Report of the voluntary inter-laboratory study on the effect of the enrichment temperature of 41.5 °C on the detection and isolation of STEC in sprouts organized as an appendix to the EURL-VTEC PT21 (2018)

Edited by:

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1. INTRODUCTION

The European Union Reference Laboratory for *E. coli* (EURL-VTEC) is chairing the activities of TAG18 in the framework of CEN/TC275/WG6 for the revision of the standard method for the detection of STEC in food ISO TS 13136:2012 (Microbiology of food and animal feed -- Real-time polymerase chain reaction (PCR)-based method for the detection of food-borne pathogens -- Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (STEC) and the determination of O157, O111, O26, O103 and O145 serogroups). The revision process has been announced in late 2015 and a group of experts (TAG18) has formally started this activity in late 2016. During the discussion among TAG18 members and based on the 5-years application of the existing standard, it has been observed that the use of mTSB supplemented with Novobiocin or Acryflavin as enrichment broth seems not to be adequate, as it seems to hamper the growth of certain STEC strains. The experts agreed that the enrichment of the food samples in Buffered Peptone Water (BPW) would be more appropriate. The use of BPW as enrichment broth without any supplements raises the issue on how to contain the background microflora present in food matrices. To address this issue, it was discussed the appropriateness of raising the incubation temperature to 41.5 °C for the enrichment step, aiming at reducing the growth of background microflora. Several promising experiences and data about the use of this temperature for STEC enrichment and detection were provided by TAG18 experts and by some of the NRLs representatives during the EURL-VTEC annual workshops. In order to gain more solid data on the effect of the enrichment temperature of 41.5 °C, EURL-VTEC organized a voluntary study to be run in parallel to the application of ISO TS 13136:2012 during the PT21 on the detection of STEC in sprouts (report available at: http://old.iss.it/binary/vtec/cont/Report_PT21_def.pdf). This inter-laboratory exercise was extended also to Italian Official Laboratories (OLs) and this document is meant to present the results of this study.

2. DESIGN AND OBJECTIVES OF THE STUDY

The study consisted in the detection and isolation of STEC O26 present in sprout samples in different amounts by using the enrichment temperature of 41.5 °C. The results were compared to those obtained in PT21 with the samples enriched at 37 °C in order to assess the effect of the increased enrichment temperature in the application of ISO TS 13136:2012.

3. PARTICIPANTS

A total of 48 Laboratories including 31 NRLs and 17 Italian OLs participated in the voluntary study. Each participant received its own individual Laboratory code, reported in the result tables and in the individual reports.

The NRLs participating in the study were:

- Belgium, Scientific Institute of Public Health, *Direction Opérationnelle Maladies Transmissibles et Infectieuses*
- Bulgaria, National Diagnostic and Research Veterinary Institute
- Chile, *Sección Microbiología de Alimentos y Aguas, Departamento de Salud Ambiental, Instituto de Salud Pública de Chile*
- Cyprus, Laboratory for the Control of Food of Animal Origin (LCFAO), Cyprus Veterinary Services
- Czech Republic, Veterinary Research Institute
- Denmark, FVST, *Mikrobiologisk Laboratorium*
- Egypt, Central Lab of Residue Analysis of Pesticides and Heavy Metals in Foods
- Estonia, Veterinary and Food Laboratory
- Finland, Finnish Food Safety Authority Evira, Research and Laboratory Department, Food and Feed Microbiology Research Unit
- France, *VetAgro Sup Campus Vétérinaire de Lyon*
- Germany, Federal Institute for Risk Assessment, Unit Food Technologies, Supply Chains and Food Defense, Department Biological safety
- Hungary, National Food Safety Office, Food and Feed Safety Directorate, National Food Microbiological Reference Laboratory
- Iceland, Matís ohf. / Icelandic Food and Biotech R&D
- Ireland, Veterinary Public Health Regulatory Laboratory, Department of Agriculture, Food and the Marine
- Italy, *Istituto Superiore di Sanità*
- Latvia, Microbiological Division, Laboratory of Food and Environmental Investigations, Institute of Food Safety, Animal Health and Environment (BIOR)
- Luxembourg, *Ministère de l'Agriculture, de la Viticulture et de la Protection des consommateurs, Administration des services vétérinaires*, LMVE
- Norway, Norwegian Veterinary Institute, Section for microbiology
- Poland, National Veterinary Research Institute, Department of Hygiene of food of animal origin

- Portugal, *Laboratório de Microbiologia dos Alimentos, Instituto Nacional de Investigação Agrária e Veterinária, I.P, Unidade Estratégica de Investigação e Serviços de Tecnologia e Segurança Alimentar* (LNIV)
- Romania, Department of Microbiology - Molecular Biology Unit, Institute for Hygiene and Veterinary Public Health
- Russia, International Department State Research Center for Microbiology and Biotechnology, Obolensk
- Slovenia, Veterinary Faculty UL, Nacional Veterinary Institute – Unit for Food of Animal Origin
- Spain, Microbiology Food Department, *Agencia Española de Consumo, Seguridad Alimentaria y Nutrición,* Spanish Agency for Consumers Affairs, Food Safety and Nutrition, National Center for Food -*Centro Nacional de Alimentación* (CNA)
- Sweden, *Livsmedelsverket*/The National Food Agency
- Switzerland, Institute for food safety and hygiene, University of Zurich
- The Netherlands, RIVM, Centre for Zoonoses and Environmental Microbiology
- The Netherlands, Food and Consumer Product Safety Authority (NVWA)
- UK, FW&E Laboratory – London, Public Health England
- UK, FW&E Laboratory – Porton, Public Health England
- UK, FW&E Laboratory – York, The Food and Environmental Research Agency (FERA), Public Health England

The Italian OLs participating in the study were:

- *ATS della Brianza, Laboratorio di Prevenzione, Oggiono (LC)*
- *ATS della Città Metropolitana di Milano, Sezioni Biologia Molecolare e Microbiologia Clinica, Laboratorio di Prevenzione, Milano*
- *Azienda USL Toscana Centro, Laboratorio di Sanità Pubblica Area Vasta Toscana Centro, Firenze*
- *IZS Abruzzo e Molise "G. Caporale", Reparto di Igiene delle Tecnologie Alimentari e dell'Alimentazione Animale, Laboratorio Nazionale di Riferimento per L. monocytogenes, Teramo*
- *IZS Puglia e Basilicata, UO Ricerca e Sviluppo Scientifico, Foggia*
- *IZS Lombardia ed Emilia Romagna, Reparto Microbiologia, Brescia*
- *IZS Lombardia ed Emilia Romagna, Sezione di Bologna*

- *IZS Lazio e Toscana, Dir. Op. Controllo degli Alimenti, Centro di Rif. Reg. Enterobatteri Patogeni, Roma*
- *IZS del Mezzogiorno, UO Microbiologia degli Alimenti, Sezione di Salerno, Fuorni (SA)*
- *IZS del Mezzogiorno, UOS Biotecnologie applicate agli alimenti-OGM, Portici (NA)*
- *IZS della Sicilia, Laboratorio Alimenti ad uso zootecnico, Area Microbiologia degli alimenti, Palermo*
- *IZS della Sicilia "A. Mirri", Area Catania, Dip. Sanità Territoriale Interprovinciale CT-RG, Catania*
- *IZS della Sardegna, Laboratorio di Microbiologia e Terreni Colturali, Sassari*
- *IZS Piemonte Liguria e Valle d'Aosta, S.C. Biotecnologie, Torino*
- *IZS Umbria e Marche, Laboratorio Controllo Alimenti, Centro Regionale Autocontrollo, Pesaro*
- *IZS delle Venezie, Sezione di Cordenons (PN)*
- *IZS delle Venezie, OIE/National Reference Laboratory for Salmonellosis, Legnaro (PD)*

4. MATERIALS AND METHODS

4.1. Sample preparation

The characteristics of the matrix used and the description of the sample preparation have been described in the Report of PT21, available at the EURL *E. coli* webpage (http://old.iss.it/binary/vtec/cont/Report_PT21_def.pdf).

A total of six test samples, corresponding to two sets of three samples (1, 2 and 3), each consisting of 25 g of red radish sprouts potentially contaminated with STEC, were sent in the blind to the participants. The Laboratories were requested to use Buffered Peptone Water (BPW) as enrichment medium. One set of samples was analyzed by the participating Laboratories following the prescription of the ISO TS 13136, using the indicated enrichment temperature of 37 °C for the PT21, and the other set was assayed using 41.5 °C as enrichment temperature.

The samples were spiked with three different levels of contamination: zero, low and high level. In detail, the set of samples sent to the NRLs contained 0, 2 and 20 estimated CFU per gram, respectively (Table 1). Serial dilutions of the inoculum suspensions of strain C1188-02 (STEC O26) contaminating the samples were plated onto MacConkey agar plates to check their actual titer.

The test samples were labeled with randomly generated numerical codes different for each NRL and each label indicated the enrichment temperature to be applied. The samples were

immediately refrigerated and transferred into refrigerated safety packages and shipped on 16 April 2018 by courier. The NRLs were requested to record date of delivery and sample temperature and to start the analyses immediately upon receipt.

Table 1: Characteristics of the set of three red radish sprout samples assessed in the study

Contaminant (*Genotype*)	**Contamination level in:**		
	Sample 1	**Sample 2**	**Sample 3**
Strain C1188-02, STEC O26 *(stx1+, stx2+, eae+)*	-	Low: 2 CFU/g	High: 20 CFU/g

4.2. Assessment of the stability and homogeneity of the samples

The stability and homogeneity of the samples were evaluated according to the requirements of ISO 17043:2010.

The stability was assessed using either the enrichment temperature of 37°C or of 41.5 °C on samples spiked on the 26th of January 2018 with 2 and 20 CFU/g of STEC O26 and tested by ISO TS 13136:2012 after 3, 5 and 10 days since the initial contamination. The Real Time PCR screening was positive for the STEC target genes even after 10 days from the spiking for both the set of samples enriched at 37 °C or 41.5 °C. The threshold cycles (Ct) obtained for the target genes were generally lower for samples enriched at 41.5 °C when compared with those at 37 °C (about 5 Ct difference). The isolation of the STEC O26 from the samples incubated at 37 °C was successful for all the samples spiked with the high level of contamination at all the time points, whereas it was never achieved for the low level of contamination, even after 3 days form spiking. When the 41.5 °C temperature was applied for enrichment, isolation was always achieved also from the low-level contamination samples. A scheme of the isolation results obtained during stability testing is reported in Table 2.

The homogeneity tests have been already described in the PT21 Report (http://old.iss.it/binary/vtec/cont/Report_PT21_def.pdf).

Table 2: Results of the isolation attempt during the stability testing carried out at EURL-VTEC

	T1 (3 days)	T2 (5 days)	T3 (10 days)
Samples	**Isolation (no. of positive pools)**	**Isolation (no. of positive pools)**	**Isolation (no. of positive pools)**
2 CFU/g @37 °C	-	-	-
2 CFU/g @41.5 °C	+ (2 pools)	+ (1 pool)	+ (1 pool)
20 CFU/g @37 °C	+ (1 pool)	+ (1 pool)	-
20 CFU/g @41.5 °C	+ (2 pools)	+ (1 pool)	+ (1 pool)

4.3. Laboratory methods

Laboratories were requested to identify the presence of STEC using the method ISO TS 13136:2012 using Buffered Peptone Water as enrichment broth and using the temperature of 37 °C (PT21) and 41.5 °C for the enrichment of the two set of samples.

4.4. Collection and elaboration of the NRL results

The results were submitted through an online 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 procedure and a step-by-step procedure 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.

At the end of the study, 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.

4.5. Analysis of the results

The proficiency of the participating laboratories was only calculated based on the results of the PT21 (37 °C) and can be found in the corresponding Reports (http://old.iss.it/binary/vtec/cont/Report_PT21_def.pdf for the NRLs study and http://old.iss.it/binary/coli/cont/Report_PT21_ITA_finale.pdf for the National OLs study).

4.6. Evaluation of the performance of the method

The performance of the method ISO TS 13136:2012 when applying the temperature of 41.5 °C as enrichment was evaluated and compared to that obtained when applying the

standard method. In particular, Sensitivity (Se) and Specificity (Sp) were calculated for the various features of the STEC considered in the study and for the different steps of the ISO TS 13136:2012. Therefore, Se and Sp were calculated for the PCR screening for *stx1*, *stx2, eae* and wzx_{O26} genes, and for the isolation of the STEC O26 strain.

The sensitivity and specificity were calculated according to the following formulas:

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) has been calculated for the isolation step using the procedure described by Wilrich and Wilrich (Journal of AOAC international, Vol. 92 No. 6, 2009, 1763-1772).

5. RESULTS

All the 48 Laboratories receiving the samples returned results via the web platform.

As for the delivery conditions, they can be found in the corresponding PT21 Reports (http://old.iss.it/binary/vtec/cont/Report_PT21_def.pdf for the NRLs study and http://old.iss.it/binary/coli/cont/Report_PT21_ITA_finale.pdf for the National OLs study).

Almost all the participants could assay the samples in the estimated time of stability and received the samples in good storage conditions. An exception was represented by L400 which received and tested the samples after 10 days from the shipment, with a recorded temperature of 25 °C. The results reported by this Laboratory are shown in Tables 3 and 4, but were excluded from the following evaluations as the analyses were conducted out of the determined stability.

5.1. Real-time PCR detection of STEC virulence and serogroup-associated genes in the enrichment cultures

The results reported by the participating Laboratories in the screening are shown in Table 3.

Several participants reported in the "note" field that the Ct observed during the screening phase when using 41.5 °C as enrichment temperature were generally lower than those obtained for the same samples enriched at 37 °C.

As for the negative sample, two Laboratories incorrectly reported the detection of *stx1, stx2, eae* and wzx_{O26} genes for the Sample 1 enriched at 37 °C. In one case, L391, this was likely due to the exchange of two samples, as Sample 2, corresponding to the low-level of contamination was found as negative, whereas in the other case, L649, cross-contamination may have occurred during the analysis. All the Laboratories correctly reported Sample 1 as negative for the set enriched at 41.5 °C.

Concerning the analysis of Sample 2 and Sample 3, a few laboratories (L295, L446 and L940), which reported incorrect negative results for some of the target genes in one or both the samples enriched at 37 °C, reported the expected results when the enrichment temperature applied was 41.5 °C (Table 3). L400, that received the samples after 10 days, could detect the presence of *stx1* and *stx2* genes only in the high level-contamination sample (Sample 3) analyzed after enrichment at 41.5 °C.

5.2. Isolation of the STEC O26 strain from PCR-positive samples.

L391 could isolate the STEC O26 strain from Sample 1 when enriching at 37 °C, confirming the exchange of samples 1 and 2.

A few Laboratories reported in the “note” field that following enrichment at 41.5 °C there was less background microflora on the plates and more colonies resembling *E. coli* were available for further confirmation.

The isolation of the contaminating STEC strain was achieved in Sample 2 from 29 (64.4 %) out of the 45 Laboratories detecting the presence of STEC in this sample, when enriching at 37 °C. At 41.5 °C, 43 Laboratories (91.5 %) out of the 47 identifying the presence of STEC in the screening phase, succeeded in isolating the STEC O26 strain.

As for the high-level contamination sample (Sample 3), 35 (76.1 %) out of 46 Laboratories could isolate the STEC O26 strain when applying the ISO TS 13136:2012 as such, whereas when the enrichment temperature applied was 41.5 °C, the isolation was successful for 44 (93.6 %) out of 47 Laboratories.

Table 3. Real time PCR detection of virulence and serogroup-associated genes in the enrichment cultures when the temperature of 37 °C or 41.5 °C were used for enrichment. The green boxes indicate the correct results, in agreement with the true value reported on the top of each column. The red boxes indicate values non-matching with the expected results and the orange boxes correspond to 'test not done'.

Lab	Detection of virulence and serogroup-associated genes in:																							
	Sample 1 (37 °C)				Sample 1 (41.5 °C)				Sample 2 Low level contamination (37 °C)				Sample 2 Low level contamination (41.5 °C)				Sample 3 High level contamination (37 °C)				Sample 3 High level contamination (41.5 °C)			
	stx1	*stx2*	*eae*	*wzx*$_{O26}$	*stx1*	*stx2*	*eae*	*wzx*$_{O26}$	*stx1*	*stx2*	*eae*	*wzx*$_{O26}$	*stx1*	*stx2*	*eae*	*wzx*$_{O26}$	*stx1*	*stx2*	*eae*	*wzx*$_{O26}$	*stx1*	*stx2*	*eae*	*wzx*$_{O26}$
True value	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L144																								
L257																								
L266																								
L269																								
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L351																								
L391	+	+	+	+					-	-														
L400									-	-			-	-			-	-					-	ONT
L439																								
L441																								
L446									-	-							-	-						
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Table 4. Isolation and genotyping of STEC O26 strain from the radish sprout samples (37 °C vs 41.5 °C enrichment temperature). The green boxes indicate the correct results, in agreement with the true value reported on the top of each column. The red boxes indicate results non-concordant with the gold standard. Orange boxes indicates that the test was not done even in the presence of *stx*-positive signals in the screening.

Lab	STEC strain isolation and genotyping from:																	
	Sample 1 (37 °C)	Sample 1 (41.5 °C)	Sample 2 (37 °C)				Sample 2 (41.5 °C)				Sample 3 (37 °C)				Sample 3 (41.5 °C)			
	-	-	STEC O26 Isolation	Genotype			STEC O26 Isolation	Genotype			STEC O26 Isolation	Genotype			STEC O26 Isolation	Genotype		
				stx1	*stx2*	*eae*		*stx1*	*stx2*	*eae*		*stx1*	*stx2*	*eae*		*stx1*	*stx2*	*eae*
True value	None	None	+	+	+	+					+	+	+	+				
L144																		
L257																		
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L351																		
L391	O26 *stx1 stx2 eae*																	
L400																		
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L521																		
L542																		
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L940				-														
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5.3. Comparison of the performance of the method

5.3.1. PCR screening step

The Sensitivity (Se) and Specificity (Sp) of the method was calculated for the detection of the *stx1, stx2, eae* and *wzx$_{O26}$* genes in the screening step following the two different enrichment conditions (temperature of 37 °C vs 41.5 °C). The results of L400, which clearly reflected a problem with the stability of the samples received, have been excluded from all the analyses.

	Enrichment at 37 °C				Enrichment at 41.5 °C			
	stx1	***stx2***	***eae***	***wzx$_{O26}$***	***stx1***	***stx2***	***eae***	***wzx$_{O26}$***
Se (low level)	93.9 %*	97.9 %*	100 %	100 %	100 %	100 %	100 %	100 %
Se (high level)	97.9 %	97.9 %	100 %	100 %	100 %	100 %	100 %	100 %

*The results provided by L391 were excluded

As for the specificity, the results submitted by L391 and L649 were not considered and was 100 % for both the enrichment temperatures applied.

5.3.2. STEC O26 isolation

The following values of Sensitivity of the isolation procedure were calculated:

	Enrichment at 37 °C	Enrichment at 41.5 °C
Se (low level)	73.3 %	92.2 %
Se (high level)	80.7 %	94 %

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

37 °C

No. of matrix	Name of matrix	Matrix effect	SD of log matrix effect	LOD$_{50\%}$ [1]			LOD$_{95\%}$ [2]			Test statistic matrix effect
				Detection limit	Lower conf. limit	Upper conf. limit	Detection limit	Lower conf. limit	Upper conf. limit	
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		0,005	0,169	5,435	3,880	7,615	23,491	16,767	32,911	0,000
Combined data		0,005	0,169	5,435	3,880	7,615	23,491	16,767	32,911	0,000

41.5 °C

No. of matrix	Name of matrix	Matrix effect	SD of log matrix effect	$LOD_{50\%}$ [1]			$LOD_{95\%}$ [2]			Test statistic matrix effect
				Detection limit	Lower conf. limit	Upper conf. limit	Detection limit	Lower conf. limit	Upper conf. limit	
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		0,016	0,197	1,692	1,141	2,509	7,312	4,929	10,845	0,000
Combined data		0,016	0,197	1,692	1,141	2,509	7,312	4,929	10,845	0,000

6. CONCLUDING REMARKS

The present study aimed at assessing the influence of the temperature of 41.5 °C for the detection and isolation of STEC following the application of the ISO TS 13136:2012 on sprout samples.

The analysis of the results induces the following conclusions:

1. a high participation rate was observed: 48 Laboratories including 31 NRLs and 17 Italian OLs agreed to participate in the voluntary study, confirming the consolidation of the network and the willingness of the Laboratories working in the field of pathogenic *E. coli* to collaborate with the EURL-VTEC;
2. the sensitivity of the method in the screening step slightly increased when the enrichment temperature applied was 41.5 °C, in the analysis of either the low level or high level comtamination samples;
3. a strong improvement of the sensitivity was observed for the isolation step when the enrichment temperature was raised at 41.5 °C, being particularly beneficial for the low level contamination samples; as a matter of fact, the estimanted LOD_{50} for the isolation step was much lower when the enrichment was carried out at 41.5 °C (1.692 CFU), compared to the enrichment at 37 °C (5.435 CFU);
4. the enrichment at 41.5 °C proved effective in improving the isolation of the STEC O26 strain used in this study.