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Report of the fourth inter-laboratory study on the enumeration of *E. coli* (PT36) – 2023 (Ver. 4)

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1. INTRODUCTION AND OBJECTIVES OF THE STUDY

As an effect of Regulation (EU) 222/2018, the European Union Reference Laboratory for *Escherichia coli* (EURL-VTEC) has taken over the activities carried out by the former EU reference laboratory for monitoring the bacteriological contamination of bivalve molluscs as regards the analytical tests for *E. coli*. In this specific case, the EURL has the task (i) to assess the performance of the designated National Reference Laboratories (NRLs) for the microbiological contamination of bivalve molluscs in the EU and (ii) to assist the EFTA Member States, EU Candidate Countries and third countries in using the method for the enumeration of *E. coli* in live bivalve molluscan shellfish.

The EU reference method for enumeration of *E. coli* in live bivalve molluscs is represented by ISO 16649-3 ***"Microbiology of the food chain - Horizontal method for the enumeration of β-glucuronidase-positive* Escherichia coli *Part 3: Detection and most probable number technique using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide"***; in particular, this scheme is intended to provide proficiency testing (PT) samples for laboratories performing the analysis of live bivalve molluscs, from production areas in accordance with Regulation (EC) N° 854/2004 and from throughout the production chain in accordance with Regulation (EC) N° 2073/2005.

In contrast to the previous proficiency rounds, PT36 employed a freeze-dried culture of the ATCC strain (25922).

This document represents the evaluation report of the PT36 study. The study was conducted according to the International Standard ISO/IEC 17043:2010 "Conformity assessment – General requirements for proficiency testing".

2. PARTICIPANTS

Thirty NRLs, representing 19 EU Member States plus two, accepted the invitation to participate.

Each NRL received its own individual laboratory numerical code, which was used to label the laboratories in the result tables.

The Laboratories participating in the study were:

Austria	Austrian Agency for Health and Food Safety, Institute for Food Safety (AGES)
Belgium	Laboratory of Foodborne Pathogens (SCIENSANO)
Bulgaria	National Diagnostic and Research Veterinary Institute (NDRVMI)
Bulgaria	Regional Food Safety Directorate – VARNA Testing laboratory for diagnostic and control

Bulgaria	Regional Food Safety Directorate – SLIVEN Testing laboratory “Laboratory activities” department
Croatia	Croatian Veterinary Institute Laboratory for food and feed microbiology (RIJEKA)
Croatia	Croatian Veterinary Institute HVI - VETERINARSKI ZAVOD SPLIT
Croatia	Laboratory for Food Microbiology, Croatian Veterinary Institute
Cyprus	Laboratory for the Control of Food of Animal Origin (LCFAO)
Denmark	Microbiological laboratory Ringsted (FVST)
Germany	NRL *E. coli*, Bundesinstitut für Risikobewertung (BfR)
Greece	Department of Food Hygiene of Athens (NRL Greece for *E.coli* in LBM)
Greece	Veterinary Laboratory of Kavala
Greece	NRL-*Salmonella*, The Veterinary Laboratory of Chalkida, Hellenic Ministry of Rural Development and Food
Ireland	Shellfish Microbiology Unit, Marine Institute (MARINE)
Italy	Istituto Superiore di Sanità - ISS
Italy	IZS Umbria e Marche, Sezione di Ancona
Italy	IZS del Mezzogiorno Sezione di Portici
Latvia	Institute of Food safety, Animal health and environment - BIOR
Netherlands	RIVM
Netherlands	Wageningen Food Safety Research - WUR
Norway	Institute of Marine Research - HMR
Poland	National Veterinary Research Institute (NVRI)
Romania	Institute for Diagnoses and Animal Health (IDAH)
Romania	Institute for Hygiene and Veterinary Public Health - IISPV
Slovakia	State veterinary and food institute - SVPU
Slovenia	University of Ljubljana, Veterinary Faculty (Unit for food safety)
Spain	Centro Nacional de Alimentación - AESAN
Sweden	Swedish Food Agency, The Biology department
UK	CEFAS

We report the analysis of the twenty-five laboratories that submitted results. Five didn’t return the results (L129, L130, L140, L142 and L150).

MATERIALS AND METHODS

3.1. Sample preparation

The process used to generate the freeze-dried strain was as follows: a live culture of the ATCC strain (25922), refreshed at 10^8 cells/ml, was pelleted and re-suspended in 5% sucrose with a bacterial load of 10^{10}/ml. A volume of 800 µl of the resuspended culture was distributed into each vial and frozen at -20 °C for a minimum of two days. The vials were then placed into the "Alpha 1-2 LSC Basic" lyophilizer apparatus and freeze-dried for a minimum of three days under the following conditions: (i) -60°C; (ii) 0.01 mbar. The operation was completed with the resultant vials being tightly sealed and closed. The resulting samples were evaluated using the Part 3 of the ISO 16649 method. An acceptable MPN value (5.6 x 10^5) was achieved by diluting the freeze-dried samples 1:2000 before starting the ISO 16649-3 analytical procedure.

On 20th of November 2023, the samples were shipped to the participating laboratories by courier. Participants were requested to analyze the samples, according to the following procedure:

(i) reconstitute the lyophilized culture with 1 ml of TSB (Tryptone Soya broth); (ii) equilibrate the culture for 5 min then prepare a 1:20 dilution putting the 1 ml of the reconstituted culture in a falcon tube containing 19 ml of TSB, from this dilution perform other two serial dilutions 1:10 to the final dilution rate 1:2000; (iii) put 10 ml of the 1:2000 diluted culture in 200 ml of MRD medium and proceed according to the Part 3 of the ISO 16649 method.

3.2. Collection and Elaboration of the NRLs Results

The results were submitted using the on-line service of the EURL for *E. coli*. The participants were requested to fill in both (i) the Evaluation form (notes field in order to specify any problem with the samples delivery/packaging) and (ii) the Sample Results section.

3.3. Analysis of the NRLs' results

3.3.1 Parameters used for the assignment of the scores

A scoring system is used to assess the participant's performance. *E. coli* MPN scores allocated to participants are detailed in the Table 1.

Table 1: *E. coli* MPN scores; dark green boxes represent the maximum value' score (12), the dark red ones the minimum (0) (graduation colour bar).

Results	Returning of results	Score allocated		Score
		Replicate 1	Replicate 2	
Both replicates MPN results are within the expected range*	2	5	5	12
One replicate MPN result reported is outside the expected range and falls between the median ± 3 SD and the median ± 5 SD value	2	5	2	9
Both replicates MPN results reported are outside the expected range and fall between the median ±3SD and the median ± 5 SD value	2	2	2	6
One replicate MPN result reported is outside the median ± 5 SD value	2	5	0	7
Both replicates MPN results reported are outside the median ± 5 SD value	2	0	0	2
Single MPN result reported only	2	5	0	7
Tube combination incosistent with MPN reported (only one replicate)	2	7		9
Tube combination incosistent with MPN reported (both replicates)	2	5		7
Sample not examined or results returned late, or no explanation received	0	0	0	0
High censored result (*i.e.* MPN => 18000 per 100g)	Score not assigned			

***expected range**: Participants' Median ±3SD – SD stands for Theoretical Standard Deviation = 0,24
The expected range values are reported in detail in **Table 3** (Results Section).

3. RESULTS

4.1. Reference results

Ten samples from the freeze-dried batch were analyzed in duplicate on 13rd of November 2023 following the ISO 16649-3 method. Sample homogeneity was assessed according with the requirements of ISO 17043:2010. The reference results are reported in Table 2.

Table 2: *E. coli* MPN/100 g reference results.

Sample Number - Type	Range (*E.coli* MPN/100g)		Median	Median±3SDT*		Median±5SDT*	
	Minimum Value	Maximum Value					
Sample 1	24000	160000	92000	4,83E+05	17530,24	1,46E+06	5804,81

SDT stands for Theoretical Standard Deviation = 0,24
Note: 4,28 E+03 stands for 4,28 x 10^3 which is 4,28 times 10 (E) to the 3rd power (+03)

4.2. Participants' results

Performance assessment was carried out according to the scoring parameters reported in Table 1 – Section Materials and Methods. Participants' results and scores are shown in Tables 3, 4, 5 and Figure 1.

Table 3: Summary statistics of participants' results (total results received 25 laboratories).

***E. coli* MPN – summary statistics'**	**Sample 1**
Participants reporting duplicate results for *E. coli* MPN	25
Participants reporting a single MPN result	0
Participants reporting both replicate MPN results within expected range*	22/25
Participants reporting both replicate MPN results outside expected range	1/25
Participants reporting one replicate MPN result outside expected range	2/25
Participants reporting one replicate MPN results as censored results	0
Participants reporting both replicate MPN results as censored results	0
Participants reporting tube combination and/or MPN results inconsistent with ISO 7218*	0

****expected range**: Participants' Median ±3SD – SD stands for Theoretical Standard Deviation = 0,24
****points deducted from participants returning results with incorrect tube combinations and/or inconsistent with ISO 7218.

Table 4: *E. coli* MPN/100 g participants' results.

Sample Number	Range (*E.coli* MPN/100g)		Median	Median±3SDT*		Median±5SDT*	
	Minimum Value	Maximum Value					
Sample 1	20	3,5E+06	135950	7,13E+05	25905	2,2E+06	8,6E+03

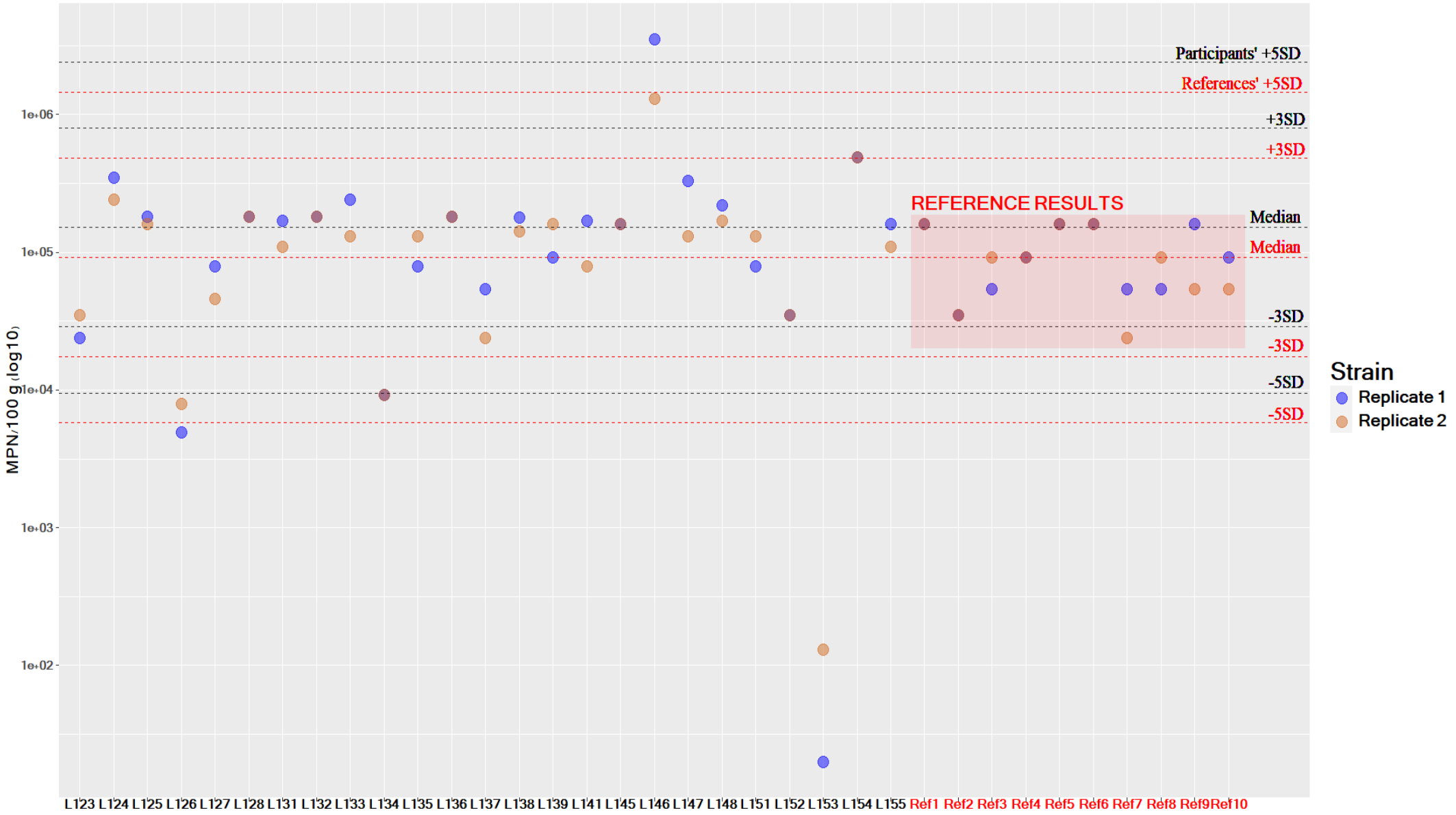
Note: The median and upper and lower limits (± 3 SD and ± 5 SD) were calculated from participants' results. SDT calculations were based on the inherent variability of the 5 x 3 MPN method (0.24 $\log_{10}$). **Reference values were excluded from the calculation of the participants' median.**

Table 5. Details of the analysis performed by the Laboratories and scores obtained; dark green boxes represent the maximum value' score (12), the dark red ones the minimum (0) (graduation colour bar

Lcode	*E.coli* MPN/100g				Score
	Replicate 1	Rarity Category	Replicate 2	Rarity Category	
L123	24000	1	35000	1	9
L124	350000	1	240000	1	12
L125	180000	1	160000	1	12
L126	4900	1	7900	1	2
L127	79000	1	46000	1	12
L128	180000	1	180000	1	12
L131	170000	1	110000	1	12
L132	180000	1	180000	1	12
L133	240000	1	130000	1	12
L134	9200	1	9200	1	6
L135	79000	1	130000	1	12
L136	180000	1	180000	1	12
L137	54000	1	24000	1	9
L138	178200	1	141900	1	12
L139	92000	1	160000	1	12
L141	170000	1	79000	1	12
L145	160000	1	160000	1	12
L146	3500000	1	1300000	1	4
L147	330000	1	130000	1	12
L148	220000	1	170000	1	12
L151	79000	1	130000	1	12
L152	35000	1	35000	1	12
L153	20	1	130	1	2
L154	490000	1	490000	1	12

Figure 1: Results' Dot Graph - lyophilized culture-lenticule

Reference results are shown in red, participants' results in grey



5. CONCLUDING REMARKS

1.Thirty laboratories joined the study and 25 returned the results.

2. Twenty-two of the 25 participants obtained the best score (12), with both replicates falling within the expected range of median ± the theoretical 3 SD.

3. The procedure for analyzing the freeze-dried ATCC strain (25922) cultures provided by the EURL was not immediately clear to the participants that requested support for the correct application of the proposed dilution scheme. This aspect will be improved in the next editions of the PTs on the enumeration of Beta glucuronidase *E. coli*. Nevertheless, most participants gave an excellent performance.

4. Some laboratories had issues with the samples' treatment prior to the application of the method. As this could be attributed to a problem of communication of the procedure, the scoring scheme in this report is proposed for the laboratory self-evaluation and will not be used to identify the underperformance.

4. In the upcoming edition of the PTs on the enumeration of Beta glucuronidase *E. coli*, the use of a mixed preparation including both beta - glucuronidase positive and negative strains will be considered to facilitate the application of the ISO 16649-3 method.

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