Cyprus, Laboratory for the Control of Food of Animal Origin (LCFAO), Cyprus Veterinary Services
6. Denmark, Ministry of Food, Agriculter and Fisheries of Denmark, Microbiological laboratory, Ringsted
7. Finland, Finnish Food Authority Laboratory and Research Division (Evira), Microbiology Unit (Food), Helsinki
8. Germany, Federal Institute for Risk Assessment (BfR), Unit Food Technologies, Supply Chains and Food, Defense
9. Ireland, Department of Agriculture, Food and the Marine, Backweston Laboratory Campus
10. Italy, *Istituto Superiore di Sanità*
11. Poland, National Institute of Public Health-National Institute of Hygiene, Warsaw
12. Romania, Institute for Hygiene and Veterinary Public Health
13. Slovakia, Department of Food Hygiene, State veterinary and food institute, Dolný Kubín

14. Slovenia, Veterinary Faculty/ National Veterinary Institute
15. Spain, *Laboratorio Central de Veterinaria, Departamento de Bacteriología-2, Algete* (Madrid)
16. Sweden, Swedish Food Agency/*Livsmedelsverket, Biologiavdelningen*
17. The Netherlands, National Institute for Public Health and the Environment (RIVM)
18. The Netherlands, Wageningen Food Safety Research (WUR)
19. UK, Public Health England, FWE Microbiology Network, Porton
20. UK, Public Health England, FWEM Laboratory, York

Non EU-NRLs

1. Egypt, Central laboratory of residues analysis (QCAP)
2. Iceland, Matís ohf. / Icelandic Food and Biotech R&D
3. Norway, Norwegian Veterinary Institute, Food Bacteriology, Ås

Italian OLs

1. ARPA FVG, *Laboratorio Alimenti e Microbiologia di Udine*
2. *ARPA-BZ, Laboratorio biologico provinciale*
3. *Agenzia di Tutela della Salute (ATS) della Brianza, Laboratorio di Prevenzione, Oggiono*
4. *Agenzia di Tutela della Salute (ATS) della Città Metropolitana di Milano, Laboratorio Prevenzione - Biologia Molecolare*
5. *Azienda USL Toscana Centro, Laboratorio di Sanità Pubblica Area Vasta Toscana Centro, Firenze*
6. *Istituto Zooprofilattico Sperimentale Abruzzo e Molise "G. Caporale", Teramo*
7. *Istituto Zooprofilattico Sperimentale Abruzzo e Molise "G. Caporale", Sezione di Campobasso*
8. *Istituto Zooprofilattico Sperimentale Puglia e Basilicata, Sede di Foggia*
9. *Istituto Zooprofilattico Sperimentale Lombardia ed Emilia Romagna, Brescia*
10. *Istituto Zooprofilattico Sperimentale Lombardia ed Emilia Romagna, Sezione di Bologna*
11. *Istituto Zooprofilattico Sperimentale Lazio e Toscana, Roma*
12. *Istituto Zooprofilattico Sperimentale Lazio e Toscana, UOT Toscana Nord - Sede Pisa*
13. *Istituto Zooprofilattico Sperimentale del Mezzogiorno, Sezione di Fuorni (SA)*
14. *Istituto Zooprofilattico Sperimentale del Mezzogiorno, Portici (NA)*
15. *Istituto Zooprofilattico Sperimentale Piemonte, Liguria e Valle d'Aosta, Laboratorio Controllo Alimenti, Torino*
16. *Istituto Zooprofilattico Sperimentale Piemonte, Liguria e Valle d'Aosta, S.C. Biotecnologie Applicate e Produzioni, Torino*
17. *Istituto Zooprofilattico Sperimentale Piemonte, Liguria e Valle d'Aosta, SS Genova-Portualità*
18. *Istituto Zooprofilattico Sperimentale Piemonte, Liguria e Valle d'Aosta, Sezione di Novara*
19. *Istituto Zooprofilattico Sperimentale della Sicilia, Palermo*
20. *Istituto Zooprofilattico Sperimentale della Sardegna, Laboratorio Microbiologia e Terreni Colturali, Sassari*
21. *Istituto Zooprofilattico Sperimentale Umbria e Marche, Perugia*
22. *Istituto Zooprofilattico Sperimentale delle Venezie, Sezione Territoriale di Pordenone, Cordenons (PN)*
23. *Istituto Zooprofilattico Sperimentale Venezie, Microbiologia generale e sperimentale, Legnaro (PD)*

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

The water specimens contained the background microflora ($4x10^5$ CFU/ml) naturally present and were negative at the PCR screening for the genes targeted by the ISO/TS 13136:2012 method. Beside the bacterial components of the background microflora, the water used in this study was heavily contaminated with protozoa. Also the protozoan contamination was naturally occurring and included different genera of free-living amoebae, such as *Amoeba* and *Naegleria*.

Two specimens, each consisting of 200 ml of water in sterile plastic bottles, potentially contaminated with STEC, were sent in the blind to the laboratories.

The artificial contamination of the samples was carried out on 1st of October 2021, 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 0.27 log CFU/ml was associated to the standardized inoculum, using the procedure described in the ISO/TS 19036:2006. 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 included in the study

Contaminant (*Genotype*)	**Contamination level in:**	
	Sample 1	**Sample 2**
C210-03 STEC O157 *(stx1+, stx2+, eae+)*	50 CFU/ml	-

Stability tests were carried out on spent irrigation water of the same nature but collected during another cycle of sprout production (red radish sprouts, collected after 48 h) using different levels of contaminations and showed that the STEC O157 strain could be isolated from the 50 CFU/ml spiking level after seven days from contamination.

On October the 4th 2021, six bottles of the water collected for the PT30 for each of the two contamination levels were randomly selected and immediately tested for homogeneity according to the PT laboratory procedure. One out of the six selected sample 1 bottles proved negative for the detection of STEC, whereas all the bottles corresponding to sample 2 assayed gave the expected negative results.

The test samples were labeled with randomly generated numerical codes different for each NRL, immediately refrigerated and transferred into refrigerated safety packages that were shipped on 4th October 2021 by courier. The NRLs were requested to start the analyses immediately upon receiving the test samples and to record the date of delivery and sample temperature upon reception.

3.2. Collection and elaboration of the results

The results were submitted directly through a dedicated Microsoft Form.

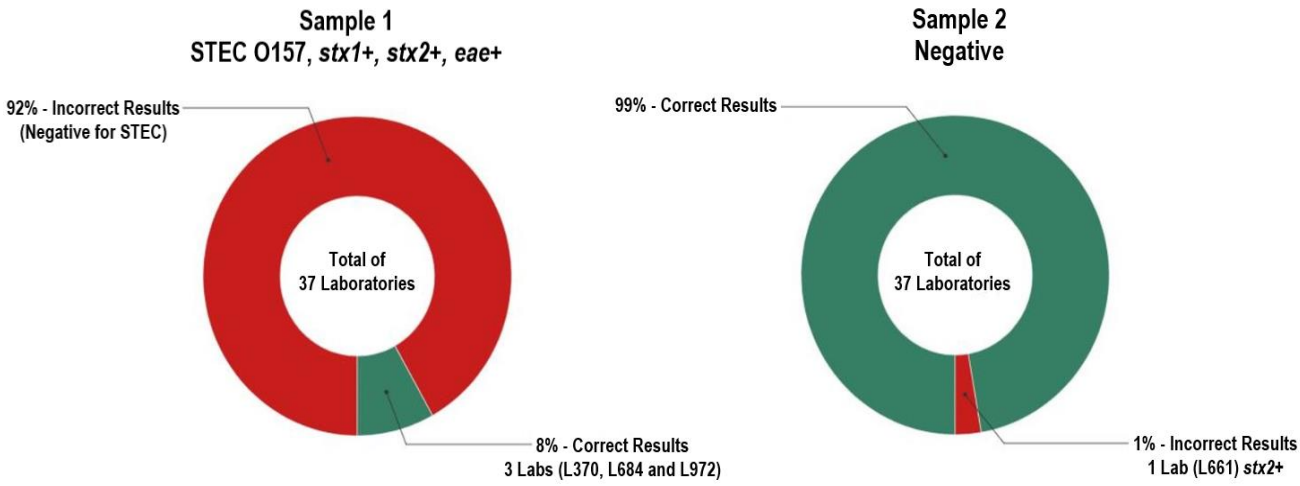
The laboratories were requested to provide in the Form their Lab code, provided in advance to each of the participating laboratories, the information on the arrival date, temperature and quality of the sample, as well as the results obtained for each blind test sample.

4. RESULTS

Test samples were sent to 46 laboratories and 37 returned the results. The parcel containing the specimens sent on the 4th October 2021 were received by the majority of the participants on the 5th October 2021, except two labs which received the samples on 6th October 2021 and 7th October 2021, respectively. As far as the shipment conditions were concerned, the temperature at delivery ranged between 1.2 °C and 13 °C for most of the laboratories. Two participants recorded the temperature of the parcel as 15.3 °C and 16°C.

The results of the screening of the two test samples submitted by the 37 participants are reported in **Figure 1** (Real-time PCR detection of STEC virulence and serogroup-associated genes in the enrichment cultures). Two out of the three labs detecting the presence of STEC in sample 1 could isolate the STEC O157 *eae*+ *stx1*+ *stx2*+ contaminating strain.

Figure 1. Real-time PCR detection of virulence and serogroup-associated genes in the enrichment cultures (Sample 1 and Sample 2). In green the NRLs submitting the correct results, in red the incorrect results.



5. CONCLUSIONS

As with other interlaboratory studies on spent irrigation water, PT30 was not meant to assess the proficiency of the laboratories but it was rather a collaborative study to verify the appropriateness of the procedure for the pre-treatment and analysis of spent irrigation water samples for the presence of STEC. The results obtained in this study confirmed the complexity of the sprout spent irrigation water testing and highlighted the necessity of fine-tuning the pre-treatment procedure. The preliminary testing of the matrix used in this study showed that the water samples contained a large number of free-living amoebae. Such contamination from protozoa may have introduced some hindrances in the conduction of the test for the detection of STEC with the ISO/TS 13136:2012 method. As a matter of fact, experiments carried out at the EURL for *E. coli*, have shown that amoebae, and particularly those belonging to *Acantamoeba* genus are able to internalize pathogenic *E. coli*, as observed with other bacterial pathogens, concealing the target of the PCR and causing the contaminating STEC to escape the diagnostic procedure (https://www.iss.it/documents/5430402/0/NRL+Italy+2021_Montalbano+di+Filippo.pdf/9ac036b4-6941-112c-89ee-64dc4a18f7fd?t=1637750542979).

All the information coming from this study will be used to improve the testing process as we did with other rounds of PT based on this matrix. In particular, EURL for *E. coli* will continue to devote efforts in improving the procedure for testing spent irrigation water.