EU Reference Laboratory for *E. coli*

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Report of the 18th inter-laboratory study (PT18) on the identification and typing of Shiga toxin-producing *E. coli* (STEC) and other pathogenic *E. coli* strains - 2016

Edited by:

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1. INTRODUCTION AND OBJECTIVES

The duties of the EU Reference Laboratory for *Escherichia coli* (EURL-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 EURL-VTEC organized seven PT rounds (PT1, PT2, PT5, PT6, PT10, PT11 and PT13) on the identification and typing of STEC, 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 STEC and other pathogenic *E. coli*. The reports of these PTs are available at the EURL-VTEC website, www.iss.it/vtec, in the “Proficiency Tests” section.

PT18 was dedicated to the identification and typing of pathogenic *E. coli* strains including STEC, as well as other diarrhoeagenic *E. coli* strains: namely, Enteropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAEC), Enterotoxigenic *E. coli* (ETEC) and Enteroinvasive *E. coli* (EIEC).

The objectives of the study were:

1. The detection of the main STEC/EPEC virulence genes.
2. The detection of the EAEC marker genes.
3. The identification of *E. coli* pathotypes belonging to ETEC and EIEC types.
4. The identification of a range of relevant STEC serogroups.
5. The subtyping of Shiga Toxins (Stx)-coding genes.
6. The 5th round of external quality assessment (EQA) for PFGE, in view of the start of the program for the collection of molecular typing data on STEC 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 PT18 study, as far as the results on the identification of the *E. coli* pathotypes and the typing of STEC virulence genes and the determination of 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* pathotypes by PCR amplification of the following target virulence genes:

- *stx1* group, *stx2* group and the intimin-coding *eae* gene for STEC
- the *eae* gene for EPEC
- the *aaiC* and *aggR* genes for EAEC
- *lt,* st_h and st_p for ETEC
- *ipaH* for EIEC

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 s*tx* genes harbored by the STEC strains identified. Participants were requested to identify the subtypes of the s*tx1* group (s*tx1a, stx1c and stx1d*) and s*tx2* group (from s*tx2a* to s*tx2g*) genes detected.

3. PARTICIPANTS

Thirty-six NRLs, representing all the 28 EU Member States, as well as Egypt, Norway, Russia, Switzerland and Turkey participated in the study. Each NRL received its own individual laboratory numerical code, which is 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
- 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 Lab of Residue Analysis of Pesticides and Heavy Metals in Foods
- 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)
- Germany, Friedrich-Loeffler-Institut, *Institut für molekulare Pathogenese*, Federal Research Institute for Animal Health
- Greece, National School of Public Health & Central Laboratory of Public Health
- Hungary, Food and Feed Safety Directorate, National Food Chain Safety Office
- Italy, *Istituto Superiore di Sanità*
- Latvia, Institute of Food Safety, Animal Health and Environment BIOR
- Lithuania, National Food and Veterinary Risk Assessment Institute
- Luxembourg, *Ministère de l'Agriculture, de la Viticulture et de la Protection des consommateurs, Administration des services vétérinaires* (LMVE)
- 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 (MAPAMA)
- Spain, *Centro Tecnológico Agroalimentario* (CETAL), Microbiology Unit
- Sweden, National Food Agency (SLV)
- Sweden, National Veterinary Institute (SVA)
- Switzerland, Institute for Food Safety and Hygiene, University of Zurich
- Turkey, Public Health Institution of Turkey, Microbiology Reference Laboratories Dept, Nat. Ref. Lab. for Enteric Pathogens
- 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 10 *E. coli* strains (samples 1 to 10), selected among those present in the EURL-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 (*stx* subtypes)						
			stx1	***stx2***	***eae***	***aggR***	***aaiC***	***lt***	***st$_h$***
1	STEC	O111	*stx1a*	-	+	-	-	-	-
2	STEC	O26	*stx1a*	-	+	-	-	-	-
3	STEC	O103	*stx1a*	-	+	-	-	-	-
4	EAEC	O78	-	-	-	+	+	-	-
5	EAEC	O104	-	-	-	+	+	-	-
6	ETEC	O6	-	-	-	-	-	+	+
7	EPEC	O128	-	-	+	-	-	-	-
8	STEC	O157	-	*stx2a*	+	-	-	-	-
9	STEC	O113	*stx1c*	*stx2b*	-	-	-	-	-
10	STEC	O91	-	*stx2a* *stx2d*	-	-	-	-	-

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 (up to three weeks).

The test samples were prepared on the 20th of October 2016. They consisted of freshly prepared bacterial cultures seeded into soft (0.3 %) nutrient agar in borosilicate 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. On the 25th of October 2016, the homogeneity test was performed on a set of 10 randomly selected samples, in order to assess the presence of the target genes in each sample. The test samples were stored at room temperature until 2nd of November 2016, when the samples were sent to the participating laboratories by courier.

4.2. Laboratory methods

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

As far as the determination of the serogroup is concerned, participants were requested to identify the O-group of the STEC strains identified. In particular, they were requested to identify the strains that belonged to any of the following 13 serogroups selected on the basis of their prevalence in human infections in Europe, if present in the provided set: O26, O45, O55, O91, O103, O104, O111, O113, O121, O128, O145, O146, O157. Participating labs could apply any serological or molecular method 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 EURL website, "Laboratory Methods" section.

The subtypes of the *stx* genes of the STEC 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 EURL-VTEC website, "Laboratory Methods" section.

4.3. Collection and elaboration of the NRL results

The results were submitted directly through an online system, using a dedicated page in the "Restricted Area" of the EURL-VTEC website. 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 samples, and with the possibility to write notes and to specify any problem with the samples 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 EURL-VTEC website.

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 *stx* genes that represent the main virulence determinants of STEC. The detection of these genes was the objective of several previous PTs.
- **2 penalty points** were assigned to each incorrect result concerning the identification of the other virulence genes considered in the PT: *eae*, *aggR*, *aaiC*. The same penalty is assigned to each incorrect result concerning the identification of the target genes for the ETEC strains *lt* and st_h.
- **1 penalty point** was assigned when reporting the detection of a certain virulence gene as “Not Done”. Results that were not uploaded (“null” field) were also considered as “Not Done”. If the “Not Done” concerned the genes *aggR*, *aaiC, lt and* st_h referred to *eae* positive strains, no penalty point was assigned.

The sum of the penalty points for each lab 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. Only the penalty points accumulated due to incorrect results provided were considered to assess the underperformance of the NRLs. Although penalties were assigned also to the results reported as “Not Done” or “null”, the latter were not considered for the assessment of the performance but as indicators to identify areas of improvement. The NRLs that summed up a score of 4 without making errors in the identification of *stx* 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 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 assignment of the penalties took into account the pathotype of strain. As a matter of fact, the determination of the serogroup was mandatory only for the STEC isolates in this PT scheme. The incorrect results reported in the determination of the serogroup of the STEC included in the set of strains contributed to the identification of the underperformance. Any error reported for the non-STEC, was not considered for the assessment of the performance but as an indicator to identify areas of improvement. The following distinction was made, according to the public health relevance of the serogroups, based on the data on human STEC infections published yearly by the ECDC-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”), included in the Reg. EU 209/2013.
– **2 penalty points:** assigned to each incorrect result concerning the typing of the strains belonging to the other eight serogroups that were in the scope of this study: O45, O55, O91, O104, O113, O121, O128, O146.
– **1 penalty point:** assigned to each incorrect result concerning the typing of the strains belonging to pathotype other than STEC regardless the serogroup they belonged to. An exception was made for serogroup O104, as the reporting of an incorrect result was assigned 2 penalty points, even if the strain was an EAEC.

No penalty points were assigned to the results reported as “ONT” for the strains belonging to the O6 and O78 serogroup that were out of the scope of this study, but one penalty point was assigned in case of the reporting of an incorrect serogroup.

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” + O104 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 stx gene subtypes

The performance of each NRL in identifying the subtypes of the *stx* genes in the six STEC strains included in the test materials was evaluated by assigning one penalty point for *stx* 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 underperforming laboratories. The calculation of the threshold did not take into account the penalties accumulated with the “Not Done” or “null” results, which will be investigated individually with the laboratories concerned.

5. RESULTS

Results were submitted by all the 36 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 (1-5), while Table 3 summarizes the results of the tests performed for all the 10 test strains, by virulence gene.

Overall, 21 laboratories reported correct results for all the characters objective of the study. All the laboratories carried out the tests for the detection of *stx* and *eae* genes. The detection of the EAEC target virulence genes was not performed at all by two NRLs, whereas two additional labs didn’t perform it for strain 10. Regarding the identification of the ETEC pathotype, six NRLs didn’t carry out the detection of the *lt* gene and seven for the st_h. The EIEC pathotype was correctly reported as negative, for the absence of the *ipaH* gene from all participating laboratories and this target was not included in the results tables.

Twenty-one NRLs (58 %) identified correctly the presence/absence of all the target genes in all the test strains, 11 NRLs provided a total of 17 incorrect results, while seven NRLs provided “Not Done” or “null” results for at least one target.

As for the detection of *stx* genes, 32 NRLs (89 %) identified correctly their presence/absence in all the test strains, while the other four NRLs provided a total of five incorrect results: four for *stx1* (three false positive and one false negative) and one for *stx2* (false positive). Regarding a false positive result for *stx1* gene, the lab L328 reported the use of a RT-PCR kit which does not distinguish between the two *stx* types.

As for the other virulence genes, 34 NRLs (94 %) identified correctly the presence/absence of the *eae* gene. The other two NRLs provided a total of two incorrect results (one false positive and one false negative).

The EAEC target genes were identified correctly by the all the laboratories carrying out the test, with the exception of a false negative result for both *aggR* and *aaiC* reported by one NRL (L959) in strain 5. Four NRLs didn't perform this test for at least one of the *eae* negative strains and one penalty point was assigned for each result reported as Not Done.

As for the ETEC target genes, 5 participants (14 %) submitted a total of 8 incorrect results, all false negative for the strain 6. Other 7 Laboratories (19 %) didn't perform the tests in the five *eae* negative strain.

Table 2 (1). Identification of the *E. coli* virulence genes (strains 1 and 2). 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) and the white boxes indicate *null* results. The orange boxes indicate ND or *null* results that caused the assignment of penalty points.

NRL	Detection of virulence genes in:													
	Sample 1							Sample 2						
	stx1	*stx2*	*eae*	*aggR*	*aaiC*	*lt*	*st$_h$*	*stx1*	*stx2*	*eae*	*aggR*	*aaiC*	*lt*	*st$_h$*
True value	+	-	+	-	-	-	-	+	-	+	-	-	-	-
L148														
L170														
L181														
L208		+												
L280														
L328						ND	ND						ND	ND
L343														
L360														
L405														
L417														
L427														
L435														
L444														
L492														
L524														
L597														
L630														
L647						ND	ND						ND	ND
L653														
L675														
L705														
L721														
L731														
L782														
L788						ND	ND						ND	ND
L789														
L817														
L826														
L838														
L844														
L879														
L886							ND							ND
L907														
L912														
L959						ND	ND						ND	ND
L973						ND	ND						ND	ND

Table 2 (2). Identification of the *E. coli* virulence genes (strains 3 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. ND indicates that the test was Not Done (ND) and the white boxes indicate *null* results. The orange boxes indicate ND or *null* results that caused the assignment of penalty points.

NRL	Detection of virulence genes in:													
	Sample 3							Sample 4						
	stx1	*stx2*	*eae*	*aggR*	*aaiC*	*lt*	*st$_h$*	*stx1*	*stx2*	*eae*	*aggR*	*aaiC*	*lt*	*st$_h$*
True value	+	-	+	-	-	-	-	-	-	-	+	+	-	-
L148														
L170														
L181														
L208														
L280														
L328						ND	ND						ND	ND
L343														
L360														
L405														
L417														
L427														
L435														
L444														
L492														
L524														
L597														
L630														
L647						ND	ND				ND	ND	ND	ND
L653														
L675														
L705														
L721														
L731														
L782														
L788						ND	ND						ND	ND
L789														
L817														
L826														
L838														
L844														
L879														
L886							ND							ND
L907														
L912														
L959						ND	ND							
L973							ND						ND	ND

Table 2 (3). Identification of the *E. coli* virulence genes (strains 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. ND indicates that the test was Not Done (ND) and the white boxes indicate *null* results. The orange boxes indicate ND or *null* results that caused the assignment of penalty points.

NRL	Detection of virulence genes in:													
	Sample 5							Sample 6						
	stx1	*stx2*	*eae*	*aggR*	*aaiC*	*lt*	st_h	*stx1*	*stx2*	*eae*	*aggR*	*aaiC*	*lt*	st_h
True value	-	-	-	+	+	-	-	-	-	-	-	-	+	+
L148														
L170														
L181														
L208														
L280														
L328						ND	ND						ND	ND
L343														
L360														
L405														
L417														
L427														
L435														
L444													-	-
L492														
L524													-	-
L597														
L630														
L647				ND	ND	ND	ND				ND	ND	ND	ND
L653														
L675														
L705														
L721													-	-
L731														-
L782														
L788						ND	ND						ND	ND
L789														
L817														
L826													-	
L838														
L844														
L879														
L886							ND							ND
L907														
L912														
L959				-	-	ND	ND						ND	ND
L973						ND	ND						ND	ND

Table 2 (4). Identification of the *E. coli* virulence genes (strains 7 and 8). 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) and the white boxes indicate *null* results. The orange boxes indicate ND or *null* results that caused the assignment of penalty points.

NRL	Detection of virulence genes in:													
	Sample 7							Sample 8						
	stx1	*stx2*	*eae*	*aggR*	*aaiC*	*lt*	*st$_h$*	*stx1*	*stx2*	*eae*	*aggR*	*aaiC*	*lt*	*st$_h$*
True value	-	-	+	-	-	-	-	-	+	+	-	-	-	-
L148														
L170														
L181														
L208														
L280														
L328						ND	ND						ND	ND
L343														
L360														
L405														
L417								+		-				
L427														
L435														
L444														
L492														
L524														
L597														
L630														
L647						ND	ND						ND	ND
L653														
L675														
L705														
L721														
L731														
L782														
L788						ND	ND						ND	ND
L789														
L817														
L826														
L838														
L844														
L879														
L886							ND							ND
L907														
L912														
L959						ND	ND						ND	ND
L973						ND	ND						ND	ND

Table 2 (5). Identification of the *E. coli* virulence genes (strains 9 and 10). 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) and the white boxes indicate *null* results. The orange boxes indicate ND or *null* results that caused the assignment of penalty points.

NRL	Detection of virulence genes in:													
	Sample 9							Sample 10						
	stx1	*stx2*	*eae*	*aggR*	*aaiC*	*lt*	*st$_h$*	*stx1*	*stx2*	*eae*	*aggR*	*aaiC*	*lt*	*st$_h$*
True value	+	+	-	-	-	-	-	-	+	-	-	-	-	-
L148														
L170														
L181														
L208								+						
L280														
L328						ND	ND	+			ND	ND	ND	ND
L343	-													
L360														
L405														
L417														
L427														
L435														
L444														
L492														
L524														
L597														
L630														
L647				ND	ND	ND	ND				ND	ND	ND	ND
L653														
L675														
L705			+											
L721														
L731														
L782														
L788						ND	ND						ND	ND
L789														
L817														
L826														
L838														
L844														
L879														
L886							ND							ND
L907														
L912														
L959						ND	ND				ND	ND	ND	ND
L973						ND	ND						ND	ND

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 10 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 10 test strains:						
	stx1	***stx2***	***eae***	***aggR***	***aaiC***	***lt***	***st_h***
L148							
L170							
L181							
L208	**1**	**1**		**5**	**5**	**5**	**5**
L280							
L328	**1**			**1**	**1**	**5**	**5**
L343	**1**						
L360							
L405							
L417	**1**		**1**				
L427							
L435							
L444						**1**	**1**
L492							
L524						**1**	**1**
L597							
L630							
L647				**5**	**5**	**5**	**5**
L653							
L675							
L705			**1**				
L721						**1**	**1**
L731							**1**
L782							
L788						**5**	**5**
L789							
L817							
L826						**1**	
L838							
L844							
L879							
L886							**5**
L907							
L912							
L959				**1** **1**	**1** **1**	**4**	**4**
L973						**5**	**5**

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. Four NRLs obtained a score equal (in case of incorrect result for the detection of *stx* genes) or higher than 4 due to incorrect results, as well as other five NRLs because they did not perform the detection of all or part of the virulence genes (targets for EAEC and ETEC pathotypes). The performance of the four laboratories providing incorrect results was not considered as satisfactory.

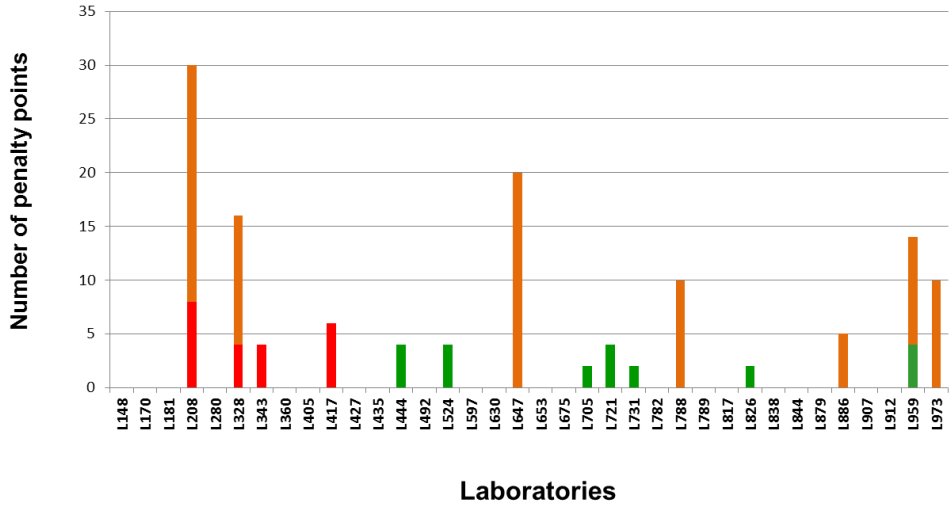

Figure 1. Evaluation of the PCR results for the detection of virulence genes, by NRL. The score was calculated according to the criteria described in section 4.4.1. The red bars indicate the penalty points assigned for incorrect results in the four NRLs whose performance was not considered as satisfactory.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

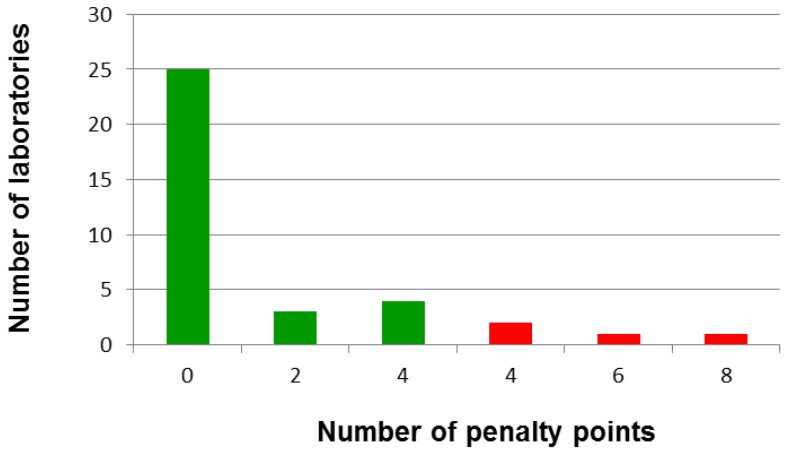

Figure 2. Evaluation of the PCR results for the detection of virulence genes: number of NRLs within each penalty score. The red bars indicate the number of NRLs whose performance was not considered as satisfactory. Only the penalty points accumulated due to incorrect results provided were considered.

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

The identification of the O-groups was mandatory only for the STEC strains. This analysis was carried out by 35 NRLs (97.2 %) out of a total of 36 participants. One NRL (L647) declared that serogroup determination was not performed as the laboratory had the intention to do it by WGS, which was not done. Thirty-one NRLs reported the use of molecular methods for the determination of the serogroup. The results are shown in Table 4. Six Laboratories reported correct results for all the 10 test strains. Seventeen NRLs correctly identified all the serogroups included in the top 13 and reported ONT for the strains belonging to O6 and O78, obtaining no penalty points, as specified in section 4.4.2.

The other 12 NRLs provided a total of 25 incorrect results. In particular, three NRLs (L653, L886 and L959), representing the 9 % of NRLs, failed to identify the O104. Two NRLs (6 %) were unsuccessful in the identification of the O103 strain. Two different laboratories reported as “Non typeable” the O111 strain (L959) and O26 strain (L597), respectively. Moreover, six NRLs (17 %) attempted the determination of the O-group of the O128 strain, but reported it as “Non Typeable”, five NRLs (14 %) couldn’t identify the O113 strain, whereas three NRLs (9 %) were not able to identify the O91 strain.

The number of penalty points assigned to each NRL according to the criteria described in section 4.4.2, is shown in Figure 3. A total of three NRLs obtained a score higher than 4 and their performance was considered as unsatisfactory. Figure 4 shows the number of NRLs grouped according to their score.

Table 4. Identification of the serogroups of the test strains. Results provided from the NRLs concerning the O-group determination. The green boxes indicate the correct results. The red boxes indicate the incorrect results. The orange boxes indicate the incorrect results reported for strains belonging to other pathotypes.

NRL	Serogroup identification in sample:									
	1	2	3	4	5	6	7	8	9	10
True value	O111	O26	O103	O78	O104	O6	O128	O157	O113	O91
L148				O137						
L170				NT		NT				
L181				NT		NT				
L208			NT	NT		NT	NT		NT	NT
L280										
L328				NT						
L343				NT		NT	O128ab			
L360				NT		NT				
L405				NT						
L417				NT		NT				
L427				NT		NT				
L435				NT		NT				
L444										
L492						NT				
L524				NT		NT	NT		NT	
L597		NT		NT		NT	NT			
L630				NT		NT				
L653				NT	NT	NT				
L675				NT		NT	NT		NT	NT
L705				NT		NT				
L721				NT		NT				
L731										
L782										
L788				O55		NT				
L789				NT		NT				
L817				NT		NT				
L826										
L838				NT		NT				
L844				NT		NT				
L879							O128ab			
L886				O91	NT	NT				
L907						NT	NT			
L912						NT				
L959	NT		NT		NT	NT	NT		NT	NT
L973				O138		NT	O128ab		NT	

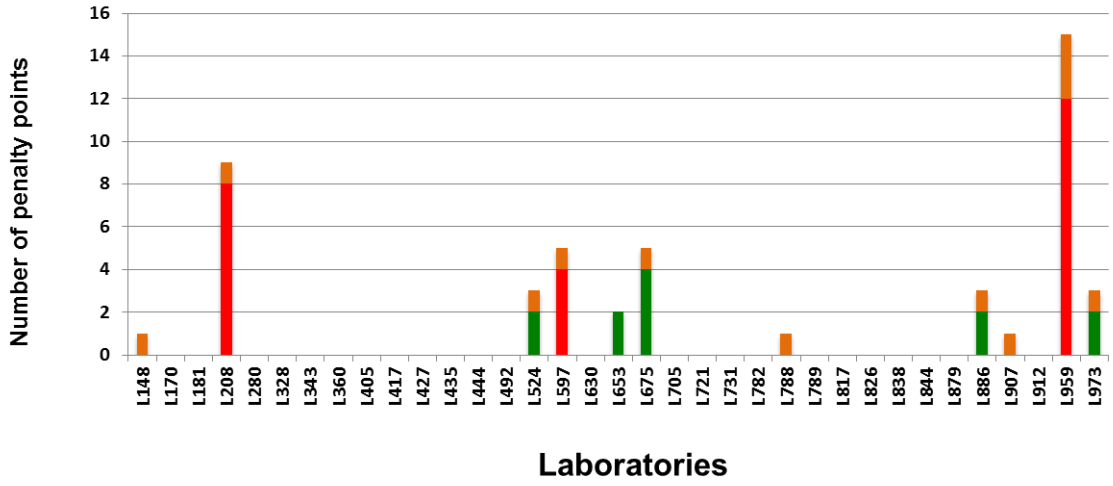

Figure 3. Evaluation of the results on serogroup identification, by NRL. The score was calculated according to the criteria described in section 4.4.2. The orange bars indicate the number of penalties obtained for errors reported for non-STEC strains, for which the serogrouping was not mandatory. The red bars indicate the NRLs whose performance was not considered as satisfactory.

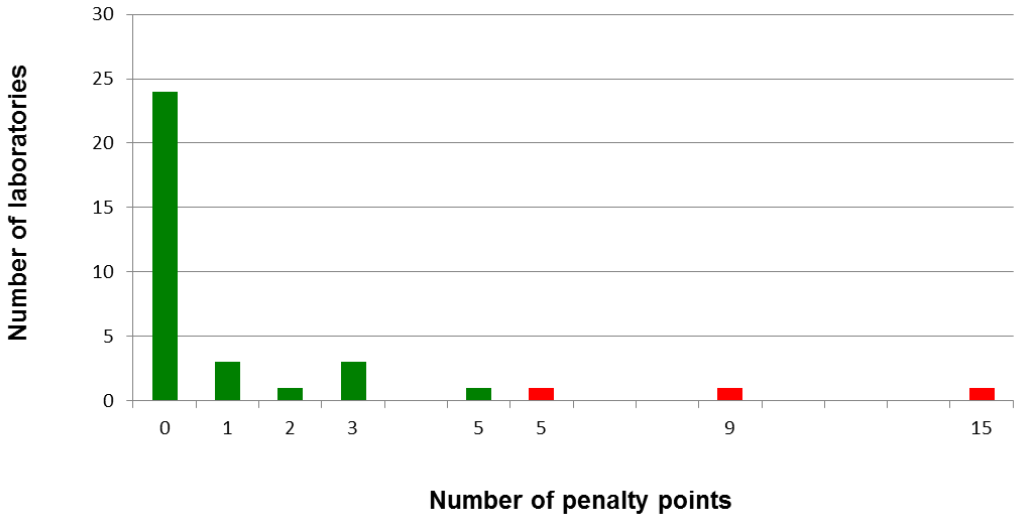

Figure 4. Evaluation of the results on serogroup identification: number of NRLs within each penalty score. The red bars indicate the NRLs whose performance was not considered as satisfactory.

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

The identification of the *stx* gene subtypes harbored by the six STEC strains was performed by 33 of the 36 NRLs that participated in the study (95 %), in detail L208, L647 and L959 didn't perform the *stx* gene subtyping and have been excluded by the analysis of the results.

The results of *stx1* subtyping are reported in Table 5 (1-2).

The presence/absence of the *stx1* gene subtypes in the four *stx1*-positive strains was identified correctly by 30 (91 %) of the 33 NRLs that carried out the subtyping for all the gene variants. Two NRLs (L328 and L838) reported a total of three incorrect results on the strain 9. In detail, two false positive results for *stx1a* and 1 false negative result for *stx1c* were reported. Two NRLs (L328 and L343) reported a total of 8 “Not Done” results for some *stx1* subtypes (five and three, respectively).

The results of *stx2* subtyping in each *E. coli* strain are reported in Table 6 (1-2).

Sixteen NRLs (48 %) identified correctly the subtyping for all the gene variants in the three *stx2* positive strains. The other 17 NRLs (51 %) reported a total of 33 incorrect results: 21 false positive results (14 for s*tx2c,* 2 for s*tx2a,* 2 for s*tx2b,* 2 for s*tx2g* and 1 for *stx2d*), 12 of which concerning the *stx2c* variant in the strain 10. A total of 12 false negative results were reported (6 for s*tx2d*, 5 for s*tx2a* and 1 for *stx2b*).

The majority of the incorrect results were reported for strain number 10.

Table 7 summarizes the results of all the *stx* subtyping tests.

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.

Two NRLs (L148 and L170) resulted as unsatisfactory, obtaining a score equal to or higher than 4 for wrong results reported. Moreover two additional laboratories (L328 and L417) obtained a score equal to or higher than 4, considering also the “Not Done” reporting tests and their performance was considered as satisfactory.

One laboratory reported the use of the WGS approach to subtype the *stx* genes of all the 10 strains of the study, this resulted in some incorrect results, indicating that particular attention should be paid, especially when extracting this information from the WGS data.

Table 5 (1). Subtyping of the *stx1* genes by PCR (strains 1 and 2). 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 *stx1* gene subtypes in:					
	Strain 1			Strain 2		
	stx1a	***stx1c***	***stx1d***	***stx1a***	***stx1c***	***stx1d***
True value	**+**	**-**	**-**	**+**	**-**	**-**
L148						
L170						
L181						
L280						
L328			**ND**			**ND**
L343						
L360						
L405						
L417						
L427						
L435						
L444						
L492						
L524						
L597						
L630						
L653						
L675						
L705						
L721						
L731						
L782						
L788						
L789						
L817						
L826						
L838						
L844						
L879						
L886						
L907						
L912						
L973						

Table 5 (2). Subtyping of the *stx1* genes by PCR (strains 3 and 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 incorrect results. The white boxes indicate that the test was Not Done (ND).

NRL	Detection of *stx1* gene subtypes in:					
	Strain 3			Strain 9		
	stx1a	***stx1c***	***stx1d***	***stx1a***	***stx1c***	***stx1d***
True value	**+**	**-**	**-**	**-**	**+**	**-**
L148						
L170						
L181						
L280						
L328			ND	+		
L343				ND	ND	ND
L360						
L405						
L417						
L427						
L435						
L444						
L492						
L524						
L597						
L630						
L653						
L675						
L705						
L721						
L731						
L782						
L788						
L789						
L817						
L826						
L838				+	-	
L844						
L879						
L886						
L907						
L912						
L973						

Table 6 (1). Subtyping of the *stx2* genes by PCR (strains 8 and 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 incorrect results. The white boxes indicate that the test was Not Done (ND).

NRL	Detection of *stx2* gene subtypes in:													
	Strain 8							Strain 9						
	stx2a	*stx2b*	*stx2c*	*stx2d*	*stx2e*	*stx2f*	*stx2g*	*stx2a*	*stx2b*	*stx2c*	*stx2d*	*stx2e*	*stx2f*	*stx2g*
True value	+	-	-	-	-	-	-	-	+	-	-	-	-	-
L148			+	+										+
L170	-	+						+	-					
L181														
L280														
L328		+						+						
L343														
L360														
L405														
L417				ND							ND			
L427														
L435														
L444														
L492														
L524														
L597														
L630														
L653														
L675			+											
L705														
L721														
L731														
L782														
L788														
L789														
L817														
L826														
L838														
L844														+
L879														
L886														
L907														
L912														
L973														

Table 6 (2). Subtyping of the *stx2* genes by PCR (strain 10).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 *stx2* gene subtypes in: Strain 10						
	stx2a	*stx2b*	*stx2c*	*stx2d*	*stx2e*	*stx2f*	*stx2g*
True value	+	-	-	+	-	-	-
L148			+				
L170				-			
L181							
L280	-		+	-			
L328	ND	ND	ND	ND	ND	ND	ND
L343							
L360							
L405				-			
L417			+	ND			
L427							
L435			+				
L444							
L492							
L524							
L597							
L630	-						
L653			+				
L675			+				
L705							
L721	-		+	-			
L731			+	-			
L782							
L788							
L789							
L817			+				
L826			+				
L838	-						
L844			+				
L879			+	-			
L886							
L907							
L912							
L973							

Table 7. Summary of the *stx* gene subtyping results. The green boxes indicate that the genes were identified correctly in all the 6 STEC 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 *stx* gene subtypes in the 6 STEC strains:									
	stx1a	*stx1c*	*stx1d*	*stx2a*	*stx2b*	*stx2c*	*stx2d*	*stx2e*	*stx2f*	*stx2g*
L148						2	1			1
L170				2	2		1			
L181										
L280				1		1	1			
L328	1	1	4	1 1	1 1	2	2	2	2	2
L343	1	1	1							
L360										
L405							1			
L417						1	3			
L427										
L435						1				
L444										
L492										
L524										
L597										
L630				1						
L653						1				
L675						2				
L705										
L721				1		1	1			
L731						1	1			
L782										
L788										
L789										
L817						1				
L826						1				
L838	1	1		1						
L844						1				1
L879						1	1			
L886										
L907										
L912										
L973										

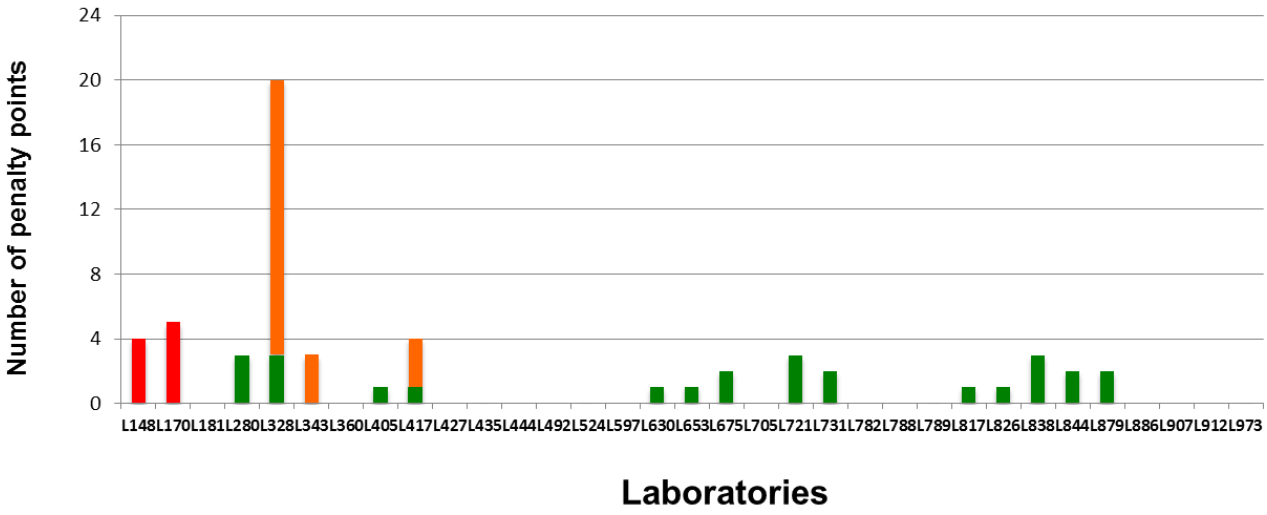

Figure 5. Evaluation of *stx* gene subtyping results, by NRL. The score was calculated according to the criteria described in section 4.4.3. The red bars indicate the penalty points assigned for incorrect results in the 2 NRLs whose performance was not considered as satisfactory. The orange bars indicate the penalty points assigned for tests Not Done.

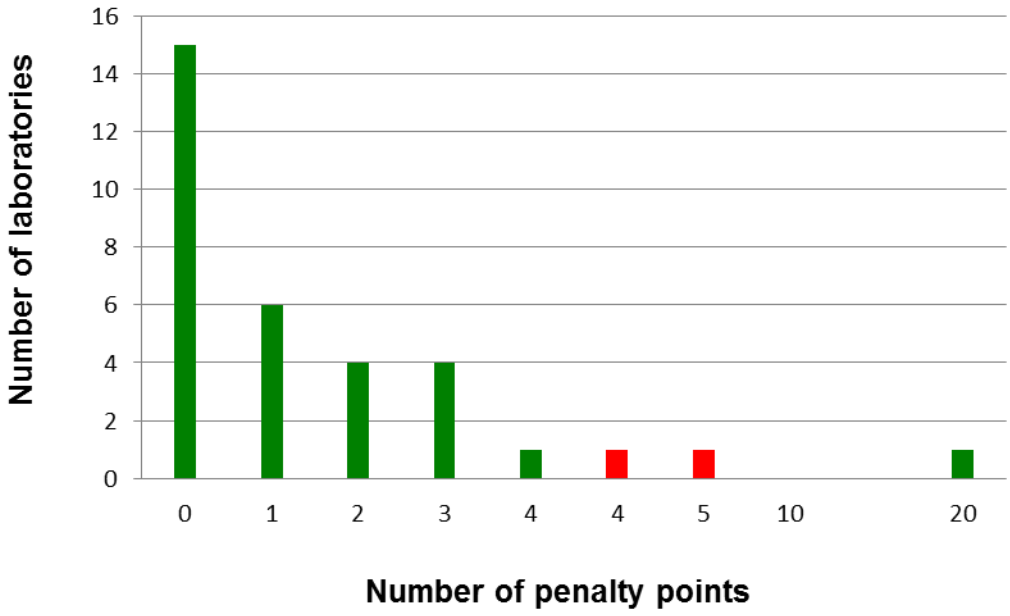

Figure 6. Evaluation of *stx* gene subtyping results: number of NRLs within each penalty score. The red bars indicate the NRLs whose performance was not considered as satisfactory.

7. CONCLUDING REMARKS

1. Thirty-six NRLs, representing all the 28 EU Member States, as well as Egypt, Norway, Russia, Switzerland and Turkey participated in the study. All the 36 NRLs submitted results for the part 1 of the study. Thirty-five and 33 laboratories submitted the result for part 2 and 3, respectively.
2. All the NRLs carried out the tests for the detection of *stx* and *eae* genes. Thirty-two NRLs (89 %) identified correctly the presence/absence of s*tx* and 34 NRLs (94 %) succeeded in the identification of *eae* gene in the test strains.
3. Target genes for EAEC were correctly identified by 32 (89 %) NRLs.
4. The ETEC target genes detection (*lt* and st_h) was successfully detected by 24 Laboratories (67 %), while five NRLs (14 %) reported total of eight incorrect results and the remaining seven did not perform the test totally or partially.
5. The performance in the detection of virulence genes was considered as “not satisfactory” for four NRLs (11 %).
6. All the NRLs but one carried out the tests for serogroup determination. Thirty-one laboratories reported the use of molecular method. A total of 24 NRLs (69 %) succeeded in the correct identification of the serogroups for all the 10 test strains, including those laboratories that reported O78 and O6 as Non Typeable. Three NRLs, representing 8 % of the total, were identified as “not satisfactory” for this part of the test.
7. The identification of the *stx* gene subtypes was carried out by 33 NRLs. Fifteen laboratories (45 %) identified correctly all the *stx* gene subtypes in the 6 STEC strains. The performance of two NRLs was considered unsatisfactory (6 %). A deeper analysis of the results showed that the majority of the incorrect results concerned a false positivity of the *stx2c* subtype, indicating that the specificity of the *stx2c* PCR reaction strongly depends on the reaction conditions and the robustness of the method still needs further adjustment. A large part of the penalties was assigned to results missing or reported as Not Done, revealing an area of improvement of this typing strategy. One laboratory declared to have used WGS for the characterization of the isolates. The results submitted indicate that attention must be paid when using such an approach for the *stx* genes subtyping.
8. In general, this study identified that eight laboratories presented unsatisfactory performance in one or more parts of the study. One laboratory (L208) showed unsatisfactory performance in part 1 and 2 and did not contribute the results for part 3.

Three laboratories (L328, L343 and L417) underperformed in accomplishing part 1 only, two (L597 and L959) in part 2 and two laboratories (L148 and L170) in part 3.

9. In conclusion, the performance of the NRLs in characterizing pathogenic *E. coli* strains was satisfactory. Some areas of improvement have been identified and will be managed through the administration of advice and, where needed, *ad hoc* training. All but one of the underperformances identified were categorized as “light”, while the remaining was considered “heavy”. All of them will be managed according to the EURL-VTEC procedure for the management of the underperformance (http://www.iss.it/binary/vtec/cont/evaluation_and_management_of_the_NRLs_performance.pdf). The overall evaluation highlighted that an excellent preparedness has been built in the EU towards the ability to identify the main virulence genes of STEC, while the capacity to detect other *E. coli* pathotypes and their most represented serogroups, although to a lesser extent, is also present with a good performance.