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Report of the fifth inter-laboratory study on the enumeration of *E. coli* (PT42) – 2024

Rev1

Edited by:

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1. INTRODUCTION AND OBJECTIVES OF THE STUDY

This exercise was intended to provide proficiency testing (PT) samples to the 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, to be assayed with the ISO 16649-3:2015 ***"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"***.

PT42 employed a freeze-dried mixed culture composed by the *beta*-glucoronidase positive ATCC strain 25922 and a field isolate of a *beta*-glucoronidase negative *E. coli* strain.

This document represents the evaluation report of the PT42 study. The study was conducted according to the International Standard ISO/IEC 17043:2010 "Conformity assessment – General requirements for proficiency testing". The most probable number of *beta*-glucoronidase positive *E. coli* was determined per millilitre (mL) or gram (g) of sample (according to the ISO 16649-3:2015 – paragraph 3.2)

2. PARTICIPANTS

Twenty-four NRLs, representing 22 EU Member States plus two third countries, 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:

Belgium	Laboratory of Foodborne Pathogens (SCIENSANO)
Bulgaria	National Diagnostic and Research Veterinary Institute (NDRVMI)
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
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)
Slovakia	State veterinary and food institute - SVPU
Slovenia	University of Ljubljana, Veterinary Faculty (Unit for food safety)
Spain	Centro Nacional de Alimentación - AESAN
Spain	National Plant Health Laboratory
Sweden	Swedish Food Agency, The Biology department
UK	CEFAS

We report the analysis of twenty-three laboratories that submitted results. One didn’t return the results (L705).

MATERIALS AND METHODS

3.1. Sample preparation

The process used to generate the freeze-dried vial was as follows: live cultures of the two *E. coli* strains (ATCC strain (25922) and the *beta*-glucoronidase negative strain (ECOR6)) were refreshed and grown at 10^8 cells/ml each, pelleted and re-suspended in 5% sucrose to a bacterial load of 10^9/ml, respectively. 800 µl of a mixture of the sucrose re-suspended cultures in the proportions of 98% of strain ECOR6 and 2% of ATCC 25922 strain was added to a lyophilization vial and frozen at -20 °C for two days. The vials were then placed into the "Alpha 1-2 LSC Basic" lyophilizer apparatus and freeze-dried for three days under the following conditions: (i) -60°C; (ii) 0.01 mbar. The vials were eventually tightly closed and sealed. A random selection of the samples was assayed using the Part 3 of the ISO 16649 method to check the MPN of the ATCC 25922 *beta*-glucoronidase positive strain.

On 18th of November 2024, the samples were shipped to the participating laboratories by courier. The participants were requested to reconstitute the lyophilized culture with 1 ml of TSB (Tryptone Soya broth) and proceed according to the ISO 16649 part 3.

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 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 - *the expected range is represented by participants' Median ±3SD (SD stands for Theoretical Standard Deviation = 0,24). The expected range values are reported in detail in Table 3 (Results Section).

Results	Returning of results	Replicate 1	Replicate 2	Score
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
One replicate MPN result reported is outside the median ± 5 SD value	2	5	0	7
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
Both replicates MPN results reported are outside the expected range: one replicate MPN results fall between the median ±3SD and the median ± 5 SD value and one replicate MPN result reported is outside the median ± 5 SD value	2	2	0	4
Both replicates MPN results reported are outside the median ± 5 SD value	2	0	0	2
Sample not examined or results returned late, or no explanation received	0	0	0	0
High censored result (i.e. MPN => 18000)	-	-	-	**Samples excluded from the analysis**

3. RESULTS

4.1. Reference results

Ten samples randomly selected from the freeze-dried batch were analyzed in duplicate on 25th of November 2024 following the ISO 16649-3:2015 method. The reference results are reported in Table 2.

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

Sample Number - Type	Range (*E.coli* MPN/g)		Median	Median±3SDT*		Median±5SDT*	
	Minimum Value	Maximum Value					
Sample	540	1600	920	4,83E+03	175,30	1,46E+04	58,05

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 23 laboratories).

***E. coli* MPN – summary statistics'**	**Sample**
Participants reporting duplicate results for *E. coli* MPN	23
Participants reporting a single MPN result	0
Participants reporting both replicate MPN results within expected range*	12/23
Participants reporting both replicate MPN results outside expected range	3/23
Participants reporting one replicate MPN result outside expected range	2/23
Participants reporting "high censored result" (i.e. MPN => 18000 per g)	6/23

****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/g participants' results.

Sample Number	Range (*E.coli* MPN/g)		Median	Median±3SDT*		Median±5SDT*	
	Minimum Value	Maximum Value					
Sample	23	9,2E+03	1600	8,40E+03	305	2,5E+04	1,0E+02

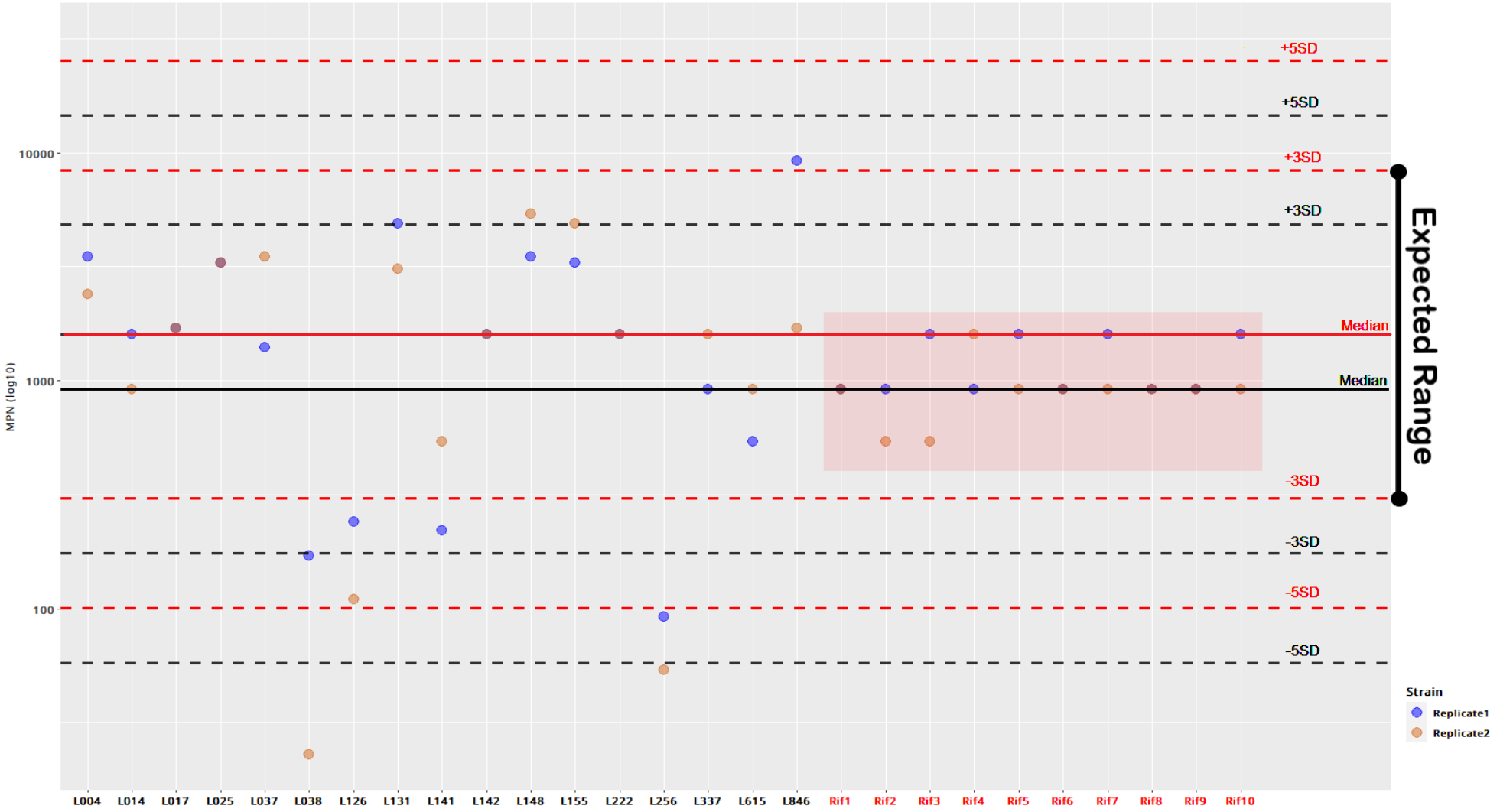
Note: The median and upper and lower limits (± 3 SD and ± 5 SD) were calculated from participants' results. SDT calculations were based on the inherent variability of the 5 x 3 MPN method (0.24 $\log_{10}$). **Reference values were excluded from the calculation of the participants' median.**

Table 5. Details of the analysis performed by the Laboratories and scores obtained; the dark red ones represent the Laboratories with both replicates MPN results reported outside the expected range.

Lcode	*E.coli* MPN/g				Score
	Replicate 1	Rarity Category	Replicate 2	Rarity Category	
L004	3500	1	2400	1	12
L014	1600	1	920	1	12
L017	1700	1	1700	1	12
L025	3300	1	3300	1	12
L037	1400	1	3500	1	12
L038	170	1	23	1	4
L126	240	1	110	1	6
L131	4900	1	3100	1	12
L141	220	1	540	1	9
L142	1600	1	1600	1	12
L148	3500	1	5400	1	12
L155	3300	1	4900	1	12
L222	1600	1	1600	1	12
L256	92	1	54	1	2
L337	920	1	1600	1	12
L615	540	1	920	1	12
L846	9200	1	1700	1	9

The six Laboratories that reported both replicates with MPN => 18000; were excluded from the analysis and are not shown.

Figure 1. Dot graph lyophilized culture lenticule - the red lines represent the Participants' results, the black ones the Reference' results; the expected range is shown and the reference results are highlighted in light red.



5. CONCLUDING REMARKS

1. Twenty-four laboratories joined the study and 23 returned the results.
2. Six laboratories reported high censored results and were excluded from the analysis. These laboratories will be contacted and offered a back-up sample to enable the assessment of their proficiency.
3. Twelve of the 16 participants with analyzable results obtained the highest score (12), with both replicates falling within the expected range of median ± the theoretical 3SD.
4. Only three participants reported results with both replicates falling outside the expected interval of the median score of the participants' results ±3SD. As above, these laboratories will be contacted and offered a back-up sample to enable the assessment of their proficiency.