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

Report of the 22nd inter-laboratory study on the detection of Shiga toxin-producing *E. coli* (STEC) in sprout spent irrigation water (PT22) - 2018

Edited by:

Silvia Arancia, Gianfranco Brambilla, Paola Chiani, Clarissa Ferreri, Fabio Galati, Federica Gigliucci, Antonella Maugliani, Valeria Michelacci, Fabio Minelli, Stefano Morabito, Rosangela Tozzoli

1. OBJECTIVES AND DESIGN OF THE STUDY

The outcome of PT19 run in 2017 on the detection and isolation of STEC in sprout spent irrigation water samples had underlined the need to revise the procedure for testing such a complex matrix. EURL-VTEC prepared a new procedure, indicating to carry out the enrichment step at 41.5 °C instead of 37°C. The purpose of PT22 was the evaluation of the performance characteristics of the procedure elaborated by the EURL-VTEC for testing sprout irrigation water, therefore no assessment of the proficiency of the participating labs was carried out.

2. PARTICIPANTS

NRLs and Italian Official Laboratories (OLs) were invited to take part to the voluntary inter-laboratory study and the 56 Laboratories who agreed were:

EU-NRLs

1. Austria, *Österreichische Agentur für Gesundheit und Ernährungssicherheit* GmbH
2. Belgium, Foodborne Pathogens/Unit Toxins and toxi-infections, Scientific Directorate Infectious Diseases in Humans (Sciensano)
3. Bulgaria, National Diagnostic and Research Veterinary Institute
4. Croatia, Laboratory for food microbiology, Croatian Veterinary Institute
5. Cyprus, Laboratory for the Control of Food of Animal Origin (LCFAO), Cyprus Veterinary Services
6. Czech Republic, Veterinary Research Institute
7. Denmark, National Food Institute, Technical University of Denmark
8. Estonia, Veterinary and Food Laboratory
9. Finland, Finnish Food Safety Authority Evira
10. France, *VetAgro Sup Campus Vétérinaire de Lyon*
11. Germany, Federal Institute for Risk Assessment (BfR), Unit Food Technologies, Supply Chains and Food, Defense
12. Hungary, National Food Microbiological Reference Laboratory
13. Ireland, Veterinary Public Health Regulatory Laboratory, Department of Agriculture, Food and the Marine
14. Italy, *Istituto Superiore di Sanità*
15. Latvia, Institute of Food Safety, Animal Health and Environment (BIOR)
16. Lithuania, National Food and Veterinary Risk Assessment Institute
17. Luxembourg, *Ministère de l'Agriculture, de la Viticulture et de la Protection des consommateurs, Administration des services vétérinaires* (LMVE)
18. Poland, National Institute of Public Health-National Institute of Hygiene, Warsaw
19. Portugal, *Instituto Nacional de Investigação Agrária e Veterinária*, Vairão
20. Romania, Institute for Hygiene and Veterinary Public Health
21. Slovakia, Department of Food Hygiene, State veterinary and food institute, Dolný Kubín
22. Slovakia, NRC of Environmental Microbiology, Public Health Authority, Bratislava

23. Slovenia, Veterinary Faculty/ National Veterinary Institute
24. Spain, *Unidad Microbiología-Centro Tecnológico Agroalimentario de Lugo* (LSA-CETAL)
25. Sweden, National Veterinary Institute (SVA)
26. Sweden, The National Food Agency
27. The Netherlands, National Institute for Public Health and the Environment (RIVM)
28. The Netherlands, Food and Consumer Product Safety Authority (NVWA)
29. UK, Public Health England, FW&E Laboratory, London
30. UK, Public Health England, FWEM Laboratory, Porton
31. UK, Public Health England, FWEM Laboratory, York

Non EU-NRLs

1. Egypt, Central Laboratory of Residue Analysis of Pesticides and Heavy Metals in Foods
2. Iceland, Matís ohf. / Icelandic Food and Biotech R&D
3. Norway, Norwegian Veterinary Institute
4. Russia, State Research Center for Microbiology and Biotechnology, Obolensk
5. Switzerland, Agroscope
6. Switzerland, Institute for food safety and hygiene, University of Zurich

Italian OLs

1. ARPA FVG, *Settore Laboratorio Unico, Laboratorio di Udine, Area Analisi Microbiologiche*
2. ARPA *Lazio, Roma*
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, Sezioni Biologia Molecolare e Microbiologia Clinica, Laboratorio di Prevenzione*
5. *Azienda USL Toscana Centro, Laboratorio di Sanità Pubblica Area Vasta Toscana Centro, Firenze*
6. *Istituto Zooprofilattico Sperimentale Abruzzo e Molise "G. Caporale", Reparto di Igiene delle Tecnologie Alimentari e dell'Alimentazione Animale, Teramo*
7. *Istituto Zooprofilattico Sperimentale Puglia e Basilicata, Sezione di Putignano (BA)*
8. *Istituto Zooprofilattico Sperimentale Reparto Microbiologia, Brescia*
9. *Istituto Zooprofilattico Sperimentale Lombardia ed Emilia Romagna, Sezione di Bologna*
10. *Istituto Zooprofilattico Sperimentale Lazio e Toscana, Laboratorio Biotecnologie applicate alla Sicurezza Alimentare*
11. *Istituto Zooprofilattico Sperimentale Lazio e Toscana, Sezione di Pisa*
12. *Istituto Zooprofilattico Sperimentale del Mezzogiorno, UO Microbiologia degli Alimenti, Sezione di Salerno, Fuorni (SA)*
13. *Istituto Zooprofilattico Sperimentale del Mezzogiorno, U.O.S."Biotecnologie applicate agli alimenti-OGM", Portici (NA)*
14. *Istituto Zooprofilattico Sperimentale Piemonte, Liguria e Valle d'Aosta, SC Genova-Savona con annesso CEROVEC e Coordinamento Liguria, Sezione di Genova*
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, Torino*
17. *Istituto Zooprofilattico Sperimentale Umbria e Marche, Centro di Riferimento Patogeni Enterici CRRPE5, Perugia*
18. *Istituto Zooprofilattico Sperimentale delle Venezie, Cordenons (PN)*
19. *Istituto Zooprofilattico Sperimentale Venezie, Sezione di 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 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 10^6 CFU/ml) and were negative at the PCR screening for the genes that were the target of the method employed in the study. 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 16 November 2018, using dilutions of an exponential liquid culture (0.5 OD read at 600 nm) of the STEC O103 strain C125-06. 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. The characteristics of the samples are reported in Table 1 and were considered as the gold standard.

The stability tests showed that it was possible to isolate the STEC O103 contaminating strain after ten days from the spiking. When the test samples were prepared, 10 bottles for each of the two contamination levels were randomly selected for homogeneity testing and analyzed according to the PT laboratory procedure. All the homogeneity tests gave the expected results.

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

Contaminant (*Genotype*)	Contamination level in:	
	Sample 1	**Sample 2**
C125-06 STEC O103 *(stx2+, eae+)*	50 CFU/ml	-

The contamination of sample 1 was set at such value since it corresponded to the lowest level used in PT19 on the detection of STEC in sprout spent irrigation water run in 2017.
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 19 November 2018 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.

3.3. Laboratory methods

The technical procedure was made available to the participants via the Restricted Area of the EURL-VTEC website and comprised the following sequential steps:

- Centrifugation of the water samples.
- Transfer of the resulting pellet presumptively containing STEC into the enrichment medium. Assuming that contaminant bacteria present in sprouts may have undergone stressing conditions, the laboratories were requested to use buffered peptone water (BPW) as enrichment medium in the proportions 1/10 vol_{pellet} or $weight_{pellet}/vol_{enrichment}$.
- Application of the ISO TS 13136:2012 standard, according to Reg. (EU) 209/2013, to identify the presence of STEC O157, O111, O26, O103, O145 and O104:H4, following the adaptation provided by the EURL-VTEC for the detection of STEC O104:H4 (available in the EURL website, http://www.iss.it/vtec, Laboratory Methods section) with the enrichment step carried out at 41.5 °C instead of 37 °C as reported in the ISO/TS 13136:2012.

3.4. Collection and elaboration of the results

The results were submitted directly through an on-line system, using a dedicated page in the Restricted Area of the EURL-VTEC website.

The laboratories received their own user ID and password for the log-in for the submission of the results. After the log-in, they had access to a dedicated section for submitting the test results. This section also contained a *Shipment form* with the list of the samples to be analyzed and the fields to collect the information on the arrival date, temperature and quality of the sample, and the possibility to write notes to specify any problem with the sample delivery/packaging.

A few days after the deadline for submitting the results, the participants could print their own instant generated individual reports, containing the submitted and expected results, directly from the secure page of the EURL-VTEC website.

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 56 laboratories and 52 returned the results.

The parcel containing the specimens were sent on the 19 November 2018 and were received by the participants on the 21st of November at latest, except one lab which received the samples on the 23rd of November and could start the analysis only on the 26th (L776). One laboratory didn't fill in the shipment form. As far as the shipment conditions were concerned, the temperature at delivery ranged between 1.2 °C and 8 °C for most of the laboratories. One participant recorded the temperature of the parcel as 14.3 °C and another one received the test samples at room temperature (L776).

The results submitted by the participating laboratories are reported in Table 2 (Real-time PCR detection of STEC virulence and serogroup-associated genes in the enrichment cultures) and Table 3 (Isolation and characterization of the contaminating STEC strain).

Table 2. Real-time PCR detection of virulence and serogroup-associated genes in the enrichment cultures. Green boxes: correct results, red boxes: incorrect results

NRL	Detection of virulence and serogroup-associated genes in:							
	Sample 1				Sample 2 Low level contamination			
	stx1	*stx2*	*eae*	*wzx*$_{O103}$	*stx1*	*stx2*	*eae*	*wzx*$_{O103}$
True value	-	+	+	+	-	-	-	-
L109								
L144								
L257								
L283								
L288								
L295								
L296								
L300								
L307		-						
L319								
L323								
L341								
L350								
L351								
L355								
L391		-				+	+	+
L400		-						
L429								
L439								
L441		-						
L446		-						
L504								
L521								
L542		-						
L576								
L590								
L598		-				+	+	+
L599								
L609								
L636								
L649								
L662								
L683								
L689								
L776	+			-				
L783								
L789								
L802								
L803								
L813								
L821		-						
L825								
L905								
L906		-						
L920		-						
L929		-						
L935								
L940								
L948								
L970								
L980								
L997								

Table 3. Isolation and genotyping of STEC strains from the spent irrigation water.

Green boxes: correct results, red boxes: incorrect results, orange box: test not done

STEC strain isolation and genotyping from:					
NRL	Sample 1				Sample 2
	STEC O103 Isolation	Genotype			-
		stx1	*stx2*	*eae*	
True value	+	-	+	+	None
L109					
L144					
L257					
L283	-				
L288					
L295					
L296					
L300					
L319					
L323					
L341					
L350					
L351	-				
L355	-				
L391					O103, vtx1-, vtx2+, eae+
L429	ND				
L439	-				
L504	-				
L521					
L576	-				
L590					
L598					O103, vtx1-, vtx2+, eae+
L599	-				
L609					
L636					
L649	-				
L662					
L683					
L689	-				
L776	-				
L783					
L789					
L802					
L803	-				
L813	-				
L825	-				
L905					
L935	-				
L940					
L948					
L970					
L980					
L997					

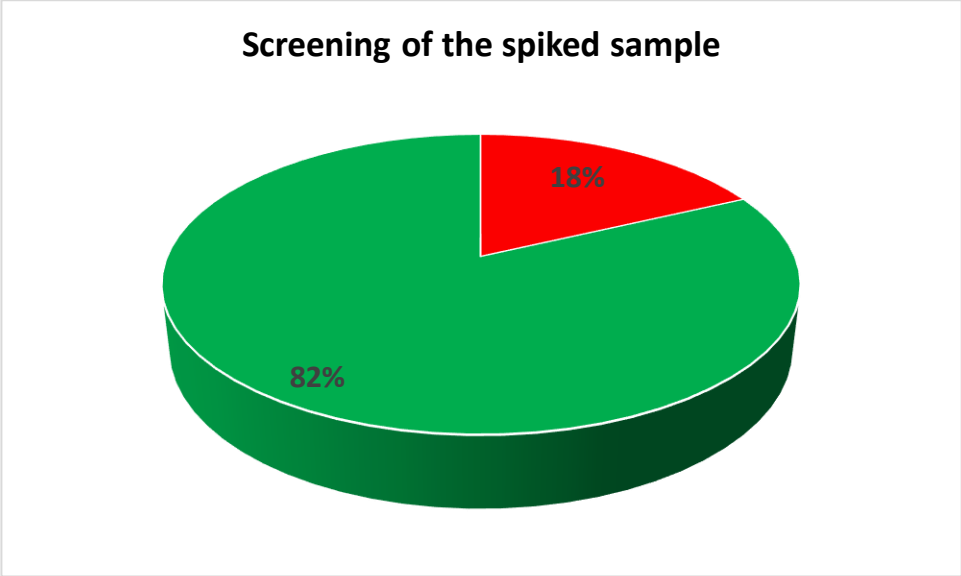

Figure 1. Screening step: Percentage of Laboratories correctly detecting STEC in the spiked sample (green: correct result; red: incorrect result). L391 and L598 have been excluded from this analysis.

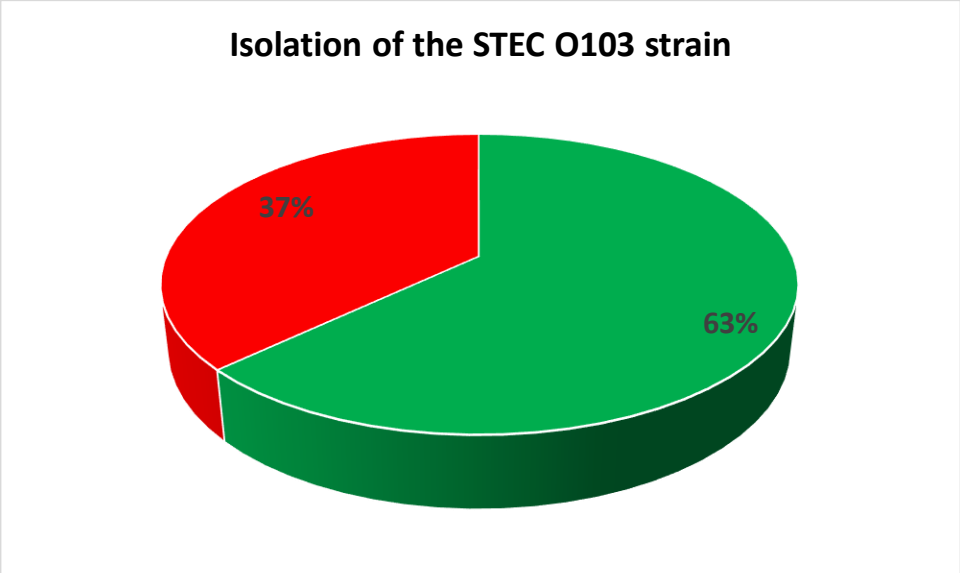

Figure 2. Isolation step: Percentage of laboratories that successfully isolated the STEC strain detected in the screening step (green: correct result; red: incorrect result). L391 and L598 have been excluded from this analysis.

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

	Se	***Sp***
stx1	N.A.	99 %
stx2	84.7 %	100 %
eae	100 %	N.A.
***wzx*$_{O103}$**	93.3 %	N.A.

Two laboratories, L391 and L598, have been excluded from this analysis, since there was a clear exchange of test samples.

The ***Se* of the isolation step** has been calculated as **74.1 %,** evaluated on the basis of the results provided by 40 laboratories (43 labs detected STEC in the screening, nonetheless for two of them, L391 and L598, there was an exchange of samples and one didn't attempt the isolation and were excluded from the analysis). Although L391 and L598 were considered as outliers and therefore excluded from the analysis of *Se* and *Sp*, they could either detect or isolate the contaminating STEC strain from the positive sample.

5. CONCLUSIONS

PT22 represented a voluntary inter-laboratory study aiming at the evaluation of a revised procedure for the analysis of spent irrigation water, which included, besides the pre-treatment procedure, the enrichment at 41.5 °C. A high participation among NRLs and Italian OLs was observed, confirming the consolidation of the network, actively participating in the initiatives proposed by EURL for *E. coli*.

The proposed modification of the procedure showed a significant improvement in the isolation of the contaminating STEC strain compared to what observed in PT19: in this PT 63.4 % of the Laboratories succeeded in the isolation step, whereas in PT19 based on the previous procedure only 17.2 % of the participants could isolate the contaminating STEC strain present in the same amount as in PT22 (50 CFU/ml).

In conclusion, the modification of the procedure increased the sensitivity of the isolation step from sprout spent irrigation water and will be proposed to the Laboratories involved in the testing of such matrix by making it available on the EURL for *E. coli* website. Nonetheless, EURL for *E. coli* will continue to devote efforts in improving the procedure for testing spent irrigation water.