European Union Reference Laboratory for *E. coli*
Department of Food Safety, Nutrition and Veterinary Public Health
Microbiological food safety and food-borne diseases Unit
Istituto Superiore di Sanità

Report of the 23rd inter-laboratory study (PT23) on the identification and typing of Shiga toxin-producing *E. coli* (STEC) and other pathogenic *E. coli* strains – 2018-2019

Edited by:

Silvia Arancia, Arianna Boni, Gianfranco Brambilla, Paola Chiani, Clarissa Ferreri, Fabio Galati, Guendalina Fornari Luswergh, Federica Gigliucci, Antonella Maugliani, Valeria Michelacci, Fabio Minelli, Margherita Montalbano Di Filippo, Stefano Morabito, Gaia Scavia, Rosangela Tozzoli

1. OBJECTIVES OF THE STUDY

The objectives of this study are:

1. The detection of the main STEC/EPEC virulence genes (*eae* and *stx* genes).
2. The detection of the EAEC marker genes (*aggR* and *aaiC*).
3. The identification of the *E. coli* pathotypes ETEC (detection of *lt*, st_h and st_p genes) and EIEC (identification of *ipaH* gene).
4. The identification of a range of relevant STEC serogroups (at least the 13 serogroups indicated in the EURL-VTEC_Method_003).
5. The subtyping of Shiga Toxins (Stx)-coding genes.
6. The 7th round of external quality assessment (EQA) for PFGE, in view 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 PT23 study, including the results on the identification of the *E. coli* pathotypes and the typing of STEC virulence genes and the determination of O antigens. 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 top-5 STEC serogroups, 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 collected by the European Centre for Disease Prevention and Control (ECDC).

3. Subtyping of the s*tx* genes present in the STEC strains identified. Participants were requested to identify the subtypes of the s*tx1* gene group (s*tx1a, stx1c* and *stx1d*) and s*tx2* gene group (from s*tx2a* to s*tx2g*).

The study was conducted on a set of six pathogenic *E. coli* strains, and the participating Laboratories submitted either the results obtained by applying the laboratory procedures available at the EURL *E. coli* website, based on conventional and Real Time PCR, or the WGS-based characterization results.

3. PARTICIPANTS

Thirty-seven NRLs, representing 27 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 has been used to label the laboratories in the result tables.

The NRLs participating in the study were:

- Austria, *Institut für Medizinische Mikrobiologie und Hygiene,* AGES
- Belgium, Scientific Directorate Infectious Diseases in Humans (SCIENSANO)
- Bulgaria, NDRVMI, BFSA
- Croatia, Laboratory for food microbiology, Croatian Veterinary Institute

- Cyprus, Laboratory for the Control of Foods of Animal Origin (LCFAO), Cyprus Veterinary Services
- Czech Republic, Veterinary Research Institute
- Denmark, Microbiological Laboratory Ringsted
- Egypt, Central Lab of Residue Analysis of Pesticides and Heavy Metals in Foods
- Estonia, Veterinary and Food Laboratory
- Finland, Finnish Food Safety Authority Evira, Research and Laboratory Services Dept.
- France, *VetAgroSup Campus Vétérinaire de Lyon*
- Germany, Federal Institute for Risk Assessment (BfR), Department Biological Safety
- Greece, National School of Public Health & Central Laboratory of Public Health, Dept. Microbiology
- Hungary, Food Microbiological National Reference Laboratory, National Food Chain Safety Office, Food and Feed Safety Directorate
- Ireland, Veterinary Public Health Regulatory Laboratory, Department of Agriculture, Food and the Marine
- Italy, *Istituto Superiore di Sanità*
- Latvia, Molecular Biology Division, Institute of Food Safety, Animal Health and Environment "BIOR"
- Lithuania, National Food and Veterinary Risk Assessment Institute
- Luxembourgh, *Service Surveillance alimentaire, Département des Laboratoires de protection de la santé, Laboratoire national de santé*
- Norway, Norwegian Veterinary Institute
- Poland, National Institute of Public Health-National Institute of Hygiene, Dept. Food Safety
- Poland, National Veterinary Research Institute, Dept. Hygiene of Food of Animal Origin
- Portugal*, Instituto Nacional de Investigação Agrária e Veterinária*, LNIV
- Romania, Institute for Hygiene and Veterinary Public Health
- Russia, International Department State Research Center for Microbiology and Biotechnology, Obolensk
- Slovakia, NRC of Environmental Microbiology, Public Health Authority of SR
- Slovenia, Veterinary Faculty UL, Nacional Veterinary Institute

- Spain, *Unidad Microbiología-Centro Tecnológico Agroalimentario de* Lugo (LSA-CETAL)
- Spain, *SG Sanidad e Higiene Animal y Trazabilidad*, *Laboratorio Central de Veterinaria de* Algete
- Sweden, *Livsmedelsverket*/The National Food Agency
- Sweden, National Veterinary Institute (SVA)
- Switzerland, AGROSCOPE
- Switzerland, Institute for food safety and hygiene, University of Zurich
- The Netherlands, Centre for Zoonoses and Environmental Microbiology (Z&O), National Institute for Public Health and the Environment (RIVM)
- The Netherlands, Laboratory Food and Feed Safety, Netherlands Food and Consumer Product Safety Authority
- Turkey, Ministry of Health, General Directorate of Public Health, Microbiology Reference Laboratories and Biological Products Department
- United Kingdom, GBRU, Public Health England

4. MATERIALS AND METHODS

4.1. Sample preparation

Six *E. coli* strains (samples 1 to 6), selected among those present in the EURL-VTEC reference collection and checked for the presence of all the required genetic and phenotypic features were sent to the NRLs. The characteristics of the strains reported in Table 1a were considered as the gold standard. Table 1b reports the virulence genes detected by WGS-based virulotyping performed at ISS in the six test *E. coli* strains.

The test strains were prepared on 5 November 2018 as 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 13th of November 2018, the homogeneity test was performed on two randomly selected sets of strain. The test samples were stored at room temperature until 19th of November 2018, when the parcels were shipped to the participating laboratories by courier. All participating Laboratories, with the exception of L906 which received the samples after four days, received the parcel containing the test material within 48 h from the shipment.

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

Strain	Pathogroup	Serotype	Target virulence genes (*stx* subtypes)								
			stx1	***stx2***	***eae***	***aggR***	***aaiC***	***lt***	***st$_h$***	***st$_p$***	***ipaH***
1	STEC	O111:H8	*stx1a*	*stx2a*	**+**	-	-	-	-	-	-
2	STEC	O91:H10	-	*stx2d*	-	-	-	-	-	-	-
3	STEC	O103:H2	*stx1a*	-	-	-	-	-	-	-	-
4	EAEC	O86:H2	-	-	-	**+**	**+**	-	-	-	-
5	ETEC/STEC	O2:H27	-	*stx2a*	-	-	-	-	-	**+**	-
6	EAEC	O104:H4	-	-	-	**+**	**+**	-	-	-	-

Table 1b: Virulence genes detected by WGS-based virulotyping in the test strains:

Strain	Virulence genes
1	*astA, cba, celB, cif, eae, efa1, ehxA, epeA, espA, espF, espI, espJ, espP, iha, katP, lpfA, nleA, nleB, nleC, prfB, stx1a, stx2a, tccP, tir*
2	*celB, espI, iha, ireA, lpfA, prfB, stx2d*
3	*cif, efa1, ehxA, espJ, etpD, katP, nleA, nleB, prfB, stx1a*
4	*aaiC, aar, aap, aatA, aggA, aggB, aggC, aggD, aggR, astA, capU, espI, iha, mchB, mchC, mchF, pic, prfB,sat*
5	*astA, ehxA, prfB, sta1, stx2a*
6	*aaiC, aap, aar, aatA, aggA, aggB, aggC, aggD, aggR, capU, sigA*

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, procedures based on end point or Real Time PCR for detecting the genes associated with the serogroups that were in the scope of the PT were available in the EURL website, “Laboratory Methods” section.

An end point PCR method for the identification of the s*tx* gene subtypes of the STEC strains, based on the paper of Scheutz *et al.* (*J. Clin. Microbiol. 2012; 50: 2951-63*), was available in the EURL-VTEC website, “Laboratory Methods” section.

The participating laboratories could also apply WGS-based characterization of the test strains and report the results obtained with such a technique.

4.3. Collection and elaboration of the NRL results

The results were submitted through a webservice 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. The same 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, after the deadline, 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 the identification of the virulence genes of pathogenic *E. coli* was evaluated by assigning penalty points for the virulence genes that were identified incorrectly according to the following scheme:

The proficiency of each NRL was evaluated by assigning penalty points as follows:

- **4 penalty points** to each incorrect or missing result concerning the identification of the *stx* genes;
- **2 penalty points** to each incorrect result concerning the identification of the other virulence genes;
- **2 penalty points** to each incorrect result concerning the identification of the 13 serogroups indicated in the EURL-VTEC_Method_003;
- **1 penalty points** to each incorrect result concerning the identification of the *stx* genes subtypes;
- **1 penalty point** when results were not uploaded ("null" field) or reported as "Not Done" for the virulence genes other than *stx*.

The sum of the penalty points received in the parts of the determination of *stx* and *eae* genes and the identification of the 13 serogroups indicated in 4.2 was used to assess the proficiency of the NRLs. A threshold of four points was set and the laboratories presenting a score higher than four were considered as under-performant.

Penalties were also assigned to incorrect or missing results reported for the other characters whose identification was requested in PT23. These features were not considered for the assessment of the laboratories' proficiency but rather as indicators to identify areas where the action of the EURL-VTEC in support to the NRLs needs to be improved.

5. RESULTS

Results were submitted by 35 Laboratories, one of which (L609) reported two sets of results, one obtained by conventional methods and the other by WGS.

5.1. Identification of the *E. coli* virulence genes

Figure 1 shows the number of participating laboratories using conventional or NGS-based methods to identify the *E. coli* virulence genes.

The results are reported in Table 2 (sections 1 to 6, each strain in a separate table's section), whereas Table 3 summarizes the correct/incorrect results submitted by each laboratory concerning the presence of the virulence genes in all the test strains. Table 4 shows the identification of the pathogroup of the different test strains as reported by the participating laboratories.

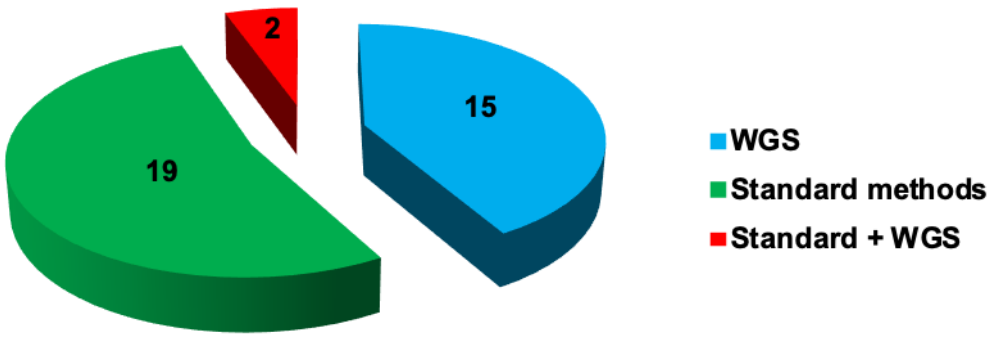


Figure 1. Number of Laboratories reporting results obtained applying NGS-based or conventional methods

Table 2 (1). Identification of the *E. coli* virulence genes (Strain 1). 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 missing (*null*) results. ND: not done.

Typing Method	NRL	Detection of virulence genes in Strain 1:								
		stx1	*stx2*	*eae*	*aggR*	*aaiC*	*lt*	*st$_h$*	*st$_P$*	*ipaH*
	True value	+	+	+	-	-	-	-	-	-
Standard	L109									
	L257									
	L266									
	L295									
	L296*									
	L341									
	L391									
	L400									
	L429									
	L609									
	L636									
	L649						ND	ND	ND	ND
	L662									
	L789									
	L804									
	L813									
	L906									
	L920*									
	L935									
	L940	-								
	L997									
WGS	L300									
	L307						ND	ND	ND	
	L319									
	L323									
	L350									
	L351									
	L355									
	L446									
	L542									
	L576									
	L598									
	L609									
	L689									
	L802									
	L803									

* The results reported by L296 and L920 were obtained by applying both WGS and conventional methods.

Table 2 (2). Identification of the *E. coli* virulence genes (Strain 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 missing (*null*) results. ND: not done.

Typing Method	NRL	Detection of virulence genes in Strain 2:								
		stx1	*stx2*	*eae*	*aggR*	*aaiC*	*lt*	*st$_h$*	*st$_P$*	*ipaH*
	True value	-	+	-	-	-	-	-	-	-
Standard	L109									
	L257									
	L266									
	L295									
	L296									
	L341									
	L391									
	L400									
	L429									
	L609									
	L636									
	L649						ND	ND	ND	ND
	L662									
	L789									
	L804									
	L813									
	L906									
	L920									
	L935				+	+				
	L940									
	L997									
WGS	L300									
	L307						ND	ND	ND	
	L319									
	L323									
	L350									
	L351									
	L355									
	L446									
	L542									
	L576									
	L598									
	L609									
	L689									
	L802									
	L803									

Table 2 (3). Identification of the *E. coli* virulence genes (Strain 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. The white boxes indicate missing (*null*) results. ND: not done.

Typing Method	NRL	Detection of virulence genes in Strain 3:								
		stx1	*stx2*	*eae*	*aggR*	*aaiC*	*lt*	st_h	st_P	*ipaH*
	True value	+	-	-	-	-	-	-	-	-
Standard	L109									
	L257									
	L266									
	L295									
	L296									
	L341									
	L391									
	L400									
	L429									
	L609									
	L636									
	L649						ND	ND	ND	ND
	L662									
	L789									
	L804									
	L813									
	L906									
	L920									
	L935									+
	L940									
	L997									
WGS	L300									
	L307						ND	ND	ND	
	L319									
	L323									
	L350									
	L351									
	L355									
	L446									
	L542									
	L576									
	L598									
	L609									
	L689									
	L802									
	L803									

Table 2 (4). Identification of the *E. coli* virulence genes (Strain 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 missing (*null*) results. ND: not done.

Typing Method	NRL	Detection of virulence genes in Strain 4:								
		stx1	*stx2*	*eae*	*aggR*	*aaiC*	*lt*	*st$_h$*	*st$_P$*	*ipaH*
	True value	-	-	-	+	+	-	-	-	-
Standard	L109									
	L257									
	L266									
	L295									
	L296									
	L341									
	L391									
	L400									
	L429									
	L609									
	L636									
	L649						ND	ND	ND	ND
	L662									
	L789									
	L804									
	L813									
	L906				-	-				
	L920									
	L935									
	L940									
	L997									
WGS	L300									
	L307						ND	ND	ND	
	L319									
	L323									
	L350									
	L351									
	L355									
	L446									
	L542									
	L576									
	L598									
	L609									
	L689									
	L802									
	L803									

Table 2 (5). Identification of the *E. coli* virulence genes (Strain 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. The white boxes indicate missing (*null*) results. ND: not done. The incorrect results concerning the absence of st_p gene when applying the conventional method were not considered for the penalty points as the primers indicated in the EURL-VTEC Real Time PCR method present a mismatch with the variant carried by this strain. The incorrect report of the absence of this gene when applying WGS was conversely taken into account for the penalty's calculation.

Typing Method	NRL	Detection of virulence genes in Strain 5:								
		stx1	*stx2*	*eae*	*aggR*	*aaiC*	*lt*	st_h	st_P	*ipaH*
	True value	-	+	-	-	-	-	-	+	-
Standard	L109							+		
	L257								-	
	L266								-	
	L295									
	L296								-	
	L341								-	
	L391								-	
	L400									
	L429								-	
	L609								-	
	L636									
	L649						ND	ND	ND	ND
	L662								-	
	L789								-	
	L804									
	L813								-	
	L906								-	
	L920									
	L935									
	L940								-	
	L997								-	
WGS	L300									
	L307						ND	ND	ND	
	L319								-	
	L323									
	L350								-	
	L351									
	L355							+	-	
	L446									
	L542									
	L576									
	L598									
	L609									
	L689								-	
	L802								-	
	L803								-	

Table 2 (6). Identification of the *E. coli* virulence genes (Strain 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 missing (*null*) results. ND: not done.

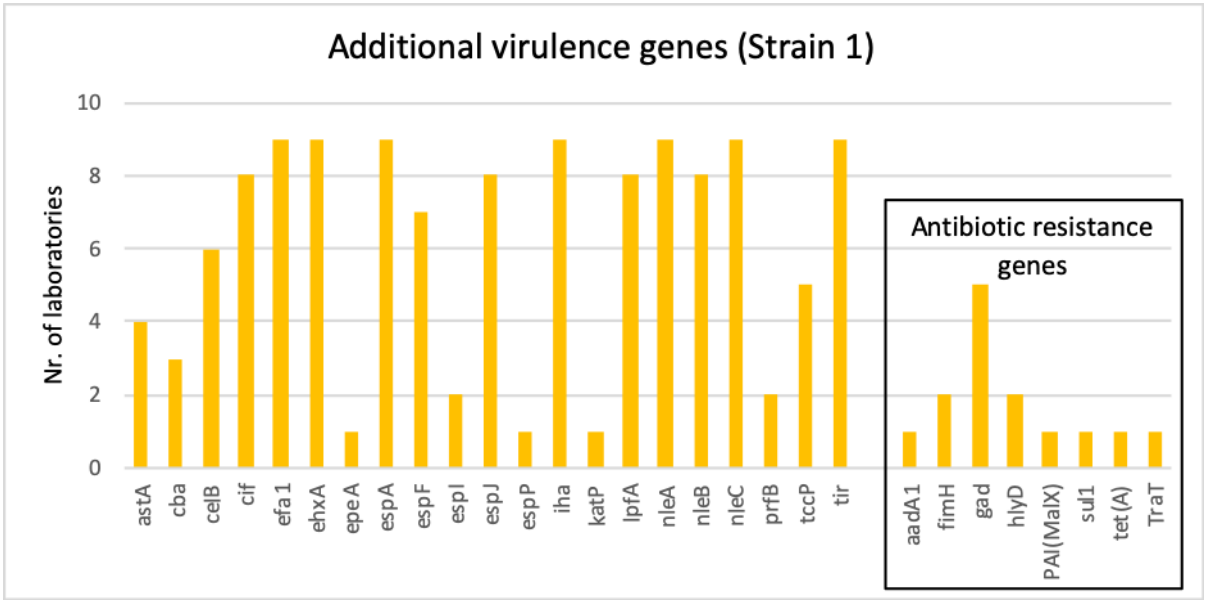
Typing Method	NRL	Detection of virulence genes in Strain 6:								
		stx1	*stx2*	*eae*	*aggR*	*aaiC*	*lt*	*st$_h$*	*st$_P$*	*ipaH*
	True value	-	-	-	+	+	-	-	-	-
Standard	L109									
	L257									
	L266									
	L295									
	L296									
	L341									
	L391									
	L400									
	L429									
	L609									
	L636									
	L649						ND	ND	ND	ND
	L662									
	L789									
	L804									
	L813									
	L906									
	L920									
	L935									
	L940									
	L997									
WGS	L300									
	L307						ND	ND	ND	
	L319									
	L323									
	L350									
	L351									
	L355									
	L446									
	L542									
	L576									
	L598									
	L609									
	L689									
	L802									
	L803									

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 6 test strains. The red and white boxes indicate incorrect results and tests Not Done reported for the given gene, respectively. The numbers in the boxes indicate the number of incorrect or "Not Done" results.

Typing Method	NRL	Detection of virulence genes in the 6 test strains:											
		stx1	*stx2*	*eae*	*aggR*	*aaiC*	*lt*	st_h	st_p	*ipaH*			
Standard	L109							1					
	L257								1				
	L266								1				
	L295												
	L296								1				
	L341								1				
	L391								1				
	L400					1	6	6	6	6			
	L429								1				
	L609								1				
	L636												
	L649						6	6	6	6			
	L662								1				
	L789								1				
	L804												
	L813								1				
	L906				1	1			1				
	L920												
	L935				1	1				1			
	L940	1							1				
	L997								1				
WGS	L300						1	1	1				
	L307						6	6	6				
	L319								1				
	L323							1	1				
	L350								1				
	L351												
	L355							1	1				
	L446												
	L542												
	L576												
	L598						5	4	4	5			
	L609												
	L689								1				
	L802								1				
	L803								1				
		1			2	2	1	24	2	24	19	24	1	17

The additional virulence genes reported by the participants are reported in the diagrams below.

Figure 2. Additional genes identified in the test strains.



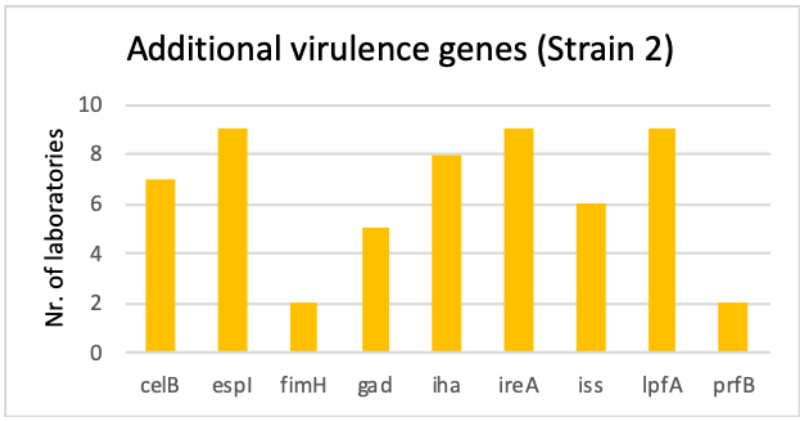
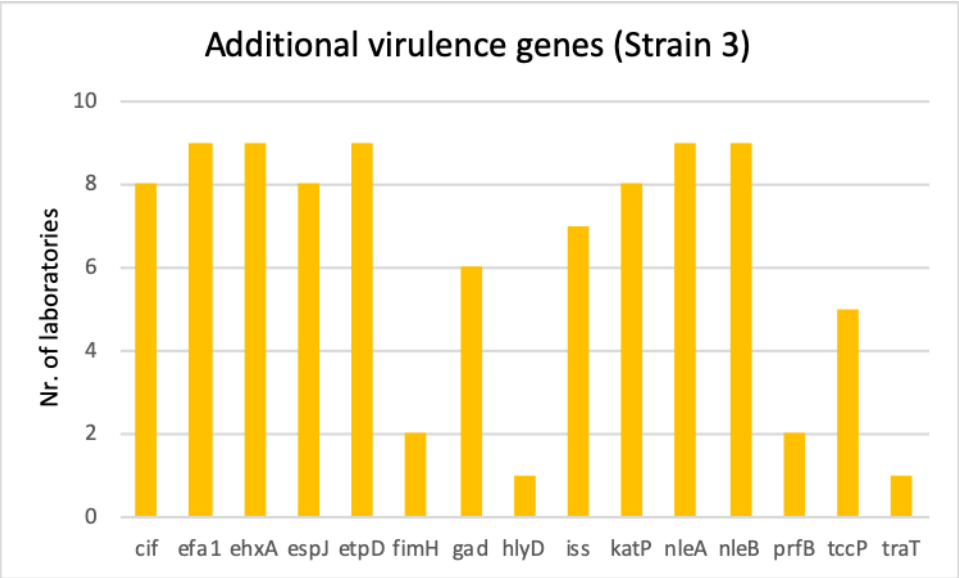


Figure 2 (continued). Additional genes identified in the test strains.



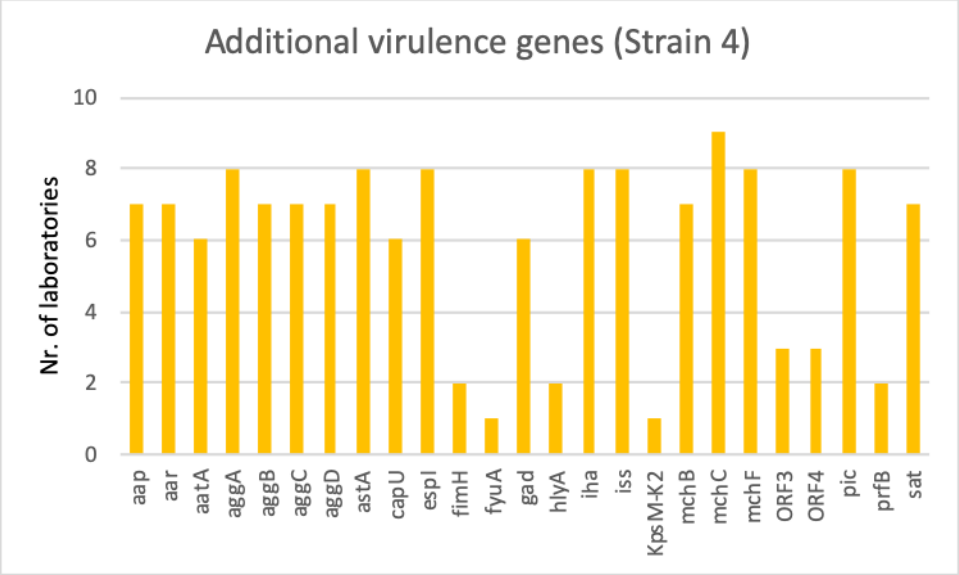
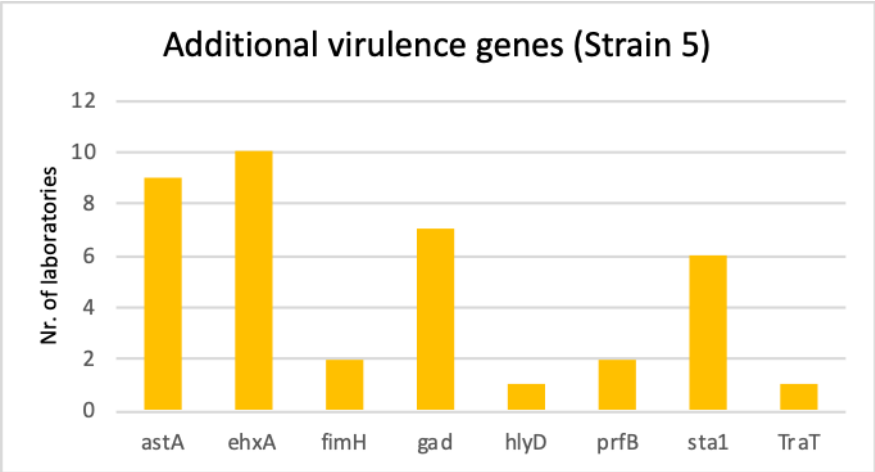


Figure 2 (continued). Additional genes identified in the test strains.



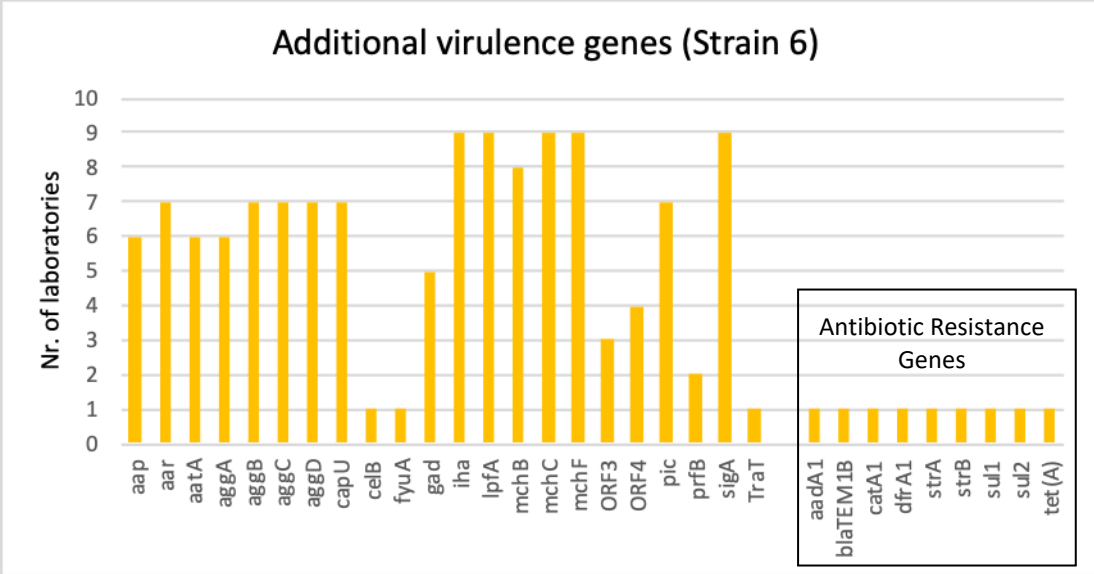


Table 4. Identification of the pathogroups of the test strains.

Typing Method	NRL	Pathogroup identification of test strain:					
		1	2	3	4	5	6
	True value	STEC	STEC	STEC	EAEC	ETEC/STEC	EAEC
Standard	L109						
	L257					STEC	
	L266					STEC	
	L295				STEC	STEC	STEC
	L296					STEC	
	L341					STEC	
	L391					STEC	
	L400				STEC	STEC	STEC
	L429					STEC	
	L609					STEC	
	L636					STEC	
	L649					STEC	
	L662					STEC	
	L789					STEC	
	L804					STEC	
	L813					STEC	
	L906				None	STEC	
	L920					STEC	
	L935					STEC	
	L940					STEC	
	L997					STEC	
WGS	L300						
	L307					STEC	
	L319					STEC	
	L323					STEC	
	L350					STEC	
	L351						
	L355					STEC	
	L446					STEC	
	L542	EPEC				ETEC	
	L576					STEC	
	L598					STEC	
	L609					Other	
	L689					STEC	
	L802					STEC	
	L803					STEC	

5.2. Identification of the serogroups of the test strains

The results of the identification of the O-groups of the six test strains are shown in table 5.

Table 5. Identification of the serogroups of the test strains. Results provided by the NRLs concerning the O-group determination. A few laboratories (13) reported also the H-type of the test strains. The green boxes indicate the correct results. The red boxes indicate the incorrect results.

Typing Method	NRL	Serogroup/Serotype identification in strain:											
		1		2		3		4		5		6	
	True value	O111:H8		O91:H10		O103:H2		O86:H2		O2:H27		O104:H4	
Standard	L109							ONT		ONT			H4
	L257							ONT		ONT			H4
	L266							ONT		ONT			H4
	L295							ONT		ONT			H4
	L296		H8		H10		H2	ONT					
	L341							ONT		ONT			
	L391							ONT		ONT			H4
	L400			O26		ONT		ONT		ONT		ONT	
	L429							ONT		ONT			
	L609							ONT		ONT			
	L636		H8		H10		H2	ONT	H2	O2/O50	H27		H4
	L649							ONT		O157			
	L662							ONT		ONT			H4
	L789									ONT			
	L804			O113				ONT		ONT			H4
	L813							ONT		ONT			
	L906							ONT		ONT			H4
	L920		H8		H10		H2		H2		H27		H4
	L935							ONT		ONT/O103			
	L940									O45			
	L997							ONT		ONT			
WGS	L300		H8		H10		H2		H2		H27		H4
	L307		H8		H10		H2		H2				H4
	L319									ONT			
	L323		H8		H10		H2				H27		H4
	L350		H8		H10		H2		H2		H27		H4
	L351		H8		H10		H2		H2		H27		H4
	L355		H8		H10		H2		H2	O50/O2	H27		H4
	L446		H8		H10		H2		H2		H27		H4
	L542									O2/O50			
	L576												
	L598									O50/O2			
	L609		H8		H10		H2		H2		H27		H4
	L689			ONT				ONT		ONT			
	L802		H8		H10		H2		H2	O50/O2	H27		H4
	L803		H8		H10		H2		H2		H27		H4

5.3. Subtyping of *stx* genes in the test strains

The results of the *stx* genes subtyping are shown in table 6 (1 to 4).

Table 6 (1). Subtyping of the *stx* genes (Strain 1). 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: not done.

Typing Method	NRL	Detection of *stx* gene subtypes in the STEC strain 1:									
		stx1a	*stx1c*	*stx1d*	*stx2a*	*stx2b*	*stx2c*	*stx2d*	*stx2e*	*stx2f*	*stx2g*
	True value	+	-	-	+	-	-	-	-	-	-
Standard	L109						+	+			
	L257										
	L266										
	L295										
	L296										
	L341										
	L391										
	L400	ND			ND						
	L429	ND			ND						
	L609										
	L636										
	L649	ND			ND						
	L662	ND			ND						
	L789										
	L804										
	L813										
	L906		+				+				
	L920										
	L935										
	L940	ND			ND						
	L997										
WGS	L300										
	L307										
	L319	ND			ND						
	L323										
	L350										
	L351										
	L355										
	L446										
	L542										
	L576	ND			ND						
	L598										
	L609										
	L689										
	L802										
	L803										

Table 6 (2). Subtyping of the *stx* genes (Strain 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.

Typing Method	NRL	Detection of *stx* gene subtypes in the STEC strain 2:									
		stx1a	*stx1c*	*stx1d*	*stx2a*	*stx2b*	*stx2c*	*stx2d*	*stx2e*	*stx2f*	*stx2g*
	True value	-	-	-	-	-	-	+	-	-	-
Standard	L109				+		+				
	L257										
	L266										
	L295										
	L296										
	L341										
	L391										
	L400							ND			
	L429							ND			
	L609				+						
	L636				+		+				
	L649										
	L662							ND			
	L789						+				
	L804										
	L813										
	L906				+		+				
	L920										
	L935				+		+				
	L940							ND			
	L997						+				
WGS	L300										
	L307										
	L319							ND			
	L323										
	L350										
	L351										
	L355										
	L446										
	L542										
	L576							ND			
	L598										
	L609										
	L689				+	+		-			
	L802										
	L803										

Table 6 (3). Subtyping of the *stx* genes (Strain 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.

Typing Method	NRL	Detection of *stx* gene subtypes in the STEC strain 3:									
		stx1a	*stx1c*	*stx1d*	*stx2a*	*stx2b*	*stx2c*	*stx2d*	*stx2e*	*stx2f*	*stx2g*
	True value	+	-	-	-	-	-	-	-	-	-
Standard	L109										
	L257										
	L266										
	L295										
	L296										
	L341										
	L391										
	L400	ND									
	L429	ND									
	L609										
	L636										
	L649										
	L662	ND									
	L789										
	L804										
	L813										
	L906		+								
	L920										
	L935										
	L940	ND									
	L997										
WGS	L300										
	L307										
	L319	ND									
	L323										
	L350										
	L351										
	L355										
	L446										
	L542										
	L576	ND									
	L598										
	L609										
	L689	stx1A, stx1B									
	L802										
	L803										

Table 6 (4). Subtyping of the *stx* genes (Strain 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.

Typing Method	NRL	Detection of *stx* gene subtypes in the STEC strain 5:									
		stx1a	*stx1c*	*stx1d*	*stx2a*	*stx2b*	*stx2c*	*stx2d*	*stx2e*	*stx2f*	*stx2g*
	True value	-	-	-	+	-	-	-	-	-	-
Standard	L109						+	+			
	L257										
	L266										
	L295										
	L296				ND						
	L341										
	L391										
	L400				ND						
	L429				ND						
	L609										
	L636										
	L649										
	L662				ND						
	L789										
	L804										
	L813										
	L906						+				
	L920										
	L935										
	L940				ND						
	L997										
WGS	L300										
	L307										
	L319				ND						
	L323										
	L350										
	L351										
	L355										
	L446										
	L542										
	L576				ND						
	L598										
	L609										
	L689					+					
	L802										
	L803										

6. Evaluation of the proficiency of the participating Laboratories

The performance of the Laboratories has been assessed using the results provided for the identification of the presence of *stx* and *eae* genes, as well as the determination of the 13 serogroups indicated in paragraph 2. In detail, the sum of the penalty points accumulated due to incorrect/missing (Not Done" or "null") results in these characters determined the laboratory score. The participating laboratories presenting a score higher than 4 were considered as under-performant, as illustrated in Figure 2.

Figure 3. Evaluation of the laboratories' performance (identification of *stx* and *eae* genes and top-13 serogroups). The red bars indicate the NRLs whose performance was considered as not satisfactory.

Penalties were assigned also to the incorrect or missing results reported for the other features part of the PT23, including the identification of the virulence genes of pathogroups other than STEC and *stx* genes subtyping. The scores obtained by each laboratory for these characters were considered to improve the EURL VTEC action in support of the NRLs.

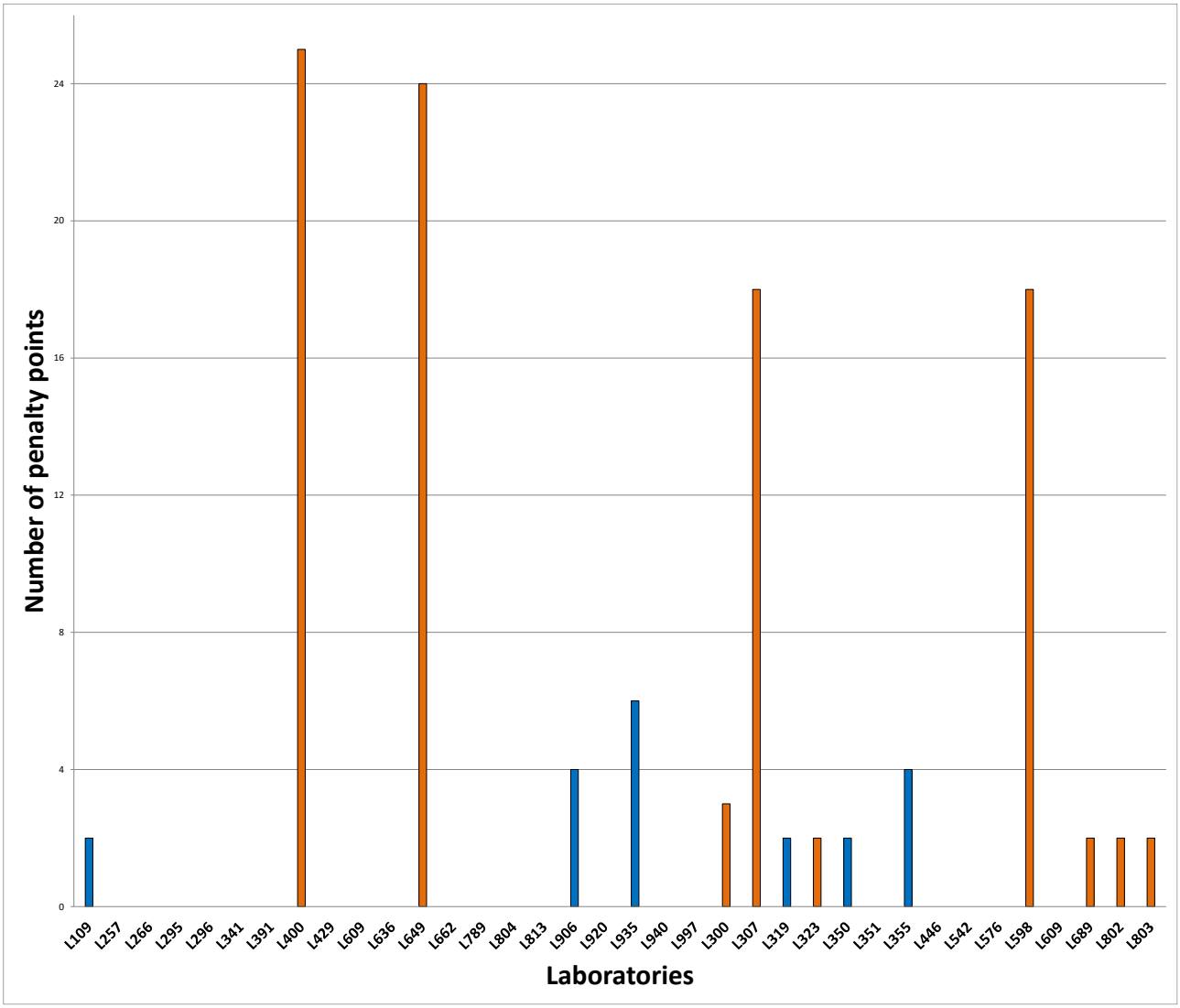


Figure 4. Evaluation of the results for the detection of the additional virulence genes (*aggR*, *aaiC*, *lt*, st_h, st_p), by NRL. The score was calculated according to the criteria described in section 4.4. The orange bars indicate the penalty points assigned for tests Not Done.

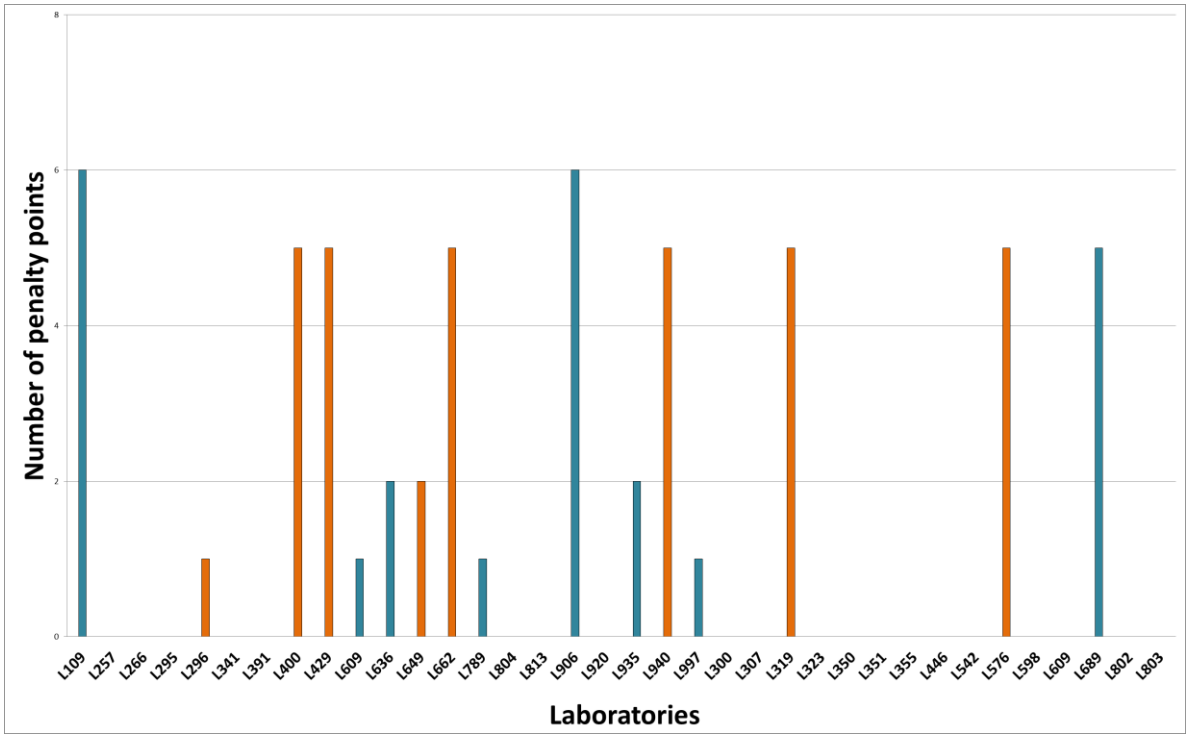


Figure 5. Evaluation of the results for the detection of the *stx* genes subtypes, by NRL. The score was calculated according to the criteria described in section 4.4. The orange bars indicate the penalty points assigned for tests Not Done.

7. CONCLUDING REMARKS

1. A high participation was recorded to PT23, with 37 NRLs, representing 27 EU Member States, as well as Egypt, Norway, Russia, Switzerland and Turkey taking part in the study.
2. Almost half of the Laboratories performed WGS on the PT23 test strains.
3. All the NRLs carried out the tests for the detection of *stx* and *eae* genes. Only one incorrect result was recorded, indicating that the capability of the NRLs in identifying the main STEC virulence genes is highly satisfactory.
4. Six laboratories didn't carry out the detection of all the requested target genes of the different *E. coli* pathogroups, representing an area where the EURL-VTEC action in support of the network may be improved through advice, reference materials and *ad hoc* training.
5. Target genes for EAEC were correctly identified by 34 NRLs (94.4 %).
6. The presence of the st_p gene in strain 5 was correctly identified by 12 Laboratories out of the 31 submitting the results. It has to be noted that the ETEC/STEC strain included in the present study possessed a particular type of st_p gene. In fact, the primers indicated in the EURL-VTEC Real Time PCR method present a mismatch with the variant carried by this strain, therefore it wouldn't succeed in detecting the presence of the st_p. Nevertheless, also a few Laboratories carrying out WGS failed to detect this gene.
7. All the participating Laboratories performed the determination of the O-group of the test strains and many submitted also the H-type. Only seven incorrect results have been reported for the serogroups identification by five NRLs.
8. It is interesting to note that several laboratories typed strain 5 (O2:H27) as O2/O50, mainly using WGS. Indeed, the sequence of the O-antigen gene *wzx* in identical in STEC belonging to O2 and O50 serogroups, but the sequence of *wzy* gene is instead different for the two serogroups and the sequences of both these genes are included in the sequences database developed for *E. coli* serotyping through WGS analysis. The analysis of both the sequences would have allowed the correct identification of the serogroup. The EURL-VTEC will take advantage of these results for refining the training activities in the area of serotyping during the next editions of courses on the use of bioinformatics to type *E. coli* strains through WGS analysis.

9. Only six laboratories out of the 36 submitting the results didn’t perform the *stx* subtyping, indicating that this assay is becoming widely adopted among the network of NRLs for *E. coli.* Based on the results provided by the NRLs, the discrimination between *stx2a* and *stx2c* genes remains a critical point of the procedure that needs to be addressed.
10. The evaluation of the performance of the Laboratories identified that only two Laboratories were under-performant.