EURL-*CAMPYLOBACTER*

REPORT

PROFICIENCY TEST NUMBER 32

Detection and species identification of *Campylobacter* spp.

Publication history

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Contents

Abbreviations

C.	*Campylobacter*
cfu	colony forming units
EU	European Union
EURL	European Union reference laboratory
FP	false positive
ISO	International Organization for Standardization
LOD_{50}	level of detection for which 50 % of tests give a positive result
$\log_{10}$	logarithm to base 10 (common logarithm)
MALDI-TOF MS	matrix-assisted laser desorption ionization–time of flight mass spectrometry
mCCD	modified charcoal cefoperazone deoxycholate
MS	Member State (of the European Union)
MS-NRL	Member State national reference laboratory
NRL	national reference laboratory (in this report used for all participating laboratories, also in non-EU Member States)
PCR	polymerase chain reaction
PT	proficiency test
SD	standard deviation
spp.	species

Summary of proficiency test number 32, 2022

The EU reference laboratory for *Campylobacter* organised proficiency test (PT) number 32 on detection and species identification of *Campylobacter* in March 2022. The objective was to assess the performance of the national reference laboratories (NRLs) to detect and identify *Campylobacter* species in pig faeces.

Participation in PT 32 was voluntary for all NRLs. Thirty-two NRLs in 25 EU Member States (some Member States have more than one NRL) and in Iceland, Norway, Switzerland, and United Kingdom received the PT, and 31 NRLs reported results.

The PT contained two sets of samples: ten core samples of pig faeces to be mixed with vials with or without freeze-dried *Campylobacter* and two educational samples of fresh, naturally contaminated pig faeces. The educational samples were distributed as an optional part of the PT, and the results were not included in the performance evaluation.

Of the 31 NRLs, 29 followed ISO 10272-1:2017 for detection of *Campylobacter* spp., and two NRLs used other methods. All except two NRL used direct plating and four used more than one procedure.

A combined five-level grading scale for performance in detection was based on the sensitivity and accuracy of detection and the number of correct identifications of the two samples without *Campylobacter* as non-*Campylobacter* samples. Twenty-nine NRLs (94 %) fulfilled the criterion for excellent or good performance in detection of *Campylobacter*, and one NRL (a MS-NRL) scored below the acceptable limit, due to two false positive results.

Of the 31 NRLs reporting results for species identification, all fulfilled the criterion for excellent performance in identification of *Campylobacter* spp.

Twenty-seven NRLs reported results of analysis of the educational samples. *Campylobacter* spp. were detected by 24 (89 %) and 17 (63 %) of the NRLs in the two samples, respectively. Five different *Campylobacter* species were identified, with *Campylobacter jejuni* and *Campylobacter hyointestinalis* as the two most frequently found species.

In summary, the majority of the NRLs met the criteria for excellent or good performance in both detection and species identification, and only one NRL scored below the acceptable limit in detection. The results of analysis of the educational samples demonstrated the presence of several *Campylobacter* species in fresh pig faeces.

Introduction

The proficiency test (PT) number 32 on detection and species identification of *Campylobacter* was organised by the EU reference laboratory (EURL) for *Campylobacter* in March 2022. Participation in the PT was voluntary. Thirty-two national reference laboratories (NRLs) in 25 EU Member States (some Member States have more than one NRL) and in Iceland, Norway, Switzerland, and United Kingdom received the PT. The test results and operational details were reported to the EURL from 31 NRLs. Twenty-eight NRLs reported that they were accredited for detection of *Campylobacter* and 18 were also accredited for enumeration of *Campylobacter*.

The PT included detection and species identification of *Campylobacter* spp. in 10 core samples of pig faeces mixed with vials with or without freeze-dried *Campylobacter* and two educational samples of fresh, naturally contaminated pig faeces (Table 1). The objective was to assess the performance of the NRLs to detect and identify *Campylobacter* species in pig faeces.

Table 1. Bacteria in the vials in proficiency test No. 32 (2022).

Vial No.	**Bacterial species in vial**	**Batch No.**	**Level *Campylobacter*** [a] **(log_{10} cfu/vial & log_{10} cfu/test portion)**			**Level *E. coli*** [b] **(log_{10} cfu/vial)**	**SD** [c] **(log_{10} cfu)**
11	*Campylobacter coli*	SVA068	4.82	1.82	(low)		0.07
12	–						
13	*Escherichia coli*	SVA061				4.80	0.07
14	*Campylobacter jejuni* [d]	SVA065	5.27	2.27	(low)		0.12
15	*Campylobacter coli*	SVA068	4.82	1.82	(low)		0.07
16	*Campylobacter coli*	SVA068	4.82	1.82	(low)		0.07
17	*Campylobacter coli*	SVA072	7.10	4.10	(high)		0.07
18	*Campylobacter jejuni* [d]	SVA065	5.27	2.27	(low)		0.12
19	*Campylobacter lari* + *Escherichia coli*	SVA070	6.66	3.66	(high)	5.85	0.04
20	*Campylobacter jejuni* [d]	SVA065	5.27	2.27	(low)		0.12

[a] Total quantity of *Campylobacter* in each vial and per test portion of 10 µl, after mixing with 6 g of pig faeces to a total volume of 10 ml. If a loop has been used to take the test portion, it may be larger than 10 µl.

[b] Total quantity of *Escherichia coli* in each vial.

[c] Standard deviation (SD) of the level defined by homogeneity test of 10 vials after the production. The maximum SD allowed was 0.15 log_{10} cfu.

[d] All *Campylobacter jejuni* strains were hippurate positive.

Terms and definitions

- *Campylobacter* spp.: Thermotolerant *Campylobacter* spp., i.e. which are able to grow at 41.5 °C, foremost (but not exclusively) *Campylobacter jejuni, Campylobacter coli, Campylobacter lari* and *Campylobacter upsaliensis*.
- Detection of *Campylobacter* spp.: Determination of the presence or absence of *Campylobacter* spp.
- Confirmation of *Campylobacter* spp.: Microorganisms suspected to be *Campylobacter* spp. are confirmed as such by biochemical tests and/or by molecular methods.
- Species identification of *Campylobacter*: Identification of thermotolerant *Campylobacter* species with biochemical tests and/or molecular methods.

Outline of the proficiency test

The PT contained 10 core samples of autoclaved pig faeces to be mixed with vials with or without *Campylobacter*, and two educational samples of fresh, naturally contaminated pig faeces. The participants were instructed to divide the faeces for core samples into 10 portions with 6 g faeces in each and mix the content of the vials with the faeces, making up a volume of 10 ml for each sample. This resulted in six samples with a low content of *Campylobacter*, two samples with a high content of *Campylobacter*, and two samples without *Campylobacter* (Table 1). The theoretical levels of contamination in the test portions of the low-level samples were estimated to be between 12 and 32 times an assumed LOD_{50} of 6.1 cfu (the LOD_{50} for chicken caecum according to ISO 10272-1:2017, annex C) and in high-level samples at least 750 × LOD_{50}. The levels were higher than recommended in ISO 22117:2019 but were selected to guarantee that any instability during transport would not have an effect of the evaluation of NRLs' performance.

Preparation of the matrix

The pig faeces used as matrix for the core samples in the PT was obtained directly from a local pig farm three months before distribution of the PT. On arrival, the pig faeces was autoclaved (4 hours, 125 °C), aliquoted and freeze-stored until dispatch. The autoclaved matrix was tested for presence of *Campylobacter* spp. by direct streak onto both modified charcoal cefoperazone deoxycholate (mCCD) and Butzler agar. The autoclaved pig faeces tested negative for presence of *Campylobacter*.

The pig faeces used for educational samples in the PT was obtained directly from two different compartments at a local pig farm three days before dispatch, aliquoted and stored cold (between 1 °C and 8 °C) until dispatch.

Production and quality control of the vials

The vials with freeze-dried bacterial cultures used in the PT were produced and tested for homogeneity and stability by the EURL in both storage and transport conditions.

To ensure that the level of instability during transport conditions did not have an effect on the performance evaluation, each combination of vial and matrix were prepared and tested under various transport conditions (Table 2). The tests were performed according to ISO 10272-1:2017, detection procedure A (enrichment in Bolton broth), procedure B (enrichment in Preston broth) and/or procedure C (direct plating), on at least five occasions. The plating was done on mCCD and Butzler agar.

The detection tests were performed before dispatch in simulated "best case" transport conditions (5 °C for 24 h) and "worst case" transport conditions (5 °C for 24 h, 15 °C for 24 h, and 5 °C for 24 h). They were also performed just after dispatch ("best case" conditions) and one week after dispatch at the last date for start of analysis by the participants (both "best case" and "worst case" conditions). At the last occasion, the stability of vials with *Campylobacter* stored under "best case" and "worst case" conditions was also checked by performing viable count on blood agar.

Table 2. Outline and results of stability testing under transport conditions for proficiency test No. 32 (2022).

Test occasion	Storage conditions [a]	Test method [b]	No. of samples tested	Result [c]
Before dispatch	Best case	B + C	Each vial with *Campylobacter* × 3	+
Before dispatch	Worst case	A + B + C	Each vial with *Campylobacter* × 3	+
Just after dispatch	Best case	C	The complete test	+
1 week after dispatch	Best case	VC + C	The complete test	+
1 week after dispatch	Worst case	VC + B + C	The complete test	+

[a] **Best case** transport conditions: 5 °C for 24 h, **worst case** transport conditions: 5 °C for 24 h, 15 °C for 24 h, and 5 °C for 24 h.

[b] Detection procedure according to ISO 10272-1:2017: **A** enrichment in Bolton broth, **B** enrichment in Preston broth, **C** direct plating. **VC**: viable count of vials with *Campylobacter*.

[c] A plus indicates *Campylobacter* could be detected in all samples with *Campylobacter* after indicated storage condition.

Distribution of the proficiency test

The PT samples were distributed from the EURL on the 7th of March, 2022. The samples were placed in foam boxes along with freezing blocks. The foam boxes were packed in cardboard boxes for transport and were sent from the EURL using courier service.

Each participant received a package containing 10 numbered vials, each containing freeze-dried material with or without *Campylobacter* spp., a plastic bag with about 120 g sterilised pig faeces, two educational sample tubes with fresh pig faeces, and small stomacher bags. A Micro-T-Log was included in each package to record the temperature every second hour during transport.

Of the 31 participating NRLs, 27 received the test one day after dispatch and four NRLs two days after dispatch. Due to logistic transportation issues, a second distribution from the EURL was done on the 21st of March, 2022. The NRL received the test one day after this second dispatch (Table 3).

The PT analyses were recommended to be started as soon as possible after the arrival and at the latest on the 11th of March, 2022 (for the second dispatch at the 25th of March). All results had to be reported in the Questback Essentials system by the 19th of April, 2022. Instructions for preparation of the samples from the vials and matrix were included in the packages, and were also sent out by e-mail a few days before the PT distribution. Until start of analysis, pig faecal material and vials were recommended to be stored at cold temperature (between 1 °C and 8 °C). If the analysis was started more than 24 hours after arrival, the vials were recommended to be stored at –20 °C or –70 °C.

Table 3. Dates of arrival and start of analysis of proficiency test No. 32, 2022.

Arrival	**Number of NRLs n=32** [a]	**Start of analysis**	**Number of NRLs n=31** [a]
8th of March	27	8th of March	8
9th of March	4	9th of March	16
22nd of March [b]	1	10th of March	2
		11th of March	2
		14th of March	2
		23rd of March [b]	1

[a] One NRL received and started analysis of the test but did not report final results.

[b] One NRL received a new package after second dispatch 21st of March.

Methods for analysis

The NRLs were recommended to follow ISO 10272-1:2017, procedure C (direct plating) for performing the PT but were allowed to use another method if their standard laboratory procedure followed a different method. The amount of faeces provided allowed the laboratories to perform enrichment, e.g. detection procedure A or B, if this was of interest to them.

Campylobacter spp. should be incubated in a microaerobic atmosphere, with oxygen content of 5 % ± 2 %, and carbon dioxide 10 % ± 3 %. The appropriate microaerobic atmosphere can be obtained by using commercially available microaerobic incubators, commercial gas-generating kits, or by using gas-jars, filled with the appropriate gas mixture prior to incubation. Of the 31 participating NRLs, 19 reported using gas-generating kits, seven microaerobic incubators, seven the Anoxomat® system and one another method (zip-lock bags filled with gas). Some of the NRLs used more than one system.

Assessment of performance in detection and identification

The NRLs' performance in sensitivity in detection, sensitivity in identification, and accuracy in detection of *Campylobacter*-positive and -negative samples were calculated from the final results as reported by each participant.

The **sensitivity** was calculated based on the NRL's ability to correctly detect *Campylobacter* spp. and identify *Campylobacter* species in the samples containing *Campylobacter*. Correct detection of all *Campylobacter*-positive resulted in a sensitivity in detection of 100 %. Correct identification of all *Campylobacter* species in positive samples in which *Campylobacter* spp. were detected resulted in a sensitivity in identification of 100 %.

The **accuracy** was also calculated, giving an overall performance of the results of correct detection of *Campylobacter* spp. in samples with *Campylobacter* and correct identification of samples without *Campylobacter* as non-*Campylobacter* samples. The accuracy was calculated as total number of correct detection results divided by total number of samples.

Since there were only two *Campylobacter*-negative samples in each set of results for which the performance assessment was done, the specificity was not assessed.

A combined five-level grading scale for **performance in detection** was based on the sensitivity and accuracy of detection and the number of correct identifications of the two samples without *Campylobacter* as non-*Campylobacter* samples, according to Table 4. Since the detection rate in PT 32 was very high for both low-level and high level-samples it was not relevant in this PT to evaluate performance based on level of contamination. The cut-off for good **performance in identification** of *Campylobacter* species was set to 85.0 %.

Table 4. The maximum number of false positive results (FP), and the lower limits of sensitivity (Se) and accuracy (Acc), applied for each combined performance grade for detection, and the lower limits for grading of species identification (Spec id) in proficiency test No. 32 (2022). Performance scoring below any of the limits for the performance grade *Needs improvement* was graded as *Poor*.

Performance grade	*for detection (combined)*			*for species identification*
	Maximum number	*Measures on the lower limit for each grade*		
	FP	**Se total**	**Acc**	**Spec id**
Excellent	0	95.1 %	95.1 %	95.1 %
Good	0	85.0 %	90.0 %	85.0 %
Acceptable	1	70.0 %	80.0 %	70.0 %
Needs improvement	2	57.0 %	70.0 %	57.0 %

Results

Detection and species identification of *Campylobacter*

Proficiency test number 32 was distributed to 32 NRLs and 31 reported the results of the analysis.

According to the instructions, analysis of the samples should be started as soon as possible after arrival and no later than four days after dispatch. Eight NRLs started the analysis the day after the samples were dispatched from the EURL, 16 NRLs two days after, two NRLs three days after, two NRLs four days after, two NRLs seven days after and one NRL two days after the second dispatch (Table 3).

Of the 31 NRLs reporting results, 29 followed ISO 10272-1:2017 for detection of *Campylobacter* spp., and two NRLs used other culture methods. All except two NRLs used direct plating. Six NRLs used enrichment, whereof four in combination with direct plating: three in Preston broth, one in Bolton broth, one in CampyFood® broth, and one in Exeter broth.

Thirty NRLs used mCCD agar and 23 plated on at least one additional medium. Other media used for plating were Butzler agar (12), Preston agar (5), Karmali agar (4), CampyFood® agar (4), Skirrow agar (3), CASA® agar (2), RAPID'*Campylobacter* agar BioRad (2), CHROMagar™ Campylobacter (1), CAT agar (1), and heart infusion sheep blood agar with filter (1).

The presumptive *Campylobacter* colonies were confirmed by typical microscopic morphology and motility, positive oxidase test, lack of aerobic growth at 25 °C and/or molecular methods, mostly matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) or polymerase chain reaction (PCR). Twenty of the 31 NRLs used microscopic examination as part of the confirmation procedure. Seventeen NRLs used oxidase test, in 13 cases in combination with aerobic growth at 25 °C, and in 12 cases in combination with MALDI-TOF and/or PCR. Eighteen NRLs used MALDI-TOF MS for confirmation, in 10 cases in combination with additional techniques other than microscopic examination. Seven NRLs used one or more PCR assays, in six cases in combination with other techniques. Three NRLs reported to have used the multiplex PCR assay published by Wang et al. (2002).

The isolated *Campylobacter* spp. were identified by biochemical tests and/or molecular methods, mostly MALDI-TOF MS or polymerase chain reaction (PCR). The biochemical tests included detection of catalase, hippurate hydrolysis, indoxyl acetate hydrolysis, sensitivity to cephalotin and hydrogen sulphide production in triple sugar iron medium.

Twenty-one of the 31 NRLs used MALDI-TOF MS for the species identification, in seven cases in combination with other techniques. Ten NRLs used one or more PCR assays, in seven cases in combination with other techniques. Four NRLs reported to have used the multiplex PCR assay published by Wang et al. (2002) and three to have used the multiplex PCR assay published by Denis et al. (1999). Twelve NRLs used biochemicals tests (at least detection of catalase), in nine cases in combination with MALDI-TOF MS and/or PCR.

Nineteen NRLs used one technique only (a set of biochemical tests and/or tests of growth regarded as one technique) and 12 NRLs combined two techniques.

Of the 31 NRLs, twenty-five reported correct results of detection, i.e. correct identification of the eight samples with *Campylobacter* and the two samples without *Campylobacter* (Figure 1). Three false positive results were reported by altogether two NRLs. All of the 31 NRLs reported correct species in all samples where *Campylobacter* spp. had been detected.

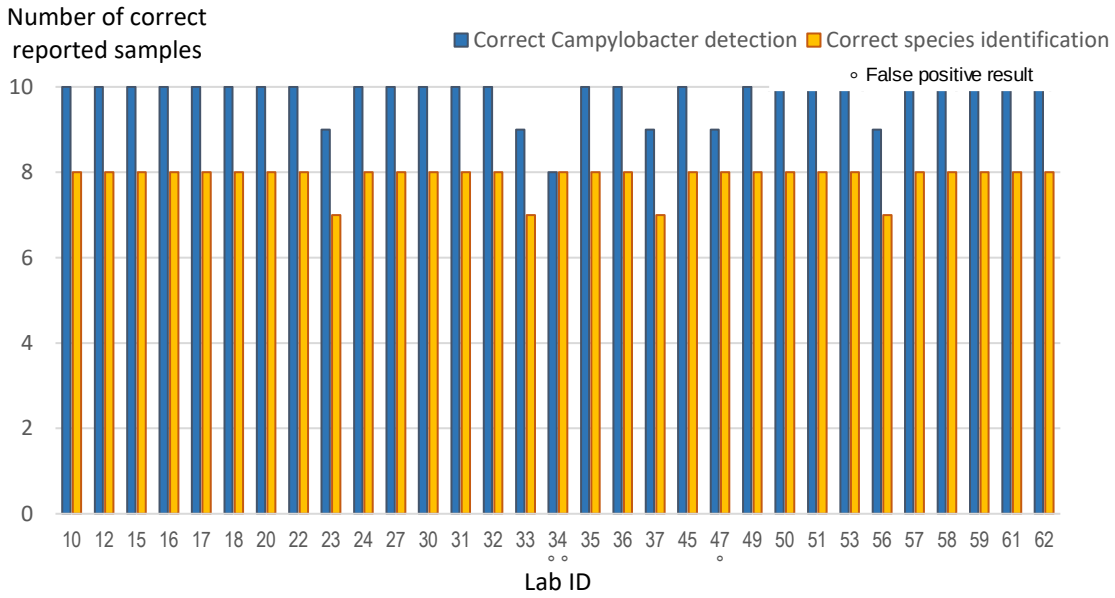

Figure 1. Distribution of correct results by 31 NRLs participating in proficiency test No. 32 (2022) in the detection and species identification of *Campylobacter* spp. in pig faeces.

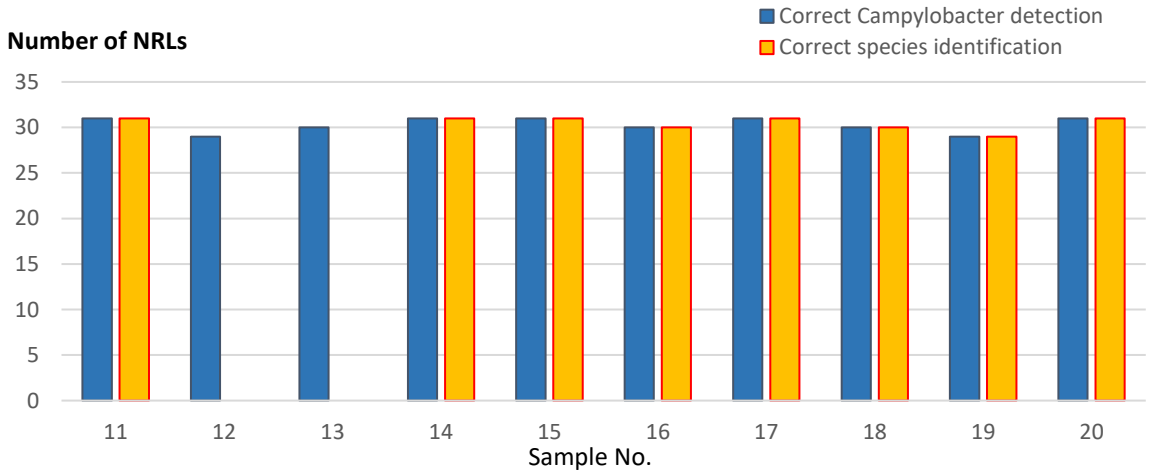

Figure 2. Number of NRLs participating in proficiency test No. 32 (2022) that correctly reported results in the detection and species identification of *Campylobacter* in 10 samples of pig faeces. In total, 31 NRLs performed detection and all performed species identification.

All 31 NRLs reported correct results of detection for five of the 10 samples (Figure 2, Table 5). The five samples containing *Campylobacter* correctly detected by all NRLs (No. 11, 14, 15, 17 and 20) were also correctly identified by all 31 NRLs performing species identification. One NRL respectively failed to detect *Campylobacter* in low-level samples No. 16 and 18 (*C. coli*) and two NRLs failed to detect *Campylobacter* in sample No. 19, which contained both *C. lari* (high-level) and *E. coli*.

Generally, the detection rate of *Campylobacter* in both low-level samples and high-level samples was very high and contamination levels used in the test could have been set lower to provide a bigger challenge to the NRLs. The test was designed to comply with some instability of the test under transport conditions for up to three days, but in the end, all NRLs received the test within two days.

Table 5. Results of detection and species identification in 10 samples of pig faeces in proficiency test No. 32 (2022). In total 31 NRLs performed species identification.

Sample No.	**Bacterial species in vial**	*C. jejuni*	*C. coli*	*C. lari*	*Campylobacter* spp. but unable to identify species	Growth of other, not *Campylobacter*	No growth at all
11	*Campylobacter coli*		31				
12			2			1	28
13	*Escherichia coli*		1			27	3
14	*Campylobacter jejuni*	31					
15	*Campylobacter coli*		31				
16	*Campylobacter coli*		30				1
17	*Campylobacter coli*		31				
18	*Campylobacter jejuni*	30					1
19	*Campylobacter lari* + *Escherichia coli*			29		2	
20	*Campylobacter jejuni*	31					

Performance in detection and species identification of *Campylobacter* spp.

Of the 31 participating NRLs, 29 NRLs (24 Member State NRLs, MS-NRLs) fulfilled the criterion for excellent or good performance in detection of *Campylobacter*, and one (an MS-NRL) scored below the acceptable limit because of low specificity (two false positive results out of two negative samples) (Table 6). All NRLs fulfilled the criterion for excellent performance in identification of *Campylobacter* spp., and none scored below the acceptable limit (Table 7).

Table 6. Combined performance grades in detection of *Campylobacter* spp. in proficiency test No. 32 (2022).

Combined performance in detection of *Campylobacter* spp.		
Grade	**Number of NRLs (%) All NRLs, n=31**	**Number of NRLs (%) MS-NRLs, n=25**
Excellent	25 (81%)	21 (84%)
Good	4 (13%)	3 (12%)
Acceptable	1 (3%)	0 (0%)
Needs improvement	1 (3%)	1 (4%)
Poor	0 (0%)	0 (0%)

Table 7. Overall performance of NRLs' sensitivity in correct species identification of *Campylobacter* in proficiency test No. 32 (2022).

Performance in identification of *Campylobacter* spp.			
Grade	**Sensitivity**	**Number of NRLs (%) All NRLs, n=35**	**Number of NRLs (%) MS-NRLs, n=25**
Excellent	95.1–100%	31 (100%)	25 (100%)
Good	85.0–95.0%	0 (0%)	0 (0%)
Acceptable	70.0–84.9%	0 (0%)	0 (0%)
Needs improvement	57.0–69.9%	0 (0%)	0 (0%)
Poor	<57.0%	0 (0%)	0 (0%)

All performance parameters for detection and identification of *Campylobacter* spp. in pig faeces for all participants are presented in Table 8.

Pre-testing by the EURL indicated that the choice of method (enrichment or direct plating) should not have a substantial impact on the result. However, one of the two NRLs performing enrichment instead of direct plating (in Preston broth) failed to detect *C. lari* but had growth of background flora in sample No. 19.

Since the detection rate was so high in PT 32, no analysis could be done of the correlation between performance and the type of selective agar used. However, four of five NRLs using only one selective plate failed to correctly detect *Campylobacter* in all eight samples with *Campylobacter*. Among the 26 NRLs using two selective plates or more, no laboratory failed to correctly detect *Campylobacter* in all eight samples with *Campylobacter*.

Table 8. The sensitivity (Se) and accuracy (Acc) in detecting *Campylobacter* and non-*Campylobacter* spp., number of false positives (FP), and the sensitivity in identification (Se id) of *Campylobacter* spp. for 31 NRLs participating in proficiency test No. 32 (2022). The performance grades in detection were based on minimum limits for sensitivity in detection, accuracy and correctly identified samples without *Campylobacter* as non-*Campylobacter* samples. Green shadowed cells indicate acceptable grades: *Excellent*, *Good* and *Acceptable*, and red shadowed cells indicate grades below the acceptable limit: *Needs improvement* and *Poor*.

Lab id	Se total	FP	Acc	Performance grade in detection	Se id
10	100%	0	100%	Excellent	100%
12	100%	0	100%	Excellent	100%
15	100%	0	100%	Excellent	100%
16	100%	0	100%	Excellent	100%
17	100%	0	100%	Excellent	100%
18	100%	0	100%	Excellent	100%
20	100%	0	100%	Excellent	100%
22	100%	0	100%	Excellent	100%
23	88%	0	90%	Good	100%
24	100%	0	100%	Excellent	100%
27	100%	0	100%	Excellent	100%
30	100%	0	100%	Excellent	100%
31	100%	0	100%	Excellent	100%
32	100%	0	100%	Excellent	100%
33	88%	0	90%	Good	100%
34	100%	2	80%	Needs improvement	100%
35	100%	0	100%	Excellent	100%
36	100%	0	100%	Excellent	100%
37	88%	0	90%	Good	100%
45	100%	0	100%	Excellent	100%
47	100%	1	90%	Acceptable	100%
49	100%	0	100%	Excellent	100%
50	100%	0	100%	Excellent	100%
51	100%	0	100%	Excellent	100%
53	100%	0	100%	Excellent	100%
56	88%	0	90%	Good	100%
57	100%	0	100%	Excellent	100%
58	100%	0	100%	Excellent	100%
59	100%	0	100%	Excellent	100%
61	100%	0	100%	Excellent	100%
62	100%	0	100%	Excellent	100%

Detection and species identification of *Campylobacter* in educational samples

Results of analysis of the educational samples No. 21 and 22 in proficiency test number 32 were reported from 27 NRLs. Twenty-five NRLs reported to have followed ISO 10272-1:2017 (in some cases with additions or modifications) for detection of *Campylobacter* spp. in the educational samples, and two NRLs used other methods. Twenty-five NRLs used direct plating on selective plates, and 11 used a procedure including enrichment. Preston broth was used for the enrichment by seven NRLs, Bolton broth by two NRLs, and three NRLs used other media for the enrichment (in one case in addition to Bolton broth). Sixteen NRLs did only direct plating. Twenty-six NRLs did the plating on mCCD agar, and 22 plated on at least one other medium. Other media used for plating were Butzler agar (13), CampyFood® agar (4), Preston agar (4), Skirrow agar (4), Karmali agar (2), CASA® agar (2), RAPID'*Campylobacter* agar BioRad (2), CAT agar (1), CHROMagar™ Campylobacter (1), and blood agar with filter (2).

Twenty-four NRLs incubated the plates at 41.5 °C and 17 NRLs at 37 °C, including 14 NRLs using both temperatures. From each sample, 0 to 25 presumptive *Campylobacter* colonies were selected and further analysed.

The presumptive colonies were confirmed by typical microscopic morphology and motility, positive oxidase test, lack of aerobic growth at 25 °C and/or molecular methods, mostly MALDI-TOF MS or PCR. Nineteen of the 27 NRLs used microscopic examination as part of the confirmation procedure. Fifteen NRLs used oxidase test, in 12 cases in combination with aerobic growth at 25 °C, and in 11 cases in combination with MALDI-TOF and/or PCR. Seventeen NRLs used MALDI-TOF MS for confirmation, in nine cases in combination with additional techniques other than microscopic examination. Seven NRLs used one or more PCR assays, in all cases in combination with other techniques.

The isolated *Campylobacter* spp. were identified by biochemical methods and/or molecular methods, PCR, MALDI-TOF MS or groEL gene sequencing. The biochemical methods included detection of catalase, hippurate hydrolysis, indoxyl acetate hydrolysis, sensitivity to cephalotin, H_2S production in triple sugar iron medium, growth in NaCl 3.5 %, growth in glycine 1 %, growth in safranin 0.05 %, growth on MacConkey agar, growth on nutrient agar, aerobic growth at 37 °C and 41.5 °C, microaerobic growth at 41.5 °C, and nitrate reduction.

Twenty NRLs reported that they used MALDI-TOF MS for the species identification, in nine cases in combination with other techniques. Ten NRLs used biochemicals methods (at least detection of catalase), in nine cases in combination with MALDI-TOF MS and/or PCR. Nine NRLs used PCR assays, in eight cases in combination with other techniques. Four NRLs reported to have used the multiplex PCR assay published by Wang *et al.* (2002). Another protocol reported to be used or adapted by more than one NRL was the PCR assay by Denis *et al.* (1999). One NRL reported to have used a latex agglutination test, and one NRL used groEL sequencing.

Thirteen NRLs used one technique only (a set of biochemical tests regarded as one technique), eight NRLs combined two techniques, and four NRLs used three techniques for

the species identification. One NRL did not find any suspected colonies on the plates and did not perform species identification.

In sample No. 21, 24 (89 %) of the 27 NRLs detected *Campylobacter* spp., and three NRLs reported growth of other bacteria only. Seventeen NRLs reported identification of *C. jejuni*, 14 of *Campylobacter hyointestinalis*, one of *Campylobacter lanienae*, and one of *C. coli*. Four NRLs reported *Campylobacter* spp. but unable to identify species or "other *Campylobacter* species" (one as the only and three as an additional result). Eight NRLs reported two and one NRL three different *Campylobacter* species in sample No. 21.

In sample No. 22, 17 (63 %) of the 27 NRLs detected *Campylobacter* spp., and 10 NRLs reported growth of other bacteria only. Seven NRLs reported identification of *C. hyointestinalis*, six of *C. jejuni*, four of *C. lanienae*, and one of *Campylobacter mucosalis*. Six NRLs reported *Campylobacter* spp. but unable to identify species or "other *Campylobacter* species" (five as the only and one as an additional result). Five NRLs reported two different *Campylobacter* species in sample No. 22.

Several NRLs reported in comments to have identified related bacteria like *Helicobacter* spp. and *Arcobacter* spp. in both samples.

Seven NRLs detected *Campylobacter* in sample No. 21 only, but no NRL detected *Campylobacter* in sample No. 22 only. Three NRLs did not detect *Campylobacter* in any of the samples. This suggests a correlation between the results from the two samples, which would be expected if differences between NRLs are due to differences in performance/level of detection. However, independence of the results could not be excluded (chi-squared test, $p = 0.078$).

In summary, the analysis of the educational samples demonstrated the presence of several *Campylobacter* species in fresh, naturally contaminated pig faeces, and that most NRLs were able to detect and identify at least one of these after collecting and transport. With careful preparation, standardisation and testing, naturally contaminated faeces may be a feasible matrix in future PTs.

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