EU Reference Laboratory for *E. coli*

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Report of the 13th inter-laboratory study (PT13) on the identification and typing of Verocytotoxin-producing *E. coli* (VTEC) and other pathogenic *E. coli* strains - 2014

Edited by:

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1. INTRODUCTION AND OBJECTIVES

The duties of the EU Reference Laboratory for *Escherichia coli* (EU-RL VTEC) include the organization of proficiency tests (PT) on the detection and typing of pathogenic *E. coli* 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 can participate in these tests on a voluntary basis.

In the past years, the EU-RL VTEC organized six PT rounds (PT1, PT2, PT5, PT6, PT10, and PT11) on the identification and typing of VTEC, as well as of strains belonging to other pathogenic groups of *E. coli*. These PTs aimed at evaluating and improving the capability of the NRLs to identify and characterize VTEC and other pathogenic *E. coli*. The reports of these PTs are available at the EU-RL VTEC www.iss.it/vtec, in the Proficiency Tests section.

The present study (PT13) was dedicated to the identification and typing of VTEC strains, as well as to the identification of *E. coli* strains belonging to the Enteropathogenic *E. coli* (EPEC) and Enteroaggregative *E. coli* (EAggEC) pathogenic groups.

Therefore, the objectives of the study were:

1. The detection of the main VTEC/EPEC virulence genes.
2. The detection of the EAggEC marker genes.
3. The identification of a range of relevant VTEC/EPEC serogroups.
4. The sub-typing of VT-coding genes.
5. The 3rd round of external quality assessment (EQA) for PFGE, in view of the start of the program for the collection of molecular typing data on VTEC strains of food and animal origin by the European Food Safety Authority (EFSA), to improve the EU preparedness to face foodborne outbreaks.

This document represents the evaluation report of the study, as far as the results on the identification of the *E. coli* pathogroups and the typing of VTEC virulence genes and O antigens are concerned. The PFGE results will be presented in a separate report.

2. 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 three parts:

1. The identification of the *E. coli* pathogroups by PCR amplification of the following target virulence genes:

- *vtx1* group, *vtx2* group, and the intimin-coding *eae* gene for VTEC
- the *eae* gene for EPEC
- the *aaiC* and *aggR* genes for EAggEC

2. Determination of the serogroups of the strains. Participants were requested to identify the strains belonging to any of the following 13 serogroups, selected on the basis of their epidemiologic or regulatory importance:

- O26, O103, O111, O145 and O157: the so called “top 5”, most involved in severe human infections worldwide.
- O45 and O121: epidemiologically relevant and considered as adulterants in beef in the USA.
- O104: relevant after the 2011 German outbreak.
- O55, O91, O113, O128, O146: selected on the basis of their prevalence in human infections in Europe in the last years, according to the data provided by the European Centre for Disease Prevention and Control (ECDC).

3. Subtyping of the *vtx* genes harbored by the VTEC strains identified. Participants were requested to identify the subtypes of the *vtx1* group (*vtx1a, vtx1c and vtx1d*) and *vtx2* group (from *vtx2a* to *vtx2g*) genes detected.

3. PARTICIPANTS

Forty-one NRLs, representing all the 28 EU Member States, as well as Argentina, Egypt, Russia, Norway, Switzerland and Turkey 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:

- Argentina, Consortium between *Instituto Nacional de Enfermedades Infecciosas* (INEI), ANLIS “Dr. Carlos G. Malbran” and *Servicio Nacional de Sanidad y Calidad Alimentaria* (SENASA)
- Argentina, *Universidad Nacional de* La Plata
- Austria, *Österreichische Agentur für Gesundheit und Ernährungssicherheit* GmbH
- Belgium, Scientific Institute of Public Health (also representing Luxembourg)
- Belgium, Veterinary & Agrochemical Research Centre (VAR - CODA – CERVA)
- Bulgaria, National Diagnostic and Research Veterinary Institute
- Croatia, 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
- Egypt, Central Lab of Residue Analysis of Pesticides and Heavy Metals in Foods
- 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, Food and Feed Safety Directorate, National Food Microbiological Reference Laboratory
- Hungary, Food and Feed Safety Directorate, Feed Investigation National Reference Laboratory
- Ireland, Central Veterinary Research Laboratory, Department of Agriculture, Food and the Marine
- Italy, *Istituto Superiore di Sanità*
- Latvia, Institute of Food Safety, Animal Health and Environment BIOR
- Lithuania, National Food and Veterinary Risk Assessment Institute
- Netherlands, RIVM, Centre for Zoonoses and Environmental Microbiology
- Netherlands, Food and Consumer Product Safety Authority (NVWA)
- Norway, Norwegian Veterinary Institute
- Poland, National Institute of Public Health-National Institute of Hygiene
- Poland, Poland National Veterinary Research Institute
- Portugal, *Instituto Nacional de Investigação Agrária e Veterinária* (INIAV)
- Romania, *Institutul de Igiena si Sanatate Publica Veterinara*
- Russia, International Department State Research Center for Microbiology and Biotechnology, Obolensk
- Slovakia, Department of Food Hygiene, State Veterinary and Food Institute
- Slovakia, NRC of Environmental Microbiology, Public Health Authority of SR
- Slovenia, Veterinary Faculty/ National Veterinary Institute
- Spain, *Laboratorio Central de Veterinaria de* Algete (MAGRAMA)

- Spain, *Centro Nacional de Alimentación, Servicio de Microbiología Alimentaria* (AESAN)
- Sweden, National Food Agency (SLV)
- Sweden, National Veterinary Institute (SVA)
- Switzerland, Institute for Food Safety and Hygiene, University of Zurich
- Turkey, *Ulusal Gida Referans Laboratuvar Müdürlügü*, National Food Reference Laboratory
- United Kingdom (UK), Public Health England (also representing Malta)

4. MATERIALS AND METHODS

4.1. Sample preparation

The test materials sent to the NRLs were constituted by 7 *E. coli* strains (samples 1 to 7), selected among those present in the EU-RL VTEC 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

Sample/Strain	Pathogroup	Serogroup	Target virulence genes (*vtx* subtypes)				
			vtx1	*vtx2*	*eae*	*aggR*	*aaiC*
1	VTEC	O145	*vtx1a*	-	+	-	-
2	VTEC	O91	-	*vtx2d*	-	-	-
3	EAggEC	O104	-	-	-	+	+
4	VTEC	O146	*vtx1c*	*vtx2a, vtx2b*	-	-	-
5	VTEC	O128	-	*vtx2b*	-	-	-
6	VTEC	O157 (sorbitol fermenting)	-	*vtx2a*	+	-	-
7	EPEC	O45	-	-	+	-	-

As for the stability of the samples, previous experiences supported the assumption that the time range between the preparation of the specimens and the deadline for submission of results was short enough to assure the detection of all the strain characteristics.

The test samples were prepared on 2 and 3 April. They consisted of freshly prepared bacterial cultures seeded into soft (0.3 %) nutrient agar in plastic vials. The cultures were incubated 18 hours at 37 °C ± 1 °C and labeled with randomly generated numerical codes (3 or 4 digits), different for each NRL. The test samples were stored at room temperature until 7 April, when the samples were sent to the participating laboratories by courier.

4.2. Laboratory methods

The identification of the *E. coli* patho-groups was carried out by PCR (end point or real time) amplification of their specific target virulence genes using the methods available in the EU-RL VTEC web site (http://www.iss.it/vtec), Laboratory Methods section.

For the determination of the serogroup, participants were allowed to use any serological or molecular method currently in use in their laboratories. However, a PCR (end point) procedure for detecting the genes associated with the serogroups that were in the scope of the PT was available in the EU-RL web site, Laboratory Methods section.

The subtypes of the *vtx* genes of the VTEC strains identified were determined by an end point PCR method based on the paper of Scheutz et al. (*J. Clin. Microbiol. 2012; 50: 2951-63*). The procedure was available in the EU-RL web site, Laboratory Methods section.

4.3. 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 VTEC 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 analyzed and the fields to collect the information on arrival date, temperature and quality of the sample, and with the possibility to write notes and to specify any problem with the sample delivery/packaging. At the end of the study, the participants could print their own instant-generated individual report, containing the submitted and the expected results, directly from the secure page of the EU-RL web site.

4.4. Analysis of the NRL results

4.4.1. Evaluation of the NRL performance in the identification of the virulence genes of pathogenic E. coli

The performance of each NRL in identifying the virulence genes of pathogenic *E. coli* was evaluated by assigning penalty points for the virulence genes that were identified incorrectly. The penalty points were assigned as follows, according to the public health relevance of the different genes:

- **4 penalty points** were assigned to each incorrect result concerning the identification of the *vtx* genes, that represent the main virulence determinants of VTEC and the main target of the ISO/TS 13136:2012, the international standard aiming at the detection of these pathogenic *E. coli* in food. The detection of these genes was the object of several previous PTs. Moreover, an EU regulation (Reg. EU 209/2013) define a microbiological criterion for the presence of VTEC in a food commodity, namely sprouts.
- **2 penalty points** were assigned to each incorrect result concerning the identification of the other virulence genes considered in the PT: *eae, AggR, aaiC*.
- **1 penalty point** was assigned to each result reported as "Not Done".

The sum of the penalty points obtained originated a total score used to evaluate the performance of the NRLs. In particular, a threshold of 4 penalty points was set in order to identify the laboratories not performing adequately for this part of the PT. The NRLs that summed up a score of 4 without making errors in the identification of *vtx* genes represented an exception and their performance was still considered as satisfactory.

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

The performance of each NRL in identifying the serogroup of the test 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 most frequently isolated from cases of hemolytic uremic syndrome in Europe: O26, O103, O111, O145, O157 (the so called "top 5").
- **2 penalty points:** assigned to each incorrect result concerning the typing of the strains belonging to the other 8 serogroups that were in the scope of this study.

The sum of the penalty points originated a score used to evaluate the performance of the NRLs. 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 still considered as satisfactory.

4.4.3. Evaluation of the NRL performance in the identification of the vtx gene subtypes

The performance of each NRL in identifying the subtypes of the *vtx* genes in the five VTEC strains included in the test materials was evaluated by assigning one penalty point for *vtx* genes that were typed incorrectly or for results reported as “Not Done”. Results that were not uploaded (“null” field) were also considered as “Not Done”. A threshold of 4 penalty points was set in order to identify the laboratories not performing adequately.

5. RESULTS

Results were submitted by 40 of the 41 NRLs that received the samples.

5.1. Identification of the *E. coli* virulence genes and evaluation of the NRL performance for these tests

The results of the PCR tests performed for the detection of virulence genes in each *E. coli* strain are reported in Table 2 (1-3), while Table 3 summarizes the results of the tests performed for all the 7 test strains, by virulence gene.

All the NRLs carried out the tests for the detection of *vtx* and *eae* genes, while the detection of the EAggEC target virulence genes was not performed by 5 NRLs for all of part of the test strains.

Twenty-seven NRLs (67 %) identified correctly the presence/absence of all the target genes in all the test strains, while 9 NRLs provided a total of 20 incorrect results.

As for the detection of *vtx* genes, 35 NRLs (87 %) identified correctly their presence/absence in all the test strains, while the other five NRLs provided a total of 12 incorrect results: 8 for *vtx1* (6 false positive and 2 false negative) and 5 for *vtx2* (4 false positive and 1 false negative).

As for the other virulence genes, 35 NRLs (87 %) identified correctly the presence/absence of the *eae* gene. The other 5 NRLs provided a total of 7 incorrect results: (5 false positive and 2 false negative).

The EAggEC target genes were identified correctly by 34 (97 %) of the 35 NRLs that carried out all the tests. The remaining NRL provided a false positive result for the *aggR* gene.

As a whole, 16 (80 %) of the 20 incorrect results on virulence gens were false positive results.

Table 2 (1). Identification of the *E. coli* virulence genes (strains 1, 2 and 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 incorrect results. ND indicates that the test was not done (ND).

NRL	Detection of virulence 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	+	-	+	-	-	-	+	-	-	-	-	-	-	+	+
L106				ND	ND				ND	ND				ND	ND
L118															
L157															
L206															
L240															
L258			-												
L273															
L285		+		ND	ND	+		+	ND	ND	+	+	+	ND	ND
L313				ND	ND				ND	ND				ND	ND
L354															
L367															
L402															
L433															
L463															
L519															
L534								+							
L544															
L551															
L553															
L566															
L605															
L608															
L633															
L674															
L679															
L687															
L691															
L692															
L699															
L712															
L715															
L737															
L768															
L775															
L776				ND	ND				ND	ND					
L905															
L947					ND					ND					ND
L949															
L986															
L987															

Table 2 (2). Identification of the *E. coli* virulence genes (strains 4 and 5). 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. ND indicates that the test was not done (ND).

NRL	Detection of virulence genes in:									
	Sample 4					Sample 5				
	vtx1	*vtx2*	*eae*	*aggR*	*aaiC*	*vtx1*	*vtx2*	*eae*	*aggR*	*aaiC*
True value	+	+	-	-	-	-	+	-	-	-
L106				ND	ND				ND	ND
L118										
L157										
L206										
L240										
L258										
L273	-									
L285			+	ND	ND	+		+	ND	ND
L313				ND	ND				ND	ND
L354										
L367										
L402										
L433										
L463									+	
L519										
L534										
L544										
L551										
L553										
L566	-									
L605										
L608										
L633										
L674										
L679										
L687										
L691										
L692										
L699										
L712										
L715										
L737										
L768										
L775										
L776				ND	ND				ND	ND
L905										
L947					ND					ND
L949										
L986										
L987										

Table 2 (3). Identification of the *E. coli* virulence genes (strains 6 and 7). 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. ND indicates that the test was not done (ND).

NRL	Detection of virulence genes in:									
	Sample 6					Sample 7				
	vtx1	*vtx2*	*eae*	*aggR*	*aaiC*	*vtx1*	*vtx2*	*eae*	*aggR*	*aaiC*
True value	-	+	+	-	-	-	-	+	-	-
L106				ND	ND				ND	ND
L118										
L157		-					+			
L206										
L240										
L258										
L273										
L285	+			ND	ND	+			ND	ND
L313				ND	ND				ND	ND
L354										
L367										
L402										
L433										
L463										
L519										
L534				ND	ND					
L544										
L551										
L553										
L566										
L605						+	+			
L608										
L633								ND		
L674										
L679										
L687										
L691										
L692										
L699								-		
L712										
L715										
L737										
L768										
L775										
L776				ND	ND				ND	ND
L905										
L947					ND					ND
L949										
L986										
L987										

Table 3. Summary of the results on the identification of *E. coli* virulence genes. The green boxes indicate that the genes were identified correctly in all the 7 test strains. The red and white boxes indicate that incorrect results and tests not done were reported for the given gene, respectively. The numbers in the boxes indicate the number of incorrect or "not done" results.

NRL	Detection of virulence genes in the 7 test strains:				
	vtx1	***vtx2***	***eae***	***aggR***	***aaiC***
L106				**7**	**7**
L118					
L157		**2**			
L206					
L240					
L258			**1**		
L273	**1**				
L285	**5**	**2**	**4**	**7**	**7**
L313				**7**	**7**
L354					
L367					
L402					
L433					
L463				1	
L519					
L534			1	**1**	**1**
L544					
L551					
L553					
L566	1				
L605	**1**	**1**			
L608					
L633			**1**		
L674					
L679					
L687					
L691					
L692					
L699			**1**		
L712					
L715					
L737					
L768					
L775					
L776				**6**	**6**
L905					
L947					**7**
L949					
L986					
L987					

For each NRL, the number of penalty points was determined using the criteria described in section 4.4.1. Figure 1 shows the score obtained by each NRL, and Figure 2 the number of NRLs grouped according to their score. Five NRLs obtained a score higher than 4 due to incorrect results, and other 4 NRLs because they did not perform the detection of all or part of the EAggEC virulence genes. The performance of these 9 NRLs was not considered as satisfactory.

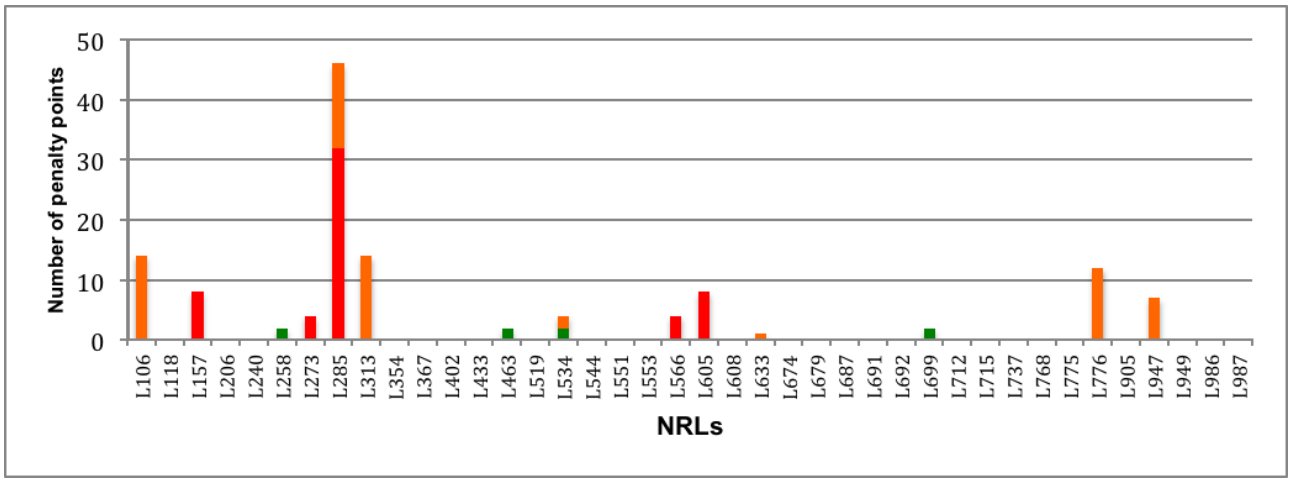

Figure 1. Evaluation of the PCR results for the detection of virulence genes, by NRL. The score was calculated according to the criteria described in section 4.4.1. The red bars indicate the penalty points assigned for incorrect results in the 5 NRLs whose performance was not considered as satisfactory. The yellow bars indicate the penalty points assigned for tests not done.

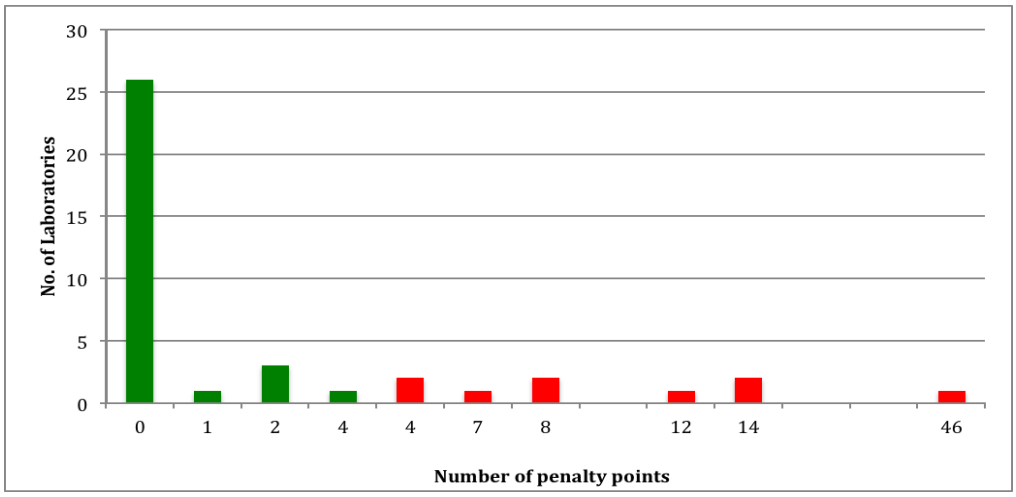

Figure 2. Evaluation of the PCR results for the detection of virulence genes: number of NRLs within each penalty score. The red bars indicate the NRLs whose performance was not considered as satisfactory.

5.2. Identification of the serogroups and evaluation of the NRL performance for this test

The identification of the O serogroups of the strains included in the PT was carried out by all the 40 NRLs, 36 of which used the PCR procedure for detecting serogroup-associated genes made available by the EU-RL.

The serotyping results are shown in Table 4.

Twenty-eight NRLs (70 %) identified correctly the serogroup of all the 7 test strains. The other 12 NRLs provided a total of 25 incorrect results. In particular, 9 NRLs (22 %) did not identify the O45 strain, which was included for the first time in the panel of O antigen to be detected, and 5 NRLs (12 %) the O146 strain. Two NRLs, L157 and L285, failed to identify the O157 and O145 strains.

The number of penalty points assigned to each NRL according to the criteria described in section 4.4.2, is shown in Figure 3.

Figure 4 shows the number of NRLs grouped according to their score.

Three NRLs obtained a score higher than 4 and their performance was not considered as satisfactory.

Table 4. Identification of the serogroups of the test strains. The green boxes indicate the correct results. The red boxes indicate the incorrect results, and display the results provided by the NRL.

NRL	Serogroup identification in sample:						
	1	2	3	4	5	6	7
True value	O145	O91	O104	O146	O128	O157	O45
L106		NT		NT			NT
L118				NT			
L157	O111					NT	O157
L206							
L240							
L258							
L273							NT
L285	O157	O104	O26	O157	O104	NT	NT
L313			O125				O86
L354							
L367							NT
L402							
L433							
L463							
L519							
L534							
L544							
L551				NT	NT		
L553							NT
L566		O146		O91			
L605							
L608							
L633							O103
L674							
L679							
L687							NT
L691							
L692							
L699							
L712							
L715							
L737							
L768							
L775							
L776							
L905							
L947							
L949							
L986							
L987							

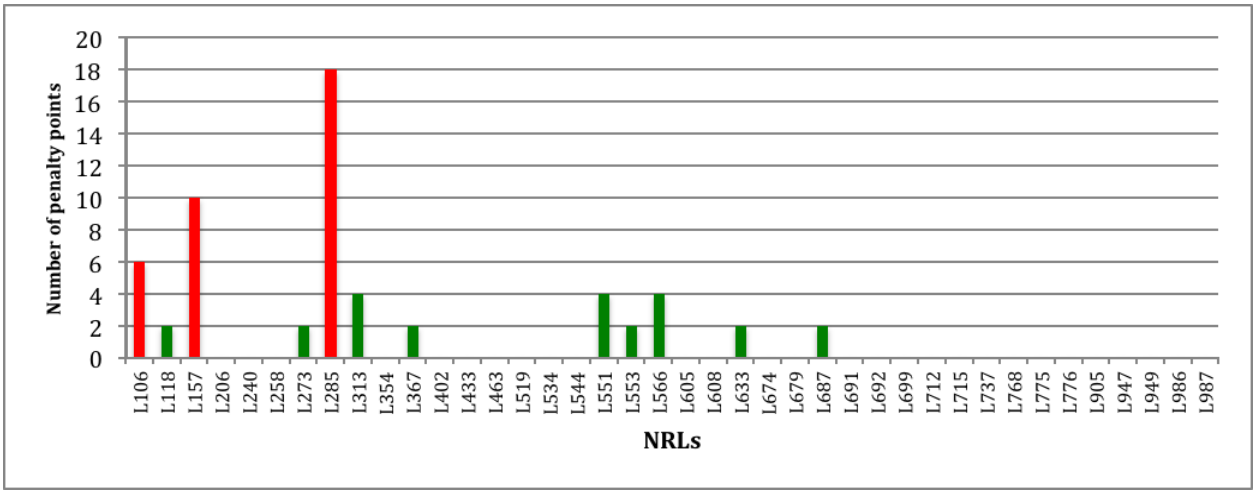

Figure 3. Evaluation of the results on serogroup identification, by NRL. The score was calculated according to the criteria described in section 4.4.2. The red bars indicate the NRLs whose performance was not considered as satisfactory.

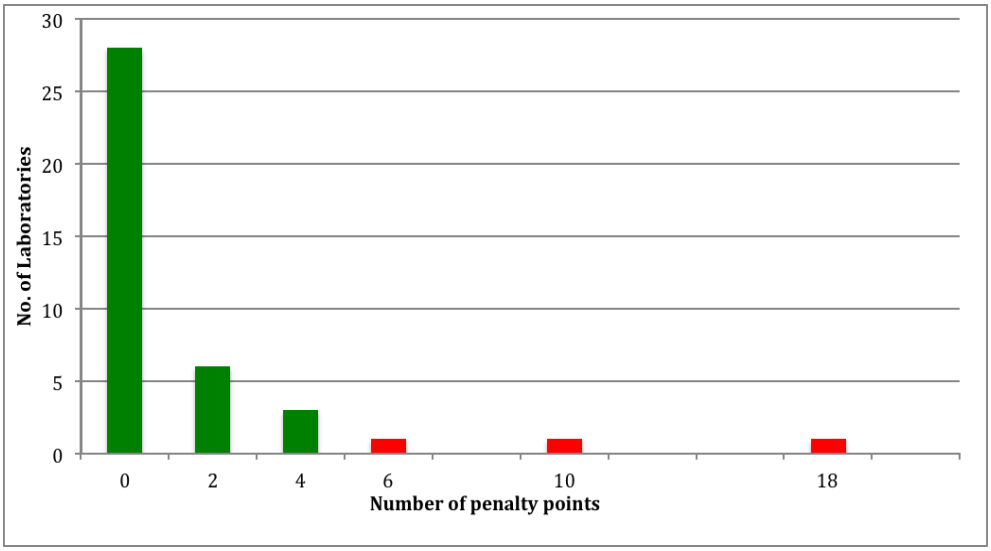

Figure 4. Evaluation of the results on serogroup identification: number of NRLs within each penalty score. The red bars indicate the NRLs whose performance was not considered as satisfactory.

5.3. Identification of the *vtx* gene subtypes by PCR

The identification of the *vtx* gene subtypes harbored by the 5 VTEC test strains was performed by 38 of the 40 NRLs that participated in the study (95 %). One NRL (L947) carried out only the subtyping of *vtx2* genes.

The results of *vtx1* subtyping are reported in Table 5.
The presence/absence of the *vtx1* gene subtypes in the two *vtx1*-positive strains was identified correctly by 24 (83 %) of the 29 NRLs that carried out the subtyping for all the gene variants. Five NRLs reported a total of 9 incorrect results: 5 false positive results (3 for *vtx1c,* 1 for *vtx1a* and 1 for *vtx1d*) and 4 false negative results (2 for *vtx1a* and 2 for *vtx1c*). Nine NRLs did not perform all the tests required.

The results of *vtx2* subtyping in each *E. coli* strain are reported in Table 6 (1-2).
Twenty-four NRLs carried out the subtyping for all the gene variants in all the strains, while the other 14 NRLs did not perform all the tests required. Eight NRLs (21 %) identified correctly the presence/absence of all the gene subtypes. The other 30 NRLs reported a total of 70 incorrect results: 59 false positive results*,* most of which concerning the *vtx2c* variant (41 for *vtx2c,* 9 for *vtx2d,* 7 for *vtx2a,* 1 for *vtx2b,* and 1 for *vtx2e*), and 11 false negative results (5 for *vtx2d,* 3 for *vtx2a* an*,* 3 for *vtx2b*). For 13 NRLs, the incorrect results were limited to the *vtx2c* variant only: 9 of them reported the false positive result for the *vtx2d*-positive strain 2 only, 2 NRLs for strains 2 and 4, and 2 NRLs for strains 2, 4 and 6.

Table 7 summarizes the results of all the *vtx* subtyping tests. Only 8 NRLs (21 %) identified correctly all the *vtx* gene subtypes in the 5 VTEC strains. Another NRL (L519) did not report incorrect results but did not perform all the tests required.

For each NRL, the number of penalty points was determined using the criteria described in section 4.4.3. Figure 5 shows the score achieved by each NRL, and Figure 6 the number of NRLs grouped according to their score.
Twelve NRLs obtained a score higher than 4 due to both incorrect results and test not done, and their performance was not considered as satisfactory.

Table 5. Subtyping of the *vtx1* genes by PCR in samples 1 and 4. 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. The white boxes indicate that the test was not done (ND).

NRL	Detection of *vtx1* gene subtypes in:					
	Strain 1			Strain 4		
	vtx1a	*vtx1c*	*vtx1d*	*vtx1a*	*vtx1c*	*vtx1d*
True value	+	-	-	-	+	-
L106						
L118						
L157		+				
L206					-	+
L240						
L258				ND		ND
L273				ND	ND	ND
L313		ND	ND	ND		ND
L354						
L367	-					
L402						
L433						
L463						
L519						
L534						
L544		ND	ND	ND		ND
L553			ND	ND	ND	ND
L566				ND	ND	ND
L605						
L608						
L633	-	+		+	-	
L674						
L679						
L687			ND			ND
L691						
L692						
L699						
L712						
L715						
L737		+				
L768						
L775						
L776		ND	ND			
L905						
L947	ND	ND	ND	ND	ND	ND
L949						
L986						
L987						

Table 6 (1). Subtyping of the *vtx2* genes by PCR in samples 2 and 4. 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. The white boxes indicate that the test was not done (ND).

NRL	Detection of *vtx2* gene subtypes in:													
	Strain 2							Strain 4						
	vtx2a	*vtx2b*	*vtx2c*	*vtx2d*	*vtx2e*	*vtx2f*	*vtx2g*	*vtx2a*	*vtx2b*	*vtx2c*	*vtx2d*	*vtx2e*	*vtx2f*	*vtx2g*
True value	-	-	-	+	-	-	-	+	+	-	-	-	-	-
L106			+							+				
L118														
L157		ND	+		ND	ND	ND		ND		+	ND	ND	ND
L206			+											
L240														
L258	ND	ND	+		ND	ND	ND			ND	ND	ND	ND	ND
L273			+						-					
L313	+	ND	+		ND	ND	ND			+	+	ND	ND	ND
L354			+											
L367		ND	+						ND	+	+			
L402			+											
L433			+											
L463			+							+				
L519					ND	ND	ND							
L534			+											
L544	+	ND	ND		ND	ND	ND			ND	ND	ND	ND	ND
L553				-										
L566	+		+	-						+				
L605	+		+	-						+				
L608			+											
L633														ND
L674			+											
L679														
L687				-										
L691			+	-						+				
L692			+											
L699			+											
L712	+		+		ND		ND					ND		ND
L715														
L737			+							+				
L768	+		+							+	+			
L775														
L776														
L905														
L947			+											
L949														
L986														
L987			+							+				

Table 6 (2). Subtyping of the *vtx2* genes by PCR in samples 5 and 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 incorrect results. The white boxes indicate that the test was not done (ND).

	Detection of *vtx2* gene subtypes in:													
	Strain 5							Strain 6						
NRL	*vtx2a*	*vtx2b*	*vtx2c*	*vtx2d*	*vtx2e*	*vtx2f*	*vtx2g*	*vtx2a*	*vtx2b*	*vtx2c*	*vtx2d*	*vtx2e*	*vtx2f*	*vtx2g*
True value	-	+	-	-	-	-	-	+	-	-	-	-	-	-
L106										+				
L118														
L157		-		ND	ND	ND	ND	-	ND			ND	ND	ND
L206														
L240														
L258									ND	ND	ND	ND	ND	ND
L273														
L313	ND		ND	ND	ND	ND	ND		ND	+	+	ND	ND	ND
L354								-		+				
L367		-			ND	ND	ND		ND	+	+	ND	ND	ND
L402														
L433														
L463														
L519														
L534														
L544	ND		ND	ND	ND	ND	ND		ND	ND	ND	ND	ND	ND
L553														
L566												+		
L605			+						+					
L608														
L633														
L674	+													
L679														
L687														
L691														
L692														
L699														
L712					ND		ND					ND		ND
L715														
L737										+				
L768				+						+	+			
L775														
L776								-			+			
L905														
L947														
L949														
L986														
L987														

Table 7. Summary of the *vtx* gene subtyping results. The green boxes indicate that the genes were identified correctly in all the 5 VTEC test strains. The red and white boxes indicate that incorrect results and tests not done were reported for the given gene, respectively. The numbers in the boxes indicate the number of incorrect or "not done" results.

NRL	Detection of *vtx* gene subtypes in the 5 VTEC strains:									
	vtx1a	***vtx1c***	***vtx1d***	***vtx2a***	***vtx2b***	***vtx2c***	***vtx2d***	***vtx2e***	***vtx2f***	***vtx2g***
L106						3				
L118										
L157		1		1	1 3	1	1 1	4	4	4
L206		1	1			1				
L240										
L258	1		1	1	2	1 2	2	3	3	3
L273	1	1	1		1	1				
L313	1	1	2	1 1	2	3 1	2 1	4	4	4
L354				1		2				
L367	1				1 3	3	2	2	2	2
L402						1				
L433						1				
L463						2				
L519								1	1	1
L534						1				
L544	1	1	2	1 1	2	4	3	4	4	4
L553			2				1			
L566	1	1	1	1		2	1	1		
L605				1	1	3	1			
L608						1				
L633	2	2								1
L674				1		1				
L679										
L687			2				1			
L691						2	1			
L692						1				
L699						1				
L712				1		1		4		4
L715										
L737		1				3				
L768				1		3	3			
L775										
L776		1	1	1			1			
L905										
L947	2	2	2			1				
L949										
L986										
L987						2				

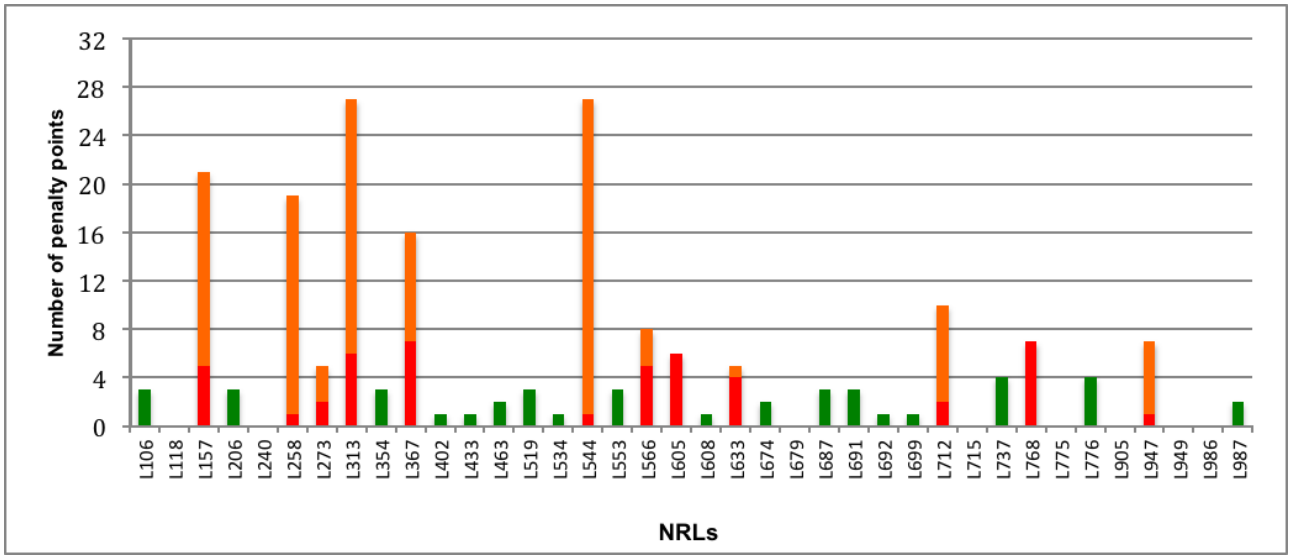

Figure 5. Evaluation of *vtx* gene subtyping results, by NRL. The score was calculated according to the criteria described in section 4.4.3. The red bars indicate the penalty points assigned for incorrect results in the 12 NRLs whose performance was not considered as satisfactory. The yellow bars indicate the penalty points assigned for tests not done.

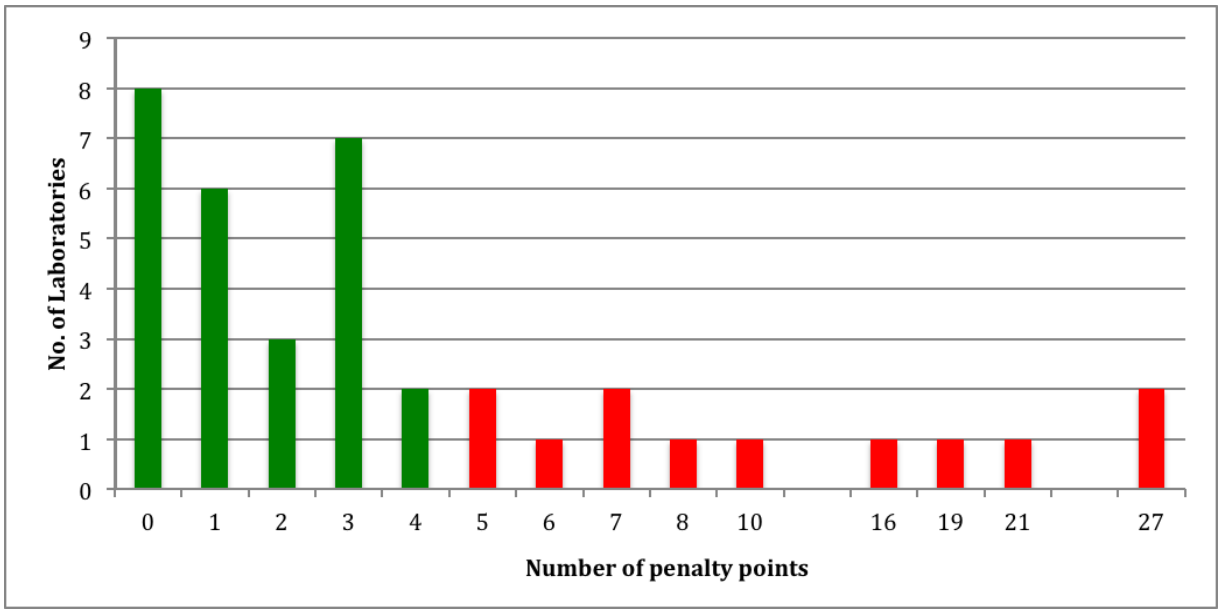

Figure 5. Evaluation of *vtx* gene subtyping results: number of NRLs within each penalty score. The red bars indicate the NRLs whose performance was not considered as satisfactory.

7. CONCLUDING REMARKS

1. Forty-one NRLs, representing all the 28 EU Member States, as well as Argentina, Egypt, Russia, Norway, Switzerland and Turkey participated in the study. Forty NRLs submitted results.
2. Thirty-five NRLs (87 %) identified correctly the presence/absence of *vtx* and *eae* target genes in all the test strains.
3. Testing of EAggEC target genes was carried out correctly by 35 (97 %) of the 36 NRLs that carried out the tests.
4. The performance in the detection of virulence genes was considered as “not satisfactory” for the 5 NRLs that provided incorrect results for *vtx* genes and for other 4 NRLs that did not perform the detection of all or part of the EAggEC genes.
5. As a whole, 16 (80 %) of the 20 incorrect results on virulence gens were false positive results, suggesting the occurrence of cross contamination between samples during the PCR procedures.
6. Twenty-eight NRLs (70 %) identified correctly the serogroup of the 7 test strains, including the O104 strain, which was misidentified by only 2 NRLs. For three NRLs, the performance was considered as “not satisfactory”.
7. The identification of the *vtx* gene subtypes was carried out by 38 NRLs, but only 8 of them (21 %) identified correctly all the *vtx* gene subtypes in the 5 VTEC strains. This negative result was mainly due to *vtx2* typing: while *vtx1* subtyping was carried out correctly by 30 (79 %) NRLs, only 9 laboratories (24 %) typed correctly all the *vtx2* variants in all the strains. This was mainly due to false positive results for *vtx2c,* which were reported as the only incorrect results by 13 NRLs. Many (as many as 21) of those false positive results referred to sample 2, which was positive for the *vtx2d* gene. This indicates that the specificity of the *vtx2c* PCR reaction strongly depends on the reaction conditions and the robustness of the method still needs further adjustment.
8. In conclusion, most NRLs performed satisfactorily in the PCR identification of the main virulence genes of VTEC, confirming that a very good preparedness has been established in the EU towards these foodborne pathogens, now included as a food safety microbiological criterion for sprouts in Reg. (EC) 209/2013. Moreover, most NRLs are able to identify the virulence and serogroup-associated genes of the VTEC/EAggEC O104, as prescribed by the same Reg. (EC) 209/2013.