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Report of the 45th inter-laboratory study (PT45) on the identification and typing of pathogenic *E. coli* - 2025

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1. OBJECTIVES OF THE STUDY

The objectives of PT45 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_rev2).
3. The detection of virulence genes of diarrheagenic *E. coli* other than STEC (*ipaH* for EIEC, *sth, stp* and *lt* for ETEC, *aggR* and *aaiC* for EAEC, methods available at the EURL for *E. coli* website - https://www.iss.it/en/vtec-laboratory-methods).
4. Subtyping of Shiga Toxins (Stx)-coding genes.
5. The identification of clusters of isolates based on genomic analysis. In this PT, the exercise on cluster analysis was mandatory for the laboratories that used WGS for strain characterisation.

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”.

PT45 was conducted on a set of six *E. coli* 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 (conventional or Real Time PCR) or WGS, using any method applied in the routine testing by the participant laboratories. Methods for all the assays were also available in the EURL-VTEC website. Participants were requested to detect the following targets: *stx1*, *stx2* and the intimin-coding *eae* gene.

2. The identification of virulence genes associated with other Diarrhoeagenic *E. coli* (DEC) pathotypes by PCR amplification (conventional or Real Time PCR) or WGS: *ipaH* for EIEC, *sth, stp* and *lt* for ETEC, *aggR* and *aaiC* for EAEC

3. The determination of a range of relevant STEC serogroups. Participating laboratories could choose to apply any serological or molecular method in use in their laboratories, including WGS. Procedures based on conventional or Real Time PCR for detecting the genes associated with

the serogroups that were in the scope of the PT were made anyway available in the EURL website. Participants were requested to identify the O-group 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 by using the standard PCR amplification based on the method described by Scheutz *et al.* (*J. Clin. Microbiol. 2012; 50: 2951-63*) (available in the EURL-VTEC website), and/or WGS. The s*tx* genes subtyping exercise was restricted to the identification of the three subtypes of the s*tx1* gene (s*tx1a, stx1c* and *stx1d*) and seven of s*tx2* gene subtypes (from s*tx2a* to s*tx2g*).

Participants using WGS were requested to perform a cluster analysis on the PT45 strains. A specific procedure for this analysis was not indicated, and each laboratory could use softwares and/or webservices of their choice, for carrying out either SNPs or cgMLST analysis. The results to be reported included information on the strains belonging to a cluster and the range of number of SNPs or allelic differences between the genomes forming a cluster. The laboratories also had the possibility to additionally provide a phylogenetic tree.

3. PARTICIPANTS

Thirty-four NRLs from 23 EU MS and three EFTA countries accepted the invitation to participate in the study. Each NRL was identified with a different numerical code, used to identify the laboratories in the results' tables.

The NRLs participating in the study were the following:

1. Agency for Health and Food Safety (AGES), IMED Graz, Austria
2. Agroscope, Switzerland
3. Centro Nacional de Alimentación-AESAN, Majadahonda, (Madrid), Spain
4. Croatian Veterinary Institute
5. Danish Veterinary and Food Administration
6. Federal Institute for Risk Assessment, Berlin, Germany
7. Finnish Food Authority, Helsinki, Finland
8. Food Microbiology Division, Department of Agriculture, Food and the Marine, Kildare, Ireland
9. Institute For Hygiene and Veterinary Public Health, Romania
10. Institute of Food Safety, Animal Health and Environment BIOR, Riga, Latvia
11. Instituto Nacional de Investigação Agrária e Veterinária, I. P., Portugal
12. Instituto Nacional de Investigação Agrária e Veterinária, Vairão, Portugal
13. Istituto Superiore di Sanità, Rome, Italy
14. Laboratoire National de Santé, Dudelange, Luxemburg
15. Laboratorio Central de Veterinaria, Altete (Madrid), Spain
16. Matís ohf, Reykjavík, Iceland
17. Ministry of Rural Development and Food, Greece
18. National Centre for Laboratory Research and Risk Assessment (LABRIS), Tartu, Estonia
19. National Diagnostic and Research Veterinary Medical Institute, Sofia, Bulgaria
20. National Food Chain Safety Office, Food Chain Safety, Budapest, Hungary
21. National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands

22. National Institute of Public Health, Warsaw, Poland
23. National Plant Health Laboratory, Spain
24. National Veterinary Institute, Uppsala, Sweden
25. National Veterinary Research Institute (NVRI), Department of Food Safety, Pulawy, Poland
26. Norwegian Veterinary Institute
27. Public Health Authority of the Slovak Republic, Bratislava
28. Sciensano, Brussels, Belgium
29. State Veterinary and Food Institute, Dolny Kubin, Slovakia
30. Swedish Food Agency, Uppsala, Sweden
31. University of Ljubljana, Veterinary faculty, National Veterinary Institute, Slovenia
32. University of Zurich, Switzerland
33. VetAgro-Sup, Marcy L'Etoile, France
34. Wageningen Food Safety Research, The Netherlands

4. MATERIALS AND METHODS

4.1. Sample preparation

Six *E. coli* test strains (reported as strains A to F in Table 1a) were sent to the NRLs. The isolates were selected among those present in the EURL-VTEC reference collections and the presence of all the genetic and/or phenotypic features included in the study design was confirmed before shipment. Table 1a shows the strains' characteristics used as the gold standard for the assessment of the participating laboratories' performance. Table 1b reports additional virulence genes detected by the WGS-based virulotyping performed at the EURL-VTEC.

The test strains were prepared on the 25th of September 2025, as fresh bacterial cultures inoculated into 0.3 % nutrient agar in borosilicate vials. The cultures were incubated for 18 hours at 37°C ± 1°C and labelled with randomly generated alphabetical codes, 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 September 29th and 30th, 2025, 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 13th 2025, when the parcels were shipped to the participating laboratories by courier.
The characteristics of the provided genomes are reported in Tables 2a and 2b.

Table 1a. Characteristics of the *E. coli* strains included in the study

ID PT45	Serotype	MLST	Virulence genes profile	*stx1* subtype	*stx2* subtype	Cluster
Strain 1	O174:H8	13	*stx1;stx2*	*stx1c*	*stx2b*	Yes
Strain 2	O174:H8	13	*stx1;stx2*	*stx1c*	*stx2b*	Yes
Strain 3	O174:H21	677	*stx2*	-	*stx2c*	No
Strain 4	O174:H8	13	*stx2*	-	*stx2b*	No
Strain 5	O103:H2	17	*stx1;stx2;eae*	*stx1a*	*stx2a*	No
Strain 6	O113:H4	10	*stx2*	-	*stx2d*	No

Table 1b. Additional virulence genes detected in the test strains by WGS-based virulotyping

ID PT45	Additional virulence genes
Strain 1	*asta, cba, celb, cia, cma, ehxa, espi, gad, iha, irea, iss, iucc, iuta, kpse, kpsmii, lpfa, ompt, senb, suba, terc, tia, trat*
Strain 2	*asta, celb, cia, cma, ehxa, espi, gad, iha, irea, iss, iucc, iuta, kpse, lpfa, senb , suba, terc, tia, trat*
Strain 3	*cea, ehxa, espi, espp, gad, iha, iss, lpfa, papc, saa, suba, terc, tia, trat*
Strain 4	*cba, cea, cia, cma, ehxa, espi, gad, iha, irea, iss, lpfa, ompt, senb, suba, terc, tia, trat*
Strain 5	*cia, cib, cif, efa1, ehxa, espa, espb, espf, espj, etpd, gad, iha, iss, iucc, nlea, nleb, nlec, ompt, tccp, terc, tir, trat*
Strain 6	*asta, espi, gad, iha, terc, tia*

4.2. Collection and elaboration of the results

The results were submitted through a dedicated website developed by the EURL for *E. coli* with the submission deadline set at November 24th, 2025.

4.4. Evaluation of the NRLs performance in the identification of the *E. coli* virulence genes and the serogroups

The performance of each NRL in the identification of the characteristics of the *E. coli* test strains was evaluated by assigning penalty 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.
- **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 O174 serogroup for strain 1-4, as it is not included in the 14 serogroups indicated in the EURL-VTEC_Method_003_rev2.
- **2 penalty point** to each missing result or incorrect result concerning the identification of the *stx* genes subtypes, calculated separately for *stx1* and *stx2* 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.

As far as the cluster analysis is concerned, two penalty points were assigned to any misassignment of an isolate to the cluster. The laboratories receiving two or more penalties were considered under-performant.

5. RESULTS

Results were submitted by 34 Laboratories. **Figure 1** shows the number of participating laboratories aggregated according to the methods used to characterise the isolates.

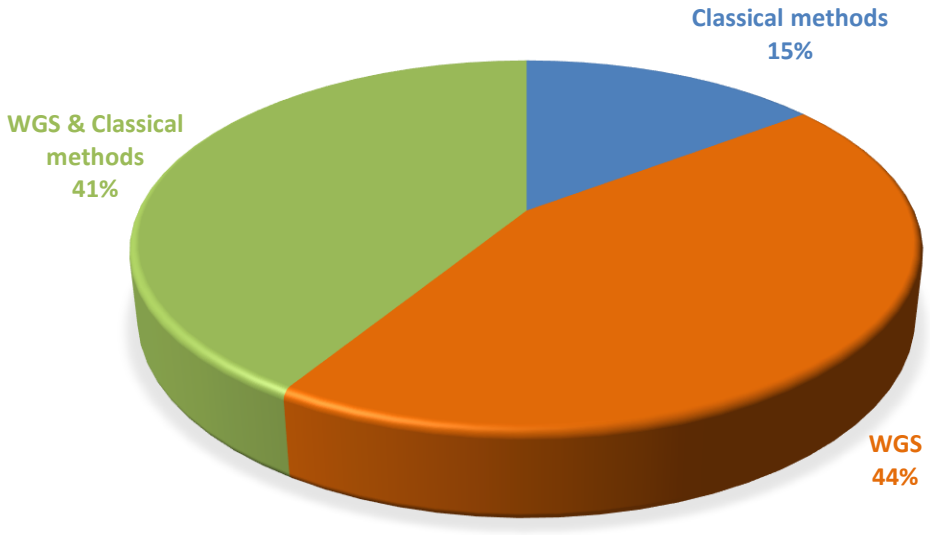

Figure 1. Methods applied by the Laboratories to type *E. coli* test strains

5.1. Characterisation of the test strains

The aggregated 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 3a. Characterisation of test strain 1

Strain 1	Virulence genes	Serogroup/serotype	*stx* genes subtyping	Penalties assigned to each lab	WGS	Classical	WGS & Classical
Expected result	*stx1;stx2*	O174:H8	*stx1c;stx2b*				
Number of Labs	Reported result	Reported result	Reported result				
1	*stx1;stx2*	ND	-	4	0	1	0
1	*stx1;stx2*	NR:H8	*stx1c;stx2b*	0[1]	0	4	0
4	*stx1;stx2*	ND	*stx1c;stx2b*	0[1]	0	0	1
28	*stx1;stx2*	O174:H8	*stx1c;stx2b*	0	15	0	13

[1] No penalty points were assigned to the mis-identification of O174 serogroup, as it was not included in the 14 serogroups indicated in the EURL-VTEC_Method_003_rev2.

Table 3b. Characterisation of test strain 2

Strain 2	Virulence genes	Serogroup/serotype	*stx* genes subtyping	Penalties assigned to each lab	WGS	Classical	WGS & Classical
Expected result	***stx1;stx2***	**O174:H8**	***stx1c;stx2b***				
Number of Labs	Reported result	Reported result	Reported result				
4	*stx1;stx2*	ND	*stx1c;stx2b*	0[1]	0	4	0
1	*stx1;stx2*	ND:H8	*stx1c;stx2b*	0[1]	1	0	0
1	*stx1;stx2*	NR:H8	*stx1c;stx2b*	0[1]	0	0	1
27	*stx1;stx2*	O174:H8	*stx1c;stx2b*	0	14	0	13
1	*stx2*	ND	-	8[1]	0	1	0

[1] No penalty points were assigned to the missing identification of O174 serogroup, as it was not included in the 14 serogroups indicated in the EURL-VTEC_Method_003_rev2.

Table 3c. Characterisation of test strain 3

Strain 3	Virulence genes	Serogroup/serotype	*stx* genes subtyping	Penalties assigned to each lab	WGS	Classical	WGS & Classical
Expected result	***stx2***	**O174:H21**	***stx2c***				
Number of Labs	Reported result	Reported result	Reported result				
1	-	O174:H21	-	6[1]	1	0	0
1	*stx2*	ND	-	2	0	1	0
2	*stx2*	ND	*stx2a;stx2c*	2[1]	0	2	0
1	*stx2*	ND	*stx2c*	0[1]	0	1	0
1	*stx2*	ND	*stx2c;stx2d*	2[1]	0	1	0
28	*stx2*	O174:H21	*stx2c*	0	14	0	14

[1] No penalty points were assigned to the missing identification of O174 serogroup, as it was not included in the 14 serogroups indicated in the EURL-VTEC_Method_003_rev2.

Table 3d. Characterisation of test strain 4

Strain 4	Virulence genes	Serogroup/serotype	*stx* genes subtyping	Penalties assigned to each lab	WGS	Classical	WGS & Classical
Expected result	***stx2***	**O174:H8**	***stx2b***				
Number of Labs	Reported result	Reported result	Reported result				
1	*stx2*	ND	-	2[1]	0	1	0
4	*stx2*	ND	*stx2b*	0[1]	0	4	0
1	*stx2*	ND:H8	*stx2b*	0[1]	1	0	0
28	*stx2*	O174:H8	*stx2b*	0	14	0	14

[1] No penalty points were assigned to the missing identification of O174 serogroup, as it was not included in the 14 serogroups indicated in the EURL-VTEC_Method_003_rev2.

Table 3e. Characterisation of test strain 5

Strain 5	Virulence genes	Serogroup/serotype	*stx* genes subtyping	Penalties assigned to each lab	WGS	Classical	WGS & Classical
Expected result	***stx1;stx2;eae***	**O103:H2**	***stx1a;stx2a***				
Number of Labs	Reported result	Reported result	Reported result				
1	*stx1;stx2*	O103:H2	*stx1a;stx2a*	2	0	0	1
1	*stx1;stx2;eae*	ND	-	5	0	1	0
4	*stx1;stx2;eae*	O103	*stx1a;stx2a*	0	0	3	1
1	*stx1;stx2;eae*	O103:-	*stx1a;stx2a*	0	0	1	0
27	*stx1;stx2;eae*	O103:H2	*stx1a;stx2a*	0	15	0	12

Table 3f. Characterisation of test strain 6

Strain 6	Virulence genes	Serogroup/serotype	*stx* genes subtyping	Penalties assigned to each lab	WGS	Classical	WGS & Classical
Expected result	***stx2***	**O113:H4**	***stx2d***				
Number of Labs	Reported result	Reported result	Reported result				
1	*stx2*	ND	-	3	0	1	0
1	*stx2*	ND:H4	*stx2d*	1	1	0	0
1	*stx2*	O113	*stx2c*	2	0	1	0
1	*stx2*	O113	*stx2c;stx2d*	2	0	1	0
1	*stx2*	O113	*stx2d*	0	0	1	0
1	*stx2*	O113:-	*stx2c;stx2d*	2	0	1	0
1	*stx2*	O113:H14	*stx2d*	0	1	0	0
27	*stx2*	O113:H4	*stx2d*	0	13	0	14

5.3 Cluster analysis

Twenty-seven NRLs out of the twenty-nine total laboratories applying WGS reported cluster analysis results. Figure 2 shows the methods used in this exercise, with the proportion of laboratories applying each method.

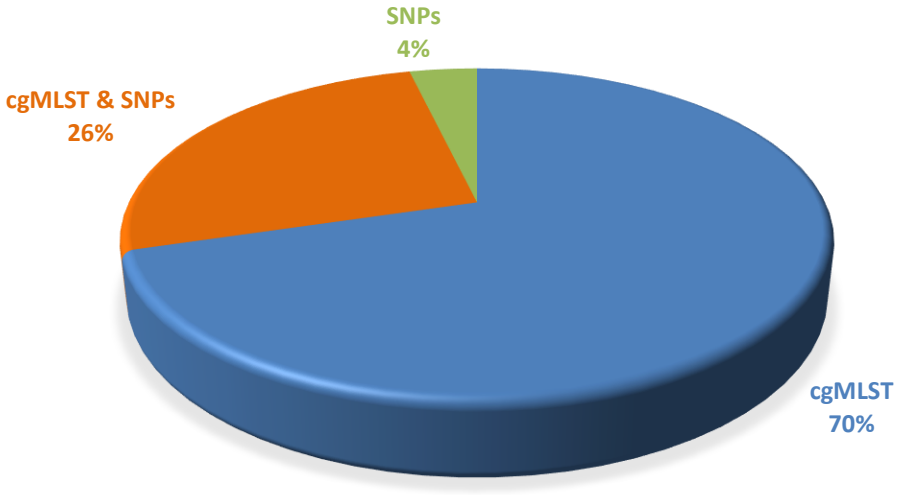

Figure 2. Methods applied by the 27 NRLs performing cluster analysis

The results of the cluster analysis exercise are reported in Table 5. All the participants could correctly identify the cluster composed by test strain 1 and 2.

Table 5. Cluster analysis results

	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5	Strain 6		
Expected result	**Yes**	**Yes**	**No**	**No**	**No**	**No**		
Number of Labs	**Reported results**						**Distance**	**Method**
16	Yes	Yes	No	No	No	No	0-5 AD	cgMLST
2	Yes	Yes	No	No	No	No	0-10 AD	cgMLST
1	Yes	Yes	No	No	No	No	15 AD	cgMLST
4	Yes	Yes	No	No	No	No	0-1 AD; 0-2 SNPs	cgMLST & SNPs
1	Yes	Yes	No	No	No	No	0-1 AD; 0-4 SNPs	cgMLST & SNPs
2	Yes	Yes	No	No	No	No	0-1 AD; 0-13 SNPs	cgMLST & SNPs
1	Yes	Yes	No	No	No	No	0-2 SNPs	SNPs

AD: Allelic differences, SNPs: SNP differences.

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 rows in **Figure 3**).

None of the laboratories participating in the cluster analysis received penalty points.

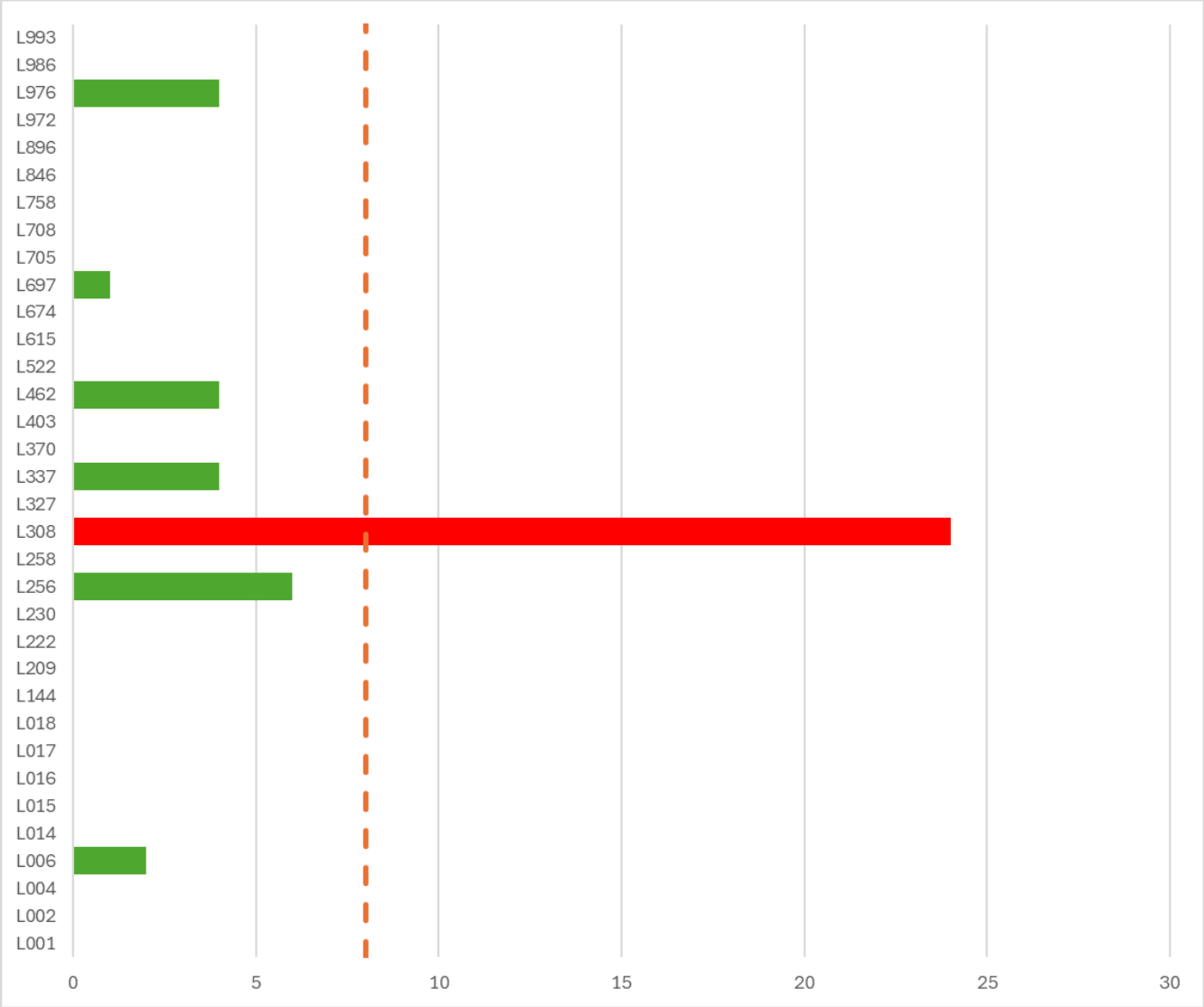

Figure 3. Number of penalty points per NRL

7. CONCLUDING REMARKS

1. A high level of participation was recorded with 34 NRLs from 23 EU MS and three EFTA countries that accepted the invitation.
2. WGS was carried out by 85% (N=29) of the laboratories participating in the study, most of which exhibited an excellent performance. These data confirm the high level of adoption of NGS and the high proficiency of the NRLs for *E. coli* network.
3. The proficiency of all the participating laboratories was satisfactory, with the only exception of L308, that accumulated most of the penalty points due to lack of reporting of *stx* subtyping results. This laboratory has already been contacted for a follow up and has been invited to participate in a training course at the EURL for E. coli.
4. A few laboratories accumulated the majority of penalty points for missing or incorrect results on *stx* subtyping, mainly by applying conventional methods. It should be noted that a procedure for *stx* subtyping through conventional PCR is available on EURL website (EURL-VTEC_Method_006_Rev 2) and that the EURL organizes training courses every year where the application of such procedure is illustrated, which the NRLs can apply to participate in.
5. Two laboratories (L256 and L896) did not perform the cluster analysis, despite applying WGS. They will be contacted for a follow up.
6. All the laboratories that performed the cluster analysis could correctly identify the expected cluster, proving an excellent level of preparedness of the network on this activity.