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Report of the 25th inter-laboratory study on the detection of Shiga toxin-producing *E. coli* (STEC) in flour (PT25) - 2019

Edited by:

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1. OBJECTIVES AND DESIGN OF THE STUDY

Recently, flour has been identified as a vehicle of STEC infection. Therefore, EURL-VTEC decided to organize an inter-laboratory study, Proficiency Test 25 (PT25), on this particular matrix, in order to enhance the preparedness of NRLs in testing flour for the presence of STEC by applying the ISO TS 13136:2012. The present document represents the full evaluation report of PT25.

2. PARTICIPANTS

NRLs were invited to take part to the inter-laboratory study and the 43 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. Malta, Department for Health Regulation, Environmental Health, Valletta
19. Poland, National Institute of Public Health-National Institute of Hygiene, Warsaw
20. Poland, National Veterinary Research Institute (NVRI), Dept. Hygiene of Food of Animal Origin, Pulawy
21. Portugal, *Instituto Nacional de Investigação Agrária e Veterinária*, Vairão
22. Romania, Institute for Hygiene and Veterinary Public Health
23. Slovakia, Department of Food Hygiene, State veterinary and food institute, Dolný Kubín
24. Slovakia, NRC of Environmental Microbiology, Public Health Authority, Bratislava

25. Slovenia, Veterinary Faculty/ National Veterinary Institute
26. Spain, *Unidad Microbiología-Centro Tecnológico Agroalimentario de Lugo* (LSA-CETAL)
27. Spain, Bacteriology Department -2, Central Veterinary Laboratory-Animal health, Ministry of Agriculture, Fisheries and Food, Algete (Madrid)
28. Spain, Microbiology Food Department, Spanish Food Safety and Nutrition Agency, National Center for Food (CNA), Majadahonda (Madrid)
29. Sweden, National Veterinary Institute (SVA)
30. Sweden, The National Food Agency
31. The Netherlands, National Institute for Public Health and the Environment (RIVM)
32. The Netherlands, Wageningen Food Safety Research (WFSR), Wageningen University & Research

Non EU-NRLs

1. Chile, *Sección Microbiología de Alimentos y Aguas, Departamento de Salud Ambiental, Instituto de Salud Pública de Chile*, Ñuñoa, Santiago
2. Egypt, Central Laboratory of Residue Analysis of Pesticides and Heavy Metals in Foods
3. Iceland, Matís ohf. / Icelandic Food and Biotech R&D
4. Norway, Norwegian Veterinary Institute
5. Russia, State Research Center for Microbiology and Biotechnology, Obolensk
6. Switzerland, Agroscope
7. Switzerland, Institute for food safety and hygiene, University of Zurich
8. UK, Public Health England, FW&E Laboratory, London
9. UK, Public Health England, FWEM Laboratory, Porton
10. UK, Public Health England, FWEM Laboratory, York
11. Uruguay, Department of Bacteriology and Virology, Faculty of Medicine, Institute of Hygiene, University of the Republic, Montevideo

3. MATERIALS AND METHODS

3.1. Sample preparation

The flour used in the study was purchased from a local retailer.

The presence of a natural background microflora has been evaluated by plating serial dilutions of flour homogenized in Buffered Peptone Water (BPW) on TSA and MacConkey agar, but no growth was observed. Two samples consisting of 25 g of flour have been assayed for the presence of STEC according to ISO TS 13136:2012 and were negative at the PCR screening for the STEC-associated gene targets.

Stability tests were conducted in September 2019 and the results obtained are reported in Table 1.

Table 1. Results obtained in the stability testing assays.

STEC O121 concentration	T0 Replicate 1		T0 Replicate 2		T1 (4 days) Replicate 1		T1 (4 days) Replicate 2	
Test	Real Time PCR	Isolation	Real Time PCR	Isolation	Real Time PCR	Isolation	Real Time PCR	Isolation
1 CFU/25 g	+	+	+	+	+	+	+	+
5 CFU/25 g	+	+	+	+	+	+	+	+
10 CFU/25g	+	+	+	+	+	+	+	+

STEC O121 concentration	T2 (7 days) Replicate 1		T2 (7 days) Replicate 2		T3 (11 days) Replicate 1		T2 (11 days) Replicate 2	
Test	Real Time PCR	Isolation	Real Time PCR	Isolation	Real Time PCR	Isolation	Real Time PCR	Isolation
1 CFU/25 g	-	Not done	-	Not done	+	+	-	Not done
5 CFU/25 g	+	+	+	+	+	+	+	+
10 CFU/25g	+	+	+	+	+	+	+	+

Based on the stability tests results, the contamination levels were selected and the characteristics of the samples are reported in Table 2 and were considered as the gold standard. Three specimens, each consisting of 25 g of flour in sterile stomacher bags, potentially contaminated with STEC, were sent in the blind to the laboratories.

Table 2: Characteristics of the flour samples included in the study

Contaminant (*Genotype*)	**Contamination level in:**		
	Sample 1	**Sample 2**	**Sample 3**
ED898 STEC O121 *(stx2+, eae+)*	0 CFU	1 CFU/25 g	5 CFU/25 g

The artificial contamination of the samples was carried out on 11 October 2019, using dilutions of an exponential liquid culture (0.5 OD read at 600 nm) of the STEC O121 strain ED898. An uncertainty of measurement of 0.4 log CFU/ml was associated to the standardized inoculum, using the procedure described in the ISO/TS 19036:2006.

When the test samples were prepared, two negative samples were tested, giving the expected results. Eight samples for each of the two contamination levels were randomly selected for homogeneity testing and analyzed: 8 out of 8 of the 5 CFU/25 g samples and only 2 out of 8 samples contaminated with 1 CFU/g gave expected results.

The test samples were labeled with randomly generated numerical codes different for each NRL and were shipped at room temperature on 14 October 2019 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 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.

3.2.1. 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 virulence genes, *stx1* and *stx2*. Two penalty points have been assigned to the laboratories not identifying the presence of *eae* gene. Based on the results of the homogeneity testing, no penalties were assigned to the negative results in the PCR for the *stx2* in the samples with the lowest level of contamination (Sample 2).

3.2.2. 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 strains from the enrichment cultures of the positive samples was evaluated by assigning two penalty points to the lack of isolation from the sample 3. No penalty points were instead assigned to the lack of isolation from sample 2 (lower level of contamination), as the contamination level was determined as being close to the limit of detection of the procedure.

As for strain characterization, two penalty points were assigned to the laboratories not identifying the O121 serogroup in the STEC isolated strain.

3.2.3. 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.

3.2.4. 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

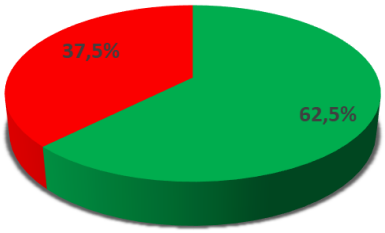
The limit of detection (LOD) was calculated for the isolation step using the procedure described by Wilrich and Wilrich (Journal of AOAC international, Vol. 92 No. 6, 2009, 1763-1772).

4. RESULTS

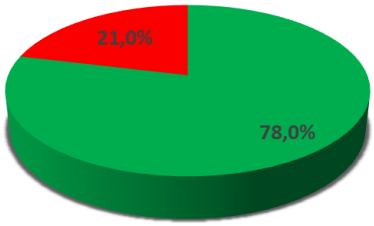
Test samples were sent to 43 laboratories and 41 returned the results.

The parcel containing the specimens were sent on the 14 October 2019 and were received by the participants on the 22nd of October at latest. Three laboratories didn't fill in the shipment form. As far as the shipment conditions were concerned, the temperature at delivery ranged between 4 °C and 27 °C for most of the laboratories. Eight participants recorded the temperature of the parcel as room temperature and 4 didn't declare it.

The results submitted by the participating laboratories are reported in Table 3 (Real-time PCR detection of STEC virulence and serogroup-associated genes in the enrichment cultures) and Table 4 (Isolation and characterization of the contaminating STEC strain). Figures 1 represents graphically the percentage of laboratories correctly identifying the presence of STEC in sample 2 and sample 3, respectively.



Sample 2



Sample 3

Figure 1. Screening step: Percentage of Laboratories correctly detecting STEC in the spiked samples (green: correct result; red: incorrect result). L417 has been excluded from the analysis for sample 2.

Table 3. Real-time PCR detection of virulence and serogroup-associated genes in the enrichment cultures. Green boxes: correct results, red boxes: incorrect results. Grey boxes: Laboratories which didn't report the results

NRL	Detection of virulence and serogroup-associated genes in:										
	Sample 1			Sample 2 Lower level contamination				Sample 3 Higher level contamination			
	stx1	*stx2*	*eae*	*stx1*	*stx2*	*eae*	*Top-5 O-genes*	*stx1*	*stx2*	*eae*	*Top-5 O-genes*
True value	-	-	-	-	+	+	-	-	+	+	-
L136											
L157									-		
L175											
L187					-						
L203					-						
L229											
L240					-						
L258									-		
L286					-						
L295											
L337									-		
L355					-				-		
L375											
L376											
L383											
L413											
L417		+	+		-						
L421											
L424					-						
L443											
L513											
L519									-		
L537					-						
L543					-						
L546											
L556					-						
L574											
L676					-						
L693											
L695									-		
L734											
L737											
L775					-						
L791					-						
L810									-		
L825											
L840											
L843					-				-		
L912											
L925					-						
L967											
L969											
L986					-				-		

Table 3. Isolation and genotyping of STEC strains from the flour samples. Green boxes: correct results, red boxes: incorrect results, orange boxes: the serogroup O121 was not identified in the isolated STEC strain. Grey boxes: isolation was not attempted as the sample was found negative for STEC

NRL	STEC strain isolation and genotyping from:								
	Sample 1	Sample 2				Sample 3			
	-	STEC O121 Isolation	Genotype			STEC O121 Isolation	Genotype		
			stx1	*stx2*	*eae*		*stx1*	*stx2*	*eae*
True value	None	+	-	+	+	+	-	+	+
L136									
L157									
L175									
L187									
L203									
L229		ONT				ONT			
L240									
L258									
L286									
L295									
L337									
L355									
L375									
L376									
L413		ONT				-			
L417									
L421									
L424									
L443									
L513									
L519									
L537									
L543									
L546									
L556									
L676									
L693									
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L734									
L737									
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L791									
L810									
L825									
L840									
L843									
L912									
L925									
L967									
L969									
L986									

All the laboratories detecting STEC in the enrichment culture of sample 2 were able to isolate the contaminating strain. The same applies for sample 3, with the exception of one laboratory (L413).

One participant (L417) presented results compatible with an exchange of sample 1 with sample 2 therefore was excluded from the sensitivity and specificity evaluation. Sensitivity of *eae* gene detection in the screening was calculated only for the tests carried out on the *stx2*-positive samples identified.

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

	***Se* (Lower level)**	***Se* (Higher level)**	***Sp***
stx1	N.A.	N.A.	100 %
stx2	72.7 %	82 %	100 %
eae	100 %	100 %	N.A.

The ***Se* of the isolation step** has been calculated as **72.7 %,** evaluated on the basis of the results provided by 40 laboratories for sample 2 and **80 %** on the basis of the results provided by 41 participants for sample 3.

The Limit of detection (LOD) of the isolation step returned the following results when combining the data from the two levels of contamination:

				$LOD_{50\%}$ [1]			$LOD_{95\%}$ [2]			
No. of matrix	Name of matrix	Matrix effect	SD of log matrix effect	Detection limit	Lower conf. limit	Upper conf. limit	Detection limit	Lower conf. limit	Upper conf. limit	Test statistic matrix effect
i	$matrix_i$	F_i	s_{fi}	$d_{0.5,i}$	$d_{0.5,i,L}$	$d_{0.5,i,U}$	$d_{0.95,i}$	$d_{0.95,i,L}$	$d_{0.95,i,U}$	$\lvert z_i \rvert$
1	Flour	0,463	0,161	0,060	0,043	0,083	0,259	0,188	0,357	4,234

4.1. Evaluation of the NRL performance in the PT procedures

For each NRL, the number of penalty points was determined using the criteria described in sections *3.4.1-3.4.3*.

Figure 2 shows the score achieved by each NRL and all the Laboratories complied the definition of satisfactory proficiency for PT25.

The results submitted by the laboratory which obtained the highest score (score of 6), L417, were compatible with samples exchange. Three Laboratories (L355, L843 and L986) did not succeed in detecting the presence of STEC in the screening step in both the

spiked. samples.

Figure 2. Evaluation of the NRL performance in the PT procedures (screening and isolation steps). The score was calculated according to the criteria described in sections 3.4.1-3.4.3. The performance of the NRLs that obtained a score higher than 8 was not considered as satisfactory (red bars).

5. CONCLUDING REMARKS

1. A high participation was recorded for PT25, confirming the eagerness and collaboration of the network.
2. Two Laboratories received the samples but did not report the results to EURL-VTEC and this represents an issue that needs to be followed up.
3. The levels of contamination used in this PT were very low and the results obtained allowed the determination of the LOD_{50} of the analyte (STEC O121) with the flour matrix as 0.043 CFU/g. This value is very close to the lowest level of contamination used in sample 2 for this PT25 (0.04 CFU/g), therefore no penalty points were assigned for the incorrect detection of STEC in Sample 2.
4. None of the participating Laboratories obtained a score equal or higher than eight.
5. One participant obtained a score of 6, but the exchange of two samples, the negative and the lower concentration of STEC, can be supposed. Therefore the incorrect results obtained for the detection of STEC-associated genes in the screening can be attributed to this and not to technical problems in applying the real-time PCR screening for STEC in this laboratory.

6. Nine Laboratories failed to detect STEC O121 in sample 3, spiked with the highest concentration of strain ED898. Although none of them resulted as underperformant, based on the criterion applied, this result was not considered as satisfactory, since the concentration of the STEC contaminating strain was far above the LOD_{50} calculated in this study. Therefore, this issue needs to be further evaluated with the participants.