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Report of the 38th inter-laboratory study (PT38) on the identification and typing of pathogenic *E. coli* - 2023

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1. OBJECTIVES OF THE STUDY

The objectives of PT38 were to assess the proficiency of the NRLs for *E. coli* 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, *sth, stp* 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”.

PT38 was conducted on a set of eight STEC strains and consisted of the following four 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, *sth, stp* 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 because of their epidemiologic or regulatory importance:

- O26, O103, O111, O145 and O157: the top-5 STEC serogroups, causing severe human infections worldwide.
- O45 and O121: epidemiologically relevant and regarded as adulterants in beef in the USA legislation.
- O104: relevant after the 2011 German outbreak.

- O55, O80, O91, O113, O128, O146: selected based on 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 detection of genomic cluster between the isolates 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 NRL received its own individual laboratory numerical code, used to identify the laboratories in the results' tables. The NRLs 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 NRLs.

The characteristics of the strains reported in Table 1a were considered as the gold standard. Table 1b reports the virulence genes detected by the WGS-based virulotyping performed at the EURL-VTEC.

The test strains were prepared on October the 3rd, 2023, 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 October the 9th 2023, a homogeneity test was performed on six randomly selected sets of test strains. The remaining test samples were stored at room temperature until October the 23rd 2023, when the parcels were shipped to the participating laboratories by courier.

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

ID PT38	Serotype	MLST	Virulence genes profile	*stx1* subtype	*stx2* subtype	Cluster
Strain 1	O104:H4	ST678	*aggR aaiC*	-	-	No
Strain 2	O9:H30	ST540	*stp(sta1) stx2*	-	*stx2e*	No
Strain 3	O157:H7	ST11	*eae stx1 stx2*	*stx1a*	*stx2c*	Yes
Strain 4	O157:H7	ST11	*eae stx1 stx2*	*stx1a*	*stx2c*	Yes
Strain 5	O157:H7	ST11	*eae stx1 stx2*	*stx1a*	*stx2a*	No
Strain 6	O26:H11	ST29	*eae*	-	-	No
Strain 7	O45:H2	ST20	*eae stx2*	-	*stx2f*	No
Strain 8	O128:H2	ST811	*stx1 stx2*	*stx1c*	*stx2b*	No

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

ID PT38	Additional virulence genes
Strain 1	*aap, aar, aata, afad, agga, aggb, aggc, aggd, capu, fyua, gad, iha, iucc, iuta, lpfa, mchb, mchc, mchf, neuc, orf3, orf4, pic, sepa, siga, terc, trat*
Strain 2	*gad, terc, trat*
Strain 3	*asta, chua, ehxa, espa, espb, espf, espj, espp, etpd, gad, iha, iss, katp, nlea, nleb, nlec, ompt, tccp, terc, tir, toxb, trat*
Strain 4	*asta, chua, ehxa, espa, espb, espf, espj, espp, etpd, gad, iha, iss, katp, nlea, nleb, nlec, ompt, tccp, terc, tir, toxb, trat*
Strain 5	*asta, chua, ehxa, espa, espb, espf, espj, espp, etpd, gad, iha, iss, katp, nlea, nleb, nlec, ompt, stx1a, stx1b, stx2a, stx2b, tccp, terc, tir, toxb, trat*
Strain 6	*asta, cia, cif, efa1, espa, espb, espf, espj, gad, iss, lpfa, mcma, nlea, nleb, nlec, ompt, papa, papc, terc, tir*
Strain 7	*asta, cba, cif, cma, espa, espb, espf, gad, hra, iss, nlea, nleb, nlec, ompt, tccp, terc, tir, trat*
Strain 8	*celb, cia, cvac, ehxa, espi, gad, iha, irea, iss, k88ab, kpse, kpsmii, lpfa, mchb, mchc, mchf, suba, terc, tia, 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 are 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 laboratories could choose to apply any serological or molecular method in use in their laboratories, including WGS. When needed, 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.

Finally, a voluntary exercise for the phylogenetic analysis of the isolates was carried out as part of PT38. The correlation between the test strains could be assessed by SNPs or 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 included in the invitation letter. The deadline for collecting the results was set at the December the 20th 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.
- **2 penalty points** to each incorrect result concerning the identification of the top-14 serogroups. No penalty points were assigned to the missing identification of O45 serogroup in strain 7, as it was not detected with EURL-VTEC_Method_0011.
- **1 penalty point** when the results of the serogroup identification were not uploaded ("null" field) or reported as "Not Done". No penalty points were assigned to the missing identification of O9 serogroup in strain 2, as it was not included in the 14 serogroups indicated in the EURL-VTEC_Method_003.
- **1 penalty point** to each missing result or incorrect result concerning the identification of the *stx* genes subtypes. No penalty points were assigned to the missing identification of *stx2e* subtype, as it was not possible to type by applying the EURL-VTEC_Method_006. In fact test strain 2 contains an *IS*3-like element of the *IS*2 family located in the intergenic region spanning *stx2A* and *stx2B* subunits coding genes.

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 three Laboratories. **Figure 1** shows the number of participating laboratories aggregated according to the methods used to characterise the isolates.

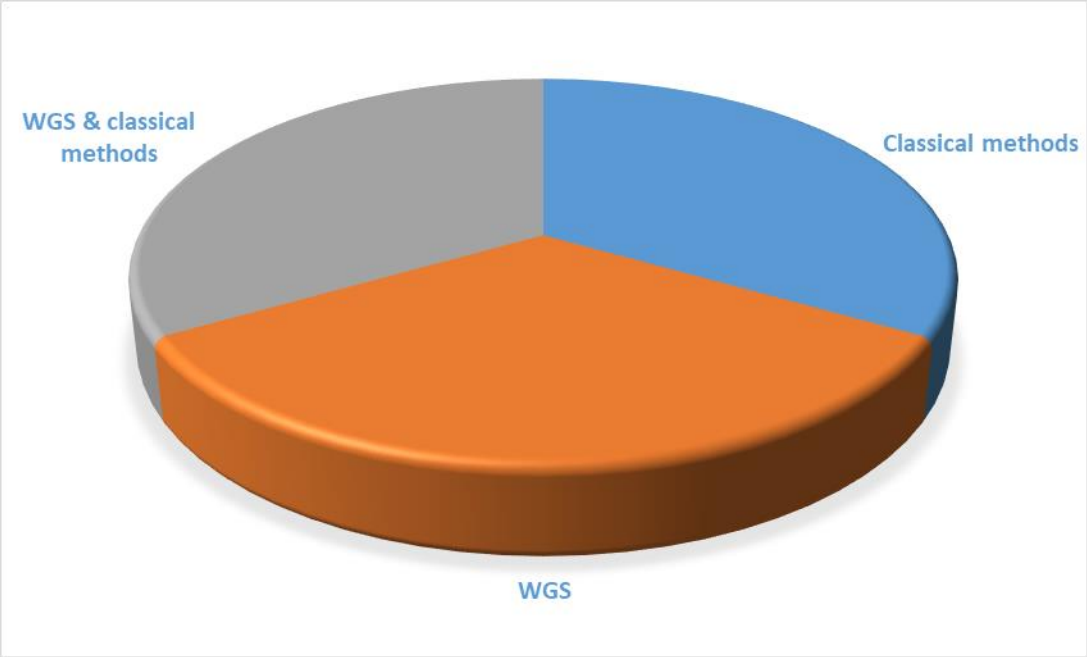


Figure 1. Methods applied by the Laboratories to type STEC test strains

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	*aaiC; aggR*	O104:H4	-		
Labcode	Reported result	Reported result	Reported result		
L005	*aaiC; aggR*	O104:H4	-	0	*
L019	*aaiC; aggR*	O104:H4	-	0	*
L563	*ipaH*	OND	-	4	

Table 2b. Characterisation of test strain 2

Strain 2	Virulence genes	Serogroup/serotype	*stx* genes subtyping	Penalties	WGS
Expected result	***stp(sta1); stx2***	**O9:H30[1]**	***stx2e[2]***		
Labcode	**Reported result**	**Reported result**	**Reported result**		
L005	*stp(sta1); stx2*	O9:H30	*stx2e*	0	*
L019	*stp(sta1); stx2*	O9a:H30	*stx2e*	0	*
L563	*lt; sth (sta2)*	OND	-	4	

[1]: No penalty points were assigned to the missing identification of O9 serogroup, as it was not included in the 14 indicated in the EURL-VTEC_Method_003.

[2] No penalty points were assigned to the missing identification of *stx2e* subtype, as it was not possible to type by applying the EURL-VTEC_Method_006. In fact test strain 2 contains an IS3-like element of the IS2 family located in the intergenic region spanning stx2A and stx2B subunits coding genes.

Table 2c. Characterisation of test strain 3

Strain 3	Virulence genes	Serogroup/serotype	*stx* genes subtyping	Penalties	WGS
Expected result	***eae; stx1; stx2***	**O157:H7**	***stx1a; stx2c***		
Labcode	**Reported result**	**Reported result**	**Reported result**		
L005	*eae; stx1; stx2*	O157:H7	*stx1a; stx2c*	0	*
L019	*eae; stx1; stx2*	O157:H7	*stx1a; stx2c*	0	*
L563	*eae; stx2*	O157	-	5	

Table 2d. Characterisation of test strain 4

Strain 4	Virulence genes	Serogroup/serotype	*stx* genes subtyping	Penalties	WGS
Expected result	***eae; stx1; stx2***	**O157:H7**	***stx1a; stx2c***		
Labcode	**Reported result**	**Reported result**	**Reported result**		
L005	*eae; stx1; stx2*	O157:H7	*stx1a; stx2c*	0	*
L019	*eae; stx1; stx2*	O157:H7	*stx1a; stx2c*	0	*
L563	*eae*	OND	-	10	

Table 2e. Characterisation of test strain 5

Strain 5	Virulence genes	Serogroup/serotype	*stx* genes subtyping	Penalties	WGS
Expected result	***eae; stx1; stx2***	**O157:H7**	***stx1a; stx2a***		
Labcode	**Reported result**	**Reported result**	**Reported result**		
L005	*eae; stx1; stx2*	O157:H7	*stx1a; stx2a*	0	*
L019	*eae; stx1; stx2*	O157:H7	*stx1a; stx2a*	0	*

L563	*eae*	O157	-	8	

Table 2f. Characterisation of test strain 6

Strain 6	Virulence genes	Serogroup/serotype	*stx* genes subtyping	Penalties	WGS
Expected result	***eae***	**O26:H11**	**-**		
Labcode	**Reported result**	**Reported result**	**Reported result**		
L005	*eae*	O26:H11	-	0	*
L019	*eae*	O26:H11	-	0	*
L563	*eae*	O26	-	0	

Table 2g. Characterisation of test strain 7

Strain 7	Virulence genes	Serogroup/serotype	*stx* genes subtyping	Penalties	WGS
Expected result	***eae; stx2***	**O45:H2**[1]	***stx2f***		
Labcode	**Reported result**	**Reported result**	**Reported result**		
L005	*eae; stx2*	O45:H2	*stx2f*	0	*
L019	*eae; stx2*	O45:H2	*stx2f*	0	*
L563	*eae*	OND	-	5	

[1] No penalty points were assigned to the missing identification of O45 serogroup, as it was not detected with EURL-VTEC_Method_0011.

Table 2h. Characterisation of test strain 8

Strain 8	Virulence genes	Serogroup/serotype	*stx* genes subtyping	Penalties	WGS
Expected result	***stx1; stx2***	**O128:H2**	***stx1c; stx2b***		
Labcode	**Reported result**	**Reported result**	**Reported result**		
L005	*stx1; stx2*	O128ac:H2	*stx1c; stx2b*	0	*
L019	*stx1; stx2*	O128ac:H2	*stx1c; stx2b*	0	*
L563	*stx2*	OND	-	7	

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 PT38. Figure 2 shows the methods used in the cluster analysis exercise, with the proportion of laboratories applying each method.

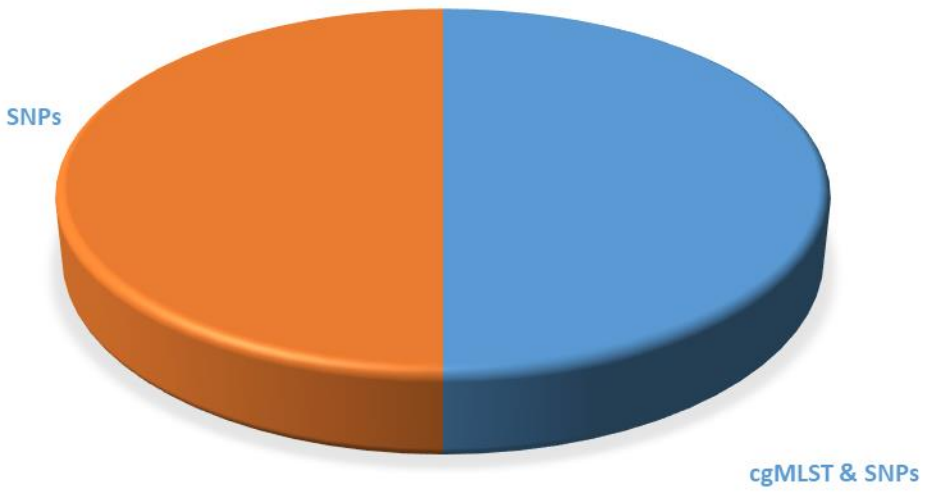


Figure 2. Methods applied by the two NRLs performing WGS for the cluster analysis

The results of the cluster analysis exercise are reported in Table 3. Both the laboratories correctly identified the cluster composed by test strains 3 and 4.

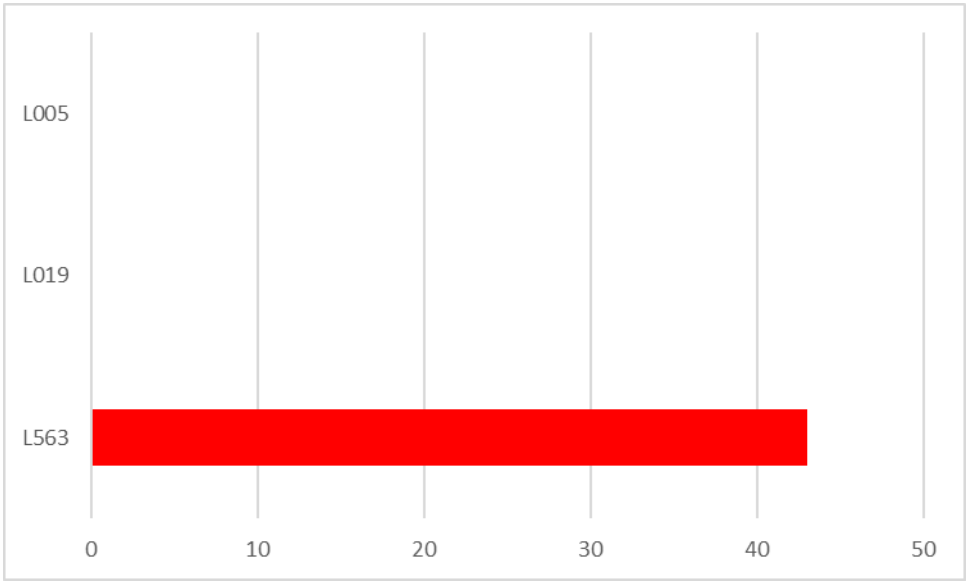
Table 3. Cluster analysis

Labcode	Expected result (strains belonging to a cluster-1;2;3;4;5;6;7;8;): No; No; Yes;Yes;No;No;No;No;	Distance	Method
L005	No; No; Yes; Yes; No; No; No; No	4 allelic differences or 0 SNPs	cgMLST & SNPs
L019	No; No; Yes; Yes; No; No; No; No	0-5 SNPs	SNPs

6. Evaluation of the proficiency of the participating Laboratories

The proficiency of the Laboratories has been assessed as described in 4.4. Laboratory with labcode L563 was considered as under-performant as it presented a score higher than eight (red bar in **Figure 3**).

Figure 3. Number of penalty points per NRL



7. CONCLUDING REMARKS

1. Three laboratories from non-EU countries participated in PT38.
2. WGS was carried out from two laboratories participating in the study, which exhibited an excellent performance throughout the whole study confirming the efficacy of this approach.
3. One laboratory underperformed, obtaining penalty points for errors in all the sections of this study.
4. The two laboratories carrying out WGS also participated in the cluster analysis exercise, correctly identifying the cluster.