EU Reference Laboratory for *E. coli*

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Report of the 10th inter-laboratory study (PT10) on Verocytotoxin-producing *E. coli* (VTEC) identification and typing – 2012-2013

Conducted jointly with the network of public health National Reference Laboratories for VTEC referring to the Food and Waterborne Diseases and Zoonoses Surveillance Programme of the European Center for Disease Prevention and Control

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1. INTRODUCTION

The duties of the EU Reference Laboratory for VTEC (EU-RL VTEC) include the organisation of proficiency tests (PT) on detection and typing methods for the designated National Reference Laboratories (NRLs) for *E. coli* in the EU Member States. The NRLs of the European Economic Area (EEA) countries, EU Candidate Countries, and third countries participate in these tests on a voluntary basis.

In the years 2007, 2008 and 2010, the EU-RL VTEC organized three PT schemes (PT1, PT2, and PT5) on the identification and typing of VTEC strains. These PTs aimed at evaluating and improving the capability of the NRLs to identify an *E. coli* strain as a VTEC, and to identify the VTEC serogroups most involved in severe human infections. In these PTs, the NRLs were requested to identify VTEC by detecting the presence of VT-coding genes and to distinguish between the two groups of *vtx* genes: *vtx1* and *vtx2*.

A fourth PT (PT6) was carried out between December 2010 and March 2011, and was extended to the sub-typing of VT-coding genes. The reports of the previous PTs are available at www.iss.it/vtec in the Proficiency Tests section.

PT6 was conducted jointly with the network of public health National Reference Laboratories for VTEC referring to the European Center for Disease Prevention and Control (ECDC), Food and Waterborne Diseases and Zoonoses Surveillance Programme (FWD, http://ecdc.europa.eu/en/activities/diseaseprogrammes/fwd). For this purpose, the EU-RL VTEC collaborated with the WHO Collaborating Centre for Reference and Research on *Escherichia* and *Klebsiella*, Statens Serum Institute, Copenhagen (SSI), which is in charge for the external quality assurance program for the ECDC-FWD network of the public health NRLs for VTEC. The aim of such a liaison was the harmonization of the typing methods used by both the NRL networks, to make the respective monitoring programs and databases compatible for comparison of data referring to human and non-human isolates of VTEC.

In the view of strengthening such a liaison, the EU-RL VTEC and the SSI organized a second joint inter-laboratory study on strain identification and typing. This study (PT10) was aimed at:

1. The detection of the main VTEC virulence genes: *vtx1* group, *vtx2* group and *eae*.
2. The detection of the genes that are considered the hallmark of Enteroaggregative *E. coli* (EAggEC) another group of pathogenic *E. coli* that has increased its public health importance after the large outbreak sustained by the mosaic VTEC-EAggEC O104:H4 strain, which occurred in Germany in 2011.

3. The identification of a range of 12 VTEC serogroups, selected on the basis of their relevance in human infections in Europe.
4. The sub-typing of VT-coding genes according to the revised version (recently published in *J Clin Microbiol. 2012; 50: 2951-63*) of the conventional PCR method adopted in the previous study conducted in 2010-2011 (PT6).
5. A first external quality assessment (EQA) of PFGE typing, in the framework of the ongoing initiatives that the EC is carrying out for the for the development of data bases hosting data from the molecular typing of foodborne pathogens in view of preparedness to face foodborne outbreaks.

This document represents the evaluation report of the study, as far as the results on gene detection and serogroup identification are concerned. The PFGE results will be presented in a separate report.

3. DESIGN OF THE STUDY

The study was conducted according to the International Standard ISO/IEC 17043:2010 "Conformity assessment – General requirements for proficiency testing" and consisted of four parts, 2 of which were facultative**:**

1. Mandatory. Identification and characterization of VTEC strains by amplification of the main virulence genes (*vtx1* group, *vtx2* group, *eae*) and of genes that represent markers of the enteroaggregative adhesion, characteristic of the VTEC O104:H4 strain that caused the 2011 outbreak (*aaiC* and *aggR*). The NRLs were allowed to perform the study using the methods currently in use in their own laboratories. The EU-RL released in the EU-RL web site (http://www.iss.it/vtec) PCR procedures for detecting *vtx* and *eae* genes as well as the enteroaggregative adhesion-associated genes *aaiC* and *aggR*.

2. Mandatory. Serogrouping, limited to the identification of the O antigens commonly occurring among VTEC strains pathogenic to human beings by conventional or molecular serotyping. The NRLs were requested to identify at least the strains that belongs to the following 12 serogroups, selected on the basis of their prevalence in human infections in Europe: O26, O55, O91, O103, O104, O111, O113, O121, O128, O145, O146, O157. The NRLs had the choice to perform the study using the methods currently in use in their own laboratories or according to the PCR procedure for detecting serogroup-associated genes provided by the EU-RL and available on the EU-RL website.

3. Facultative. Subtyping of the *vtx1* group (*vtx1a, vtx1c and vtx1d*) and *vtx2* group (from *vtx2a* to *vtx2g*) genes detected. The laboratories who accepted to take part to this step

were required to used the revised version of the conventional PCR method adopted in the previous study conducted in 2010-2011 (PT6), available in the web site of the WHO Collaborating Centre for Reference and Research on Escherichia and Klebsiella at the url: http://www.ssi.dk/English/HealthdataandICT/National%20Reference%20Laboratories/~/media/Indhold/EN%20-%20engelsk/Public%20Health/National%20Reference%20Laboratories/vtx%20detection%20%20subtyping%20protocolrev6final.ashx

4. Facultative. Determination of the PFGE profile of 11 strains, indicated by the EU-RL, out of the 15 strains sent out by using the Standard PulseNet PFGE protocol for *E. coli* O157 available at the Pulsenet web site (www.pulsenetinternational.org/protocols/Pages/default.aspx). The NRLs were requested to submit the pictures of the PFGE gels as TIFF files.

4. PARTECIPANTS

Thirty-four NRLs, representing 26 EU Member States, Norway, Switzerland and Egypt participated in the study. Each NRL received its own individual laboratory numerical code, which was reported in the result tables.

- The NRLs participating in the study were:
- Austria, Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH
- Belgium, Scientific Institute of Public Health
- Belgium, Veterinary and Agrochemical Research Centre (CODA-CERVA), (also representing Luxembourg)
- Bulgaria, National Diagnostic and Research Veterinary Institute
- Cyprus, Laboratory for the Control of Food of Animal Origin (LCFAO)
- Czech Republic, Veterinary Research Institute
- Denmark, National Food Institute, Technical University of Denmark
- Egypt, Central Public Health Laboratories of Ministry of Health and Population, Cairo
- Estonia, Veterinary and Food Laboratory
- Finland, Finnish Food Safety Authority Evira, Helsinki
- Finland, Finnish Food Safety Authority Evira Kuopio
- France, VetAgro Sup Campus Vétérinaire de Lyon
- Germany, Federal Institute for Risk Assessment (BfR)
- Greece, National School of Public Health, Central Laboratory of Public Health

- Hungary, National Food Safety Office, National Food Microbiological Reference Laboratory
- Hungary, Central Agricultural Office, Feed Investigation National Reference Laboratory
- Ireland, Central Veterinary Research Laboratory
- Italy, Istituto Superiore di Sanità
- Latvia, Institute of Food Safety, Animal Health and Environment BIOR
- Lithuania, National Food and Veterinary Risk Assessment Institute
- Netherlands, National Institute of Public Health and Environment, RIVM
- Norway, Norwegian Veterinary Institute
- Poland, National Institute of Hygiene, Warsaw
- Poland, National Veterinary Research Institute, Pulawy
- Portugal, Laboratório Nacional de Investigação Veterinária
- Romania, Institute for Hygiene and Veterinary Public Health, Microbiology Dept.
- Slovakia, Public Health Authority, UVSZR
- Slovakia, State Veterinary and Food Institute Dolný Kubín
- Spain, Laboratorio Central de Sanidad Animal
- Spain, University of Santiago de Campostela, Dept. of Microbiology and Parasitology, Lugo
- Sweden, National Food Administration, SLV
- Sweden, National Veterinary Institute, SVA
- Switzerland, University of Zurich
- UK, Health Protection Agency Centre for Infections, (also representing Malta).

5. MATERIALS AND METHODS

The test material sent to the NRLs was constituted by 15 strains of *E. coli* (samples 1 to 15).

5.1. Sample preparation

The test materials consisted of *E. coli* strains selected among those present in the SSI reference collection and checked for the presence of all the required genetic and phenotypic features. The characteristics of the strains are reported in Table 1 and were considered as the gold standard.

Table 1: Characteristics of the *E. coli* strains included in the study

Strain	Serogroup	*vtx1* group gene (subtype)	*vtx2* group gene (subtype)	*eae* gene	*aggR* gene	*aaic* gene
1	O113	+ (*vtx1c)*	+ (*vtx2b*)	-	-	-
2	O177	-	-	+	-	-
3	O121	-	+ (*vtx2a*)	+	-	-
4	O128	+ (*vtx1c)*	-	-	-	-
5	O41	+ (*vtx1d)*	-	-	-	-
6	O26	-	+ (*vtx2a*)	+	-	-
7	O111	+ (*vtx1a)*	-	+	-	-
8	O104	-	-	-	+	+
9	O157	-	+ (*vtx2a, vtx2c*)	+	-	-
10	O146	-	+ (*vtx2d*)	-	-	-
11	O103	+ (*vtx1a)*	-	+	-	-
12	O157	-	+ (*vtx2a*)	+	-	-
13	O166	-	+ (*vtx2d*)	-	-	-
14	O78	-	-	-	-	-
15	O124	-	-	-	-	-

A specific stability assessment was performed at SSI and consisted of 10 passages on modified Drigalski plates before re-testing the relevant phenotypic and genotypic features with satisfactory results. Furthermore, previous experiences supported the assumption that the time range between the preparation of the samples and the deadline for

submission of results by NRLs was short enough to assure the detection of all the strain characteristics.

The test samples were prepared at SSI in the period between 12 and 21 November, with the assistance of a scientist from the EU-RL VTEC. They consisted of bacterial cultures seeded into soft (0.3 %) nutrient agar in plastic vials. The cultures were incubated 18 hours at 37 °C ± 1 °C and labelled with randomly generated numerical codes (3 or 4 digits), different for each NRL. The test samples were shipped between 27 November and 4 December by courier.

5.2. Collection and elaboration of the NRL results

The results were submitted directly through an on-line system, using a dedicated page in the "Restricted Area" of the EU-RL web site. The NRLs 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 analysed 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 the expected results directly from the secure page of the EU-RL web site.

5.3. Analysis of the NRL results

The present study had two different scopes:

1. To evaluate the proficiency of the NRLs in the detection of the main VTEC virulence genes (*vtx1* group, *vtx2* group and *eae*) and in the identification of an extended range of pathogenic VTEC serogroups.
2. To assess the performance of the PCR method for the sub-typing of VT-coding genes that was slightly modified with respect to that used in PT6.

Accordingly, the results provided by the NRLs were analyzed as follows.

5.3.1. Evaluation of the NRL performance in the detection of virulence genes

The analytical performance of each NRL in the detection of the main VTEC virulence genes (*vtx1* gene group, *vtx2* gene group, and *eae),* and in the detection of the genes associated with entero-aggregative adhesion (*aggR,* and *aaiC*) was evaluated by calculating the following indicators:

- Agreement (*Cohen's kappa*)
- Sensitivity

- Specificity

Cohen's kappa refers to the level of association between two measurements of the same item. K test estimates the level of agreement beyond chance.

The threshold values described by Fleiss J.L. (*Statistical methods for rates and proportions, 1981*) were used for the evaluation of the value of Kappa. Therefore, Kappa values > 0.75 were considered as “excellent” agreement, values between 0.45 and 0.75 as “good” agreement and values <0.45 as “poor” agreement. The 95 % confidence interval (95 % CI) was also calculated. Sensitivity was defined as the proportion of positive samples correctly identified. Specificity was defined as the proportion of negative samples correctly identified.

5.3.2. Evaluation of the NRL performance in the identification of the O serogroups

The performance of each NRL in identifying the serogroup of the strains was evaluated by assigning penalty points for strains that were typed incorrectly.

The following distinction was made according to the public health importance of the serogroups, based on the data on human STEC infections published yearly by the ECD-FWD surveillance program in the EU Summary Report on Trends and Sources of Zoonoses.

- **4 penalty points:** assigned to each incorrect result concerning the typing of the strains belonging to the 5 serogroups, the “top 5”, most frequently isolated from cases of hemolytic uremic syndrome in Europe: O26, O103, O111, O145, O157.
- **2 penalty points:** assigned to each incorrect result concerning the typing of the strains belonging to the other 7 serogroups of public health relevance that were in the scope of this study: O55, O91, O104, O113, O121, O128, O146.
- **1 penalty point:** assigned to each incorrect result concerning the typing of the 5 test strains that did not belong to the 12 VTEC serogroups most common in human infections in Europe, and therefore did not fall within the scope of this study (strains 2, 5, 13, 14, and 15). For these strains, the result “not typeable” (ONT) was considered as correct.

The sum of the penalty points originated a score used to evaluate the underperformance. In particular, a threshold of 4 penalty points was set in order to identify the laboratories not performing adequately. The NRLs that summed up a score of 4 without making errors in the typing of the strains belonging to the “top 5” serogroups represented an exception and their performance was considered as satisfactory in the absence of other penalties.

5.3.3. Assessment of the performance of the PCR method for vtx-genes sub-typing

The assessment was performed separately for *vtx* genes sub-typing, using the data provided respectively by 15 and 17 NRLs, who were considered as non-outlier in their performance, based on the results obtained in the first phase of the PT. In detail, the selected NRLs (non-outliers) were those who did not report errors in the detection of the genes: *vtx1* group and *vtx2* group (see section 6.1 and Table 4).

The performance characteristics assessed were:

i) **Sensitivity**, defined as the ability of the method to detect the analyte when it is present in the sample;

ii) **Specificity**, defined as the ability of the method to not detect the analyte when it is not present in the sample;

iii) Agreement (*Cohen's kappa*).

6. RESULTS

The samples were sent to the 34 NRLs that had accepted to participate in the study in the period between 27 November and 4 December.

6.1. Detection of the virulence genes (*vtx1* group, *vtx2* group, *eae, aggR,* and *aaiC*) by PCR

PCR detection of virulence genes was performed by all the 34 NRLs and the results are reported in Table 2 (1-5).

Table 3 summarizes the results obtained for all the 15 test strains.

Table 2 (1). Detection by PCR of the virulence genes (*vtx1* group, *vtx2* group, *eae, aggR,* and *aaic*) in strains 1-3. The green boxes indicate the correct results, in agreement with the true value reported on the top of each column. The red boxes indicate the wrong results. ND indicates that the test was not done.

NRL	Detection of genes in:														
	Sample 1					Sample 2					Sample 3				
	vtx1	*vtx2*	*eae*	*aggR*	*aaiC*	*vtx1*	*vtx2*	*eae*	*aggR*	*aaiC*	*vtx1*	*vtx2*	*eae*	*aggR*	*aaiC*
True value	+	+	-	-	-	-	-	+	-	-	-	+	+	-	-
L01															
L02															
L03															
L06															
L09															
L10				+	+										
L11															
L13															
L14															
L15													-		
L18															
L22				ND	ND				ND	ND				ND	ND
L25															
L26			+	+	+										
L31															
L36															
L38															
L41															
L47															
L48															
L50															
L53															
L54				ND	ND				ND	ND				ND	ND
L58															
L60															
L63											+				
L70															
L71															
L81													-		
L89					ND					ND					ND
L97															
L108															
L110															
L117															

Table 2 (2). Detection by PCR of the virulence genes (*vtx1* group, *vtx2* group, *eae, aggR,* and *aaic*) in strains 4-6. The green boxes indicate the correct results, in agreement with the true value reported on the top of each column. The red boxes indicate the wrong results. ND indicates that the test was not done.

NRL	Detection of genes in:														
	Sample 4					Sample 5					Sample 6				
	vtx1	*vtx2*	*eae*	*aggR*	*aaiC*	*vtx1*	*vtx2*	*eae*	*aggR*	*aaiC*	*vtx1*	*vtx2*	*eae*	*aggR*	*aaiC*
True value	+	-	-	-	-	+	-	-	-	-	-	+	+	-	-
L01															
L02															
L03															
L06															
L09															
L10															
L11															
L13															
L14															
L15															
L18															
L22				ND	ND				ND	ND				ND	ND
L25															
L26															
L31															
L36															
L38															
L41															
L47														+	+
L48															
L50						-									
L53															
L54				ND	ND				ND	ND				ND	ND
L58															
L60															
L63															
L70															
L71															
L81															
L89					ND	-				ND					ND
L97															
L108			+												
L110															
L117															

Table 2 (3). Detection by PCR of the virulence genes (*vtx1* group, *vtx2* group, *eae, aggR,* and *aaic*) in strains 7-9. The green boxes indicate the correct results, in agreement with the true value reported on the top of each column. The red boxes indicate the wrong results. ND indicates that the test was not done.

NRL	Detection of genes in:														
	Sample 7					Sample 8					Sample 9				
	vtx1	*vtx2*	*eae*	*aggR*	*aaiC*	*vtx1*	*vtx2*	*eae*	*aggR*	*aaiC*	*vtx1*	*vtx2*	*eae*	*aggR*	*aaiC*
True value	+	-	+	-	-	-	-	-	+	+	-	+	+	-	-
L01															
L02															
L03															
L06															
L09															
L10															
L11															
L13															
L14															
L15															
L18											+				
L22				ND	ND				ND	ND				ND	ND
L25															
L26						+	+	+							
L31															
L36															
L38															
L41															
L47															
L48															
L50															
L53															
L54				ND	ND				ND	ND				ND	ND
L58															
L60									-	-					
L63															
L70															
L71															
L81															
L89					ND					ND					ND
L97															
L108															
L110															
L117															

Table 2 (4). Detection by PCR of the virulence genes (*vtx1* group, *vtx2* group, *eae, aggR,* and *aaic*) in strains 10-12. The green boxes indicate the correct results, in agreement with the true value reported on the top of each column. The red boxes indicate the wrong results. ND indicates that the test was not done.

NRL	Detection of genes in:														
	Sample 10					Sample 11					Sample 12				
	vtx1	*vtx2*	*eae*	*aggR*	*aaiC*	*vtx1*	*vtx2*	*eae*	*aggR*	*aaiC*	*vtx1*	*vtx2*	*eae*	*aggR*	*aaiC*
True value	-	+	-	-	-	+	-	+	-	-	-	+	+	-	-
L01															
L02															
L03															
L06															
L09															
L10													-		
L11															
L13															
L14															
L15															
L18															
L22				ND	ND				ND	ND				ND	ND
L25															
L26															
L31															
L36															
L38															
L41						-									
L47															
L48															
L50															
L53															
L54				ND	ND				ND	ND				ND	ND
L58															
L60															
L63															
L70						-	+								
L71															
L81															
L89					ND					ND					ND
L97															
L108															
L110											+				
L117															

Table 2 (5). Detection by PCR of the virulence genes (*vtx1* group, *vtx2* group, *eae, aggR,* and *aaic*) in strains 13-15. The green boxes indicate the correct results, in agreement with the true value reported on the top of each column. The red boxes indicate the wrong results. ND indicates that the test was not done.

NRL	Detection of genes in:														
	Sample 13					Sample 14					Sample 15				
	vtx1	*vtx2*	*eae*	*aggR*	*aaiC*	*vtx1*	*vtx2*	*eae*	*aggR*	*aaiC*	*vtx1*	*vtx2*	*eae*	*aggR*	*aaiC*
True value	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
L01															
L02															
L03															
L06															
L09															
L10												+			
L11													+		
L13					+										
L14															
L15															
L18															
L22				ND	ND				ND	ND				ND	ND
L25															
L26			+												
L31															
L36															
L38															
L41			+			+	+	+							
L47		-										+			
L48															
L50															
L53															
L54															
L58															
L60															
L63															
L70											+				
L71															
L81															
L89					ND					ND					ND
L97															
L108															
L110															
L117															

Table 3. Summary of the PCR results obtained for the detection of the virulence genes, by strain. The green boxes indicate that all the genes were identified correctly in the given strain. The red boxes indicate that incorrect results were reported for the given strain. The numbers in the box indicate the number of incorrect results.

NRL	Correct detection of virulence genes in strain:														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
L01															
L02															
L03															
L06															
L09															
L10	2											1			1
L11															1
L13													1		
L14															
L15			1												
L18									1						
L22															
L25															
L26	3							3					1		
L31															
L36															
L38															
L41											1		1	3	
L47						2							1		1
L48															
L50					1										
L53															
L54															
L58															
L60								2							
L63			1												
L70											2				1
L71															
L81			1												
L89					1										
L97															
L108				1											
L110												1			
L117															

Eighteen NRLs (53 %) identified correctly the presence/absence of all the target genes in the test strains. The other 16 NRLs provided a total of 35 incorrect results (25 false positive and 10 false negative),

Table 4 summarizes the results of the PCR assays according to the target virulence genes.

Table 4. Summary of the PCR results obtained for the detection of the virulence genes, by virulence gene. The green boxes indicate that the gene or the serogroup were identified correctly in all the 15 test strains. The red boxes indicate that incorrect results were reported for the given gene. The numbers in the box indicate the number of incorrect results. ND indicates that the test was not done.

NRL	Detection of virulence genes in all the 15 test strains:				
	vtx1	*vtx2*	*eae*	*aggR*	*aaiC*
L01					
L02					
L03					
L06					
L09					
L10		1	1	1	1
L11			1		
L13					1
L14					
L15			1		
L18	1				
L22				ND	ND
L25					
L26	1	1	3	1	1
L31					
L36					
L38					
L41	2	1	2		
L47		2		1	1
L48					
L50	1				
L53					
L54				ND	ND
L58					
L60				1	1
L63	1				
L70	2	1			
L71					
L81			1		
L89	1				ND
L97					
L108			1		
L110	1				
L117					

Eight NRLs provided 10 incorrect results for *vtx1* (6 false positive and 4 false negative), 5 NRLs provided 6 incorrect results for *vtx2* (5 false positive and 1 false negative), 7 NRLs provided 10 incorrect results for *eae* (7 false positive and 3 false negative), 4 NRLs provided 4 incorrect results for *aggR* (3 false positive and 1 false negative), and 5 NRLs

provided 5 incorrect results for *aaic* (4 false positive and 1 false negative). In particular, 1 NRL (L60) failed to detect the entero-aggregative strain (sample 8), providing false negative results for both the target genes. Finally, 3 NRLs did not perform the detection of one or both the entero-aggregative *E. coli* virulence genes.

The analytical performances of the NRLs in genotyping the *E. coli* strains were evaluated by calculating the accordance of the results provided with the true values of the test material's characteristics (Cohen's Kappa, K), sensitivity and specificity.

The K values for the detection of the main virulence genes of VTEC (*vtx1* group, *vtx2* group, *eae*), considered altogether, are reported in Table 5.

Table 5. Detection of the main virulence genes of VTEC (*vtx1* group, *vtx2* group, *eae)*: agreement (Cohen's Kappa) between the results of the NRLs and the true values of the samples. The green boxes highlight the values of Kappa >0.75 (excellent agreement); the yellow boxes the values between 0.45 and 0.75 (good agreement), and the red boxes the values < 0.45 (poor agreement).

NRL	K values		
	K value	95% lower limit	95% upper limit
L01	1	1	1
L02	1	1	1
L03	1	1	1
L06	1	1	1
L09	1	1	1
L10	0.91	0.79	1
L11	0.95	0.87	1
L13	1	1	1
L14	1	1	1
L15	0.97	0.86	1
L18	0.95	0.87	1
L22	1	1	1
L25	1	1	1
L26	0.78	0.60	0.96
L31	1	1	1
L36	1	1	1
L38	1	1	1
L41	0.78	0.59	0.96
L47	0.91	0.79	1
L48	1	1	1
L50	0.95	0.87	1
L53	1	1	1
L54	1	1	1
L58	1	1	1
L60	1	1	1
L63	0.95	0.87	1
L70	0.86	0.72	1
L71	1	1	1
L81	0.95	0.87	1
L89	0.95	0.87	1
L97	1	1	1
L108	0.95	0.87	1
L110	0.95	0.87	1
L117	1	1	1
Overall	0.96	0.95	0.98

The overall agreement between the results reported by the 34 NRLs and the true values of the samples was considered excellent, as well as the single agreements of all the NRLs.

The sensitivity and specificity values for each NRL in the detection of the main VTEC virulence genes (*vtx1* group, *vtx2* group, and *eae*) altogether are reported in Table 6.

Table 6. Detection of *vtx1* group, *vtx2* group, and *eae,* genes: sensitivity and specificity for each NRL. The red boxes highlight values < 100 %.

NRL	Sensitivity (Se) and Specificity (Sp) for each NRL									
	L01	**L02**	**L03**	**L06**	**L09**	**L10**	**L11**	**L13**	**L14**	**L15**
Se	100	100	100	100	100	94.7	100	100	100	94.7
Sp	100	100	100	100	100	96.2	96.2	100	100	100
NRL	**L18**	**L22**	**L25**	**L26**	**L31**	**L36**	**L38**	**L41**	**L47**	**L48**
Se	100	100	100	100	100	100	100	94.7	94.7	100
Sp	96.2	100	100	80.8	100	100	100	84.6	96.2	100
NRL	**L50**	**L53**	**L54**	**L58**	**L60**	**L63**	**L70**	**L71**	**L81**	**L89**
Se	94.7	100	100	100	100	100	94.7	100	94.7	94.7
Sp	100	100	100	100	100	96.2	92.3	100	100	100
NRL	**L97**	**L108**	**L110**	**L117**						
Se	100	100	100	100						
Sp	100	96.2	96.2	100						

The overall sensitivity and specificity values were 98.8 and 97.9, respectively

The values of Cohen's Kappa, sensitivity, and specificity for the detection of the genes associated with entero-aggregative adhesion (*aggR,* and *aaic*) were calculated for the 32 NRLs that made a number of tests for these genes that was sufficient for the evaluation.

The results are reported in Table 7 and showed that the overall agreement with the true values of the samples was excellent, as well as the single agreements of all but six the NRLs. For these latter, the agreement was considered as good.

Table 7. Detection of the genes associated with entero-aggregative adhesion (*aggR,* and *aaic*): Cohen's Kappa (K), sensitivity (Se) and specificity (Sp) for each NRL. The green boxes highlight the values of Kappa >0.75 (excellent agreement), and the yellow boxes the values between 0.45 and 0.75 (good agreement).The red boxes highlight K values < 0.45 (poor agreement) and sensitivity and specificity values < 100 %. ND: test not done.

NRL	Genes					
	aggR			*aaic*		
	K	Se	Sp	K	Se	Sp
L01	1	100	100	1	100	100
L02	1	100	100	1	100	100
L03	1	100	100	1	100	100
L06	1	100	100	1	100	100
L09	1	100	100	1	100	100
L10	0.63	100	92.9	0.63	100	92.9
L11	1	100	100	1	100	100
L13	1	100	100	0.63	100	92.9
L14	1	100	100	1	100	100
L15	1	100	100	1	100	100
L18	1	100	100	1	100	100
L25	1	100	100	1	100	100
L26	0.63	100	92.9	0.63	100	92.9
L31	1	100	100	1	100	100
L36	1	100	100	1	100	100
L38	1	100	100	1	100	100
L41	1	100	100	1	100	100
L47	0.63	100	92.9	0.63	100	92.9
L48	1	100	100	1	100	100
L50	1	100	100	1	100	100
L53	1	100	100	1	100	100
L58	1	100	100	1	100	100
L60	0.63	-	100	0.63	-	100
L63	1	100	100	1	100	100
L70	1	100	100	1	100	100
L71	1	100	100	1	100	100
L81	1	100	100	1	100	100
L89	1	100	100	ND	ND	ND
L97	1	100	100	1	100	100
L108	1	100	100	1	100	100
L110	1	100	100	1	100	100
L117	1	100	100	1	100	100
Overall	0.93	96.9	99.3	0.91	96.8	99.8

6.2. Identification of the O serogroup

The identification of the serogroup was performed by all the 34 NRLs and the results are shown in Table 8.

The result “not typable” (ONT) was considered correct for the 5 test strains belonging to serogroups that were not among the 12 most common in human infections in Europe and therefore did not fall within the scope of this study (Strains 2, 5, 13, 14, and 15).

Nineteen NRLs (56%) identified correctly the serogroup of all the 15 test strains. The other 15 NRLs provided a total of 45 incorrect results. The number of incorrect results ranged from 1 (6 NRLs) to 7 (2 NRLs).

As for the test strains belonging to the serogroups comprised among the “top-five” associated with disease, all the NRLs identified correctly the VTEC O26, O103, and O111 strains, as well as one of the two VTEC O157 strains included among the test strains, while one NRL failed to identify the second VTEC O157 strain.

The *E. coli* O104 strain was identified correctly by 31 NRLs (91%).

Among the less common serogroups, 32 NRLs (94%) identified correctly the O128 strain, 30 NRLs (88%) the O121 strain, 28 NRLs (82%) the O146 strain, and 27 NRLs (79%) the O113 strain.

As for the 5 test strains belonging to serogroups that were not in the scope of this study (strains 2, 5, 13, 14, and 15), 4 NRLs typed them correctly, and other 4 NRLs were able to type correctly at least one of them.

Conversely, 11 NRLs reported 22 incorrect results, assigning these strains to other serogroups.

Table 8. Identification of the serogroup of the test strains. The green boxes indicate the correct results, in agreement with the true value reported on the top of each column. The red boxes indicate the incorrect results, and display the results provided by the NRL. ONT = not typable.

NRL	Serogroup identification in sample:														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
True Value	O113	O177 (ONT)	O121	O128	O41 (ONT)	O26	O111	O104	O157	O146	O103	O157	O166 (ONT)	O78 (ONT)	O124 (ONT)
L01		ONT			O55								ONT	ONT	ONT
L02		O177			O41								O166	O78	O124
L03		O177			O41								ONT	O78	O124
L06		ONT			ONT								ONT	ONT	ONT
L09		O145			ONT								ONT	ONT	ONT
L10		O157		O91	O145				O91	148			O128	ONT	O146
L11		O177			ONT					ONT			ONT	ONT	ONT
L13		ONT			ONT								ONT	ONT	ONT
L14		ONT			ONT								ONT	ONT	ONT
L15		ONT			ONT								ONT	O78	O124
L18		ONT			ONT								ONT	ONT	ONT
L22	ONT	O145			O55			ONT		ONT			ONT	ONT	ONT
L25			ONT		ONT								ONT	ONT	ONT
L26		O146			ONT								ONT	ONT	ONT
L31	ONT	ONT			ONT			ONT					ONT	ONT	ONT
L36		ONT			ONT								ONT	ONT	ONT
L38		O177			O41								O166	O78	O124
L41	ONT	ONT			ONT								ONT	ONT	O113
L47		ONT			ONT								ONT	ONT	ONT
L48		ONT			ONT								ONT	ONT	ONT
L50		ONT			ONT								ONT	ONT	ONT
L53		ONT			ONT								ONT	ONT	ONT
L54	O125	ONT			O121			ONT		ONT			ONT	ONT	ONT
L58		ONT			ONT								ONT	ONT	ONT
L60	O55	O145			O91					O55			O113	O146	O26
L63		ONT			ONT								ONT	ONT	ONT
L70	O55	O145	O113		O121					O104			O146	O78	O124
L71		ONT			ONT								ONT	ONT	ONT
L81		O145	ONT		O55								ONT	ONT	ONT
L89		O145			O41								O166	O78	O124
L97		ONT			ONT								ONT	ONT	ONT
L108		ONT			ONT								ONT	ONT	ONT
L110	ONT	ONT	ONT	ONT	ONT								ONT	ONT	ONT
L117		O177			O41								O166	O78	O124

For each NRL, the number of penalty points was determined using the criteria described in section 5.3.2. Figure 1 shows the score achieved by each NRL, and Figure 2 the number of NRLs grouped according to their score. Six NRLs obtained a score higher than 4, and their performance was not considered as satisfactory. Two NRLs obtained a score equal to 4 but their performance was considered as satisfactory since they did not make errors in typing the strains belonging to the "top 5" serogroups.

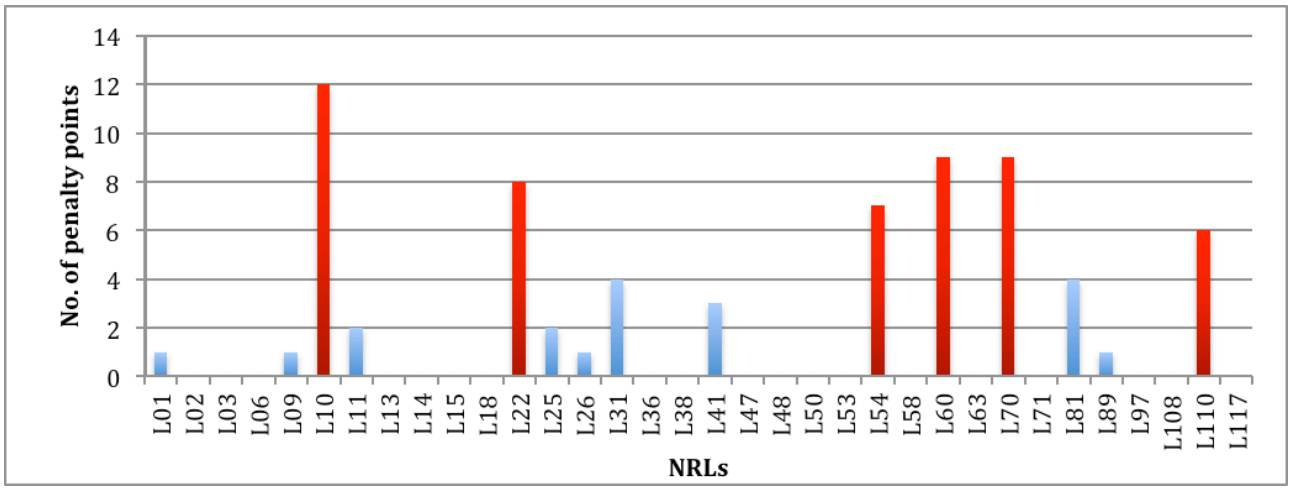

Figure 1. Evaluation of the serogrouping results for each NRL. The score was calculated according to the criteria described in section 5.3.2. The red bars indicate the NRLs whose performance was not considered as satisfactory.

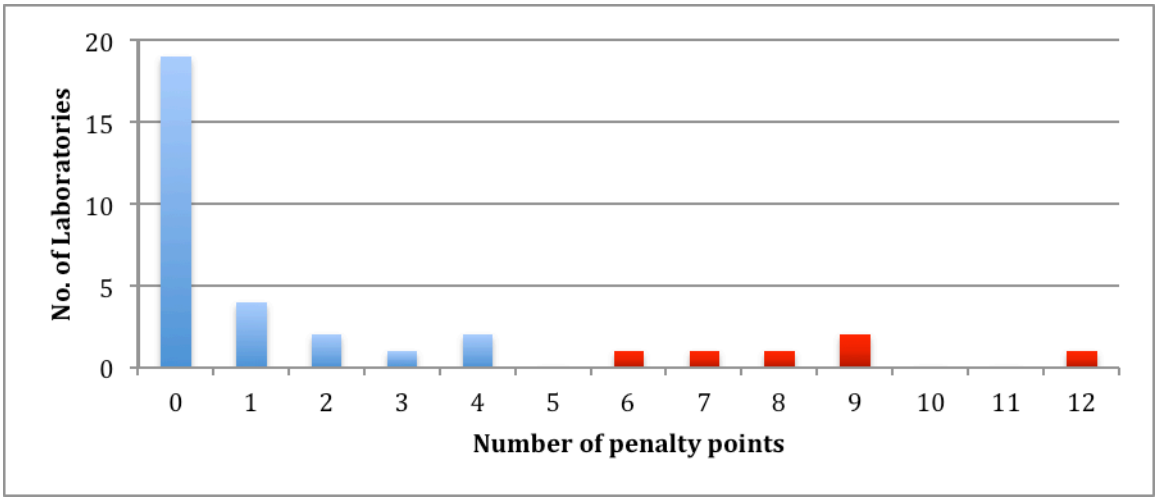

Figure 2. Evaluation of serogrouping results: number of NRLs within each penalty score. The red bars indicate the NRLs whose performance was not considered as satisfactory.

6.3. Identification of the *vtx* gene subtypes by conventional PCR

The identification of the *vtx* gene subtypes was performed by 19 of the 34 NRLs that participated in the study (56%).

6.3.1. Sub-typing of vtx1

The results of the *vtx1* sub-typing are reported in Table 9.

Table 9. Sub-typing of the *vtx1* gene, by PCR: The green boxes indicate the correct results, in agreement with the true value reported on the top of each column. The red boxes indicate the wrong results. The white boxes indicate that the test was not done (ND).

NRL	Detection of the *vtx1* gene subtype in:														
	Sample 1			Sample 4			Sample 5			Sample 7			Sample 11		
	vtx1a	*vtx1c*	*vtx1d*	*vtx1a*	*vtx1c*	*vtx1d*	*vtx1a*	*vtx1c*	*vtx1d*	*vtx1a*	*vtx1c*	*vtx1d*	*vtx1a*	*vtx1c*	*vtx1d*
True Value	-	+	-	-	+	-	-	-	+	+	-	-	+	-	-
L02															
L03															
L09															
L11															
L14															
L15															
L18							+	+							
L25															
L31															
L38															
L41									-				ND	ND	ND
L47															
L50							ND	ND	ND						
L60							ND	ND	ND						
L63		-													
L81															
L97															
L108															
L117															

The presence/absence of the *vtx1* subtypes was identified correctly in all the 5 *vtx1*-positive strains by 15 of the 19 NRLs (79 %) that performed the subtyping. The other 4 NRLs reported 2 false negative results (one for *vtx1c,* and one for *vtx1d*) and did not perform the typing for one of the strains.

6.3.2. Performance of the vtx1 sub-typing method

The performance characteristics of the proposed method were calculated using ~~only~~ the results provided by 15 non-outlier NRLs, which were defined as those who did not report errors in the PCR detection of the *vtx1* gene group performed in the first step of the study (see section 6.1, table 4). Therefore, the data provided by 4 NRLs (L18, L41, L50, L63) were excluded from the analyses. The performance characteristics of the method for subtyping the *vtx1* gene are reported in Table 10, as a whole and for the detection of the single gene subtypes.

Table 10. Performance characteristics of the method for subtyping the *vtx1* gene

Analyte	**Performance characteristics**		
	Sensitivity	**Specificity**	**Cohen's Kappa**
***vtx1a* gene**	**100 %**	**98.2%**	**0.98**
***vtx1c* gene**	**97.4 %**	**98.1 %**	**0.96**
***vtx1d* gene**	**94.1 %**	**100 %**	**0.96**
Overall typing	**97.8 %** (95 %CI 95.1 % - 100 %)	**98.9 %** (95 %CI 97.5 % - 100 %)	**0.97** (95 %CI 0.94 – 1)

6.3.3. Subtyping of vtx2

The results of the *vtx2* subtyping are reported in Table 11 (1-2).

Table 11 (1). Subtyping of the *vtx2* gene, by PCR (samples 1-9). The green boxes indicate the correct results, in agreement with the true value reported on the top of each column. The red boxes indicate the wrong results. The white boxes indicate that the test was not done (ND).

NRL	Detection of the *vtx2* gene subtype in:															
	Sample 1				Sample 3				Sample 6				Sample 9			
	vtx2a	*vtx2b*	*vtx2c*	*vtx2d*	*vtx2a*	*vtx2b*	*vtx2c*	*vtx2d*	*vtx2a*	*vtx2b*	*vtx2c*	*vtx2d*	*vtx2a*	*vtx2b*	*vtx2c*	*vtx2d*
True value	-	+	-	-	+	-	-	-	+	-	-	-	+	-	+	-
L02																
L03																
L09																
L11																
L14																
L15		ND				ND				ND				ND		
L18		ND				ND				ND				ND		
L25																
L31															-	
L38																
L41		ND				ND				ND				ND		
L47																+
L50																
L60		ND				ND				ND				ND		
L63		ND				ND				ND				ND		
L81		ND				ND				ND				ND		
L97																
L108																
L117																

Table 11 (2). Subtyping of the *vtx2* gene, by PCR (samples 10-13). The green boxes indicate the correct results, in agreement with the true value reported on the top of each column. The red boxes indicate the wrong results. The white boxes indicate that the test was not done (ND).

NRL	Detection of the *vtx2* gene subtype in:											
	Sample 10				Sample 12				Sample 13			
	vtx2a	*vtx2b*	*vtx2c*	*vtx2d*	*vtx2a*	*vtx2b*	*vtx2c*	*vtx2d*	*vtx2a*	*vtx2b*	*vtx2c*	*vtx2d*
True Value	-	-	-	+	+	-	-	-	-	-	-	+
L02												
L03												
L09												
L11												
L14												
L15		ND				ND				ND		
L18		ND				ND				ND	+	
L25												
L31												
L38												
L41		ND	+			ND				ND	+	
L47			+	ND					ND	ND	ND	ND
L50			+	-							+	
L60		ND				ND				ND		
L63		ND				ND				ND	+	
L81		ND				ND				ND		
L97												
L108												
L117											+	

The presence/absence of the *vtx2* subtypes was identified correctly in all the 7 *vtx2*-positive strains by 9 of the 19 NRLs (47%) that performed the subtyping. The other 10 NRLs reported 2 false negative results (one for *vtx2c,* and one for *vtx2d*) and 9 false positive results (8 for *vtx2c,* and one for *vtx2d*). Six NRLs did not perform the detection of the *vtx2b* gene.

6.3.4. Performance of the vtx2 sub-typing method

The performance characteristics of the proposed method were calculated using the results provided by 17 non-outlier NRLs, which were defined as those who did not report errors in the PCR detection of the *vtx2* gene group performed in the first step of the study (see section 6.1, table 4). Therefore, the data provided by 2 NRLs (L41, L47) were excluded from the analyses.

The performance characteristics of the method for subtyping the *vtx2* gene are reported in Table 12, as a whole and for the detection of the single gene subtypes.

Table 12. Performance characteristics of the method for subtyping the *vtx2* gene

Analyte	Performance characteristics		
	Sensitivity	**Specificity**	**Cohen's Kappa**
***Vtx2a* gene**	**100 %**	**100 %**	**1**
***Vtx2b* gene**	**100 %**	**100 %**	**1**
***Vtx2c* gene**	**96.3 %**	**92.9 %**	**0.81**
***Vtx2d* gene**	**97.4 %**	**99.0 %**	**0.96**
Overall typing	**98.6 %** (95 % CI 97.0 % - 100 %)	**97.4 %** (95 % CI 95.8 % - 99.1 %)	**0.95** (95 %CI 0.92 – 0.98)

7. CONCLUDING REMARKS

PT 10 was the second study on the identification and typing of VTEC that the EU-RL VTEC conducted jointly with the network of public health National Reference Laboratories for VTEC referring to the ECDC-FWD. The joint study had the aim of perfecting the harmonization of the typing methods used by both the NRL networks, to make the respective monitoring programs and databases compatible for the comparison of typing data referring to human and non-human isolates of VTEC.

With reference to this report, the study had two different scopes:

- To evaluate the proficiency of the NRLs in the detection of the main virulence genes of VTEC (*vtx1* group, *vtx2* group and *eae*) and the target genes identifying EAggEC (*aggR* and *aaic*), a group of pathogenic *E. coli* that has increased its public health importance after the large 2011 outbreak sustained by the mosaic VTEC-EAggEC O104:H4 strain.
- To evaluate the proficiency of the NRLs in the identification of 12 VTEC serogroups with high public health relevance, according to the epidemiologic data on human infections in Europe published yearly by the ECDC.
- To assess the performance of the PCR method for the sub-typing of VT-coding genes, after the revision done considering the results obtained with its first assessment in the previous EU-RL/ECDC-FWD joint PT (PT6).

While an overall report on the results produced by all the laboratories participating in the study will be elaborated jointly with SSI and published elsewhere, the present report considered only the results produced by the network of the NRLs in the EU member states

appointed according to the Reg. (EC) 882/2004 and by veterinary/food NRLs of other countries, that participate in the network activities.

The following remarks can be drawn.

1. Thirty-one EU NRLs, representing 26 EU Member States, and the NRLs of Norway, Switzerland and Egypt participated in the study.
2. Twenty NRLs (59%) identified correctly the presence/absence of the VTEC target genes in the test strains, while 18 NRLs (53 %) identified correctly the presence/absence of all the VTEC and EAggEC target genes in the test strains.
3. As for the VTEC target genes, an excellent agreement (K value > 0.75) of the results with the true values was observed for all the participating NRLs.
4. As for the EAggEC target genes, the agreement was excellent for all but six the NRLs. For these latter, the agreement was considered good.
5. A good genotyping performance of the Network in terms of sensitivity and specificity was highlighted.
6. Nineteen NRLs (56 %) did not make errors in the identification of the serogroup of all the 15 test strains.
7. All the NRLs identified correctly the 5 VTEC strains belonging to the “top-five” serogroups causing severe human infections (O157, O26, O103, and O111), with the exception of one NRL that failed to identify one of the 2 VTEC O157 strain included in the test.
8. Most NRLs (91 %) were able to identify the *E. coli* O104 strain.
9. As a whole, the results summarized at points 1 - 8 indicate that a very good preparedness has been established in the EU towards the identification of VTEC strains causing most of the HUS cases in Europe and included in the recently issued Reg. (EC) 209/2013 defining a microbiological criterion for VTEC in sprouts.
10. The proportion of the NRLs that were able to identify the less common serogroups included in the test (O113, O121, O128, and O146) ranged from 79 % to 94 %.
11. The general serotyping performance could have been negatively influenced by the number of strains included in the PT (15), higher than in the previous PTs, and by their wide range of serotypes. Nevertheless, an improvement in the capacity to identify the VTEC serogroups causing human infections in Europe was observed, with respect to the previous PTs on serotyping.
12. When the serotyping performance was evaluated by assigning a penalty score, the performance of 6 NRLs was not considered as satisfactory, and corrective actions will

be planned for those laboratories, including either the shipment of backup samples or the invitation to attend the training stages organized by the EU-RL VTEC.

13. The identification of the *vtx* gene subtypes using the revised version of the method proposed by the WHO Collaborating Centre for Reference and Research on *Escherichia* and *Klebsiella* and by the EU-RL was performed by 19 NRLs. The *vtx1* subtypes were identified correctly in all the test samples by 15 NRLs (79 %) whereas only 9 NRLs (47 %) identified correctly the *vtx2* subtypes.
14. A substantial improvement in the performance of the *vtx2* subtyping method was recorded with respect to the results obtained in PT6 by using the previous version of the method: the sensitivity passed from 95.5% to 98.6% but, mostly, the specificity passed from 79.5% to 97.4%.
15. In conclusion, most NRLs performed satisfactorily in the PCR identification of the main virulence genes of VTEC and EAggEC as well as in the typing of the serogroups most common among human infections in Europe. The method proposed for the identification of the *vtx* gene represents now a robust tool for further typing of VTEC.
16. The organization of joint inter-laboratory studies on VTEC typing with the ECDC-FWD network of public health NRLs represents an important achievement in the perspective of making the respective monitoring programs and databases compatible for comparison of human and non-human data.

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