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Report of the 26th inter-laboratory study (PT26) on the identification and typing of Shiga toxin-producing *E. coli* (STEC) 2019

Edited by:

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1. OBJECTIVES OF THE STUDY

The objectives of this study were to assess the proficiency of the NRLs for *E. coli* network in:

1. The detection of the main STEC virulence genes (*eae* and *stx* genes).
2. The identification of a range of relevant STEC serogroups (at least the 13 serogroups indicated in the EURL-VTEC_Method_003).
3. The subtyping of Shiga Toxins (Stx)-coding genes.
4. The identification of clusters of isolates based on genomic analysis (PFGE or WGS).

This document represents the evaluation report of the PT26 study.

2. DESIGN OF THE STUDY

The study was designed according to the International Standard ISO/IEC 17043:2010 “Conformity assessment – General requirements for proficiency testing”.

PT26 was conducted on a set of seven STEC strains and consisted of three parts:

1. The identification of the Shiga toxin-producing *E. coli* main virulence genes by PCR amplification. Participants were requested to detect the following targets:

- *stx1* group, *stx2* group and the intimin-coding *eae* gene.

2. Determination of the serogroups of the strains. Participants were requested to identify the serogroup of the test strains assaying at least 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. 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*).

3. PARTICIPANTS

Forty NRLs, representing 27 EU Member States, as well as Argentina (a *consortium* between SENASA and ANLIS), Chile, Iceland, Norway, Russia, Switzerland and Uruguay participated in the study. Each NRL received its own individual laboratory numerical code, indicating the participant in the result tables.

The NRLs participating in the study were:

- Argentina, Joint participation: SENASA and INEI-ANLIS
- Austria, *Institut für Medizinische Mikrobiologie und Hygiene,* AGES
- Belgium, Scientific Directorate Infectious Diseases in Humans (SCIENSANO)
- Bulgaria, NDRVMI, BFSA
- Chile, Department of Environmental Health, Institute of Public Health
- 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
- Estonia, Veterinary and Food Laboratory
- Finland, Finnish Food Authority, Research and Laboratory Services Dept., Microbiology Research Unit, Helsinki
- Finland, Finnish Food Authority, Research and Laboratory Services Dept., Veterinary Bacteriology, Kuopio
- 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
- Iceland, Matis ohf., Icelandic Food and Biotech R&D
- 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
- Luxembourg, *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
- Portugal*, Instituto Nacional de Investigação Agrária e Veterinária*, INIAV
- Romania, Institute for Hygiene and Veterinary Public Health
- Russia, International Department State Research Center for Microbiology and Biotechnology, Obolensk
- Slovakia, Dept. of Food Hygiene, Veterinary and Food Institute, Dolny Kubin
- Slovakia, National Reference Center for Environmental Mirobiology, Public Health Authority, Bratislava
- Slovenia, Veterinary Faculty UL, National 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
- United Kingdom, GBRU, Public Health England
- Uruguay, Department of Bacteriology and Virology, Faculty of Medicine, Institute of Hygiene, University of the Republic

4. MATERIALS AND METHODS

4.1. Sample preparation

Seven *E. coli* strains (samples 1 to 7), 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. The test strains were prepared on 5th October 2019 as fresh 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 set of strains sent to the NRLs. Previous data produced by the EURL-VTEC indicate that bacterial cultures prepared in this way are stable at least up to one month. On the 8th of October 2019, the homogeneity test was performed on five randomly selected sets of strain. The remaining test samples were stored at room temperature until the 14th of October 2019, when the parcels were shipped to the participating laboratories by courier. Twenty-eight laboratories received the parcel containing the test material within 24 h from the shipment, five in 48 hours and the remaining participants from 72 h up to one week (one laboratory, L375).

Table 1a: Characteristics of the STEC strains included in the study

Strain	Serotype	ST	Target virulence genes (*stx* subtypes)		
			stx1	***stx2***	***eae***
1	O121:H19	655	-	*stx2a*	+
2	O121:H19	655	-	*stx2a*	+
3	O128ab:H2	25	*stx1c*	*stx2b*	-
4	O91:H14	33	*stx1a*	*stx2b*	-
5	O55:H7	335	-	*stx2a*	+
6	O121:H19	655	-	*stx2a*	+
7	O145:H28	137	*stx1a*	-	+

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

Strain	Virulence genes
1	*cba, cma, eae, efa1, ehxA, espA, espB, espF, espI, espJ, espP, lpfA, nleA, nleB, nleC, stx2a, tir, toxB*
2	*cba, cma, eae, efa1, ehxA, espA, espB, espF, espI, espJ, espP, lpfA, nleA, nleB, nleC, stx2a, tir, toxB*
3	*ehxA, espI, iha, ireA, lpfA, mchB, mchC, mchF, stx1c, stx2b, subA*
4	*ehxA, espI, iha, lpfA, mchB, mchC, mchF, mcmA, senB, stx1a, stx2b, subA, tia*
5	*astA, efa1, espA, espB, espJ, etpD, nleA, nleB, nleC, stx2a, tir*
6	*efa1, ehxA, espA, espB, espI, espJ, espP, lpfA, nleA, nleB, nleC, stx2a, tir, toxB*
7	*celB, cif, efa1, ehxA, espA, espB, espJ, iha, nleA, nleB, nleC, stx1a, tir*

4.2. Laboratory methods

The laboratories were requested to identify the main STEC virulence genes by PCR (end point or Real Time PCR) using any method applied in the routine testing. Methods for all the assays were also made available in the EURL-VTEC website in the “Laboratory Methods” section. (http://old.iss.it/vtec/index.php?lang=2&tipo=3). The participating Laboratories were also allowed to submit results obtained with WGS.

As far as the determination of the serogroups is concerned, participants were requested to identify the O-group of the STEC strains by testing at least for the following 13 serogroups: O26, O45, O55, O91, O103, O104, O111, O113, O121, O128, O145, O146, O157. Participating labs could choose to apply any serological or molecular method in use in their laboratories, including WGS. 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 made available in the EURL website, “Laboratory Methods” section.

As for the *stx* genes subtyping, an end point PCR method for the identification of the *stx* gene subtypes of the STEC strains, based on the method described by Scheutz *et al.* (*J. Clin. Microbiol. 2012; 50: 2951-63*), was made available in the EURL-VTEC website, “Laboratory Methods” section. Also in this case, the participating laboratories could also choose to characterize the strains through WGS and to report the results obtained with such a technique.

Finally, an exercise for the phylogenetic analysis of the isolates was also carried out as part of PT26. The correlation between the test strains could be assessed by PFGE or SNPs/wg/cgMLST analysis if WGS was performed. For PFGE analysis, the Laboratories participating in this part of the study were requested to provide the number of total bands observed *per* strain and the number bands shared between each test strain and one of the isolates of the panel chosen as reference by the laboratory. In case WGS was used to subtype the strains, the laboratories were requested to submit the number of SNPs or allelic differences observed between each strain and one test strain selected as reference. In both cases the laboratories were requested to interpret their own results by indicating which strains were part of the same cluster.

4.3. Collection and elaboration of the NRLs' 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, containing a *Shipment form* to collect the information on samples' arrival date, temperature and quality, 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 NRLs' results

4.4.1. Evaluation of the NRLs performance in the identification of the STEC virulence genes and the serogroups

The performance of each NRL in the identification of the virulence genes of STEC was evaluated by assigning penalty points for each incorrect result in the STEC virulence genes detection according to the following scheme:

- **4 penalty points** to each incorrect or missing result concerning the identification of the *stx* genes;
- **2 penalty points** to each incorrect or missing result concerning the identification of the *eae* gene;
- **2 penalty points** to each incorrect result concerning the identification of the serogroups, carried out either by conventional or molecular methods, including WGS, if they fell within the 13 serogroups indicated in the EURL-VTEC_Method_003;
- **1 penalty point** when the results of the serogroup identification were not uploaded ("null" field) or reported as "Not Done".
- **1 penalty points** to each incorrect result concerning the identification of the *stx* genes subtypes;

The sum of the penalty points received was used to assess the proficiency of the NRLs. A threshold of four points was set and the laboratories presenting a higher score were considered as under-performant.

Penalties accumulated following incorrect or missing results reported for the *stx* genes subtyping were not considered for the assessment of the laboratories' proficiency but rather as indicators to identify areas where the method should be improved or the action of the EURL-VTEC in support to the NRLs enhanced.

5. RESULTS

Results were submitted by 39 Laboratories. Eighteen provided results obtained by conventional methods, 20 by WGS and one (L543) carried out both the conventional and WGS-based methods. One laboratory did not submit any result (L643). Some of the NRLs did not specify to have used WGS (L424, L912 and L969) as requested, but have been included in the group of laboratories that used this technology as they have reported a number of additional genes not plausible with the use of PCR.

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

Figure 1 shows the number of participating laboratories aggregated according to the methods used to identify the *E. coli* virulence genes.

The individual results concerning the identification of the *stx1*, *stx2* and *eae* genes are reported in Table 2a and Table 2b, whereas Table 3 summarizes the results submitted by each laboratory related with the presence of the additional virulence genes in the test strains.

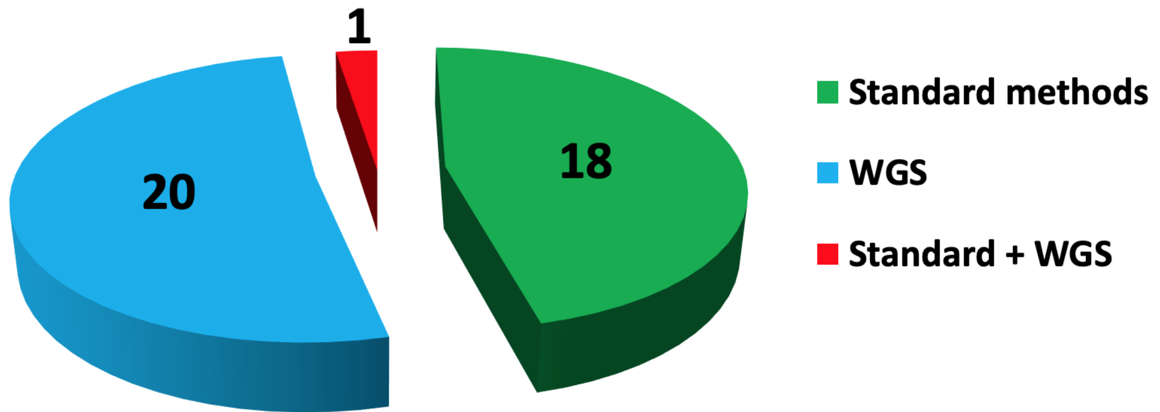


Figure 1. Number of Laboratories reporting results obtained with the different analytical methods

Table 2a. Identification of the *E. coli* virulence genes by standard typing method. 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.

NRL	Detection of virulence genes in:																				
	Strain 1			Strain 2			Strain 3			Strain 4			Strain 5			Strain 6			Strain 7		
	stx1	*stx2*	*eae*	*stx1*	*stx2*	*eae*	*stx1*	*stx2*	*eae*	*stx1*	*stx2*	*eae*	*stx1*	*stx2*	*eae*	*stx1*	*stx2*	*eae*	*stx1*	*stx2*	*eae*
True value	-	+	+	-	+	+	+	+	-	+	+	-	-	+	+	-	+	+	+	-	+
L136																					
L187																					
L258																					
L295																					
L337																					
L355																					
L375																					
L413																					
L417																					
L443												+									
L543																					
L546																					
L556								-			-										
L676																					
L693																					
L775																					
L893																					
L925																					
L986																					

Table 2b. Identification of the *E. coli* virulence genes by WGS. 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.

NRL	Detection of virulence genes in:																				
	Strain 1			Strain 2			Strain 3			Strain 4			Strain 5			Strain 6			Strain 7		
	stx1	*stx2*	*eae*	*stx1*	*stx2*	*eae*	*stx1*	*stx2*	*eae*	*stx1*	*stx2*	*eae*	*stx1*	*stx2*	*eae*	*stx1*	*stx2*	*eae*	*stx1*	*stx2*	*eae*
True value	-	+	+	-	+	+	+	+	-	+	+	-	-	+	+	-	+	+	+	-	+
L175																					
L203																					
L229															-						
L286																					
L376																					
L424																					
L513																					
L519																					
L537																					
L543																					
L597																					
L734																					
L737																					
L791																					
L810																					
L825		-			-										-		-	-			
L840																					
L843																					
L912																					
L967																					
L969																					

The distribution of laboratories reporting additional virulence genes is represented in the bar charts below.

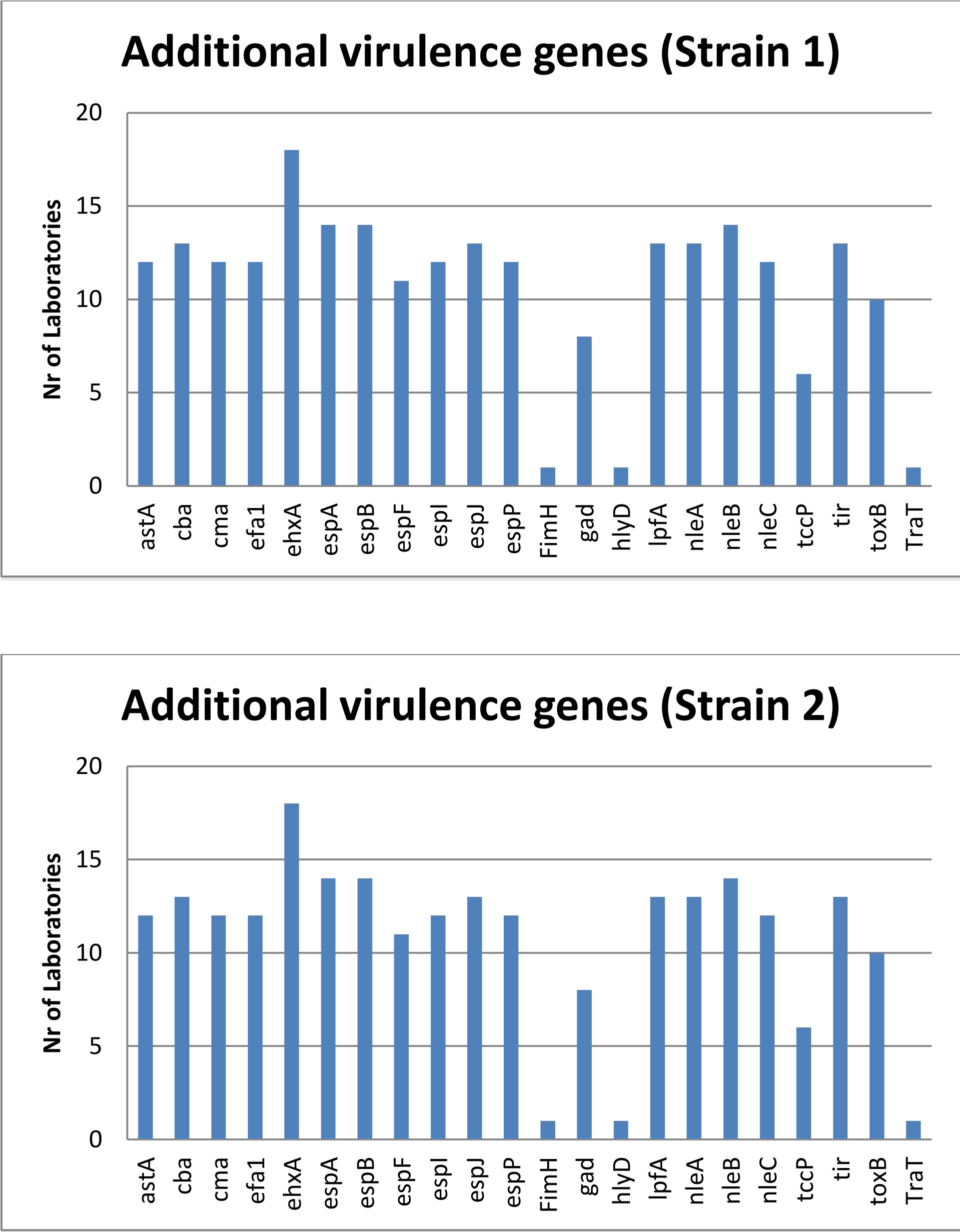
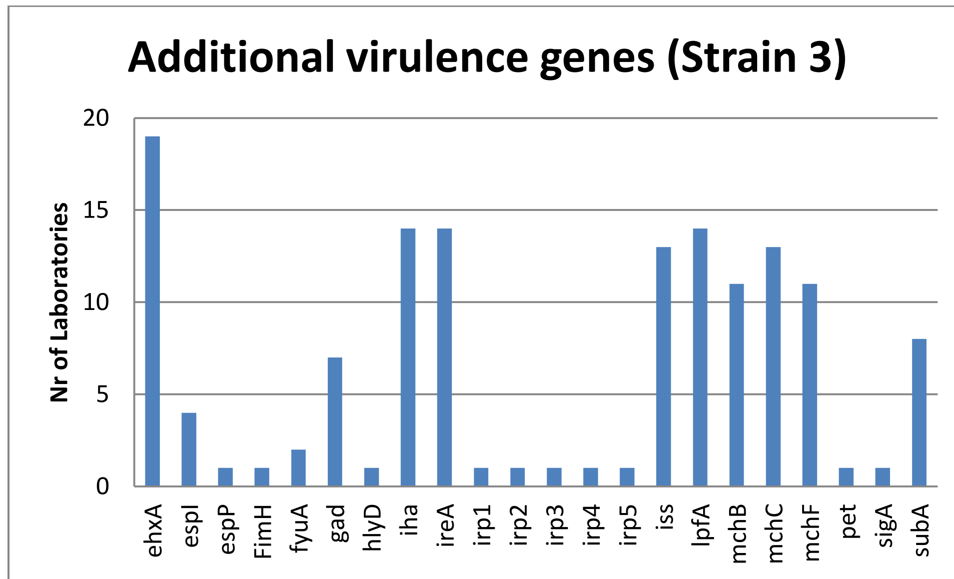


Figure 2a. Additional genes identified in the test strains by the different participating laboratories (test strains 1 and 2).



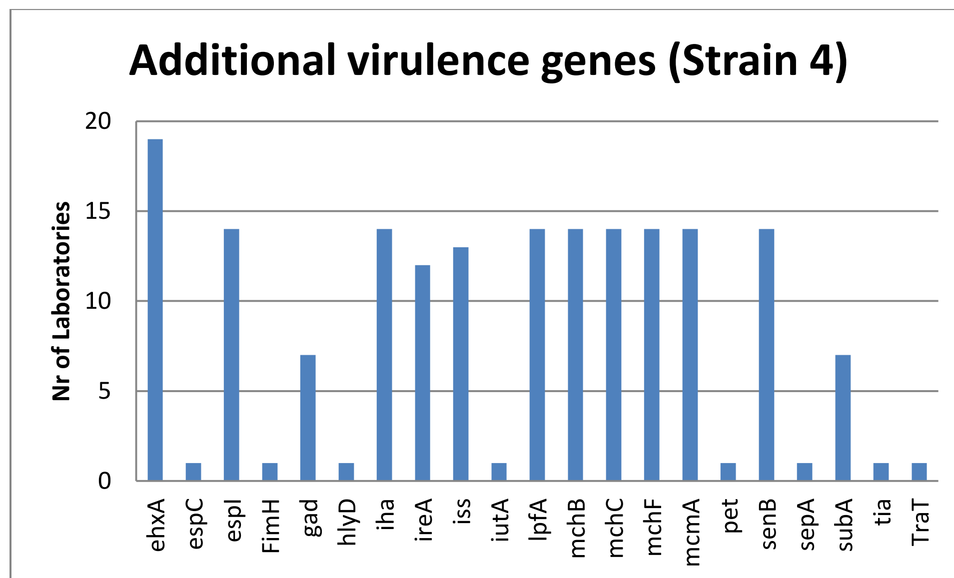
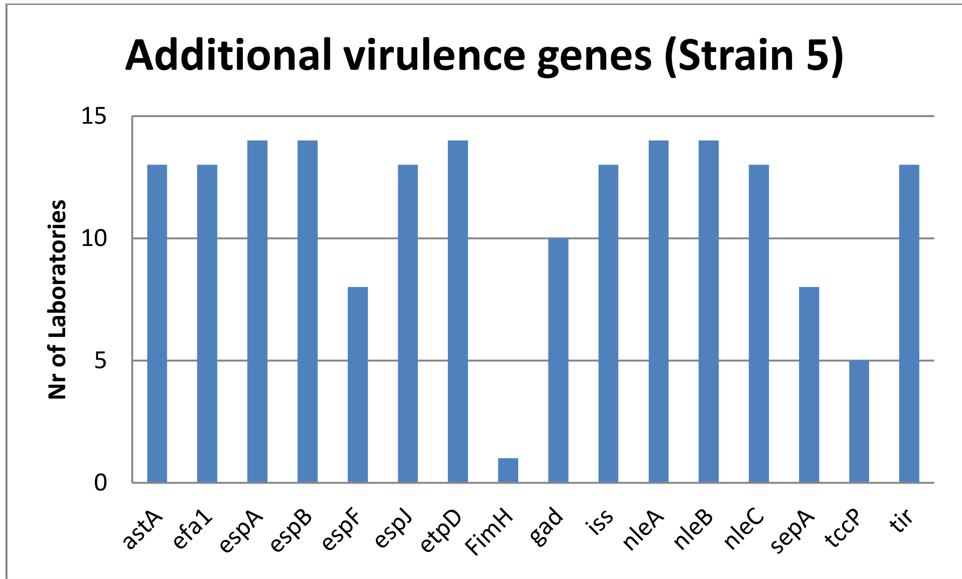


Figure 2b. Additional genes identified in the test strains by the different participating laboratories (test strains 3 and 4).



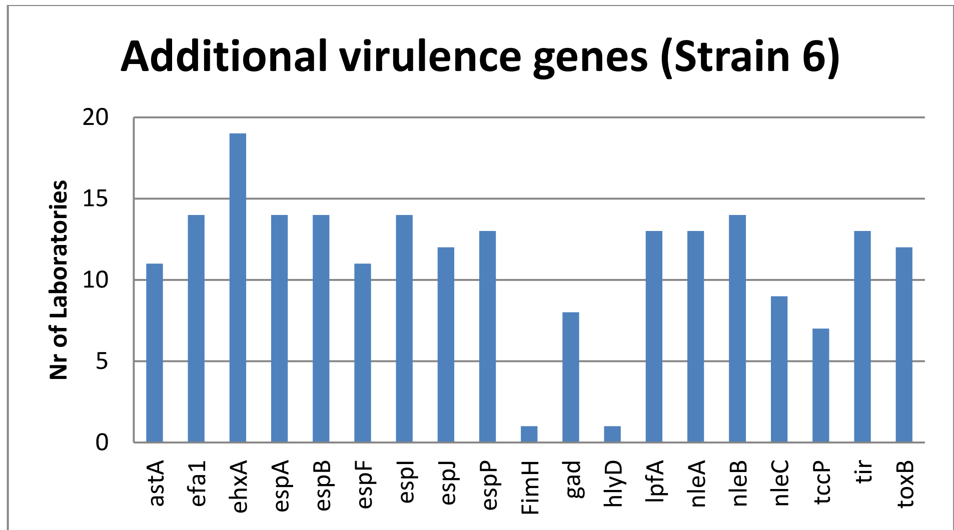


Figure 2c. Additional genes identified in the test strains by the different participating laboratories (test strains 5 and 6).

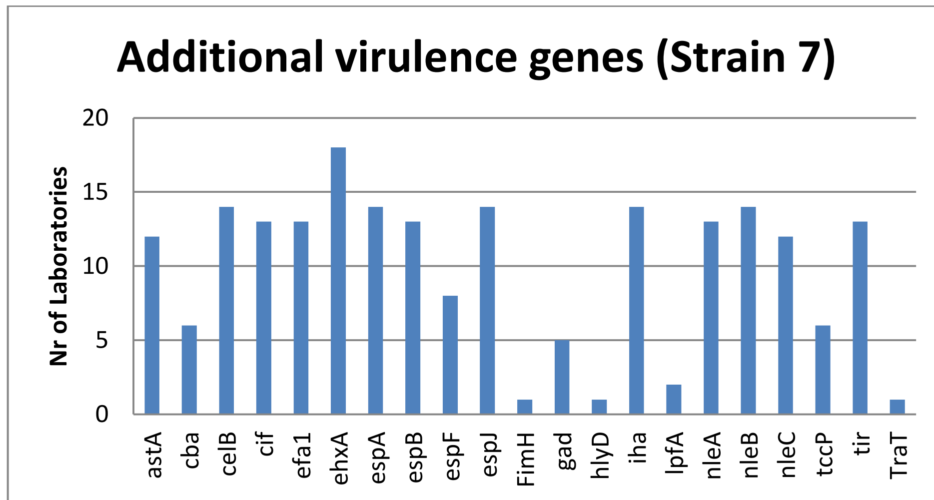


Figure 2d. Additional genes identified in the test strains by the different participating laboratories (test strain 7).

5.2. Identification of the serogroups of the test strains

The results of the identification of the O-groups of the seven test strains are shown in Table 3a and 3b.

Table 3a. Identification of the serogroups of the test strains. Results provided by the NRLs concerning the O-group determination obtained with standard methods (PCR or agglutination). The green boxes indicate the correct results. The red boxes indicate the incorrect results.

NRL	Serogroup identification in Strain:						
	1	2	3	4	5	6	7
True value	O121	O121	O128	O91	O55	O121	O145
L136							
L187			O NT				
L258							
L295							
L337				O146			
L355	ONT					ONT	
L375		O103					
L413							
L417			ONT	ONT			
L443							
L543							
L546							
L556			ONT	ONT			
L676							
L693							
L775							
L893							
L925							
L986				ONT			

Table 3b. Identification of the serogroups/serotypes of the test strains by WGS. Results provided by the NRLs concerning the O-group determination obtained with WGS. Thirteen laboratories reported also the correct H-type of the test strains. The green boxes indicate the correct results. The red boxes indicate the incorrect results.

NRL	Serogroup / Serotype identification in Strain:													
	1		2		3		4		5		6		7	
True value	O121:H19		O121:H19		O128ab:H2		O91:H14		O55:H7		O121:H19		O145:H28	
L175														
L203														
L229														
L286														
L376														
L424														
L513														
L519														
L537														
L543														
L597														
L734														
L737					ONT									
L791														
L810														
L825														
L840														
L843														
L912														
L967														
L969														

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

The results of the *stx* genes subtyping are shown in Table 4 (panels 1-14, one test strain divided for the typing technology in each panel). The laboratories that didn't perform the subtyping for all the strains (L417 and L825) have not been included in the tables.

Table 4 panel 1. Subtyping of the *stx* genes in strain 1- Standard typing method (PCR). The green boxes indicate the correct results, in agreement with the true value reported on the top of each column. The red boxes indicate the incorrect results.

NRL	*stx* genes subtypes									
	stx1a	***stx1c***	***stx1d***	***stx2a***	***stx2b***	***stx2c***	***stx2d***	***stx2e***	***stx2f***	***stx2g***
True Value	-	-	-	+	-	-	-	-	-	-
L136										
L187						+				
L258										
L295										
L337										
L355										
L375										
L413					+					
L443						+				
L543										
L546										
L556										
L676										
L693										
L775										
L893										
L925										
L986										

Table 4 panel 2. Subtyping of the *stx* genes in strain 1 - Typing based on WGS. 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.

NRL	***stx* genes subtypes**									
	stx1a	***stx1c***	***stx1d***	***stx2a***	***stx2b***	***stx2c***	***stx2d***	***stx2e***	***stx2f***	***stx2g***
True Value	-	-	-	+	-	-	-	-	-	-
L175										
L203										
L229										
L286										
L376										
L424										
L513										
L519										
L537										
L543										
L597										
L734										
L737										
L791										
L810										
L840										
L843										
L912										
L967										
L969										

Table 4 panel 3. Subtyping of the *stx* genes in strain 2 - Standard typing method (PCR).

The green boxes indicate the correct results, in agreement with the true value reported on the top of each column. The red boxes indicate the incorrect results.

NRL	*stx* genes subtypes									
	stx1a	*stx1c*	*stx1d*	*stx2a*	*stx2b*	*stx2c*	*stx2d*	*stx2e*	*stx2f*	*stx2g*
True Value	-	-	-	+	-	-	-	-	-	-
L136										
L187						+				
L258										
L295										
L337										
L355										
L375										
L413					+					
L443						+				
L543										
L546										
L556										
L676										
L693										
L775										
L893										
L925										
L986										

Table 4 panel 4. Subtyping of the *stx* genes in strain 2 -Typing based on WGS. 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.

NRL	*stx* genes subtypes									
	stx1a	*stx1c*	*stx1d*	*stx2a*	*stx2b*	*stx2c*	*stx2d*	*stx2e*	*stx2f*	*stx2g*
True Value	-	-	-	+	-	-	-	-	-	-
L175										
L203										
L229										
L286										
L376										
L424										
L513										
L519										
L537										
L543										
L597										
L734										
L737										
L791										
L810										
L840										
L843										
L912										
L967										
L969										

Table 4 panel 5. Subtyping of the *stx* genes in strain 3 - Standard typing method (PCR).

The green boxes indicate the correct results, in agreement with the true value reported on the top of each column. The red boxes indicate the incorrect results. ND: not done.

NRL	*stx* genes subtypes									
	stx1a	*stx1c*	*stx1d*	*stx2a*	*stx2b*	*stx2c*	*stx2d*	*stx2e*	*stx2f*	*stx2g*
True Value	-	+	-	-	+	-	-	-	-	-
L136										
L187										
L258					-					
L295					-	+				
L337					-	+				
L355										
L375										
L413	+	-		+						
L443	+									
L543										
L546										
L556					ND					
L676										
L693										
L775										
L893										
L925										
L986										

Table 4 panel 6. Subtyping of the *stx* genes in strain 3 - Typing based on WGS. 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.

NRL	***stx* genes subtypes**									
	stx1a	***stx1c***	***stx1d***	***stx2a***	***stx2b***	***stx2c***	***stx2d***	***stx2e***	***stx2f***	***stx2g***
True Value	-	+	-	-	+	-	-	-	-	-
L175										
L203										
L229	+	-								
L286										
L376										
L424										
L513										
L519										
L537										
L543										
L597										
L734										
L737										
L791										
L810										
L825	+	-		+						
L840				+						
L843										
L912										
L967										
L969		-	+							

Table 4 panel 7. Subtyping of the *stx* genes in strain 4 - Standard typing method (PCR).

The green boxes indicate the correct results, in agreement with the true value reported on the top of each column. The red boxes indicate the incorrect results. ND: not done.

NRL	***stx* genes subtypes**									
	stx1a	***stx1c***	***stx1d***	***stx2a***	***stx2b***	***stx2c***	***stx2d***	***stx2e***	***stx2f***	***stx2g***
True Value	+	-	-	-	+	-	-	-	-	-
L136										
L187							+			
L258					-					
L295					-	+				
L337										
L355										
L375										
L413				+						
L443		+								
L543										
L546							+			
L556	-	+			ND					
L676										
L693		+								
L775										
L893										
L925										
L986										

Table 4 panel 8. Subtyping of the *stx* genes in strain 4 - Typing based on WGS. 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.

NRL	***stx* genes subtypes**									
	stx1a	***stx1c***	***stx1d***	***stx2a***	***stx2b***	***stx2c***	***stx2d***	***stx2e***	***stx2f***	***stx2g***
True Value	+	-	-	-	+	-	-	-	-	-
L175										
L203										
L229										
L286										
L376										
L424										
L513					-		+			
L519										
L537										
L543										
L597										
L734										
L737										
L791										
L810										
L825				+						
L840				+						
L843										
L912										
L967										
L969										

Table 4 panel 9. Subtyping of the *stx* genes in strain 5 - Standard typing method (PCR).

The green boxes indicate the correct results, in agreement with the true value reported on the top of each column. The red boxes indicate the incorrect results.

NRL	*stx* genes subtypes									
	stx1a	*stx1c*	*stx1d*	*stx2a*	*stx2b*	*stx2c*	*stx2d*	*stx2e*	*stx2f*	*stx2g*
True Value	-	-	-	+	-	-	-	-	-	-
L136										
L187						+				
L258										
L295										
L337										
L355										
L375										
L413					+					
L443						+				
L543										
L546										
L556										
L676										
L693										
L775										
L893										
L925										
L986										

Table 4 panel 10. Subtyping of the *stx* genes in strain 5 - Typing method based on WGS. 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.

NRL	***stx* genes subtypes**									
	stx1a	***stx1c***	***stx1d***	***stx2a***	***stx2b***	***stx2c***	***stx2d***	***stx2e***	***stx2f***	***stx2g***
True Value	-	-	-	+	-	-	-	-	-	-
L175										
L203										
L229										
L286										
L376										
L424										
L513										
L519										
L537										
L543										
L597										
L734										
L737										
L791										
L810										
L825					+					
L840										
L843										
L912										
L967										
L969										

Table 4 panel 11. Subtyping of the *stx* genes in strain 6 - Standard typing method (PCR). The green boxes indicate the correct results, in agreement with the true value reported on the top of each column. The red boxes indicate the incorrect results.

NRL	***stx* genes subtypes**									
	stx1a	***stx1c***	***stx1d***	***stx2a***	***stx2b***	***stx2c***	***stx2d***	***stx2e***	***stx2f***	***stx2g***
True Value	**-**	**-**	**-**	**+**	**-**	**-**	**-**	**-**	**-**	**-**
L136										
L187										
L258										
L295										
L337										
L355										
L375										
L413					**+**					
L443						**+**				
L543										
L546										
L556										
L676										
L693										
L775										
L893										
L925										
L986										

Table 4 panel 12. Subtyping of the *stx* genes in strain 6 - Typing based on WGS. 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.

NRL	***stx* genes subtypes**									
	stx1a	*stx1c*	*stx1d*	*stx2a*	*stx2b*	*stx2c*	*stx2d*	*stx2e*	*stx2f*	*stx2g*
True Value	-	-	-	+	-	-	-	-	-	-
L175										
L203										
L229										
L286										
L376										
L424										
L513										
L519										
L537										
L543										
L597										
L734										
L737										
L791										
L810										
L840					+					
L843										
L912										
L967										
L969										

Table 4 panel 13. Subtyping of the *stx* genes in strain 7 - Standard typing method (PCR). The green boxes indicate the correct results, in agreement with the true value reported on the top of each column. The red boxes indicate the incorrect results.

NRL	*stx* genes subtypes									
	stx1a	*stx1c*	*stx1d*	*stx2a*	*stx2b*	*stx2c*	*stx2d*	*stx2e*	*stx2f*	*stx2g*
True Value	+	-	-	-	-	-	-	-	-	-
L136										
L187										
L258										
L295										
L337										
L355										
L375										
L413										
L443		+								
L543										
L546										
L556										
L676										
L693		+								
L775										
L893										
L925										
L986										

Table 4 panel 14. Subtyping of the *stx* genes in strain 7 - Typing based on WGS. 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.

NRL	*stx* genes subtypes									
	stx1a	*stx1c*	*stx1d*	*stx2a*	*stx2b*	*stx2c*	*stx2d*	*stx2e*	*stx2f*	*stx2g*
True Value	+	-	-	-	-	-	-	-	-	-
L175										
L203										
L229										
L286										
L376										
L424										
L513										
L519										
L537										
L543										
L597										
L734										
L737										
L791										
L810										
L825		stx1b								
L840										
L843										
L912										
L967										
L969										

5.4 Cluster analysis

A total of 24 NRLs participated in the cluster analysis exercise and performed the phylogenetic analysis on the strains received for PT26. Four NRLs used PFGE, 17 NRLs applied WGS-based methods and three used both the approaches (Figure 3a). Among the laboratories applying WGS, the majority (12 labs) used cgMLST and seven NRLs calculated the SNPs differences in the whole genome to infer on the relatedness among strains. One laboratory applied both the strategies (Figure 3b).

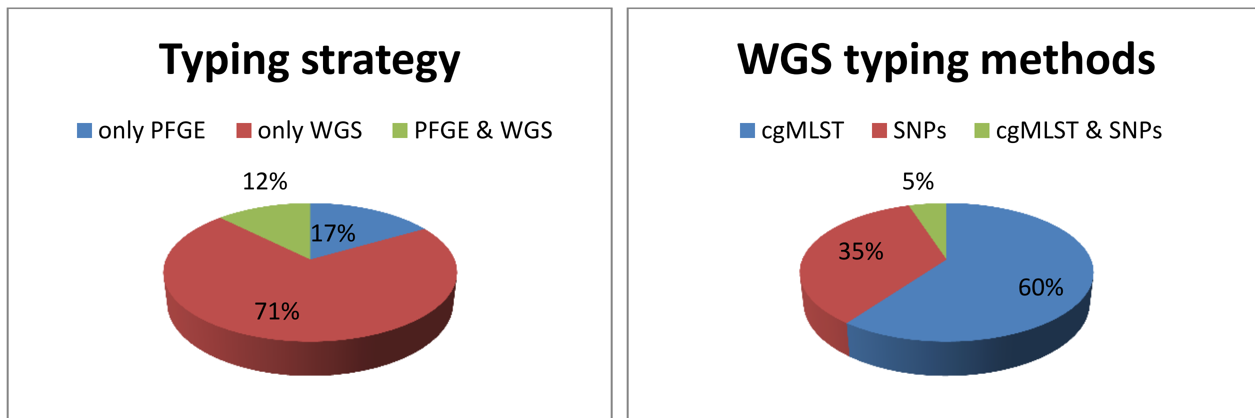


Figure 3. Typing approaches and WGS-based methods used by the NRLs participating in the cluster analysis exercise.

The results of the cluster analysis carried out by PFGE showed that two out of the seven NRLs who submitted these results observed a number of total bands differing for at least two bands from the expected value for more than three strains (L175 and L893), while three NRLs showed this discrepancy only for one strain (L295, L546, L556 and L843). Nevertheless, all the participating laboratories correctly identified that strains 1 and 2 clustered together. These two test strains were actually two cultures of the same isolate. Despite this common origin, a genetic instability of the original strain, resulting in the change in two bands was frequently observed at EURL-VTEC. For this reason, the identification of two maximum differing bands among strains 1 and 2 was considered correct.

As far as the WGS-based typing is concerned, all the laboratories applying WGS typing could identify the correlation among strains 1 and 2. L791 reported results obtained with two different schemes for cgMLST and could identify the correlation only when applying one of them. Other two laboratories reported errors in identifying the cluster: L737 could correctly

identify allelic distances but wrongly reported all the test strains as part of the cluster, probably due to a misunderstanding of the question, while L413, L843 and L967, all applying SNPs analysis, wrongly reported strain 6 as being part of the cluster. This error in the interpretation could be due to the absence of an agreed thresholds for identifying the clusters with a SNPs-based strategy.

Table 5. Results obtained by NRLs applying PFGE for the cluster analysis exercise. The green boxes correspond to correct results, while orange and red boxes correspond to results differing from the expected for one band and for two or more bands, respectively.

NRL	Strain number							Different bands between strains 1 and 2
	1	2	3	4	5	6	7	
N. of expected bands	**18**	**19**	**15**	**18**	**14**	**19**	**16**	**≤2**
L175	19	19	20	21	16	19	17	0
L295	18	18	15	17	13	19	14	0
L519	18	19	15	18	14	19	16	2
L546	18	19	16	18	16	18	15	2
L556	19	19	15	16	15	18	15	0
L843	17	17	16	19	15	18	17	0
L893	18	18	19	20	16	19	18	0

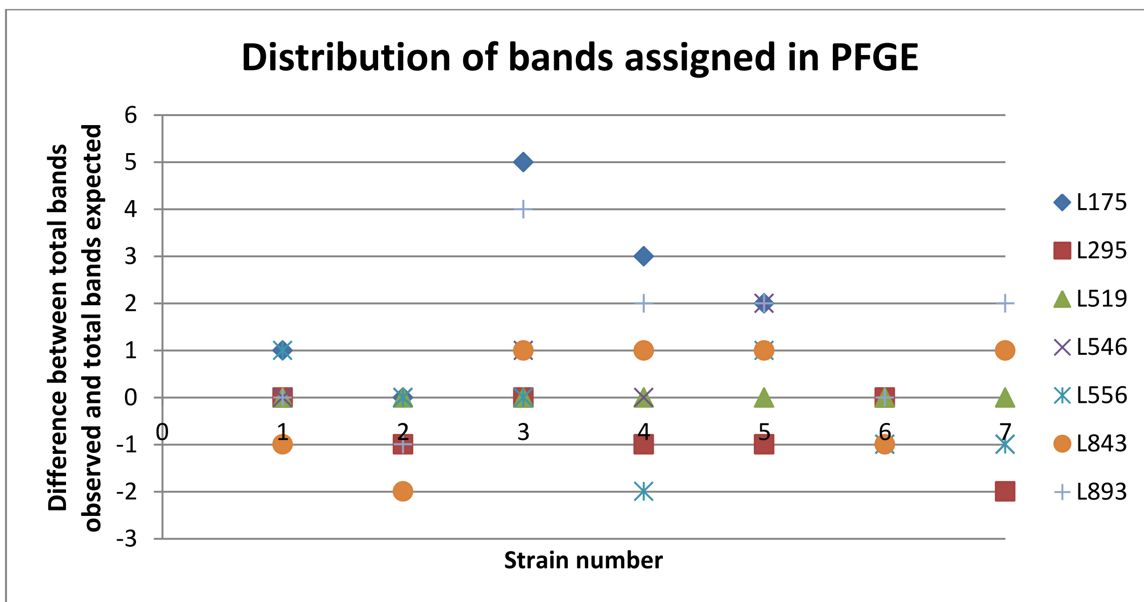


Figure 4. Distribution of the bands assigned by the NRLs for each strain, expressed as difference from the total number of bands expected.

Table 6. Results obtained by NRLs that used WGS for the cluster analysis exercise. The green boxes correspond to correct results in the identification of the expected cluster, composed of strains 1 and 2.

		NRL	Strain number						
			1	2	3	4	5	6	7
Strategy	**Tool details**	**Clustering strains**	**Yes**	**Yes**	**No**	**No**	**No**	**No**	**No**
Alleles-based, cgMLST	Enterobase 09-12-2019	L175							
	chewBBACA	L286							
	SeqSphere+, Enterobase 2513 loci	L424							
	SeqSphere+, Enterobase 2513 loci	L513							
	chewBBACA, INNUENDO 2360 loci	L519							
	SeqSphere+, Enterobase 2531 loci	L537							
	in house, SeqSphere, 1734 loci	L597							
	chewBBACA, INNUENDO 2360 loci	L676							
	SeqSphere+	L734							
	chewBBACA	L737							
	chewBBACA, INNUENDO 2360 loci	L791_1							
	SeqSphere+ v.1 scheme	L791_2							
	CGE cgMLST Finder v1.1, Enterobase scheme 2513 loci	L840_1							
	Enterobase	L912							
SNPs	CGE NDtree	L229							
	CGE CSI Phylogeny 1.4	L376							
	CGE CSI Phylogeny 1.4	L413							
	CGE CSI Phylogeny 1.4	L810							
	CGE CSI Phylogeny 1.4	L840_2							
	FDA SNP Pipeline	L843							
	Snippy 4.4.5	L967							
	in house	L969							

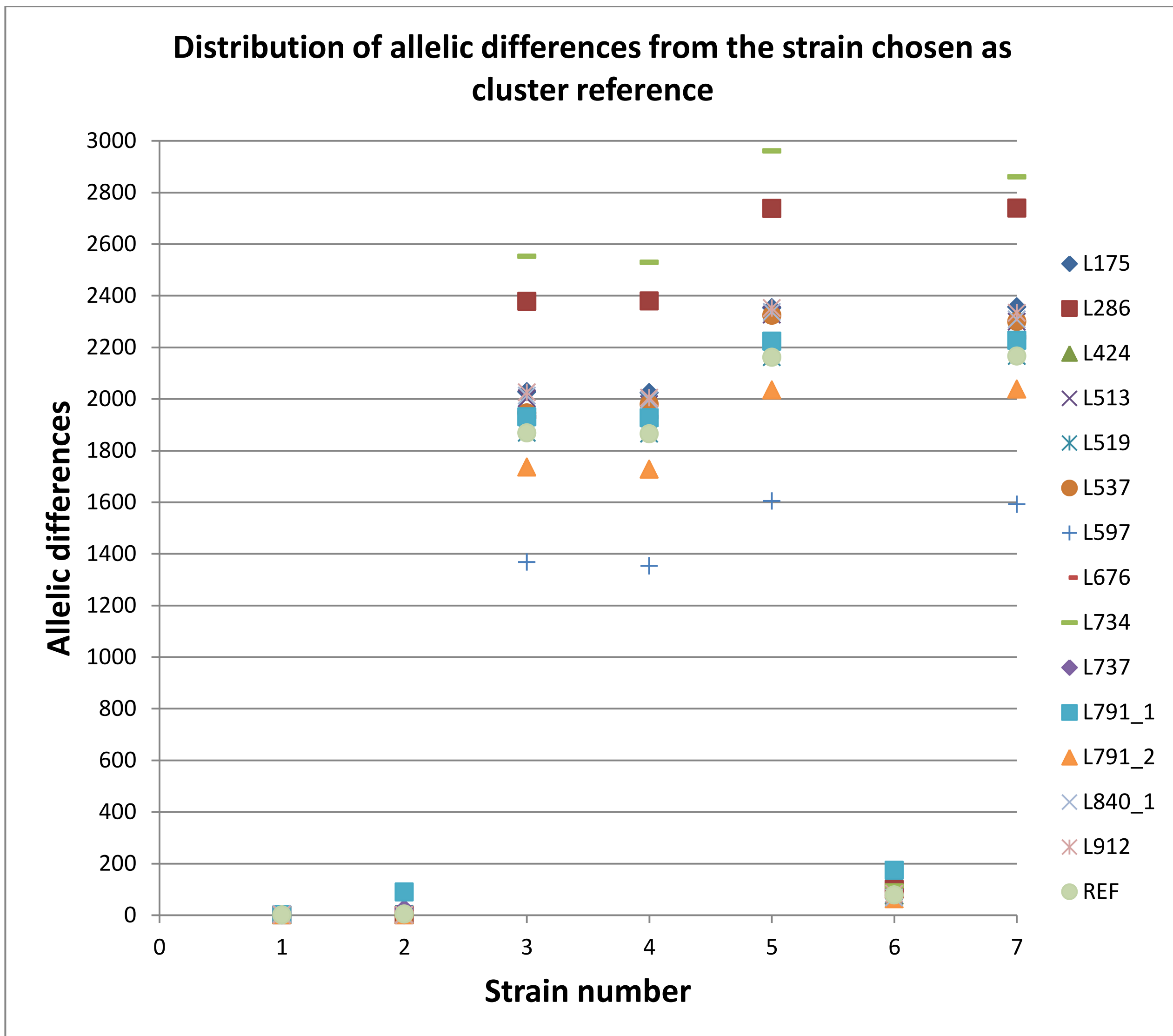


Figure 5. Distribution of the allelic differences from the cluster strains reported by NRLs applying cgMLST tools for WGS cluster investigation.

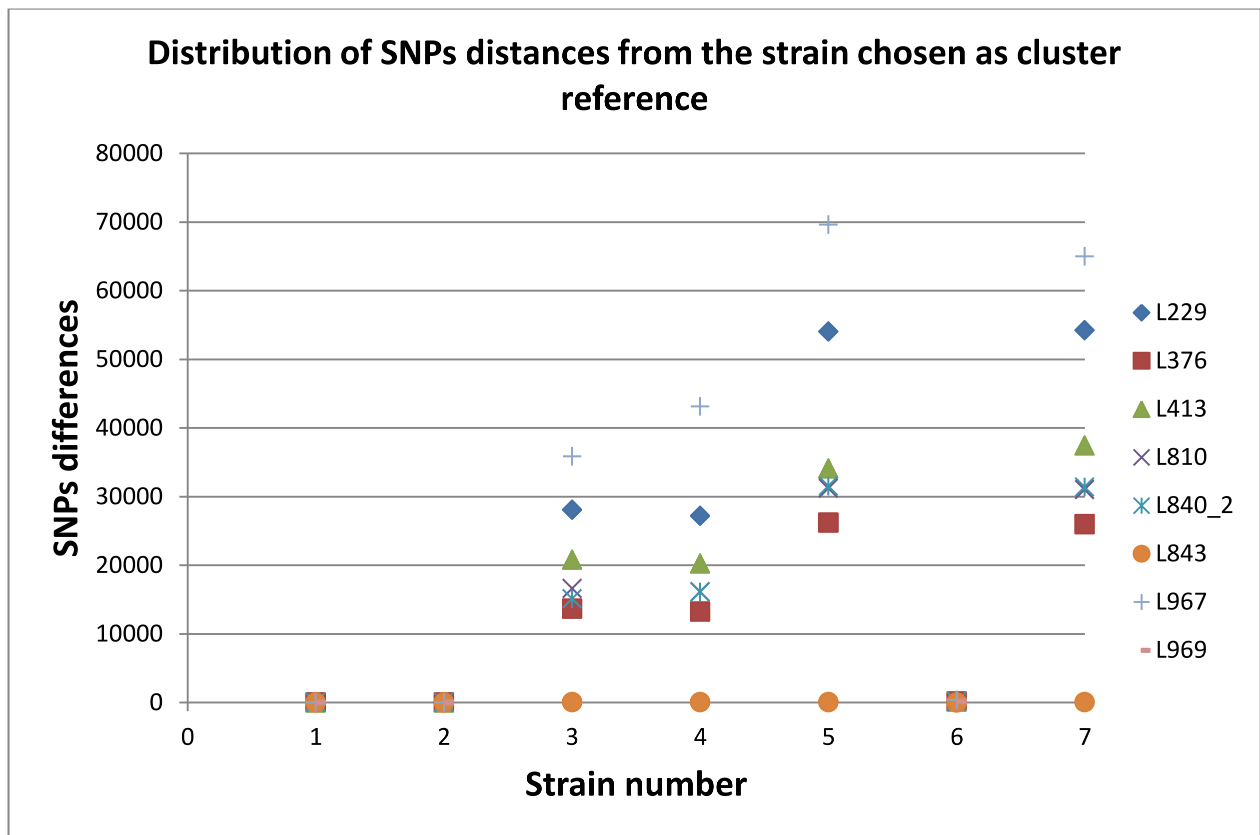


Figure 6. Distribution of the SNPs differences from the cluster strains reported by NRLs applying SNPs analysis tools for WGS cluster investigation

6. Evaluation of the proficiency of the participating Laboratories

The proficiency of the Laboratories has been assessed using the results on the identification of the *stx* and *eae* genes, as well as on the determination of the serogroups. The participating laboratories presenting a score higher than four were considered as under-performant (red bars in Figure 7).

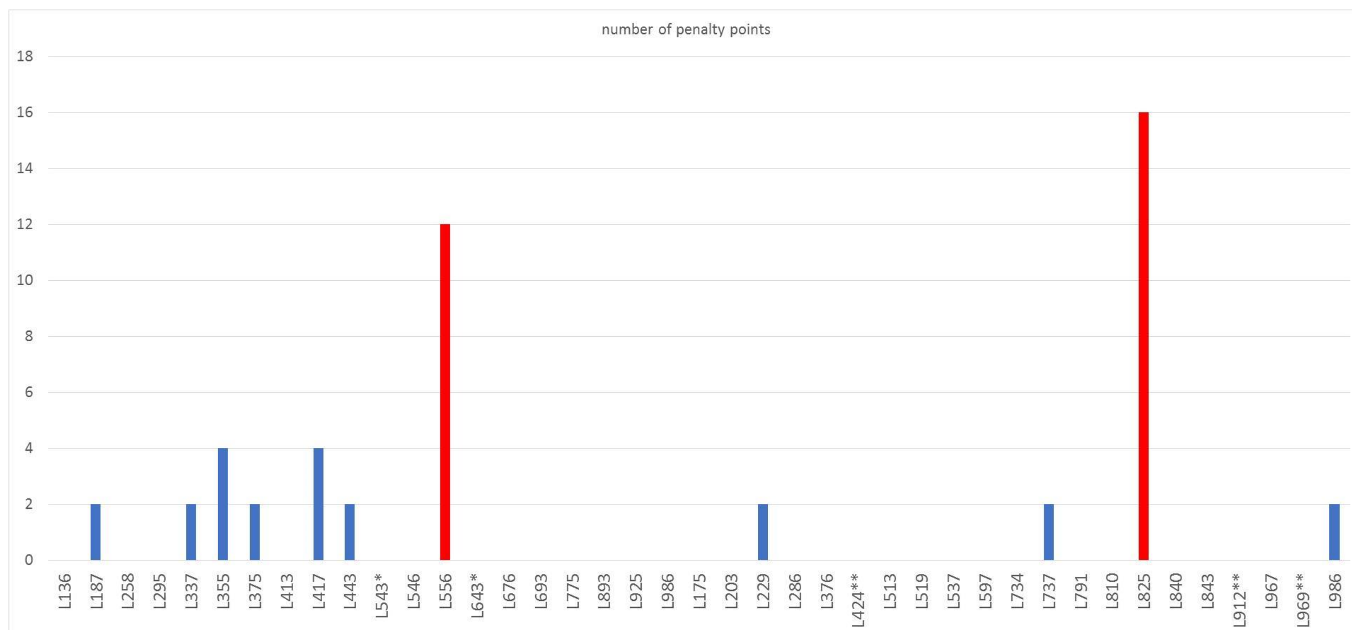


Figure 7. Evaluation of the laboratories' performance (identification of *stx* and *eae* genes and top-13 serogroups). The red bars indicate the NRLs whose performance was considered as not satisfactory. Penalties were assigned as described in 4.4.

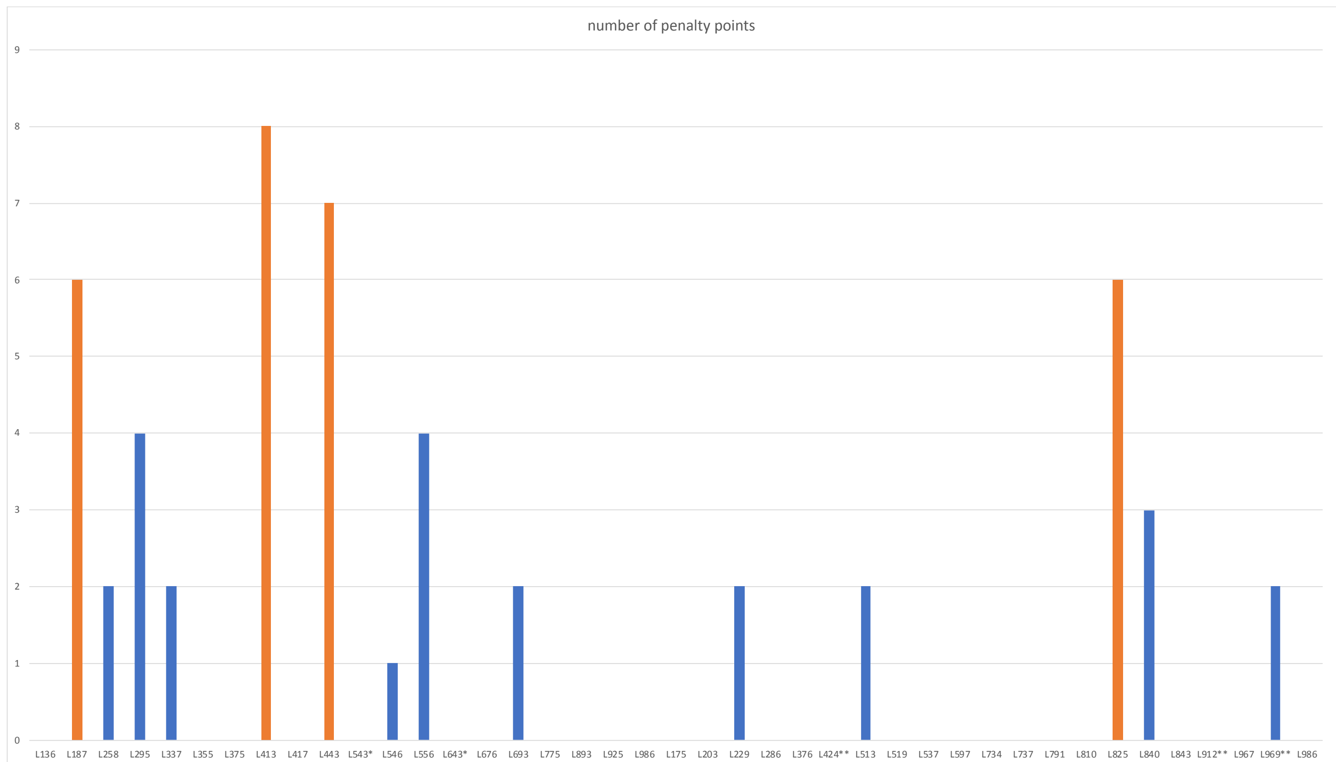


Figure 8. Evaluation of the results for the detection of the *stx* genes subtypes, by NRL.
The score was calculated according to the criteria described in section 4.4. The orange bars indicate the laboratories accumulating a number of penalties over the threshold of four. In this case, the threshold was not used to identify underperformance but to pinpoint that the method has areas of improvement.

7. CONCLUDING REMARKS

1. A high participation to PT26 was recorded, with 40 NRLs representing 27 EU Member States, as well as Argentina (a *consortium* between SENASA and ANLIS), Chile, Iceland, Norway, Russia, Switzerland and Uruguay taking part in the study.
2. Almost half of the laboratories performed WGS, which displayed an excellent performance in both the characterization and subtyping of the STEC isolates.
3. Two laboratories underperformed in the characterization of STEC strains (detection of *stx*, *eae* and serogroups). This result indicates that the capability of the NRLs in identifying the main STEC virulence genes is highly satisfactory (95 % of laboratories with correct results), but still identifies an area where the technical skill of the network must be improved through provision of advice and training.
4. Although the serogroups of the STEC strains fell all outside the group of the most searched ones and out of the scope of the standard method ISO TS 13136:2012, the participating Laboratories performed well in their identification when the standard analytical approach was used and in an excellent way when WGS was used.
5. Most of the laboratories submitted results for the *stx* subtyping, indicating that this assay is becoming widely adopted among the network of NRLs for *E. coli*, with a good performance on average. Apart from known criticalities of the typing method (*e.g.* discrimination between *stx2a* and *stx2c* genes in the PCR assay) the network responded well to the *stx* subtyping exercise, particularly when the results were obtained through WGS. This represents undoubtedly an area where the EURL for *E. coli* will intervene by devoting efforts in refining the PCR method and providing training. Additionally, stimulating the adoption of WGS and developing bioinformatics procedure easy to interrogate will be one focus of the EURL-VTEC action.
6. More than 50 % of the laboratories performed the cluster analysis exercise. The results showed that the participating laboratories that used PFGE were all able to identify the two related strains. Similarly, the majority of laboratories that used WGS performed well in this part of the PT26 regardless the method used (cgMLST or SNP analysis). Among the points to be highlighted there are the wide variability displayed by the PFGE with respect to the number of bands detected in the different strains and the need to fine-tune some of the algorithms used to call the alleles or the SNPs, which did not work uniformly in all the

laboratories. Additionally, the need to define a threshold to call the clusters using the SNP analysis should be defined.