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

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1. OBJECTIVES OF THE STUDY

The objectives of PT35 were:

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 for the participating laboratories from non-EU Countries.

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

3. Determination of a range of relevant STEC serogroups. 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).

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

Three Laboratories from non-EU Countries participated in the study. Each participant received its own individual laboratory numerical code, reported in the tables of results.

The Laboratories participating in the study were:

1. Argentina, SENASA / INEI-ANLIS, Buenos Aires
2. Egypt, Central laboratory of residues analysis of pesticides and heavy metals in food, Giza
3. United Kingdom, UK Health Security Agency, 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 participating Laboratories.

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

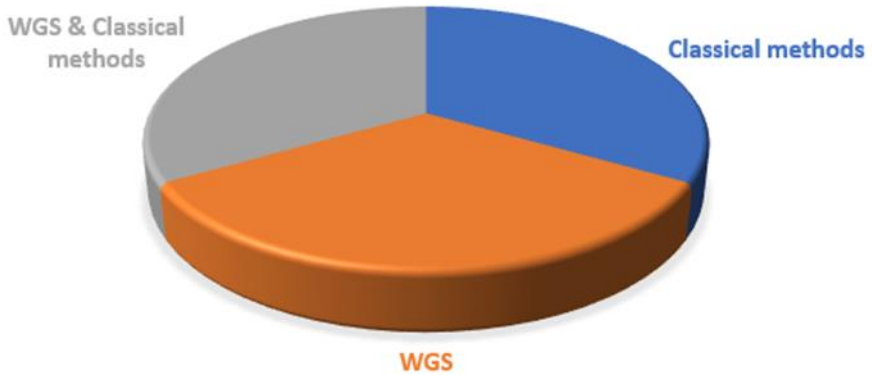
- **4 penalty points** to each incorrect or missing result concerning the identification of the *stx* genes.
- **2 penalty points** to each incorrect or missing result concerning the identification of the *eae* and the additional DEC virulence genes. No penalty points have been assigned to the missing detection of *aaiC* in strain 6, since the allelic variant of the *aaiC* gene present in this isolate 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 strain (test strains 5 and 7) was not included in the 14 indicated in the EURL-VTEC methods 03 and 11 (available at the EURL-VTEC website, Laboratory Methods (https://www.iss.it/web/iss-en/vtec-laboratory-methods).
- **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 participants. A threshold of eight points was set and the laboratories presenting a higher score were considered as under-performant.

5. RESULTS

Figure 1 shows the number of participating laboratories applying each method 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/missing 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		
L005	*eae; stx2*	O26:H11	*stx2a*	0	*
L019	*eae; stx2*	O26:H11	*stx2a*	0	*
L563	*eae; stx2*	O26	-	1	

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		
L005	*eae; stx2*	O26:H11	*stx2a*	0	*
L019	*eae; stx2*	O26:H11	*stx2a*	0	*
L563	*eae; stx2*	O26	-	1	

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**		
L005	*aaiC; aggR*	O111:H21	-	0	*
L019	*aaiC; aggR*	O111:H21	-	0	*
L563	-	O111	-	4	

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**		
L005	*eae; stx2*	O26:H11	*stx2d*	0	*
L019	*eae; stx2*	O26:H11	*stx2d*	0	*
L563	*eae; stx2*	O26	-	1	

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**		
L005	*st; stx2*	O187:H28	*stx2g*	0	*
L019	*lt; stx2*	O187:H28	*stx2g*	4	*
L563	*stx2*	Non typeable	-	3	

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**		
L005	*aaiC; stx1*	O104:H7	*stx1c*	0	*
L019	*stx1*	O104:H7	*stx1c*	0	*
L563	*stx1*	Non typeable	-	1	

* 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**		
L005	*ipaH*	O124:H30	-	0	*
L019	*ipaH*	O124:H30	-	0	*
L563	*ipaH*	Non typeable	-	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**		
L005	*eae; stx2*	O26:H11	*stx2a*	0	*
L019	*eae; stx2*	O26:H11	*stx2a*	0	*
L563	*eae; stx2*	O26	-	1	

5.2 Cluster analysis

The two laboratories 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.

Figure 2. Methods applied by the two Laboratories performing WGS for the cluster analysis

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

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

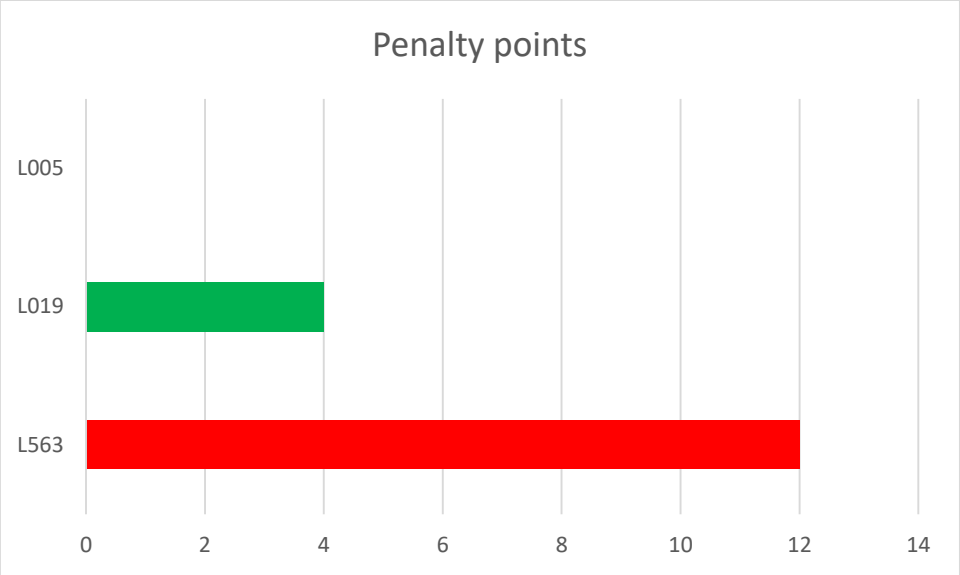
Table 3. Cluster analysis

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
L005	Yes;Yes;No;No;No;No;No;Yes	0-5 (2 allelic differences)	cgMLST
L019	Yes;Yes;No;No;No;No;No;Yes	0-5	SNP

6. Evaluation of the proficiency of the participating Laboratories

The proficiency of the Laboratories has been assessed as described in 4.4. One participating laboratory, L563, obtained a score higher than eight, and was considered as under-performant (red bars in **Figure 3**). This laboratory obtained penalty points particularly for the lack of *stx* genes subtyping, which consisted in a mandatory request in this PT.

Figure 3. Number of penalty points per participant



7. CONCLUDING REMARKS

1. Three laboratories from non-EU countries participated in PT35.
2. WGS was carried out from 2 laboratories participating in the study, which exhibited an excellent performance confirming the efficacy of this approach.
3. Some incorrect results were reported from the participants for 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. One laboratory underperformed, obtaining penalty points for lack of *stx* genes subtyping and missing results for the detection of ETEC and EAEC virulence genes.
5. Two test strains belonged to serogroups out of the top-14 serogroups causing human diseases, and both laboratories carrying out WGS could correctly identify them.

6. The laboratories carrying out WGS also participated in the cluster analysis exercise and 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.