Report of the 31st inter-laboratory study (PT31) on the identification and typing of Shiga toxin-producing *E. coli* (STEC)

2021

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1. OBJECTIVES OF THE STUDY

The objectives of PT31 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 14 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.

This document represents the evaluation report of this 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".

PT31 was conducted on a set of eight STEC strains and consisted of the following three mandatory sections:

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, O80, 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 *stx* 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*).

In addition, a voluntary exercise consisting in the comparison of the genomic signatures of the isolates for the identification of the genomes belonging to a cluster was carried out. The participants were requested to characterise the isolates by whole genome sequencing and to determinate the relatedness between genomes using cgMLST or SNPs-based methods

3. PARTICIPANTS

Thirty-two NRLs, representing 24 EU Member States, as well as Argentina, Egypt, Iceland Russia, Switzerland and UK participated in the study. Each NRL received its own individual laboratory numerical code, reported in the tables of results.

The NRLs participating in the study were:

1. Argentina, Consortium between Instituto Nacional de Enfermedades Infecciosas (INEI), ANLIS “Dr. Carlos G. Malbran” and Servicio Nacional de Sanidad y Calidad Alimentaria (SENASA)
2. Austria, Austrian Agency for Health and Food Safety (AGES), IMED, Graz
3. Belgium, Foodborne Pathogens Service, Scientific Directorate Infectious Diseases in Humans (Sciensano)
4. Croatia, Laboratory for Food Microbiology, Croatian Veterinary Institute Zagreb
5. Cyprus, Laboratory for the Control of Food of Animal Origin (LCFAO), Cyprus Veterinary Services
6. Denmark, Danish Veterinary and Food Administration, Ringsted
7. Egypt, Central laboratory of residues analysis (QCAP)
8. Estonia, Veterinary and Food Laboratory
9. Finland, Finnish Food Authority Laboratory and Research Division (Evira), Microbiology Unit (Food), Helsinki
10. Finland, Finnish Food Authority Laboratory and Research Division (Evira), Kuopio
11. France, VetAgroSup, LMAP/LNR E. coli STEC
12. Germany, Federal Institute for Risk Assessment (BfR), Unit Food Technologies, Supply Chains and Food, Defense
13. Hungary, National Food Chain Safety Office
14. Iceland, Matís ohf. / Icelandic Food and Biotech R&D

15. Ireland, Food Microbiology Division, Department of Agriculture, Food and the Marine
16. Italy, Istituto Superiore di Sanità
17. Latvia, Food Safety Animal Health and Environment Institute (BIOR)
18. Lithuania, National Food dnd Veterinary Risk Assessment Institute
19. Luxembourg, Service EPIGEM, Laboratoire National de Santé
20. Poland, National Institute of Public Health (NIH) - National Research Institute
21. Poland, National Veterinary Research Institute (NVRI), Department of Hygiene of Food of Animal Origin
22. Portugal, Instituto Nacional de Investigação Veterinária (National Institute for Agrarian and Veterinary Research-INIAV)
23. Romania, Institute for Hygiene and Veterinary Public Health
24. Russia, FBIS State Research Center for Applied Microbiology (SRCAMB) Obolensk
25. Slovakia, Department of Food Hygiene, State veterinary and food institute, Dolný Kubín
26. Slovenia, University of Ljubljana, Veterinary Faculty, National Veterinary Institute
27. Spain, Laboratorio Central de Veterinaria, Departamento de Bacteriología-2, Algete (Madrid)
28. Spain, Centro Nacional de Alimentación, Agencia Española de Seguridad Alimentaria y Nutrición (AESAN), Majadahonda (Madrid)
29. Sweden, Swedish Food Agency/Livsmedelsverket, Biologiavdelningen
30. Switzerland, AGROSCOPE
31. The Netherlands, National Institute for Public Health and the Environment (RIVM)
32. UK, Public Health England, Bacterial Reference Department, London

4. MATERIALS AND METHODS

4.1. Sample preparation

Eight *E. coli* strains (test strains 1 to 8), selected among those present in the EURL-VTEC reference collections and checked for the presence of all the required genetic and/or 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 the EURL VTEC.

The test strains were prepared on November the 3rd, 2021, 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 labelled 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 November the 4th 2021, a homogeneity test was performed on five randomly selected sets of strain. The remaining test samples were stored at room temperature until 8th of November 2021, when the parcels were shipped to the participating laboratories by courier.

The delivery of some parcels underwent a delay, due to bad weather conditions in Rome. The conditions of most of the parcels was reported as good despite the delay, with the exception of one laboratory (L226) receiving the test samples at 30°C and L697 reporting the stab agar cultures in bad conditions (gas production and liquefied agar) but could anyway participate in the study.

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

ID PT31	O-group	H-type	Virulence genes profile	*stx1* subtype	*stx2* subtype
Test strain 1	O111	H8	*stx1+ stx2+ eae+*	*stx1a*	*stx2a*
Test strain 2	O111	H8	*stx1+ stx2+ eae+*	*stx1a*	*stx2a*
Test strain 3	O111	H8	*stx2+ eae+*	-	*stx2a*
Test strain 4	O111	H8	*stx1+ stx2+ eae+*	*stx1a*	*stx2a*
Test strain 5	O145	H28	*stx1- stx2+ eae+*	-	*stx2a*
Test strain 6	O26	H11	*stx1+ stx2+ eae+*	*stx1a*	*stx2a*
Test strain 7	O113	H4	*stx1+ stx2+*	*stx1a*	*stx2d*
Test strain 8	O174	H21	*stx2+*	-	*stx2c*

Table 1b: Virulence genes and MLST 7-genes detected in the test strains by WGS-based virulotyping

ID PT31	additional virulence genes	ST
Test strain 1	*astA;celB;cif;efa1;espA;espF;espJ;gad;iha;iucC;iutA;lpfA;nleA;nleB;nleC;terC;tir;traT*	ST16
Test strain 2	*astA;celB;cif;efa1;espA;espF;espJ;gad;iha;iucC;iutA;lpfA;nleA;nleB;nleC;terC;tir;traT*	ST16
Test strain 3	*astA;cif;efa1;ehxA;espA;espF;espJ;gad;iha;iss;iucC;iutA;lpfA;nleA;nleB;nleC;terC;tir;traT*	ST16
Test strain 4	*astA;celB;cif;efa1;ehxA;espA;espF;espJ;gad;iha;iss;lpfA;nleA;nleB;nleC;tccP;terC;tir;traT*	ST16
Test strain 5	*astA;chuA;cif;ehxA;espA;espB;espI;espJ;espP;gad;iha;iss;neuC;nleA;nleB;nleC;senB;terC;tir;toxB;traT;vat*	ST32
Test strain 6	*astA;celB;cif;efa1;ehxA;espA;espB;espF;espJ;espP;gad;iha;iss;iucC;katP;lpfA;nleA;nleB;nleC;tccP;terC;tir;toxB;traT*	ST21
Test strain 7	*celB;espI;gad;iha;iss;papC;terC;tia*	ST10
Test strain 8	*cea;ehxA;espI;espP;gad;iha;iss;lpfA;papC;saa;subA;terC;tia;traT*	ST677

4.2. Laboratory methods

The laboratories were requested to identify the main STEC virulence genes by PCR (endpoint or Real Time PCR) using any method applied in the routine testing. Methods for all the assays were also available in the EURL-VTEC website.

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 14 serogroups:

O26, O45, O55, O80, O91, O103, O104, O111, O113, O121, O128, O145, O146, and O157. Participating labs could choose to apply any serological or molecular method in use in their laboratories, including WGS. However, procedures based on endpoint 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.

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. The participating laboratories could choose to characterise the strains through WGS as well, and to report the results obtained with such a technique.

Finally, a voluntary exercise for the phylogenetic analysis of the isolates was carried out as part of PT31. The correlation between the test strains could be assessed by SNPs/wg/cgMLST analysis: in particular, the laboratories were requested to submit the number of SNPs or allelic differences observed between each of the strains assayed and one of the test strains selected as reference. In addition, 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 an on-line form prearranged by the EURL for *E. coli*. The link to access the form was sent by E-mail to all the participants' laboratories.

4.4. 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.

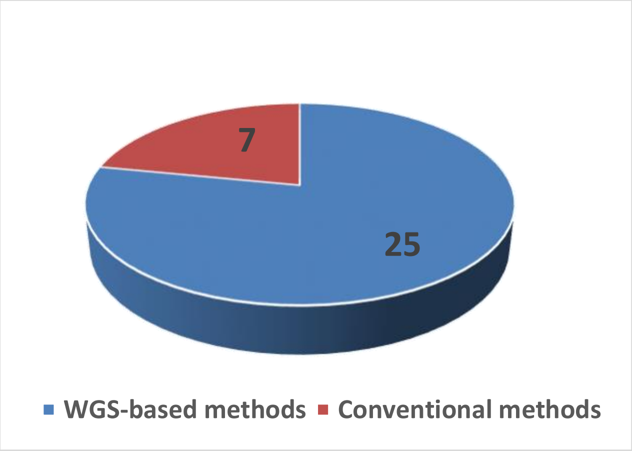
- **1 penalty point** when the results of the serogroup identification were not uploaded ("null" field) or reported as "Not Done". No penalty points were instead assigned to the laboratories reporting the serogroup of the STEC isolated strain as not typeable (ONT) when the serogroup of the test starin was not included in the 14 indicated in the EURL-VTEC_Method_003.
- **1 penalty points** to each incorrect result concerning the identification of the *stx* genes subtypes.

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

5. RESULTS

Results were submitted by all the 32 Laboratories. **Figure 1** shows the number of participating laboratories aggregated according to the methods used to characterise the isolates.

Figure 1. Methods applied by the participating laboratories to type STEC test strains



5.1. Characterisation of the test strains

The individual results reported by the participating laboratories on the characterisation of the test strains are reported in the tables below. The incorrect results are highlighted in red.

Table 2a. Characterisation of test strain 1

Strain 1	**Virulence genes (*stx1; stx2; eae*)**	**Serogroup/serotype**	***stx* genes subtyping**	**Penalties**	**WGS**
Expected result	***stx1;stx2;eae;***	**O111:H8**	***stx1a;stx2a;***		
Labcode	**Reported result**	**Reported result**	**Reported result**		
L144	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L180	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L222	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L226	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L230	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L256	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L262	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L327	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L358	*stx1;stx2;eae;*	O111	*stx1a;stx2a;*	0	
L370	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L389	*stx1;stx2;eae;*	O111	*stx1a;stx2a;*	0	*
L403	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L407	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L462	*stx1;stx2;eae;*	O111	*stx1a;stx2a;*	0	
L494	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L497	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L522	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L561	*stx1;stx2;eae;*	O111	*stx1a;stx1c;stx2a;*	1	
L563	*stx1;stx2;eae;*	O111	*stx2c;*	1	
L615	*stx1;stx2;eae;*	ND	*stx1a;stx2a;*	1	
L635	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L640	*stx1;stx2;eae;*	O111	*stx1a;stx2a;*	0	
L674	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;stx2b;*	1	*
L685	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L697	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L705	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L708	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L735	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L758	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L814	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L886	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L926	*stx1;stx2;eae;*	O111	*stx1a;stx2a;*	0	

Table 2b. Characterisation of test strain 2

Strain 2	**Virulence genes (*stx1; stx2; eae*)**	**Serogroup/serotype**	***stx* genes subtyping**	**Penalties**	**WGS**
Expected result	***stx1;stx2;eae;***	**O111:H8**	***stx1a;stx2a;***		
Labcode	**Reported result**	**Reported result**	**Reported result**		
L144	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L180	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L222	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L226	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L230	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L256	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L262	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L327	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L358	*stx1;stx2;eae;*	O111	*stx1a;stx2a;*	0	
L370	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L389	*stx1;stx2;eae;*	O111	*stx1a;stx2a;*	0	*
L403	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L407	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L462	*stx1;stx2;eae;*	O111	*stx1a;stx2a;*	0	
L494	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L497	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L522	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L561	*stx1;stx2;eae;*	O111	*stx1a;stx1c;stx2a;*	1	
L563	*stx1;stx2;eae;*	O111	*stx2c;*	1	
L615	*stx1;stx2;eae;*	ND	*stx1a;stx2a;*	1	
L635	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L640	*stx1;stx2;eae;*	O111	*stx1a;stx2a;*	0	
L674	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;stx2b;*	1	*
L685	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L697	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L705	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L708	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L735	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L758	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L814	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L886	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L926	*stx1;stx2;eae;*	O111	*stx1a;stx2a;*	0	

Table 2c. Characterisation of test strain 3

Strain 3	**Virulence genes (*stx1; stx2; eae*)**	**Serogroup/serotype**	***stx* genes subtyping**	**Penalties**	**WGS**
Expected result	***stx2;eae;***	**O111:H8**	***stx2a;***		
Labcode	**Reported result**	**Reported result**	**Reported result**		
L144	*stx2;eae;*	O111:H8	*stx2a;*	0	*
L180	*stx2;eae;*	O111:H8	*stx2a;*	0	*
L222	*stx2;eae;*	O111:H8	*stx2a;*	0	*
L226	*stx2;eae;*	O111:H8	*stx2a;*	0	*
L230	*stx2;eae;*	O111:H8	*stx2a;*	0	*
L256	*stx2;eae;*	O111:H8	*stx2a;*	0	*
L262	*stx2;eae;*	O111:H8	*stx2a;*	0	*
L327	*stx2;eae;*	O111:H8	*stx2a;*	0	*
L358	*stx2;eae;*	O111	*stx2a;*	0	
L370	*stx2;eae;*	O111:H8	*stx2a;*	0	*
L389	*stx2;eae;*	O111	*stx2a;*	0	*
L403	*stx2;eae;*	O111:H8	*stx2a;*	0	*
L407	*stx2;eae;*	O111:H8	*stx2a;*	0	*
L462	*stx2;eae;*	O111	*stx2a;*	0	
L494	*stx2;eae;*	O111:H8	*stx2a;*	0	*
L497	*stx2;eae;*	O111:H8	*stx2a;*	0	*
L522	*stx2;eae;*	O111:H8	*stx2a;*	0	*
L561	*stx2;eae;*	O111	*stx2a;*	1	
L563	*stx2;eae;*	O111	*stx2b;*	1	
L615	*stx2;eae;*	O111	*stx2a;*	1	
L635	*stx2;eae;*	O111:H8	*stx2a;*	0	*
L640	*stx2;eae;*	O111	*stx2a;*	0	
L674	*stx2;eae;*	O111:H8	*stx2a;stx2b;*	1	*
L685	*stx2;eae;*	O111:H8	*stx2a;*	0	*
L697	*stx2;eae;*	O111:H8	*stx2a;*	0	*
L705	*stx2;eae;*	O111:H8	*stx2a;*	0	*
L708	*stx2;eae;*	O111:H8	*stx2a;*	0	*
L735	*stx2;eae;*	O111:H8	*stx2a;*	0	*
L758	*stx2;eae;*	O111:H8	*stx2a;*	0	*
L814	*stx2;eae;*	O111:H8	*stx2a;*	0	*
L886	*stx2;eae;*	O111:H8	*stx2a;*	0	*
L926	*stx2;eae;*	O111	*stx2a;*	0	

Table 2d. Characterisation of test strain 4

Strain 4	**Virulence genes (*stx1; stx2; eae*)**	**Serogroup/serotype**	***stx* genes subtyping**	**Penalties**	**WGS**
Expected result	***stx1;stx2;eae;***	**O111:H8**	***stx1a;stx2a;***		
Labcode	**Reported result**	**Reported result**	**Reported result**		
L144	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L180	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L222	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L226	*stx1;eae;*	O111:H8	*stx1a;*	5	*
L230	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L256	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L262	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L327	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L358	*stx1;stx2;eae;*	O111	*stx1a;stx2a;*	0	
L370	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L389	*stx1;stx2;eae;*	O111	*stx1a;stx2a;*	0	*
L403	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L407	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L462	*stx1;stx2;eae;*	O111	*stx1a;stx2a;*	0	
L494	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L497	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L522	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L561	*stx1;stx2;eae;*	O111	*stx1a;stx1c;stx2a;*	1	
L563	*stx1;stx2;eae;*	O111	*stx2a;*	1	
L615	*stx1;stx2;eae;*	ND	*stx1a;stx2a;*	1	
L635	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L640	*stx1;stx2;eae;*	O111	*stx1a;stx2a;*	0	
L674	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2b;*	1	*
L685	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L697	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L705	*stx1;eae;*	O111:H8	*stx1a;*	5	*
L708	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L735	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L758	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L814	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L886	*stx1;stx2;eae;*	O111:H8	*stx1a;stx2a;*	0	*
L926	*stx1;stx2;eae;*	O111	*stx1c;stx2a;*	1	

Table 2e. Characterisation of test strain 5

Strain 5	Virulence genes (*stx1; stx2; eae*)	Serogroup/serotype	*stx* genes subtyping	Penalties	WGS
Expected result	***stx2;eae;***	**O145:H28**	***stx2a;***		
Labcode	**Reported result**	**Reported result**	**Reported result**		
L144	*stx2;eae;*	O145:H28	*stx2a;*	0	*
L180	*stx2;eae;*	O145:H28	*stx2a;*	0	*
L222	*stx2;eae;*	O145:H28	*stx2a;*	0	*
L226	*stx2;eae;*	O145:H28	*stx2a;*	0	*
L230	*stx2;eae;*	O145:H28	*stx2a;*	0	*
L256	*stx2;eae;*	O145: (no H-type was found)	*stx2a;*	0	*
L262	*stx2;eae;*	O145:H28	*stx2a;*	0	*
L327	*stx2;eae;*	O145:H28	*stx2a;*	0	*
L358	*stx2;eae;*	O145	*stx2a;*	0	
L370	*stx2;eae;*	O145:H28	*stx2a;*	0	*
L389	*stx2;eae;*	O145	*stx2a;*	0	*
L403	*stx2;eae;*	O145:H28	*stx2a;*	0	*
L407	*stx2;eae;*	O145:H28	*stx2a;*	0	*
L462	*stx2;eae;*	O145	*stx2a;*	0	
L494	*stx2;eae;*	O145:H28	*stx2a;*	0	*
L497	*stx2;eae;*	O145:H28	*stx2a;*	0	*
L522	*stx2;eae;*	O145:H28	*stx2a;*	0	*
L561	*stx2;eae;*	O145	*stx2a;*	0	
L563	*stx2;eae;*	O145	*stx2a;stx2b;*	1	
L615	*stx2;eae;*	O145	*stx2a;*	0	
L635	*stx2;eae;*	O145:H28	*stx2a;*	0	*
L640	*stx2;eae;*	O145	*stx2a;*	0	
L674	*stx2;eae;*	O145:H28	*stx2a;stx2b;*	1	*
L685	*stx2;eae;*	O145:H28	*stx2a;*	0	*
L697	*stx2;eae;*	O145:H28	*stx2a;*	0	*
L705	*stx2;eae;*	O145:H28	*stx2a;*	0	*
L708	*stx2;eae;*	O145:H28	*stx2a;*	0	*
L735	*stx2;eae;*	O145:H28	*stx2a;*	0	*
L758	*stx2;eae;*	O145:H28	*stx2a;*	0	*
L814	*stx2;eae;*	O145:H28	*stx2a;*	0	*
L886	*stx2;eae;*	O145:H28	*stx2a;*	0	*
L926	*stx2;eae;*	O145	*stx2a;*	0	

Table 2f. Characterisation of test strain 5

Strain 6	**Virulence genes (*stx1; stx2; eae*)**	**Serogroup/serotype**	***stx* genes subtyping**	**Penalties**	**WGS**
Expected result	***stx1;stx2;eae;***	**O26:H11**	***stx1a;stx2a;***		
Labcode	**Reported result**	**Reported result**	**Reported result**		
L144	*stx1;stx2;eae;*	O26:H11	*stx1a;stx2a;*	0	*
L180	*stx1;stx2;eae;*	O26:H11	*stx1a;stx2a;*	0	*
L222	*stx1;stx2;eae;*	O26:H11	*stx1a;stx2a;*	0	*
L226	*stx1;stx2;*	O26:H11	*stx1a;stx2a;*	2	*
L230	*stx1;stx2;eae;*	O26:H11	*stx1a;stx2a;*	0	*
L256	*stx1;stx2;eae;*	O26:H11	*stx1a;stx2a;*	0	*
L262	*stx1;stx2;eae;*	O26:H11	*stx1a;stx2a;*	0	*
L327	*stx1;stx2;eae;*	O26:H11	*stx1a;stx2a;*	0	*
L358	*stx1;stx2;eae;*	O26	*stx1a;stx2a;*	0	
L370	*stx1;stx2;eae;*	O26:H11	*stx1a;stx2a;*	0	*
L389	*stx1;stx2;eae;*	O26	*stx1a;stx2a;*	0	*
L403	*stx1;stx2;eae;*	O26:H11	*stx1a;stx2a;*	0	*
L407	*stx1;stx2;eae;*	O26:H11	*stx1a;stx2a;*	0	*
L462	*stx1;stx2;eae;*	O26	*stx1a;stx2a;*	0	
L494	*stx1;stx2;eae;*	O26:H11	*stx1a;stx2a;*	0	*
L497	*stx1;stx2;eae;*	O26:H11	*stx1a;stx2a;*	0	*
L522	*stx1;stx2;eae;*	O26:H11	*stx1a;stx2a;*	0	*
L561	*stx1;stx2;eae;*	O26	*stx1a;stx1c;stx2a;*	1	
L563	*stx1;stx2;eae;*	O26	*stx2c;*	2	
L615	*stx1;stx2;eae;*	O26	*stx1a;stx1c;stx2a;*	1	
L635	*stx1;stx2;eae;*	O26:H11	*stx1a;stx2a;*	0	*
L640	*stx1;stx2;eae;*	O26	*stx1a;stx2a;*	0	
L674	*stx1;stx2;eae;*	O26:H11	*stx1a;stx2a;stx2b;*	1	*
L685	*stx1;stx2;eae;*	O26:H11	*stx1a;stx2a;*	0	*
L697	*stx1;stx2;eae;*	O26:H11	*stx1a;stx2a;*	0	*
L705	*stx1;stx2;eae;*	O26:H11	*stx1a;stx2a;*	0	*
L708	*stx1;stx2;eae;*	O26:H11	*stx1a;stx2a;*	0	*
L735	*stx1;stx2;eae;*	O26:H11	*stx1a;stx2a;*	0	*
L758	*stx1;stx2;eae;*	O26:H11	*stx1a;stx2a;*	0	*
L814	*stx1;stx2;eae;*	O26:H11	*stx1a;stx2a;*	0	*
L886	*stx1;stx2;eae;*	O26:H11	*stx1a;stx2a;*	0	*
L926	*stx1;stx2;eae;*	O26	*stx1a;stx2a;*	0	

Table 2g. Characterisation of test strain 7

Strain 7	**Virulence genes (*stx1; stx2; eae*)**	**Serogroup/serotype**	***stx* genes subtyping**	**Penalties**	**WGS**
Expected result	***stx1;stx2;***	**O113:H4**	***stx1a;stx2d;***		
Labcode	**Reported result**	**Reported result**	**Reported result**		
L144	*stx1;stx2;*	O113:H4	*stx1a;stx2d;*	0	*
L180	*stx1;stx2;*	O113:H4	*stx1a;stx2d;*	0	*
L222	*stx1;stx2;*	O113:H4	*stx1a;stx2d;*	0	*
L226	*stx1;stx2;*	O113:H4	*stx1a;stx2d;*	0	*
L230	*stx1;stx2;*	O113:H4	*stx1a;stx2d;*	0	*
L256	*stx1;stx2;*	O113:H4	*stx1a;stx2d;*	0	*
L262	*stx1;stx2;*	O113:H4	*stx1a;stx2d;*	0	*
L327	*stx1;stx2;*	O113:H4	*stx1a;stx2d;*	0	*
L358	*stx1;stx2;*	O113	*stx1a;stx2c;*	1	
L370	*stx1;stx2;*	O113:H4	*stx1a;stx2d;*	0	*
L389	*stx1;stx2;*	O113	*stx1a;stx2d;*	0	*
L403	*stx1;stx2;*	O113:H4	*stx1a;stx2d;*	0	*
L407	*stx1;stx2;*	O113:H4	*stx1a;stx2d;*	0	*
L462	*stx1;stx2;*	O113	*stx1a;stx2d;*	0	
L494	*stx1;stx2;*	O113:H4	*stx1a;stx2d;*	0	*
L497	*stx1;stx2;*	O113:H4	*stx1a;stx2d;*	0	*
L522	*stx1;stx2;*	O113:H4	*stx1a;stx2d;*	0	*
L561	*stx1;stx2;*	O113	*stx1a;stx1c;stx2c;stx2d;*	2	
L563	*stx1;stx2;*	NA	*stx2c;stx2d;*	1	
L615	*stx1;stx2;*	O113	*stx1a;stx2c;stx2d;*	1	
L635	*stx1;stx2;eae;*	O113:H4	*stx1a;stx2d;*	2	*
L640	*stx1;stx2;*	O113	*stx1a;stx2c;stx2d;*	1	
L674	*stx1;stx2;*	O113:H4	*stx1a;stx2a;stx2b;*	2	*
L685	*stx1;stx2;*	O113:H4	*stx1a;stx2d;*	0	*
L697	*stx1;stx2;*	O113:H4	*stx1a;stx2d;*	0	*
L705	*stx1;stx2;*	O113:H4	*stx1a;stx2d;*	0	*
L708	*stx1;stx2;*	O113:H4	*stx1a;stx2d;*	0	*
L735	*stx1;stx2;*	O113:H4	*stx1a;stx2d;*	0	*
L758	*stx1;stx2;*	O113:H4	*stx1a;stx2d;*	0	*
L814	*stx1;stx2;*	O113:H4	*stx1a;stx2d;*	0	*
L886	*stx1;stx2;*	O113:H4	*stx1a;stx2d;*	0	*
L926	*stx1;stx2;*	O113	*stx2d;*	1	

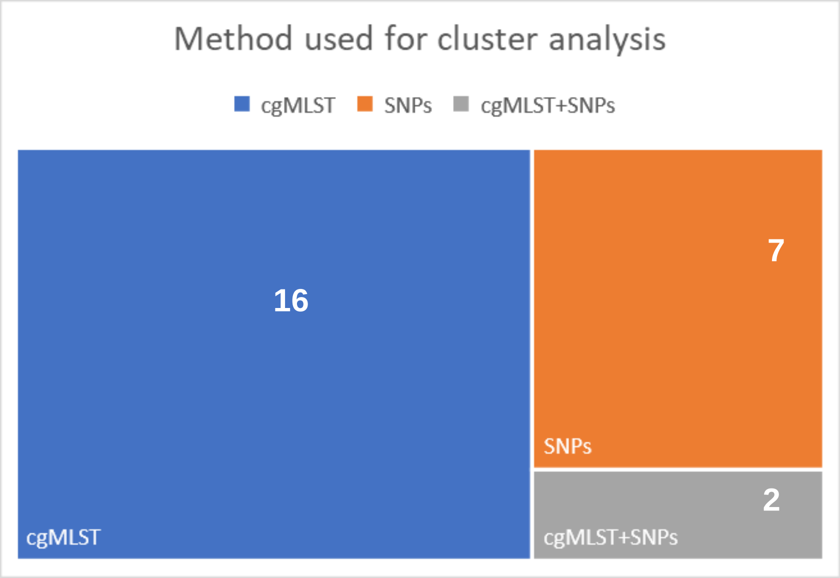
Table 2h. Characterisation of test strain 8

Strain 8	**Virulence genes (*stx1; stx2; eae*)**	**Serogroup/serotype**	***stx* genes subtyping**	**Penalties**	**WGS**
Expected result	***stx2;***	**O174:H21**	***stx2c;***		
Labcode	**Reported result**	**Reported result**	**Reported result**		
L144	*stx2;*	O174:H21	*stx2c;*	0	*
L180	*stx2;*	O174:H21	*stx2c;*	0	*
L222	*stx2;*	O174:H21	*stx2c;*	0	*
L226	*stx2;*	O174:H21	*stx2c;*	0	*
L230	*stx2;*	O174:H21	*stx2c;*	0	*
L256	*stx2;*	O174:H21	*stx2c;*	0	*
L262	*stx2;*	O174:H21	*stx2c;*	0	*
L327	*stx2;*	O174:H21	*stx2c;*	0	*
L358	*stx2;*	ONT-H21	*stx2c;*	0	
L370	*stx2;*	O174:H21	*stx2c;*	0	*
L389	*stx2;*	O174	*stx2c;*	0	*
L403	*stx2;*	O174:H21	*stx2c;*	0	*
L407	*stx2;*	ONT-H21	*stx2c;*	0	*
L462	*stx2;*	ONT	*stx2c;*	0	
L494	*stx2;*	O174:H21	*stx2c;*	0	*
L497	*stx2;*	O174:H21	*stx2c;*	0	*
L522	*stx2;*	O174:H21	*stx2c;*	0	*
L561	*stx2;*	ONT	*stx2a;stx2c;stx2d;*	2	
L563	*stx2;*	O157	*stx2c;stx2d;*	3	
L615	*stx2;*	ONT	*stx2c;stx2d;*	1	
L635	*stx2;*	O174:H21	*stx2c;*	0	*
L640	*stx2;*	O113	*stx2c;*	2	
L674	*stx2;*	O174:H21	*stx2b;*	1	*
L685	*stx2;*	O174:H21	*stx2c;*	0	*
L697	*stx2;*	O?:H21	*stx2c;*	0	*
L705	*stx2;*	O174:H21	*stx2c;*	0	*
L708	*stx2;*	O174:H21	*stx2c;*	0	*
L735	*stx2;*	O174:H21	*stx2c;*	0	*
L758	*stx2;*	O174:H21	*stx2c;*	0	*
L814	*stx2;*	O174:H21	*stx2c;*	0	*
L886	*stx2;*	O174:H21	*stx2c;*	0	*
L926	*stx2;*	ONT	*stx2c;*	0	

5.2 Cluster analysis

All the 25 NRLs carrying out WGS participated in the cluster analysis exercise and performed the phylogenetic analysis on the strains received for PT31. Figure 2 shows the methods used in the cluster analysis exercise, with the number of laboratories applying each method.

Figure 2. Methods applied by the 25 NRLs performing WGS for the cluster analysis



Below, in Table 3 the results of the cluster analysis exercise carried out by the participating labs are reported.

Table 3. Cluster analysis

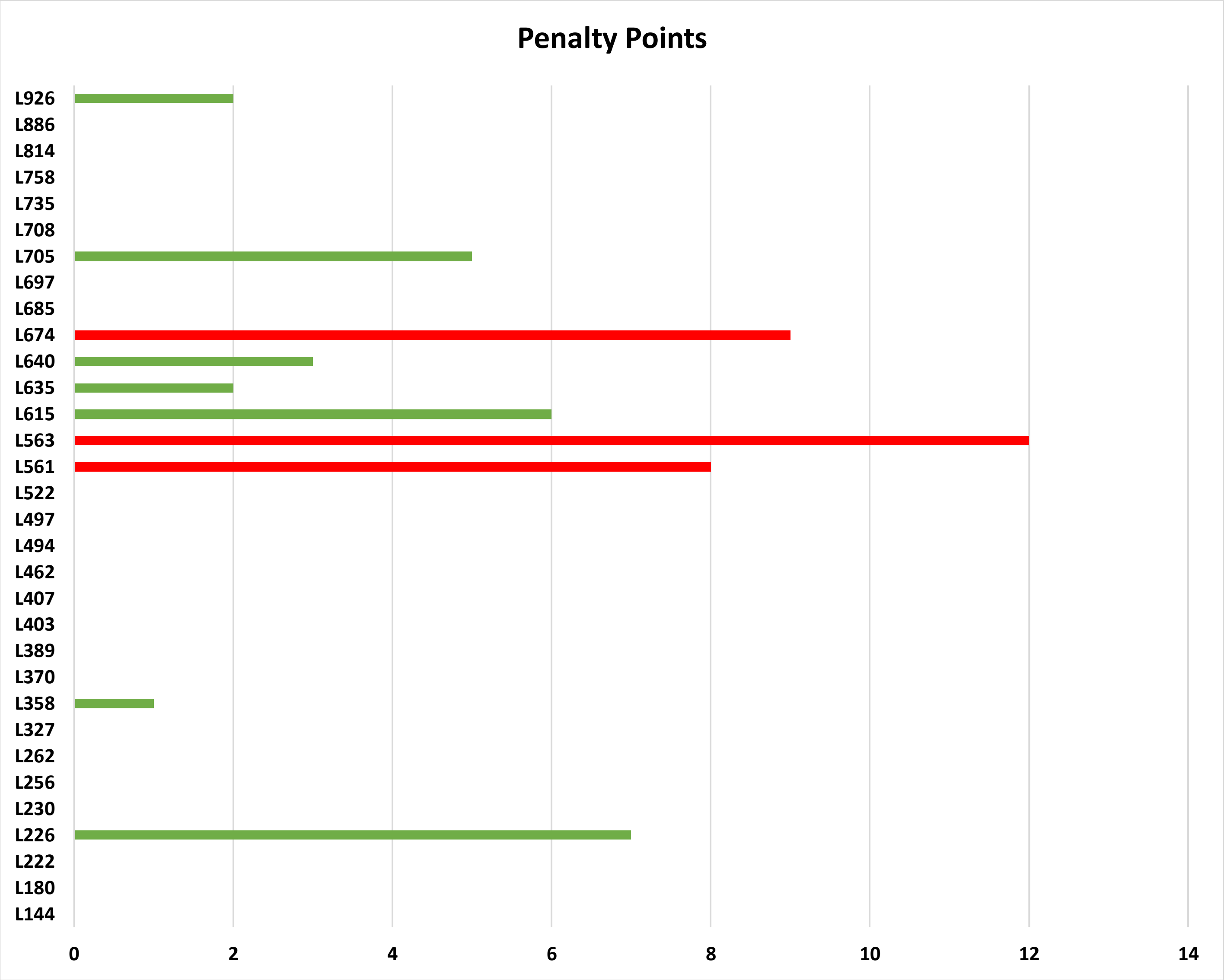
	Expected result (strains belonging to a cluster-1;2;3;4;5;6;7;8;): Yes;Yes;No;No;No;No;No;No;	Distance	Method
Labcode	**Reported result**		
L144	Yes;Yes;No;No;No;No;No;No;	0 SNPs	SNP;
L180	Yes;Yes;No;No;No;No;No;No;	0 allelic differences	cgMLST;
L222	Yes;Yes;No;No;No;No;No;No;	7 allelic differences	cgMLST;
L226	Yes;Yes;No;No;No;No;No;No;	29	cgMLST;
L230	Yes;Yes;No;No;No;No;No;No;	1 allele	cgMLST;
L256	Yes;Yes;Yes;Yes;No;No;No;No;	10-503 SNPs	SNP;
L262	Yes;Yes;No;No;No;No;No;No;	0-5 allelic differences	cgMLST;
L327	Yes;Yes;No;No;No;No;No;No;	0 allelic differences	cgMLST;
L370	Yes;Yes;No;No;No;No;No;No;	0 alleles difference between 4849 and 4643	cgMLST;
L389	Yes;Yes;No;No;No;No;No;No;	0-15 SNPs	SNP;
L403	Yes;Yes;No;No;No;No;No;No;	0 allelic differences	cgMLST;
L407	Yes;Yes;No;No;No;No;No;No;	0 allelic differences (cgMLST) and 3 SNPs	cgMLST;SNP;
L494	Yes;Yes;No;No;No;No;No;No;	AD = 0	cgMLST;
L497	Yes;Yes;No;No;No;No;No;No;	0 allelic differences	cgMLST;
L522	Yes;Yes;No;No;No;No;No;No;	SNP: 0 SNPs between 1745 and 4190; cgMLST 1 allelic difference between 1745 and 4190	cgMLST;SNP;
L635	Yes;Yes;No;No;No;No;No;No;	0	cgMLST;
L674	Yes;Yes;No;No;No;No;No;No;	1	SNP;
L685	Yes;Yes;Yes;Yes;Yes;Yes;Yes;Yes;	0-25	SNP;
L697	Yes;Yes;No;No;No;No;No;No;	1 allelic difference	cgMLST;
L705	Yes;Yes;No;No;No;No;No;No;	0 allelic differences	cgMLST;
L708	Yes;Yes;No;No;No;No;No;No;	0 allelic difference	cgMLST;
L735	Yes;Yes;No;No;No;No;No;No;	2 SNPs	SNP;
L758	Yes;Yes;No;No;No;No;No;No;	0 AD	cgMLST;
L814	Yes;Yes;No;No;No;No;No;No;	0-1	SNP;
L886	Yes;Yes;No;No;No;No;No;No;	1 allelic difference	cgMLST;

All the laboratories except two correctly identified the cluster composed by test strain 1 and test strain 2. Both laboratories reporting incorrect results were applying SNPs analysis, and the incorrect assignment of the clusters may be possibly due to misinterpretation of the results obtained. No penalty points have been assigned to the cluster analysis exercise.

6. Evaluation of the proficiency of the participating Laboratories

The proficiency of the Laboratories has been assessed as described in 4.4. The participating laboratories presenting a score higher than eight were considered as under-performant (red bars in **Figure 3**).

Figure 3. Number of penalty points per NRL



7. CONCLUDING REMARKS

1. A high level of participation was recorded for PT31 despite the COVID-19 pandemic added substantial burden to the laboratories' workflow.
2. WGS was carried out from 78% of the laboratories participating in the study, most of which exhibited an excellent performance confirming the efficacy of this approach.
3. Three laboratories underperformed, and most incorrect results were reported for *stx* genes subtyping. Only one laboratory typed the isolates by WGS*.*
4. Two test strains belonged to serogroups out of the scope of the standard method ISO TS 13136:2012 (O113 and O174). O113 is one of the top 14 serogroups causing human diseases and is included in the method issued by the EURL for the determination of O-groups. All but one laboratory could identify it. As for the serogroup O174, the participating Laboratories performed well in identifying it when WGS-based methods were used.
5. All the laboratories using WGS also participated in the cluster analysis exercise. Most laboratories performed well regardless the method used (cgMLST or SNP analysis). The need to refine the threshold to call the clusters has been highlighted.
6. The use of WGS should be encouraged throughout the network, as it demonstrated better performances in comparison to the standard methods used to type STEC.