EU Reference Laboratory for *E. coli*

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Report of the 6th inter-laboratory study on Verocytotoxin-producing *E. coli* (VTEC) identification and typing – 2010-2011

(Conducted jointly with the network of medical National Reference Laboratories for VTEC referring to the European Center for Disease Prevention and Control)

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, EU Candidate Countries and certain third countries.

In the years 2007, 2008 and 2010, the EU-RL VTEC organized three rounds of PT (PT1, PT2, and PT5, reports available at www.iss.it/vtec, section Proficiency Tests) 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. Both the number of the participating NRLs and their performance were satisfactory and increased by time (Figure 1).

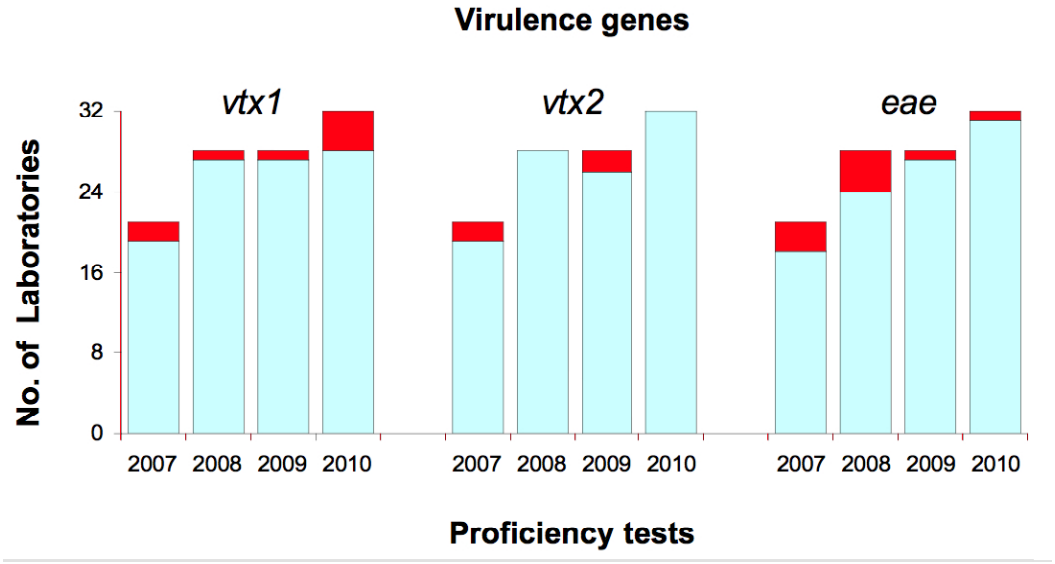

Figure 1: Proficiency tests organised by the EU-RL on the detection of virulence genes in VTEC strains by PCR. For each gene, blue bars represent the number of NRLs obtaining correct results for all the strains included in the test.

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*. However, several variants of these toxin genes have been described and some of them have been shown to be more clinically relevant than others. Therefore, this study was aimed at the sub-typing of VT-coding genes.

The study was conducted jointly with the network of medical National Reference Laboratories for VTEC referring to the European Center for Disease Prevention and Control (ECDC), Food- and Waterborne Diseases and Zoonoses Surveillance Programme (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), that is in charge for the external quality assurance program for the ECDC network of the medical 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.

This document represents the evaluation report of the results produced by the network of VTEC NRLs in the veterinary and food safety field (Regulation EC 882/2004). An overall report considering the results produced by all the laboratories that participated in the study will be elaborated jointly with SSI and published elsewhere.

2. RATIONALE OF THE STUDY

Verocytotoxins (VT), synonymous Shiga toxins, are a toxin family characterized by an elevated degree of diversity. The VT family is divided into two branches, VT1 and VT2, based on their antigenic differences. The terms “VT1” and “VT2” were also used to describe the prototypic toxins first described in each branch. Many toxin variants have been described in either branch and it has been recommended that VT family members be classified based on phenotypic differences, biologic activity and hybridization properties.

Classification of VT variants does not represent only a taxonomic exercise: some of the variants are clinically relevant in that they are produced by strains isolated from cases of hemolytic uremic syndrome (HUS), while some others are primarily associated with milder course of disease or are probably not produced by *E. coli* strains causing human disease. Different systems of nomenclature have been proposed and used for VT variants and their coding genes (*vtx*). A consensus on a comprehensive proposal of nomenclature has been reached during the 7th International Symposium on VTEC held in 2009. This proposal is going to be published and introduces three levels of designations:

Types: the two major branches VT1 and VT2 that share structure and function but that are not cross neutralized with heterologous antibodies. The terms VT1 and VT2 should only be used when the subtype is unknown.

Subtypes: the antigenically related members of the two main types, which are suffixed with small Arabic letters (e.g. VT1a).

Variants: variants include the subtype specific prototypic toxins or related toxins within a subtype (that differ by one or more AAs from the prototype). The variants are designated by toxin subtype, O group of the host *E. coli* strain, followed by the strain name or number from which that toxin was described (*e.g.* VT1a-O157-EDL933 or VT2c-O157-E32511). Nucleotide variants within a given VT subtype are italicised *e.g. vtx2c*-O157-E32511 is a nucleotide variant that encodes VT2c-O157-E32511.

The present study had two different scopes:

1. To assess the performance of a recently developed PCR method for the sub-typing of VT-coding genes, according to the above mentioned scheme.
2. To exploit the set of VTEC strains sent for the *vtx* subtyping to further 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.

3. DESIGN OF THE STUDY

At present, 10 *vtx* subtypes have been identified. However, the study was restricted to the identification of the six subtypes considered to be more relevant for laboratories involved in the veterinary and food safety fields: all three *vtx1* subtypes (*vtx1a*, *vtx1c* and *vtx1d*) and the three *vtx2* subtypes most involved in severe human infections (*vtx2a, vtx2c, vtx2d*). A conventional PCR method for the identification of these subtypes was provided to the participants by the EU-RL VTEC. The method had been previously agreed with the SSI.

In summary, the study consisted of 3 parts:

1. Identification and first characterization of VTEC by amplification of the main virulence genes (*vtx1* group, *vtx2* group, *eae*).
2. Identification of all three *vtx1* subtypes (*vtx1a*, *vtx1c* and *vtx1d*) and the three *vtx2* subtypes most involved in severe human infections (*vtx2a, vtx2c, vtx2d*) by using the conventional PCR method.
3. Identification of the O antigens of the test strains: it was limited to 11 serogroups comprised among those frequently reported as cause of human infections in Europe by the Food and Waterborne Diseases (FWD) surveillance programme of the ECDC, with

the addition of O121, a serogroup epidemiologically important in Japan, some EU countries, and North America.

Together with the test strains, the participating NRLs received a complete set of 10 reference control strains covering all the 10 established subtypes of *vtx1* and *vtx2* genes.

4. PARTECIPANTS

Thirty NRLs, representing 25 EU Member States, Croatia, Norway and Switzerland 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
- Croatia, Poultry Center of the Croatian 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
- Estonia, Veterinary and Food Laboratory
- Finland, Finnish Food Safety Authority Evira, Kuopio
- France, VetAgro Sup Campus Vétérinaire de Lyon
- Germany, Federal Institute for Risk Assessment (BfR)
- 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
- Lithuania, National Food and Veterinary Risk Assessment Institute
- Netherlands, Food and Consumer Product Safety Authority
- Norway, National Veterinary Institute
- Poland, National Veterinary Research Institute
- Portugal, Laboratório Nacional de Investigação Veterinária
- Slovakia, Public Health Authority, UVSZR

- Slovakia, State Veterinary and Food Institute Dolný Kubín
- Slovenia, National Veterinary Institute
- Spain, Agencia Española de Seguridad Alimentaria y Nutrición, AESAN
- Spain, Laboratorio Central de Sanidad Animal
- 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 five strains of *E. coli* (samples 1 to 5). NRLs were requested to detect the presence of *vtx1* (group), *vtx2* (group) and *eae* genes, to identify the *vtx* gene subtypes, and to identify the serogroup (O antigen or serogroup-associated genes) by either conventional or molecular serotyping.

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* (intimin) gene
1	**O103**	**+ (*vtx1a*)**	**-**	**+**
2	**O146**	**+ (*vtx1c*)**	**+ (*vtx2a*)**	**-**
3	**O154**	**+ (*vtx1d*)**	**-**	**-**
4	**O157**	**-**	**+ (*vtx2a* + *vtx2c*)**	**+**
5	**O91**	**-**	**+ (*vtx2d*)-**	**+**

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 22 and 26 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 borosilicate glass vials. The cultures were incubated 18 hrs at 37 °C +/- 1°C and labelled with randomly generated numerical codes (3 digits), different for each NRL. The test samples were shipped between 26 November and 3 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. Before uploading the serotyping results, the NRLs were requested to indicate the serogroups that they were able to identify either by immunological or molecular methods.

At the end of the study, the participants could print their own individual reports directly from the 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 a recently developed PCR method for the sub-typing of VT-coding genes.

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

5.3.1 Evaluation of the NRL performance

The analytical performance of each NRL was evaluated for the following items:

- detection of *vtx*1 and *vtx2* genes group and *eae* (altogether);
- identification of the O serogroup (within the expected panel of 11 serogroups).

For each of the items the NRL performances were 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.

The performance of each NRL in identifying the serogroup of the strains was evaluated according to the indication provided by each NRL on its capability to identify the specific serogroup (see section 5.2). Therefore a failure in the identification of a specific serogroup was evaluated as an error only if the NRL had claimed to be able to identify the specific serogroup.

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

The assessment was performed separately for *vtx1* and *vtx2*, using the data provided respectively by 23 and 24 NRLs, who were considered as non-outlier in their performance, based on the results obtained in the first phase of the PT. Therefore, the selected NRLs were those who did not report errors in the detection of the main virulence genes: *vtx1* group, *vtx2* group and *eae* (see section 6.1 and Table 2).

The performance characteristics assessed were those defined by the EN ISO 16140:2003 international standard: 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) **Accuracy**, defined as the closeness of agreement between a test result and the accepted reference value.

6. RESULTS

The samples were sent to the 30 NRLs that had accepted to participate in the study in the period between 26 November and 3 December. After receiving the samples, one of the NRLs (L31) communicated that it was not longer available to perform the tests and was not included in the result tables.

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

PCR detection of virulence genes was performed by all the 29 NRLs that made the analyses. The results are reported in Table 2.

Twenty-four NRLs (83 %) correctly identified the presence/absence of all the target genes in the test samples. Three NRLs (L02, L03, L08) failed to detect the presence of *vtx1* gene in one sample. One NRL (L15) failed to detect the *eae* gene in one sample an another one (L51) reported 3 false positive results for the *eae* gene.

Table 2. Detection by PCR of the main virulence genes (*vtx1* group, *vtx2* group, *eae*).

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.

NRL	Detection of genes in:														
	Sample 1			Sample 2			Sample 3			Sample 4			Sample 5		
	vtx1	*vtx2*	*eae*	*vtx1*	*vtx2*	*eae*	*vtx1*	*vtx2*	*eae*	*vtx1*	*vtx2*	*eae*	*vtx1*	*vtx2*	*eae*
True value	+	-	+	+	+	-	+	-	-	-	+	+	-	+	-
L01															
L02				-											
L03				-											
L08							-								
L10															
L12															
L14															
L15			-												
L17															
L19															
L20															
L21															
L22															
L23															
L24															
L25															
L28															
L29															
L34															
L36															
L37															
L41															
L42															
L45															
L46															
L49															
L50															
L51						+			+						+
L75															

The analytical performances of the participating NRLs in genotyping the *E. coli* strains were evaluated in terms of agreement (Cohen's Kappa), sensitivity and specificity. The agreement between the results obtained by the NRLs and the true values is reported in Tables 3.

Table 3. Detection of *vtx1* group, *vtx2* group and *eae* genes: agreement (Cohen's Kappa) between the results obtained by 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	0.49	1
L02	0.87	0.37	1
L03	0.87	0.37	1
L08	0.87	0.37	1
L10	1	0.49	1
L12	1	0.49	1
L14	1	0.49	1
L15	0.87	0.37	1
L17	1	0.49	1
L19	1	0.49	1
L20	1	0.49	1
L21	1	0.49	1
L22	1	0.49	1
L23	1	0.49	1
L24	1	0.49	1
L25	1	0.49	1
L28	1	0.49	1
L29	1	0.49	1
L34	1	0.49	1
L36	1	0.49	1
L37	1	0.49	1
L41	1	0.49	1
L42	1	0.49	1
L45	1	0.49	1
L46	1	0.49	1
L49	1	0.49	1
L50	1	0.49	1
L51	0.59	0.13	1
L75	1	0.49	1
Overall	0.97	0.88	1

The overall agreement between the results reported by the 29 NRLs and the true values of the samples was considered excellent, as well as the single agreements of all but one the NRLs.

The values of sensitivity and specificity for each NRL are reported in Table 4.

Table 4. Detection of *vtx1* group, *vtx2* group and *eae* genes: sensitivity and specificity for each NRL. The red boxes highlight values < 100 %.

	Sensitivity (Se) and Specificity (Sp) for each NRL									
NRL	**L01**	**L02**	**L03**	**L08**	**L10**	**L12**	**L14**	**L15**	**L17**	**L19**
Se	100	87,5	87,5	87,5	100	100	100	87,5	100	100
Sp	100	100	100	100	100	100	100	100	100	100
NRL	**L20**	**L21**	**L22**	**L23**	**L24**	**L25**	**L28**	**L29**	**L34**	**L36**
Se	100	100	100	100	100	100	100	100	100	100
Sp	100	100	100	100	100	100	100	100	100	100
NRL	**L37**	**L41**	**L42**	**L45**	**L46**	**L49**	**L50**	**L51**	**L75**	
Se	100	100	100	100	100	100	100	100	100	
Sp	100	100	100	100	100	100	100	57,1	100	

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

The identification of the *vtx* gene subtypes using the method proposed by the study guidelines was performed by 27 NRLs for *vtx1* and by 28 NRLs for *vtx2*.

One of the NRL (L29) performed the *vtx1* subtyping using a different method and the corresponding results were therefore not included in the analysis.

6.2.1. Sub-typing of vtx1

The results of the *vtx1* sub-typing are reported in Table 5.

Twenty-one of the 27 NRLs (78 %) that performed the subtyping identified correctly the presence/absence of the *vtx1* subtypes in the test samples.

Six NRLs reported a total of 11 errors: four errors (false positive) involved the detection of *vtx1a*, six errors (false positive) involved *vtx1c,* and one (false negative) involved *vtx1d.*

Table 5. 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.

NRL	Detection of the gene subtype in:								
	Sample 1			Sample 2			Sample 3		
	vtx1a	*vtx1c*	*vtx1d*	*vtx1a*	*vtx1c*	*vtx1d*	*vtx1a*	*vtx1c*	*vtx1d*
True Value	+	-	-	-	+	-	-	-	+
L01									
L02								+	
L03									
L08				+					-
L10									
L12									
L14								+	
L15									
L17									
L19									
L20									
L21		+		+			+	+	
L22									
L23									
L24									
L25									
L28									
L29									
L34									
L36									
L37		+							
L41									
L42							+	+	
L45									
L46									
L49									
L50									
L51									
L75									

6.2.2. Performance of the vtx1 sub-typing method

The performance characteristics of the proposed method were calculated using only the data from the 23 non-outlier NRLs, which were defined as those who did not report errors in the first step of the study, involving the PCR detection of the main virulence genes (see

also sections 5.3.2 and 6.1, and also table 2). Therefore, the data provided by 4 NRLs (L02, L03, L08, L15) were excluded from the analyses.

The performance characteristics of the method for subtyping the *vtx1* gene are reported in Table 6, as a whole and for the detection of the single gene subtypes.

Table 6. Performance characteristics of the method for subtyping the *vtx1* gene

Analyte	Performance characteristics		
	Sensitivity	**Specificity**	**Accuracy**
***vtx1a* gene**	**100 %**	**93.5 %**	**95.7 %**
***vtx1c* gene**	**100 %**	**89.1 %**	**92.8 %**
***vtx1d* gene**	**100 %**	**100 %**	**100 %**
Overall typing	**100 %** (95 %CI 100 % - 100 %)	**94.2 %** (95 %CI 90.3 % - 98.1 %)	**96.1 %** (95 %CI % 93.5 – 98.7 %)

6.2.3. Subtyping of vtx2

The results of the *vtx2* subtyping are reported in Table 7.

Ten of the 28 NRLs (36 %) that performed the subtyping identified correctly the presence/absence of the *vtx2* subtypes in all the test samples.

18 NRLs reported a total of 37 errors: 7 errors (3 false positive and 4 false negative) involved the detection of *vtx2a*, 18 errors (all false positive) involved *vtx2c,* and 12 (11 false positive and 1 false negative) involved *vtx2d.*

Table 7. Subtyping of the *vtx2* 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.

NRL	Detection of the gene subtype in:								
	Sample 2			Sample 4			Sample 5		
	vtx2a	*vtx2c*	*vtx2d*	*vtx2a*	*vtx2c*	*vtx2d*	*vtx2a*	*vtx2c*	*vtx2d*
True Value	+	-	-	+	+	-	-	-	+
L01								+	
L02						+		+	
L03						+			
L08						+	+	+	
L10								+	
L12									
L14			+					+	-
L15						+		+	
L17	-						+		
L19		+						+	
L20									
L21	-					+		+	
L22									
L23									
L24									
L25									
L28								+	
L29	-								
L34									
L36		+					+	+	
L37		+							
L41		+				+		+	
L42			+	-		+		+	
L45									
L46									
L49		+				+		+	
L50						+			
L51									
L75									

6.2.4. Performance of the vtx2 sub-typing method

The performance characteristics of the proposed method were calculated using only the data from the 24 non-outlier NRLs, which were defined as those who did not report errors in the first step of the study, involving the PCR detection of the main virulence genes (see also sections 5.3.2 and 6.1, and also table 2). Therefore, the data provided by 4 NRLs (L02, L03, L08, L15) were excluded from the analyses. The performance characteristics of

the method for subtyping the *vtx2* gene are reported in Table 8, as a whole and for the detection of the single gene subtypes.

Table 8. Performance characteristics of the method for subtyping the *vtx2* gene

Analyte	Performance characteristics		
	Sensitivity	**Specificity**	**Accuracy**
***Vtx2a* gene**	**93.3 %**	**90.9 %**	**92.5 %**
***Vtx2c* gene**	**100 %**	**68.9 %**	**79.1 %**
***Vtx2d* gene**	**95.5 %**	**84.8 %**	**88.1 %**
Overall typing	**95.5 %** (95 % CI 90.3 % - 99.3 %)	**79.5 %** (95 % CI 72.8 % - 87.2 %)	**86.6 %** (95 % CI 82.0 % - 91.1 %)

6.3. Identification of the O serogroup

Information on the availability of reagents for serogroup identification was provided by all the 29 NRLs and is summarized in Table 9. The methods included slide or tube agglutination with commercial or in house prepared antisera, or PCR amplification of serogroup-associated genes.

Table 9. Availability of reagents for serogroup identification, based on the NRLs' declarations in the entry form.

Number (%) of NRLs potentially able to identify serogroup:										
O157	**O26**	**O103**	**O145**	**O111**	**O91**	**O121**	**O55**	**O128**	**O113**	**O146**
29	29	29	29	28	27	21	20	17	16	14
(100 %)	(100 %)	(100 %)	(100 %)	(97 %)	(93 %)	(72 %)	(69 %)	(59 %)	(55 %)	(48 %)

Samples 1 and 4 contained VTEC strains belonging to one of the "top-five" serogroups commonly causing severe disease in humans (O103 and O157). Sample 2 and 5 contained VTEC strains belonging to O146 and O91, two serogroups comprised in the extended list of VTEC serogroups involved in human infections, according to the ECDC surveillance data. Sample 3 contained a VTEC strain belonging to a serogroup (O154) not comprised among the 11 serogroups included in the diagnostic panel considered for the

test, and was therefore classified as O N.T. (O antigen not typeable) with regard to the "true value" for the NRL performance evaluation. The identification of the serogroup was performed by all the 29 NRLs and the results are shown in Table 10.

Table 10. Results of serogroup identification. The green and red boxes indicate correct and wrong results, respectively, according to the diagnostic capability claimed by the NRLs for each serogroup.

NRL	Serogroup identification in:				
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
True Value	O103	O146	O154 (O N.T.)	O157	O91
L01	O103	O N.T.	O N.T.	O157	O91
L02	O103	O N.T.	O N.T.	O157	O N.T.
L03	O103	O N.T.	O N.T.	O157	O91
L08	O103	O26	O26	O157	O91
L10	O103	O N.T.	O N.T.	O157	O91
L12	O103	O146	O154	O157	O91
L14	O103	O146	O N.T.	O157	O91
L15	O103	O146	O N.T.	O157	O91
L17	O103	O146	O154	O157	O91
L19	O103	O146	O N.T.	O157	O91
L20	O103	O N.T.	O N.T.	O157	O N.T.
L21	O103	O N.T.	O121	O157	O121
L22	O103	O N.T.	O N.T.	O157	O91
L23	O103	O N.T.	O N.T.	O157	O91
L24	O103	O146	O154	O157	O91
L25	O103	O N.T.	O N.T.	O157	O91
L28	O103	O N.T.	O N.T.	O157	O91
L29	O103	O146	O N.T.	O157	O91
L34	O103	O146	O55	O157	O N.T.
L36	O103	O146	O154	O157	O91
L37	O103	O N.T.	O N.T.	O157	O91
L41	O103	O N.T.	O154	O157	O91
L42	O103	O146	O N.T.	O157	O91
L45	O103	O146	O N.T.	O157	O91
L46	O103	O146	O N.T.	O157	O91
L49	O103	O146	O N.T.	O157	O91
L50	O103	O N.T.	O N.T.	O157	O91
L51	O111	O N.T.	O N.T. *	O157	O91
L75	O N.T.	O N.T.	O N.T.	O157	O N.T.

* **Note:** L51 correctly identified the strain as not belonging to the 11 serogroups considered in the test but wrongly typed it as O148.

As a whole, 23 NRLs (79 %) typed correctly all the serogroups that they had declared to be able to identify. Six NRLs reported a total of 11 errors, involving 2 samples for five of them.
Three NRLs reported a “not typeable” result for 4 samples containing strains belonging to serogroups that the laboratory had claimed to be able to identify.
Four NRLs reported another wrong O antigen for 7 samples: in particular, 4 NRLs (L08, L21, L34, and L51) misidentified the VTEC O154 strain in sample 3 as O26, O121, O55, and O148, respectively.
The O154 strain in sample 3, not comprised among the 11 serogroups considered in the test, was correctly identified as O N.T. by 21 NRLs. In addition, 5 NRLs (L12, L17, L24, L36, L41) were able to identify the O154 serogroup.

The analytical performance of the NRLs for serogroup identification was evaluated in terms of agreement (Cohen’s Kappa), sensitivity, and specificity.

The agreement between the results obtained by the NRLs and the true values is reported in Table 11.

The overall agreement between the results reported by the 29 NRLs and the true values of the samples was considered excellent, as well as the single agreements of 24 NRLs.

Table 11. Serogroup identification: agreement (Cohen's Kappa) between the results obtained by 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	1,00	0,71	1,00
L02	1,00	0,61	1,00
L03	1,00	0,78	1,00
L08	0,60	0,03	1,00
L10	1,00	0,71	1,00
L12	1,00	0,81	1,00
L14	1,00	0,74	1,00
L15	1,00	0,72	1,00
L17	1,00	0,81	1,00
L19	0,88	0,69	1,00
L20	1,00	0,60	1,00
L21	0,52	0,20	0,85
L22	1,00	0,60	1,00
L23	1,00	0,69	1,00
L24	1,00	0,81	1,00
L25	1,00	0,64	1,00
L28	1,00	0,67	1,00
L29	1,00	0,69	1,00
L34	0,73	0,45	1,00
L36	1,00	0,74	1,00
L37	1,00	0,69	1,00
L41	1,00	0,71	1,00
L42	1,00	0,71	1,00
L45	1,00	0,74	1,00
L46	1,00	0,81	1,00
L49	1,00	0,72	1,00
L50	0,85	0,59	1,00
L51	0,64	0,33	0,95
L75	0,48	0,25	0,72
Overall	0,93	0,88	0,99

The values of sensitivity and specificity for each NRL are reported in Table 12.

Table 12. Serogroup identification of *E. coli* strains: Sensitivity and Specificity for each NRL. The red boxes highlight values < 100%.

Sensitivity (Se) and Specificity (Sp) for each NRL										
NRL	**L01**	**L02**	**L03**	**L08**	**L10**	**L12**	**L14**	**L15**	**L17**	**L19**
Se	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
Sp	100 %	100 %	100 %	95 %	100 %	100 %	100 %	100 %	100 %	100 %
NRL	**L20**	**L21**	**L22**	**L23**	**L24**	**L25**	**L28**	**L29**	**L34**	**L36**
Se	100 %	67 %	100 %	100 %	100 %	100 %	100 %	100 %	75 %	100 %
Sp	100 %	94 %	100 %	100 %	100 %	100 %	100 %	100 %	98 %	100 %
NRL	**L37**	**L41**	**L42**	**L45**	**L46**	**L49**	**L50**	**L51**	**L75**	
Se	100 %	100 %	100 %	100 %	100 %	100 %	75 %	67 %	33 %	
Sp	100 %	100 %	100 %	100 %	100 %	100 %	100 %	97 %	100 %	

The capability of the NRL network to identify the serogroups of the test strains is shown in Table 13, where failures in serogroup identification were due to either wrong typing results (red boxes) or the lack of reagents for the identification of the given serogroup (white boxes).

Table 13. Capability of the NRL network to identify the serogroups of the test strains: The green and red boxes indicate correct and wrong results. The white boxes indicate the lack of reagents for the identification of the given serogroup.

NRL	Serogroup identification in:				
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
True Value	O103	O146	O154	O157	O91
L01					
L02					
L03					
L08		O26	O26		
L10					
L12					
L14					
L15					
L17					
L19					
L20					
L21			O121		O121
L22					
L23					
L24					
L25					
L28					
L29					
L34			O55		O N.T.
L36					
L37					
L41					
L42					
L45					
L46					
L49					
L50		O N.T.			
L51	O111		O148		
L75	O N.T.				O N.T.

As for the two strains belonging to the serogroups comprised among the “top-five”, all the NRLs identified correctly the VTEC O157 strain, but 2 NRLs (L51 and L75) failed to

identify the O103 strain. The strains belonging to serogroups O91 and O146 were identified respectively by 24 of 27 and 13 of 15 NRLs that had claimed to be able to identify these serogroups.

7. CONCLUDING REMARKS

The present study was conducted jointly with the network of medical National Reference Laboratories for VTEC referring to the ECDC, with the aim of harmonizing 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.

The study had two different scopes:

- 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;
- to assess the performance of a recently developed PCR method for the sub-typing of VT-coding genes.

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 veterinary and food safety NRLs. The following remarks can be drawn.

1. Twenty-six EU NRLs, representing 22 Member States, and the NRLs of Croatia, Norway and Switzerland participated in the study and performed the tests, for a total of 29 laboratories.
2. Twenty-four NRLs (83%) correctly identified the presence/absence of the main virulence genes (*vtx1* group, *vtx2* group, *eae*) in all the test samples.
3. Twenty-three NRLs (79 %) typed correctly all the serogroups that they had claimed to be able to identify. In particular, all the NRLs identified correctly the O157 strain included among the test samples and all but two (93 %) the O103 strain, the other strain belonging to the serogroups comprised among the “top-five” serogroups causing severe human infections. The less common O91 and O146 serogroups were identified respectively by 24 of 27 and 13 of 15 NRLs that had claimed to be able to identify these O antigens.
4. Considering altogether the results of genotyping and serotyping, an excellent agreement (K value > 0.75) of the results with the true values was observed for all the

participating NRLs. A good accuracy was also obtained in terms of sensitivity and specificity.

5. The identification of the *vtx* gene subtypes using the method proposed by the study guidelines was performed by 27 NRLs for *vtx1* and by 28 NRLs for *vtx2*. Twenty-one NRLs (78 %) identified correctly the *vtx1* subtypes in all the test samples, whereas only 10 NRLs (36 %) identified correctly the *vtx2* subtypes.
6. The performance parameters of the proposed subtyping method were calculated using the data from 23 non-outlier NRLs, defined as those who did not report errors in the PCR detection of the main virulence genes (Table 2). Sensitivity, specificity and accuracy were satisfactory for *vtx1* subtyping (100 %, 94.2 %, 96.1 %, respectively) but not for *vtx2* subtyping (95.5 %, 79.5 %, 86.6 %, respectively), which will require further adjustment.
7. In conclusion, most NRLs performed satisfactorily in the identification of VTEC by PCR amplification of the main virulence factors and in the typing of the serogroups most involved in severe human disease. The method proposed for the identification of the *vtx* gene subtypes lacks in specificity for the vtx2 typing and needs further adjustment.
8. The organization of a joint inter-laboratory study on VTEC typing with the ECDC network of medical 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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