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Report of the 35th inter-laboratory study (PT35) on the identification and typing of Shiga toxin-producing *E. coli* (STEC) 2022-2023

Edited by:

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1. OBJECTIVES OF THE STUDY

The objectives of PT35 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 detection of virulence genes of other pathogenic *E. coli* (*ipaH* for EIEC, *st* and *lt* for ETEC, *aggR* and *aaiC* for EAEC, methods available at the EURL for *E. coli* website).
4. Subtyping of Shiga Toxins (Stx)-coding genes.
5. 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”.

PT35 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. The identification of virulence genes associated to other Diarrhoeagenic *E. coli* (DEC) pathotypes, and in particular *ipaH* for EIEC, *st* and *lt* for ETEC, *aggR* and *aaiC* for EAEC

3. The determination of a range of relevant STEC serogroups. Participants were requested to identify the serogroup of the test strains assaying at least the following 14 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).

4. 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*).

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-three NRLs 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. Austria, Austrian Agency for Health and Food Safety (AGES), IMED, Graz
2. Belgium, NRL STEC, institute of Public Health Sciensano, Elsene
3. Bulgaria, National Diagnostic and Research Veterinary Institute /NDRVMI/, NRL "Listeria, E.coli and foodborne viruses", Sofia
4. Croatia, Croatian Veterinary Institute, Laboratory for Food Microbiology, Zagreb
5. Denmark, Danish Food and Veterinary Administration, laboratory, Ringsted
6. Estonia, Estonian Veterinary and Food Laboratory, Tartu
7. Finland, Finnish Food Authority Laboratory and Research Division (Evira), Microbiology Unit (Food), Helsinki
8. France, VetAgroSup, LMAP/LNR E. coli STEC, Marcy-l'Etoile
9. Germany, Federal Institute for Risk Assessment (BfR); German National Reference Laboratory for E. coli, Berlin
10. Germany, Friedrich-Loeffler-Institut / Federal Research Institute for Animal Health, Jena
11. Hungary, National Food Chain Safety Office, Food Chain Safety Directorate, Microbiological NRL, Budapest
12. Iceland, Matís ohf/Icelandic Food and Biotech R&D, Reykjavík
13. Ireland, Food Microbiology Division, Department of Agriculture, Food and the Marine, Celbridge Co. Kildare
14. Italy, Istituto Superiore di Sanità, Rome
15. Latvia, Institute of Food Safety, Animal Health and Environment "BIOR", Riga
16. Lithuania, National Food and Veterinary Risk Assessment Institute, Vilnius
17. Luxembourg, Service EPIGEM, Laboratoire National de Santé, Dudelange
18. Norway, Norwegian Veterinary Institute (NVI), Ås
19. Poland, National Institute of Public Health (NIH) - National Research Institute, Warsaw

20. Poland, National Veterinary Research Institute (NVRI), Department of Hygiene of Food of Animal Origin, Pulawy
21. Portugal, Instituto Nacional de Investigação Veterinária (National Institute for Agrarian and Veterinary Research-INIAV), Oeiras
22. Romania, Institute for Hygiene and Veterinary Public Health, Bucharest
23. Slovakia, State Veterinary and Food Institute, Dolný Kubín
24. Slovakia, Public Health Authority of the Slovak Republic, Bratislava
25. Slovenia, University of Ljubljana, Veterinary Faculty, National Veterinary Institute
26. Spain, Laboratorio Central de Veterinaria de Algete (MAPA), Algete (Madrid)
27. Spain, National Plant Health Laboratory, Lugo
28. Spain, Centro Nacional de Alimentación, Agencia Española de Seguridad Alimentaria y Nutrición (AESAN), Madrid
29. Sweden, Swedish Food Agency/Livsmedelsverket, Biologiavdelningen, Uppsala
30. Sweden, National Veterinary Institute (SVA), Uppsala
31. Switzerland, AGROSCOPE, Research Group of Bacteriological Food Safety, Bern
32. The Netherlands, National Institute for Public Health and the Environment (RIVM), Bilthoven
33. The Netherlands, Wageningen Food Safety Research, Wageningen

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 16th, 2022, 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 five weeks. On November the 18th 2022, a homogeneity test was performed on six randomly selected sets of test strains. The remaining test samples were stored at room temperature until 24th of November 2022, when the parcels were shipped to the participating laboratories by courier.

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

ID PT35	Serotype	MLST	Virulence genes profile	*stx1* subtype	*stx2* subtype	Cluster
1	O26:H11	ST21	*eae stx2*	-	*stx2a*	Yes
2	O26:H11	ST21	*eae stx2*	-	*stx2a*	Yes
3	O111:H21	ST40	*aaiC aggR*	-	-	No
4	O26:H11	ST396	*eae stx2*	-	*stx2d*	No
5	O187:H28	ST200	*stp (=sta1) stx2*	-	*stx2g*	No
6	O104:H7	ST2283	*stx1 aaiC**	*stx1c*	-	No
7	O124:H30	ST6	*ipaH*	-	-	No
8	O26:H11	ST21	*eae stx2*	-	*stx2a*	Yes

***** the allelic variant of the *aaiC* gene present in this strain is not detected by the method "Detection of Enteroaggregative *Escherichia coli* in food by Real Time PCR amplification of the *aggR* and *aaiC* genes" (available at: https://www.iss.it/documents/20126/0/EURL-VTEC_Method_05_Rev+2.pdf)

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

ID PT35	additional virulence genes
Test strain 1	*asta, cif, efa1, ehxa, espa, espb, espf, espj, espp, fyua, gad, iha, iss, iucc, iuta, katp, lpfa, nlea, nleb, nlec, terc, tir, toxb, trat*
Test strain 2	*asta, cif, efa1, ehxa, espa, espb, espf, espj, espp, fyua, gad, iha, iss, iucc, iuta, katp, lpfa, nlea, nleb, nlec, terc, tir, toxb, trat*
Test strain 3	*aap, aar, aata, afad, agg3b, agg3c, agg3d, agg5a, asta, espi, gad, iha, iss, iucc, iuta, lpfa, ompt, orf3, orf4, pic, sat, sepa, terc*
Test strain 4	*asta, cif, efa1, ehxa, espa, espb, espf, espj, espp, fyua, gad, iha, iss, lpfa, nlea, nleb, nlec, terc, tir, toxb, trat*
Test strain 5	*asta, celb, ehxa, gad, lpfa, terc, trat*
Test strain 6	*cba, celb, cia, epea, gad, irea, katp, lpfa, neuc, orf3, orf4, terc, trat*
Test strain 7	*capu, fyua, gad, iha, ipad, iucc, iuta, pic, senb, siga, sita, terc, trat, virf*
Test strain 8	*asta, cif, efa1, ehxa, espa, espb, espf, espj, espp, fyua, gad, iha, iss, iucc, iuta, katp, lpfa, nlea, nleb, nlec, terc, tir, toxb, trat*

4.2. Laboratory methods

The laboratories were requested to identify the main STEC and other DEC 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*), is 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 PT35. The correlation between the test strains could be assessed by SNPs/wg/cgMLST analysis: in particular, the laboratories were requested to indicate the blind codes of the strains belonging to a cluster, according to their interpretation, and to submit the range of differences (in number of SNPs or allelic differences) observed among the samples composing the cluster.

4.3. Collection and elaboration of the NRLs' results

The results were submitted through an on-line form prearranged by the EURL-VTEC. The link to access the form was sent by E-mail to all the participants' laboratories. The deadline for collecting the results was set at the 23rd of January 2023.

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* and the additional DEC virulence genes. No penalty points have been assigned to the missing detection of *aaiC* in strain 6 since the allelic variant of the *aaiC* gene present in this strain is not detected by the method "Detection of Enteroaggregative *Escherichia coli* in food by Real Time PCR amplification of the *aggR* and *aaiC* genes" (https://www.iss.it/documents/20126/0/EURL-VTEC_Method_05_Rev+2.pdf)
- **2 penalty points** to each incorrect result concerning the identification of the top-14 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 (test strains 5 and 7).
- **1 penalty points** to each missing result or 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 33 Laboratories. **Figure 1** shows the number of participating laboratories aggregated according to the methods used to characterise the isolates.

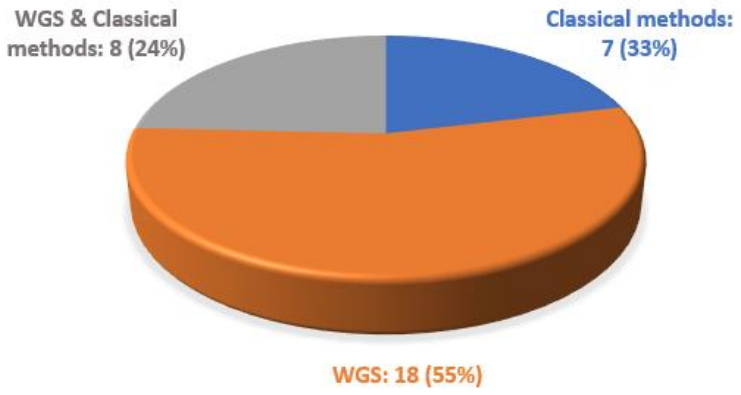


Figure 1. Methods applied by the Laboratories to type STEC test strains

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	Serogroup/serotype	*stx* genes subtyping	Penalties	WGS
Expected result	*eae; stx2*	O26:H11	*stx2a*		
Labcode	Reported result	Reported result	Reported result		
L001	*eae; stx2*	O26:H11	*stx2a*	0	*
L002	*eae; stx2*	O26:H11	*stx2a*	0	*
L004	*eae; stx2*	O26:H11	*stx2a*	0	*
L006	*eae; stx2*	O26	*stx2a*	0	
L007	*eae; stx2*	O26:H11	*stx2a*	0	*
L014	*eae; stx2*	O26:H11	*stx2a*	0	*
L015	*eae; stx2*	O26:H11	*stx2a*	0	
L016	*eae; stx2*	O26	*stx2b; stx2c*	2	
L017	*eae; stx2*	O26	*stx2a*	0	
L018	*eae; stx2*	O26:H11	*stx2a*	0	*
L144	*eae; stx2*	O26:H11	*stx2a*	0	*
L222	*eae; stx2*	O26:H11	*stx2a*	0	*
L230	*eae; stx2*	O26:H11	*stx2a*	0	*
L256	*eae; stx2*	O26:H11	*stx2a*	0	*
L258	*eae; stx2*	O26:H11	*stx2a*	0	*
L327	*eae; stx2*	O26:H11	*stx2a*	0	*
L337	*eae; stx2*	O26	*stx2a*	0	
L370	*eae; stx2*	O26:H11	*stx2a*	0	*
L403	*eae; stx2*	O26:H11	*stx2a*	0	*
L462	*eae; stx2*	O26	*stx2a*	0	
L522	*eae; stx2*	O26:H11	*stx2a; stx2b*	1	*
L615	*eae; stx2*	O26:H11	*stx2a*	0	*
L674	*eae; stx2*	O26:H11	*stx2a*	0	*
L685	*eae; stx2*	O26:H11	*stx2b*	1	*
L697	*eae; stx2*	O26:H11	*stx2a*	0	*
L705	*eae; stx2*	O26:H11	*stx2a*	0	*
L708	*eae; stx2*	O26:H11	*stx2a*	0	*
L758	*eae; stx2*	O26:H11	*stx2a*	0	*
L846	*eae; stx2*	O26:H11	*stx2a*	0	*
L972	*eae; stx2*	O26:H11	*stx2a*	0	*
L976	*eae; stx2*	O26	*stx2a*	0	
L986	*eae; stx2*	O26:H11	*stx2a*	0	*
L993	*eae; stx2*	O26:H11	*stx2a*	0	*

Table 2b. Characterisation of test strain 2

Strain 2	Virulence genes	Serogroup/serotype	*stx* genes subtyping	Penalties	WGS
Expected result	***eae; stx2***	**O26:H11**	***stx2a***		
Labcode	**Reported result**	**Reported result**	**Reported result**		
L001	*eae; stx2*	O26:H11	*stx2a*	0	*
L002	*eae; stx2*	O26:H11	*stx2a*	0	*
L004	*eae; stx2*	O26:H11	*stx2a*	0	*
L006	*eae; stx2*	O26	*stx2a*	0	
L007	*eae; stx2*	O26:H11	*stx2a*	0	*
L014	*eae; stx2*	O26:H11	*stx2a*	0	*
L015	*eae; stx2*	O26	*stx2a*	0	
L016	*eae; stx2*	O26	*stx2b; stx2c*	2	
L017	*eae; stx2*	O26	*stx2a*	0	
L018	*eae; stx2*	O26:H11	*stx2a*	0	*
L144	*eae; stx2*	O26:H11	*stx2a*	0	*
L222	*eae; stx2*	O26:H11	*stx2a*	0	*
L230	*eae; stx2*	O26:H11	*stx2a*	0	*
L256	*eae; stx2*	O26:H11	*stx2a*	0	*
L258	*eae; stx2*	O26:H11	*stx2a*	0	*
L327	*eae; stx2*	O26:H11	*stx2a*	0	*
L337	*eae; stx2*	O26	*stx2a*	0	
L370	*eae; stx2*	O26:H11	*stx2a*	0	*
L403	*eae; stx2*	O26:H11	*stx2a*	0	*
L462	*eae; stx2*	O26	*stx2a*	0	
L522	*eae; stx2*	O26:H11	*stx2a; stx2b*	1	*
L615	*eae; stx2*	O26:H11	*stx2a*	0	*
L674	*eae; stx2*	O26:H11	*stx2a*	0	*
L685	*eae; stx2*	O26:H11	*stx2b*	1	*
L697	*eae; stx2*	O26:H11	*stx2a*	0	*
L705	*eae; stx2*	O26:H11	*stx2a*	0	*
L708	*eae; stx2*	O26:H11	*stx2a*	0	*
L758	*eae; stx2*	O26:H11	*stx2a*	0	*
L846	*eae; stx2*	O26:H11	*stx2a*	0	*
L972	*eae; stx2*	O26:H11	*stx2a*	0	*
L976	*eae; stx2*	O26	*stx2a*	0	
L986	*eae; stx2*	O26:H11	*stx2a*	0	*
L993	*eae; stx2*	O26:H11	*stx2a*	0	*

Table 2c. Characterisation of test strain 3

Strain 3	Virulence genes	Serogroup/serotype	*stx* genes subtyping	Penalties	WGS
Expected result	*aaiC; aggR*	O111:H21	-		
Labcode	Reported result	Reported result	Reported result		
L001	*aaiC; aggR*	O111:H21	-	0	*
L002	*aaiC; aggR*	O111:H21	-	0	*
L004	*aaiC; aggR*	O111:H21	-	0	*
L006	*aaiC; aggR*	O111	-	0	
L007	*aaiC; aggR*	O111:H21	-	0	*
L014	*aaiC; aggR*	O111:H21	-	0	*
L015	*aaiC; aggR*	O111:H21	-	0	
L016	*aaiC; aggR*	O111	-	0	
L017	*aaiC; aggR*	O111	-	0	
L018	*aaiC; aggR*	O111:H21	-	0	*
L144	*aaiC; aggR*	O111:H21	-	0	*
L222	*aaiC; aggR*	O111:H21	-	0	*
L230	*aaiC; aggR*	O111:H21	-	0	*
L256	*aaiC; aggR*	O111:H21	-	0	*
L258	*aaiC; aggR*	O11:H21	-	0	*
L327	*aaiC; aggR*	O111:H21	-	0	*
L337	*aaiC; aggR*	O111	-	0	
L370	*aaiC; aggR*	O111:H21	-	0	*
L403	*aaiC; aggR*	O111:H21	-	0	*
L462	*aaiC; aggR*	O111	-	0	
L522	*aaiC; aggR*	O111:H21	-	0	*
L615	*aaiC; aggR*	O111:H21	-	0	*
L674	*aaiC; aggR*	O111:H21	-	0	*
L685	*aaiC; aggR*	O111:H21	-	0	*
L697	*aaiC; aggR*	O111:H21	-	0	*
L705	*aaiC; aggR*	O111:H21	-	0	*
L708	*aaiC; aggR*	O111:H21	-	0	*
L758	*aaiC; aggR*	O111:H21	-	0	*
L846	*aaiC; aggR*	O111:H21	-	0	*
L972	*aaiC; aggR*	O111:H21	-	0	*
L976	*aaiC; aggR*	O111	-	0	
L986	*aaiC; aggR*	O111:H21	-	0	*
L993	*aaiC; aggR*	O111:H21	-	0	*

Table 2d. Characterisation of test strain 4

Strain 4	Virulence genes	Serogroup/serotype	*stx* genes subtyping	Penalties	WGS
Expected result	*eae; stx2*	O26:H11	*stx2d*		
Labcode	Reported result	Reported result	Reported result		
L001	*eae; stx2*	O26:H11	*stx2d*	0	*
L002	*eae; stx2*	O26:H11	*stx2d*	0	*
L004	*eae; stx2*	O26:H11	*stx2d*	0	*
L006	*eae; stx2*	O26	*stx2c; stx2d*	1	
L007	*eae; stx2*	O26:H11	*stx2d*	0	*
L014	*eae; stx2*	O26:H11	*stx2d*	0	*
L015	*eae; stx2*	O26	-	1	
L016	*eae; stx2*	O26	*stx2b; stx2c*	2	
L017	*eae; stx2*	O26	*stx2d*	0	
L018	*eae; stx2*	O26:H11	*stx2d*	0	*
L144	*eae; stx2*	O26:H11	*stx2d*	0	*
L222	*eae; stx2*	O26:H11	*stx2d*	0	*
L230	*eae; stx2*	O26:H11	*stx2d*	0	*
L256	*eae; stx2*	O26:H11	*stx2d*	0	*
L258	*eae; stx2*	O26:H11	*stx2d*	0	*
L327	*eae; stx2*	O26:H11	*stx2d*	0	*
L337	*eae; stx2*	O26	*stx2c;stx2d*	1	
L370	*eae; stx2*	O26:H11	*stx2d*	0	*
L403	*eae; stx2*	O26:H11	*stx2d*	0	*
L462	*eae; stx2*	O26	-	1	
L522	*eae; stx2*	O26:H11	*stx2a; stx2b*	2	*
L615	*eae; stx2*	O26:H11	*stx2d*	0	*
L674	*eae; stx2*	O26:H11	*stx2d*	0	*
L685	*eae; stx2*	O26:H11	*stx2a;stx2b*	2	*
L697	*eae; stx2*	O26:H11	*stx2d*	0	*
L705	*eae; stx2*	O26:H11	*stx2d*	0	*
L708	*eae; stx2*	O26:H11	*stx2d*	0	*
L758	*eae; stx2*	O26:H11	*stx2d*	0	*
L846	*eae; stx2*	O26:H11	*stx2d*	0	*
L972	*eae; stx2*	O26:H11	*stx2d*	0	*
L976	*eae; stx2*	O26	*stx2d*	0	
L986	*eae; stx2*	O26:H11	*stx2d*	0	*
L993	*eae; stx2*	O26:H11	*stx2d*	0	*

Table 2e. Characterisation of test strain 5

Strain 5	Virulence genes	Serogroup/serotype	*stx* genes subtyping	Penalties	WGS
Expected result	*stp (=sta1) stx2*	O187:H28	*stx2g*		
Labcode	Reported result	Reported result	Reported result		
L001	*stx2*	O187:H28	*stx2g*	2	*
L002	*stx2*	O187:H28	*stx2g*	2	*
L004	*stx2*	O187:H28	*stx2g*	2	*
L006	*stx2*	O group not identified	*stx2g*	2	
L007	*st; stx2*	O187:H28	*stx2g*	0	*
L014	*stx2*	O187:H28	*stx2g*	2	*
L015	*stx2*	O187:H28	*stx2g*	2	
L016	*stx2*	O103	*stx2b; stx2g*	5	
L017	*stx2*	Not detected	*stx2g*	2	
L018	*st; stx2*	O187:H28	*stx2g*	0	*
L144	*stx2*	O187:H28	*stx2g*	2	*
L222	*st; stx2*	O187:H28	*stx2g*	0	*
L230	*st; stx2*	O187:H28	*stx2g*	0	*
L256	*stx2*	O187:H28	*stx2g*	2	*
L258	*st; stx2*	O187:H28	*stx2g*	0	*
L327	*stx2*	O187:H28	*stx2g*	2	*
L337	*stx2*	ONT	*stx2g*	2	
L370	*st; stx2*	O187:H28	*stx2g*	0	*
L403	*st; stx2*	O187:H28	*stx2g*	0	*
L462	*stx2*	-	-	3	
L522	*stx2*	O187:H28	*stx2a; stx2b*	4	*
L615	*stx2*	O187:H28	*stx2g*	2	*
L674	*stx2*	O187:H28	*stx2g*	2	*
L685	*stx2*	O187:H28	*stx2a; stx2b*	4	*
L697	*st; stx2*	O187:H28	*stx2g*	0	*
L705	*stx2*	O187:H28	*stx2g*	2	*
L708	*stx2*	O187:H28	*stx2g*	2	*
L758	*stx2*	O187:H28	*stx2g*	2	*
L846	*st; stx2*	O187:H28	*stx2g*	0	*
L972	*st; stx2*	O187:H28	*stx2g*	0	*
L976	*stx2*	not identified	*stx2g*	2	
L986	*st; stx2*	O187:H28	*stx2g*	0	*
L993	*st; stx2*	O187:H28	*stx2g*	0	*

Table 2f. Characterisation of test strain 6

Strain 6	Virulence genes	Serogroup/serotype	*stx* genes subtyping	Penalties	WGS
Expected result	*aaiC*[§]*; stx1*	O104:H7	*stx1c*		
Labcode	Reported result	Reported result	Reported result		
L001	*aaiC; stx1*	O104:H7	*stx1c*	0	*
L002	*aaiC; stx1*	O104:H7	*stx1c*	0	*
L004	*aaiC; stx1*	O104:H7	*stx1c*	0	*
L006	*stx1*	O104	*stx1c*	0	
L007	*aaiC; stx1*	O104:H7	*stx1c*	0	*
L014	*aaiC; stx1*	O104:H7	*stx1c*	0	*
L015	*aaiC; stx1*	O104:H7	*stx1c*	0	
L016	*stx1*	O104	*stx1a; stx1c*	1	
L017	*stx1*	O104	*stx1c*	0	
L018	*aaiC; stx1*	O104:H7	*stx1c*	0	*
L144	*aaiC; stx1*	O104:H7	*stx1c*	0	*
L222	*stx1*	O104:H7	*stx1c*	0	*
L230	*aaiC; stx1*	O104:H7	*stx1c*	0	*
L256	*aaiC; stx1*	O104:H7	*stx1c*	0	*
L258	*aaiC; stx1*	O104:H7	*stx1c*	0	*
L327	*aaiC; stx1*	O104:H7	*stx1c*	0	*
L337	*stx1*	O104	*stx1c*	0	
L370	*aaiC; stx1*	O104:H7	*stx1c*	0	*
L403	*aaiC; stx1*	O104:H7	*stx1c*	0	*
L462	*stx1*	O104	*stx1c*	0	
L522	*stx1*	O104:H7	*stx1a*	1	*
L615	*aaiC; stx1*	O104:H7	*stx1c*	0	*
L674	*aaiC; stx1*	O104:H7	*stx1c*	0	*
L685	*stx1*	O104:H7	*stx1a*	1	*
L697	*aaiC; stx1*	O104:H7	*stx1c*	0	*
L705	*aaiC; stx1*	O104:H7	*stx1c*	0	*
L708	*stx1*	O104:H7	*stx1c*	0	*
L758	*aaiC; stx1*	O104:H7	*stx1c*	0	*
L846	*aaiC; stx1*	O104:H7	*stx1c*	0	*
L972	*aaiC; stx1*	O104:H7	*stx1c*	0	*
L976	*stx1*	O104	*stx1c*	0	
L986	*aaiC; stx1*	O104:H7	*stx1c*	0	*
L993	*aaiC; stx1*	O104:H7	*stx1c*	0	*

§ No penalty points have been assigned to the missing detection of *aaiC* since the allelic variant of the *aaiC* gene present in this strain is not detected by the method "Detection of Enteroaggregative *Escherichia coli* in food by Real Time PCR amplification of the *aggR* and *aaiC* genes" (https://www.iss.it/documents/20126/0/EURL-VTEC_Method_05_Rev+2.pdf)

Table 2g. Characterisation of test strain 7

Strain 7	Virulence genes	Serogroup/serotype	*stx* genes subtyping	Penalties	WGS
Expected result	***ipaH***	**O124:H30**	**-**		
Labcode	Reported result	Reported result	Reported result		
L001	*ipaH*	O124:H30	-	0	*
L002	Other	O124:H30	-	2	*
L004	*ipaH*	O124:H30	-	0	*
L006	*ipaH*	O group not identified	-	2	
L007	*ipaH*	O124:H30	-	0	*
L014	Other	O124:H30	-	2	*
L015	*ipaH*	O124:H30	-	0	
L016	*ipaH*	-	-	0	
L017	*ipaH*	Not detected	-	0	
L018	*ipaH*	O124:H30	-	0	*
L144	*ipaH*	O124:H30	-	0	*
L222	*ipaH*	O124:H30	-	0	*
L230	*ipaH*	O124:H30	-	0	*
L256	*ipaH*	O124:H30	-	0	*
L258	Other	O124:H30	-	2	*
L327	*ipaH*	O124:H30	-	0	*
L337	*ipaH*	ONT	-	0	
L370	*ipaH*	O124:H30	-	0	*
L403	*ipaH*	O124:H30	-	0	*
L462	*ipaH*	-	-	0	
L522	Other	O124:H30	-	2	*
L615	*ipaH*	O124:H30	-	0	*
L674	Other	O124:H30	-	2	*
L685	Other	O124:H30	-	2	*
L697	*ipaH*	O124:H30	-	0	*
L705	*ipaH*	O124:H30	-	0	*
L708	*ipaH*	Or:H30; Genoserotype O124:H30	-	0	*
L758	*ipaH*	O124:H30	-	0	*
L846	Other	O124:H30	-	2	*
L972	Other	O124:H8	-	2	*
L976	*ipaH*	not identified	-	0	
L986	*ipaH*	O124:H30	-	0	*
L993	*ipaH*	O124:H30	-	0	*

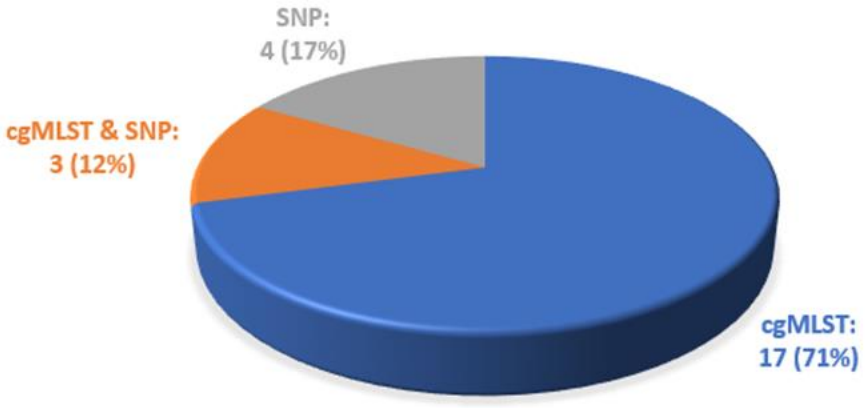
Table 2h. Characterisation of test strain 8

Strain 8	Virulence genes	Serogroup/serotype	*stx* genes subtyping	Penalties	WGS
Expected result	***eae; stx2***	**O26:H11**	***stx2a***		
Labcode	**Reported result**	**Reported result**	**Reported result**		
L001	*eae; stx2*	O26:H11	*stx2a*	0	*
L002	*eae; stx2*	O26:H11	*stx2a*	0	*
L004	*eae; stx2*	O26:H11	*stx2a*	0	*
L006	*eae; stx2*	O26	*stx2a*	0	
L007	*eae; stx2*	O26:H11	*stx2a*	0	*
L014	*eae; stx2*	O26:H11	*stx2a*	0	*
L015	*eae; stx2*	O26:H11	*stx2a*	0	
L016	*eae; stx2*	O26	*stx2b; stx2c*	2	
L017	*eae; stx2*	O26	*stx2a*	0	
L018	*eae; stx2*	O26:H11	*stx2a*	0	*
L144	*eae; stx2*	O26:H11	*stx2a*	0	*
L222	*eae; stx2*	O26:H11	*stx2a*	0	*
L230	*eae; stx2*	O26:H11	*stx2a*	0	*
L256	*eae; stx2*	O26:H11	*stx2a*	0	*
L258	*eae; stx2*	O26:H11	*stx2a*	0	*
L327	*eae; stx2*	O26:H11	*stx2a*	0	*
L337	*eae; stx2*	O26	*stx2a*	0	
L370	*eae; stx2*	O26:H11	*stx2a*	0	*
L403	*eae; stx2*	O26:H11	*stx2a*	0	*
L462	*eae; stx2*	O26	*stx2a*	0	
L522	*eae; stx2*	O26:H11	*stx2a; stx2b*	1	*
L615	*eae; stx2*	O26:H11	*stx2a*	0	*
L674	*eae; stx2*	O26:H11	*stx2a*	0	*
L685	*eae; stx2*	O26:H11	*stx2a; stx2b*	1	*
L697	*eae; stx2*	O26:H11	*stx2a*	0	*
L705	*eae; stx2*	O26:H11	*stx2a*	0	*
L708	*eae; stx2*	O26:H11	*stx2a*	0	*
L758	*eae; stx2*	O26:H11	*stx2a*	0	*
L846	*eae; stx2*	O26:H11	*stx2a*	0	*
L972	*eae; stx2*	O26:H11	*stx2a*	0	*
L976	*eae; stx2*	O26	*stx2a*	0	
L986	*eae; stx2*	O26:H11	*stx2a*	0	*
L993	*eae; stx2*	O26:H11	*stx2a*	0	*

5.2 Cluster analysis

Twenty-four out of the 26 NRLs carrying out WGS participated in the cluster analysis exercise and performed the phylogenetic analysis on the strains received for PT35. 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



The results of the cluster analysis exercise are reported in Table 3.

All the laboratories correctly identified the cluster composed by test strains 1, 2 and 8.

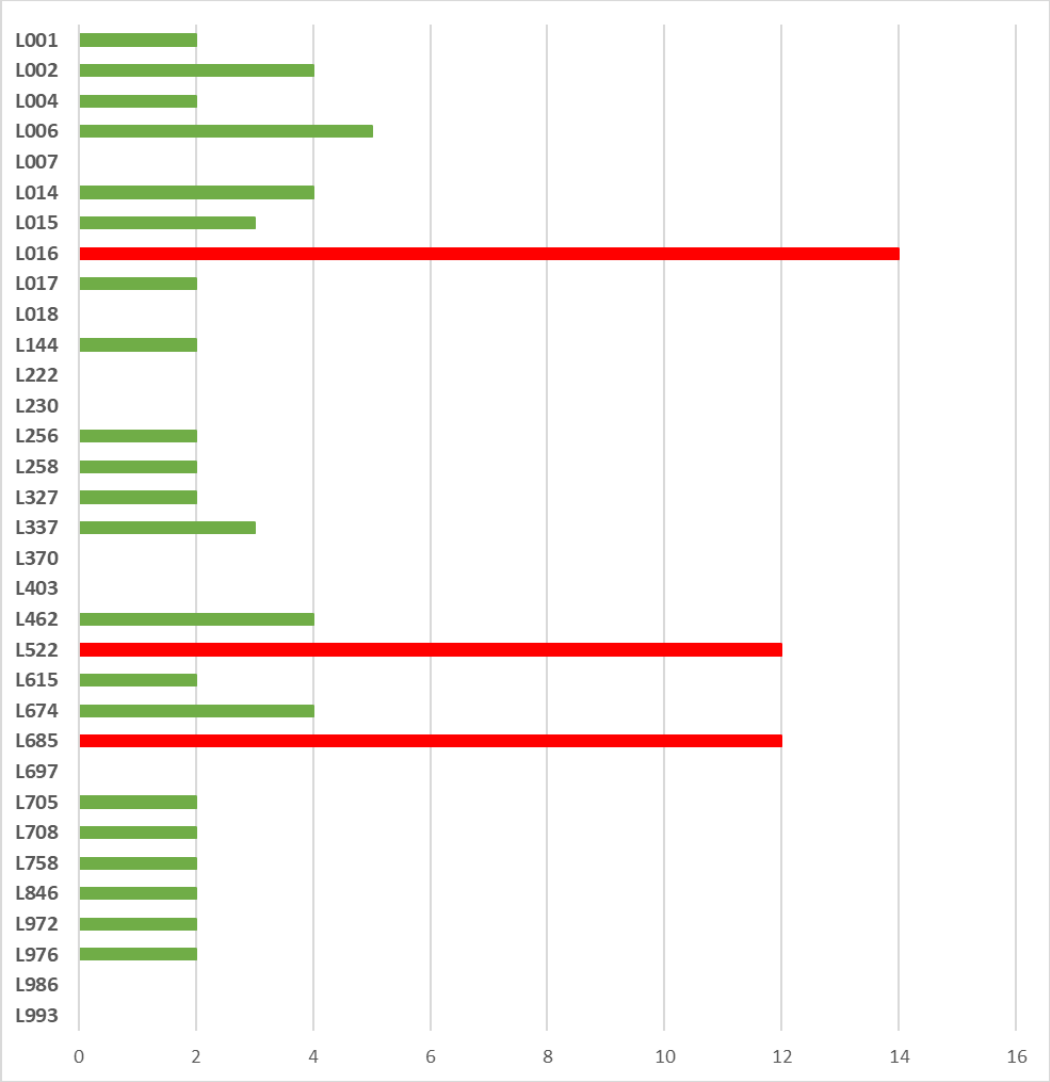
Table 3. Cluster analysis

Labcode	Expected result (strains belonging to a cluster-1;2;3;4;5;6;7;8;): Yes;Yes;No;No;No;No;No;Yes;	Distance	Method
L001	Yes;Yes;No;No;No;No;No;Yes	0	cgMLST
L002	Yes;Yes;No;No;No;No;No;Yes	0	SNP
L004	Yes;Yes;No;No;No;No;No;Yes	45085	cgMLST
L014	Yes;Yes;No;No;No;No;No;Yes	0 allelic differences	cgMLST
L018	Yes;Yes;No;No;No;No;No;Yes	0 allelic differences	cgMLST
L144	Yes;Yes;No;No;No;No;No;Yes	1-3 allelic differences	cgMLST
L222	Yes;Yes;No;No;No;No;No;Yes	0	cgMLST
L230	Yes;Yes;No;No;No;No;No;Yes	0 allelic differences, when profile size was 1671 loci	cgMLST
L256	Yes;Yes;No;No;No;No;No;Yes	0-1 SNPs	SNP
L258	Yes;Yes;No;No;No;No;No;Yes	cgMLST = 0 allelic differences; SNP = 0-8 SNPs	cgMLST; SNP
L327	Yes;Yes;No;No;No;No;No;Yes	0-2 allelic differences	cgMLST
L370	Yes;Yes;No;No;No;No;No;Yes	0 allelic differences between the 3 strains	cgMLST
L403	Yes;Yes;No;No;No;No;No;Yes	0-1 allelic differences	cgMLST
L522	Yes;Yes;No;No;No;No;No;Yes	1-2 allelic differences	cgMLST
L615	Yes;Yes;No;No;No;No;No;Yes	0 allelic differences; 0-5 SNPs	cgMLST; SNP
L674	Yes;Yes;No;No;No;No;No;Yes	0-1 SNPs; 0-1 allelic differences	cgMLST; SNP
L697	Yes;Yes;No;No;No;No;No;Yes	0 AD	cgMLST
L705	Yes;Yes;No;No;No;No;No;Yes	0 allelic differences	cgMLST
L708	Yes;Yes;No;No;No;No;No;Yes	0 AD	cgMLST
L758	Yes;Yes;No;No;No;No;No;Yes	0 allelic differences	cgMLST
L846	Yes;Yes;No;No;No;No;No;Yes	0 SNPs	SNP
L972	Yes;Yes;No;No;No;No;No;Yes	0-19	SNP
L986	Yes;Yes;No;No;No;No;No;Yes	0 allelic differences	cgMLST
L993	Yes;Yes;No;No;No;No;No;Yes	2-3 allelic differences	cgMLST

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 PT35.
2. WGS was carried out from 79% of the laboratories participating in the study, most of which exhibited an excellent performance confirming the efficacy of this approach.
3. Several laboratories did not report the presence of the additional virulence genes characteristic of other DEC pathotypes, which was a mandatory request in this PT and resulted in the assignment of penalty points.
4. Three laboratories underperformed, and most incorrect results were reported for *stx* genes subtyping, even if two of them were applying WGS.
5. Two test strains belonged to serogroups other than the 14 serogroups whose determination was mandatory in this PT, and all the laboratories carrying out WGS could correctly identify them.
6. All the laboratories participating in the cluster analysis exercise performed well, regardless the method used (cgMLST or SNP analysis).
7. The use of WGS should be encouraged throughout the network, as it demonstrated good performances to type STEC.