INTERLABORATORY STUDY – PCR FOR CONFIRMATION AND/OR IDENTIFICATION OF CAMPYLOBACTER SPP.

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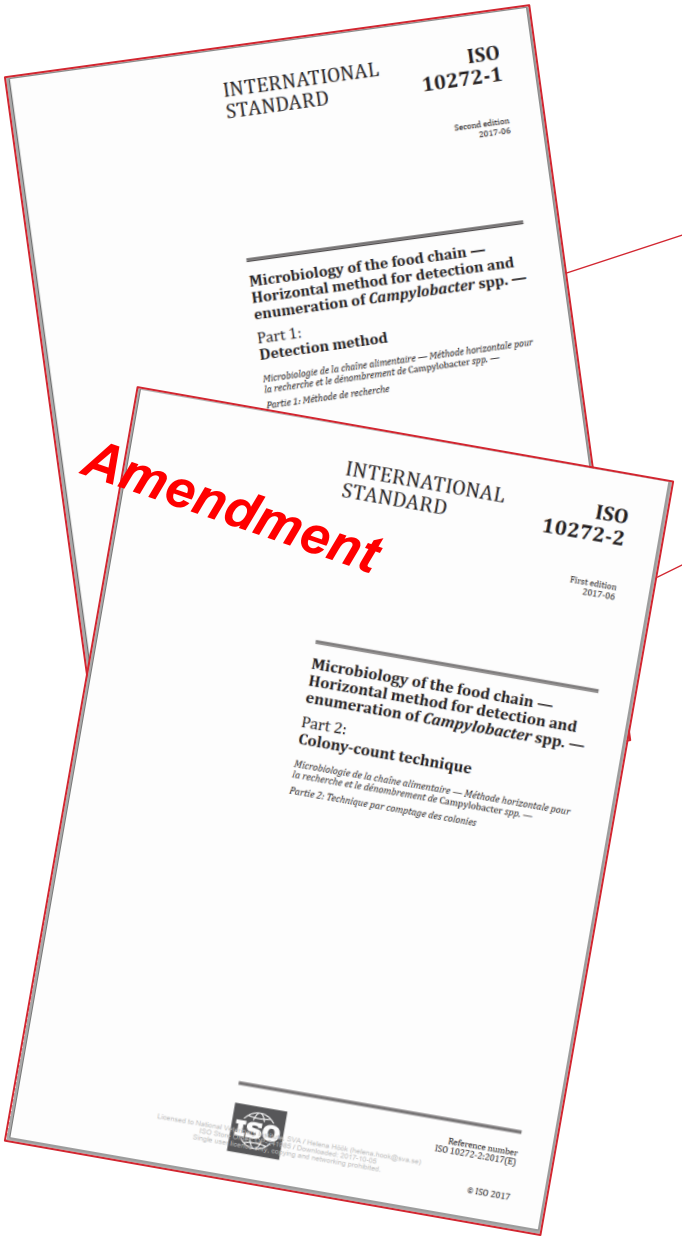
EURL-*Campylobacter* Workshop 28-29 September 2021

BACKGROUND INFORMATION



9.5 Confirmation of *Campylobacter*

9.5.1 General

As *Campylobacter* rapidly loses culturability in air, follow the procedure described in 9.5.2 to 9.5.5 without delay.

For a clear distinction between positive and negative confirmation reactions, it is helpful to verify this with well-characterized positive and negative control strains. Examples of suitable control strains are *Campylobacter jejuni* WDCM 00005 (positive control)[17] and *Escherichia coli* WDCM 00013 (negative control).

As an alternative, or in addition, to the confirmation and identification tests described in this document, other tests (PCR tests, serological methods, matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS) analysis, etc.) can be used, providing the suitability of the alternative procedure is verified (see ISO 7218).

4.4 Confirmation

The suspect *Campylobacter* colonies are examined for morphology and motility using a microscope and sub-cultured on a non-selective blood agar, and then confirmed by detection of oxidase activity and an aerobic growth test at 25 °C. Optionally, the *Campylobacter* species are identified by specific biochemical tests and/or molecular methods.

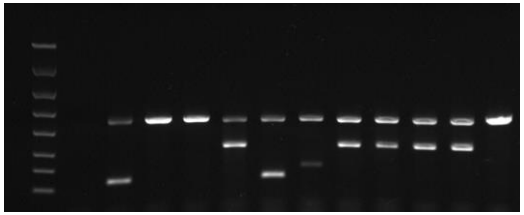
Molecular methods as alternative to biochemical tests for confirmation and species identification of *Campylobacter* spp.

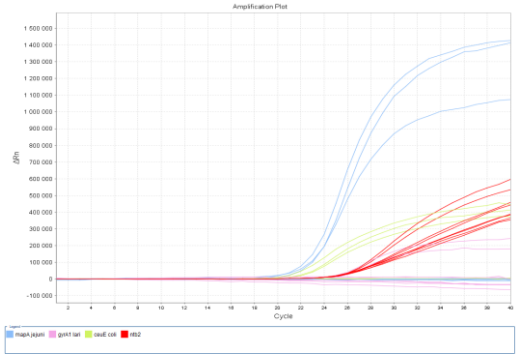
Development of amendments to **ISO 10272:2017**

Three PCR assays were chosen to be included.

- one conventional PCR method
- two qPCR methods

THE PCR METHODS





Confirmation of thermotolerant *Campylobacter*

PCR 1 - Josefsen et al., 2004 (2010) and Pacholewicz et al., 2019 (qPCR)

-Targets *C. jejuni*, *C. coli* and *C. lari*

Identification of thermotolerant *Campylobacter*

PCR 2 - Wang et al., 2002 (conventional PCR)

but C. lari primers changed to Chaban 2009 et al.,(targets both subspecies).

- Targets 23S rRNA of *Campylobacterales* and species specific targets of *C. jejuni*, *C. coli, C. lari* and *C. upsaliensis (also C. fetus)*

PCR 3 - Mayr et al., 2010 (qPCR)

- Targets *C. jejuni*, *C. coli* and *C. lari*

VALIDATION STUDY

Validation of the PCR methods according to the ISO 16140-6 standard (as far possible)

Microbiology of the food chain - Method validation - Part 6: Protocol for the validation of alternative (proprietary) methods for microbiological confirmation and typing procedures (ISO 16140-6:2019)

A method comparison study against reference method (2020)

An interlaboratory study (ILS) (2021)

SELECTION OF STRAINS

Number of strains to analyse per participant and per method:

Genus level confirmation method:

16 target strains (jejuni/coli/lari)
8 non-target strains
= 24 strains

PCR 1

Species level identification methods:

16 *C. jejuni*, 16 *C. coli*, 10 *C. lari*, *2 C. upsaliensis* (PCR 2)
8 non-target strains
= 50 strains

PCR 2
PCR 3

Selection of strains

Target strains - positive by ref. and the three PCR methods

Non-target strains - negative by ref. and the three PCR methods

Growth on mCCD agar and 41.5°C in microaerobic atmosphere

NON-TARGET STRAINS USED IN THE TEST

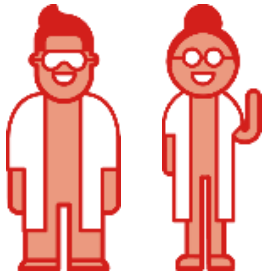
Species	Source
Campylobacter hyointestinalis	Pig (feaces)
Campylobacter hyointestinalis	Cattle farm (sock sample)
Campylobacter lanienae	Pig (faeces)
Campylobacter helveticus	Cat (faeces)
Escherichia coli	Chicken (caecum)
Acinetobacter baumannii	Chicken (caecum)
Candida rugosa	Chicken (caecum)
Pseudomonas aeruginosa	Type strain (human blood)

PREPARATIONS OF STRAINS

All selected strains were:

- freeze-dried to high concentration
- tested for growth at 41.5°C, on non-selective agar and mCCDA
- tested with the ref. and the PCR methods three weeks before and six weeks after send-off.
- stored at -70°C until send-off.

INVITATION TO ILS



- Minimum 10 valid datasets per method from different participants needed (according to ISO 16140-6).
- Experts in WG3 *Campylobacter* and all NRL-*Campylobacter* were invited to participate in March (voluntary).
- Whenever possible, the study conditions should reflect the normal variation between laboratories, so the participants were allowed to use own reagents.
- The participants were allowed to participate in a subset of methods.
- We welcomed participants that run these PCRs routinely and also those that don’t.
- Offered to send reagents for the PCR assays if required for participation.

PARTICIPANTS

- PCR 1 - 14 laboratories from 13 different countries registered for participation
- PCR 2 - 15 laboratories from 14 different countries registered for participation
- PCR 3 - 15 laboratories from 14 different countries registered for participation

PREPARATIONS OF THE ILS

- Collected information from participants on PCR instrument, IAC and preferences for fluorophores

qPCR instrument	Primers	Probes	IAC -ntb2	camp probe	ntb2 probe	reagents	comments	Primers	reagen	comments	Primers	Probes	IAC	jejuni prob	coli probe	lari probe	probe	reagent	comments
Biorad CFX 96	1	1	1	FAM	HEX	1		1	1		1	1	1	FAM	Cy5	ROX	HEX	1	
Biorad CFX 96	1	1	1	FAM	HEX	0		1	0		1	1	1	FAM	CY5	ROX	HEX	0	
	0	0	0			0		0	0		0	0	0					0	
	0	0	0			0		0	0		0	0	0					0	
	0	0	0			0		1	1		0	0	0					0	
ABI 7500	1	1	1	FAM	JOE	1		0	0		1	1	1	FAM	Cy5	TAMRA	JOE	1	
Biorad CFX 96	1	1	0	FAM		0	VIC for own IAC	1	0		1	1	0	FAM	Cy5	ROX		0	VIC for own IAC
Biorad CFX 96	1	1	0	FAM		0	Texas red For o	1	0		1	1	0	FAM	Cy5	TAMRA?		0	Texas red for own IAC
Agilent Stratagene MX3000	1	1	1	FAM	HEX	1		1	1	also need generul	1	1	1	FAM	Cy5	TAMRA	HEX	1	
ABI 7500	1	1	0	FAM		1	Cy3, Cy5 for IAC	1	1		1	1	0	FAM	HEX	TAMRA		1	Cy3, Cy5 for own IAC
ABI 7500	1	1	0	FAM		0	Cy5 for IAC	1	0	only lari primers	1	1	0	FAM	HEX	TAMRA		0	Cy5 for own IAC
	0	0	0			0		1	0	only lari primers. r	0	0	0					0	
iCycler iQ5 thermocycler (BioRad)	1	1	1	FAM	JOE	1		1	0		1	1	1	FAM	Cy5	TAMRA	JOE	1	
Biorad CFX 96	1	1	1		HEX	0	only primers an	0	0		1	1	1				HEX	0	only primers and probe for IAC
ABI 7500	1	1	1	FAM	JOE	1		0	0		1	1	1	FAM	Cy5	TAMRA	JOE	1	
Quantstudio 5	1	1	1	FAM	JOE	1		0	0		1	1	1	FAM	Cy5	TAMRA	JOE	1	
Biorad CFX 96	0	0	0			0		0	0		1	1	1	FAM	Cy5	ROX	HEX	1	
Biorad CFX 96	1	1	1	FAM	HEX	1		0	0		0	0	0						
	13	13	9	12		8	0	10	4	0	13	13	9				0	8	

PREPARATIONS OF THE ILS

- A shipping item list to each participant was created

SHIPPING ITEM LIST

Country: - **Laboratory:** - **Contact:**

	Label	Material	Concentration	Volume	PCR no.	Box no.	Storage temp.
Primers	Jos-F1	Josefsen Forward Primer	100 µM	15 µl	1	1	-20°C
	Jos-R1	Josefsen Reverse Primer	100 µM	15 µl	1	1	-20°C
	IPC-ntb2-fw	IAC forward primer	100 µM	15 µl	1 and 3	1	-20°C
	IPC-ntb2-re	IAC forward primer	100 µM	15 µl	1 and 3	1	-20°C
Probes	Jos-P-FAM	Josefsen Probe - FAM reporter	100 µM	10 µl	1	1	-20°C
	IPC-ntb2-p-HEX	IAC probe - HEX reporter	100 µM	10 µl	1 and 3	1	-20°C
Other components	IPC-ntb2 plasmid	IPC-ntb2 plasmid, stabilized and air-dried	$2x10^8$ copies	1 tube	1 and 3	1	RT
	Salmon Sperm DNA	UltraPure Salmon Sperm DNA solution (Thermo Sci. 15632011)	10 ng/ml	1.4 ml	1 and 3	1	4°C
	Water, Nuclease free	Thermo Nuclease free water (Thermo Sci. R0581)		2x 1.25 ml	All	1	-20°C
	dNTPs mix	dNTPs mix (Thermo Sci. R0192)	10 mM each	135 µl	All	1	-20°C
	$MgCl_2$	MgCl2 (Thermo Sci. R0971)	25 mM	1.25 ml	All	1	-20°C
	PCR buffer	Invitrogen Platinum Taq PCR buffer (Thermo Sci. 10966034)	10x	125 µl	All	1	-20°C
	Platinum Taq Polymerase	Invitrogen Platinum Taq polymerase (Thermo Sci. 10966034)	10 U/µl	5 µl	All	1	-20°C
	Strains 1-25	Strains 1-25, freeze-dried in vials		25 vials	All	Jar 1	-20°C

Primers and probes - Dilution table

Dilution	H20 volume	Working conc.	Volume Per 25 µl reaction	Conc. per reaction
1:10	135 µl	10 µM	1.25 µl	0.5 µM
1:10	135 µl	10 µM	1.25 µl	0.5 µM
1:10	135 µl	10 µM	0.75 µl	0.3 µM
1:10	135 µl	10 µM	0.75 µl	0.3 µM
1:10	90 µl	10 µM	0.25µl	0.1 µM
1:10	90 µl	10 µM	0.25µl	0.1 µM

Upon arrival all components should immediately be stored at the storage temperature indicated in the Shipping item list. Note that all components should be stored at -20°C except the air-dried IPC-ntb2 plasmid (RT) and the Salmon Sperm DNA solution (4°C) in box 1.

Template France Portugal Croatia Czech Italy Hungary Albania Slovakia Spain 1 Poland Rumania Rumania (2) USA Spain 2 Kosova Greece Sweden Germany 1 Germany 2

PREPARATIONS OF THE ILS

- Ordered all reagents in April
- Invitrogen Taq polymerase tested for stability (RT 4 days) for PCR 1 and 2 = ok
- The EURL performed the whole test end of April = ok, except one C. *upsaliensis* strain neg for PCR 2.
- On May 17, the ILS test packages were sent from SVA, Sweden, with ice packs and T-log to 19 participants.
- The test included a detailed SOP, a result table, randomley numbered freeze-dried strains, and reagents for the PCR assays if required. Provided instructions on how to store vials and reagents.

METHODOLOGY

PCR system	Scope	Reference method
PCR 1: Josefsen et al. 2004 qPCR	Confirmation (*C. jejuni*, *C. coli* and *C. lari*)	Morphology, motility, aerobic growth at 25°C and oxidase activity (Table 1)
PCR 2: Wang et al. 2002 Conventional PCR	Confirmation and species id (*C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis)*	Table 1 plus catalase activity, hippurate hydrolysis and indoxyl acetate hydrolysis tests (Table 2)
PCR 3: Mayr et al. 2010 qPCR	Confirmation and species id (*C. jejuni*, *C. coli*, *C. lari)*	Table 1 plus catalase activity, hippurate hydrolysis and indoxyl acetate hydrolysis tests (Table 2)

Table 1 — Characteristics of *Campylobacter*

Morphology (9.5.3)	Small curved bacilli[a]
Motility (9.5.3)	Characteristic corkscrew darting[a]
Aerobic growth at 25 °C (9.5.4)	–
Oxidase activity (9.5.5)	+

\+ Positive.

– Negative.

[a] Older cultures may rapidly lose their characteristic shape and motility and turn into less motile coccoid forms.

Table 2 — Characteristics of *Campylobacter* species

Characteristic	*C. jejuni*	*C. coli*	*C. lari*	*C. upsaliensis*
Catalase activity (9.6.2)	+	+	+	– or weak
Hippurate hydrolysis (9.6.3)	+[a]	–	–	–
Indoxyl acetate hydrolysis (9.6.4)	+	+	–	+

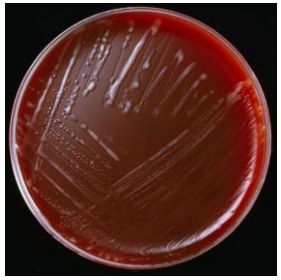
\+ Positive.

– Negative.

[a] Some hippurate-negative *C. jejuni* strains have been reported.

TEST PROCEDURES

1 ml
e.g BPW



non-selective agar
41.5 °C for 24 h – 48 h

mCCDA
41.5 °C for 44 h ± 4 h
to confirm growth

Tests according to
ISO 10272:2017

DNA extraction
through boiling

PCR 1
PCR 3
1:100 dilution

PCR 2
undiluted
1:50 dilution if only one band

pos and neg controls in all test
also IAC in qPCR (Cq 32-38)

SUBMISSION OF RESULTS

- Participants submitted filled-in result table
  - ➢ Control questions to make sure SOP and ISO 10272 was followed
  - ➢ Control questions to make sure biochemical tests were valid

- Participants submitted raw data (gel pictures, data from qPCR - evaluate curves)

EXCLUDED DATA

- Strains that grew poorly or not at all (mainly non-target *Campylobacter* spp.)
- Some strains did not grow in 41.5°C but in 37°C in some laboratories (considered neg)
- Incomplete or missing data for biochemical tests or PCR assays
- Deviations to the SOP
- Improper handling of the controls (e.g controls not run at the same time as the PCR test, or pos controls being neg and neg controls being pos)
- One of the two *C. upsaliensis* strains gave negative result in PCR 2 in the final test by EURL

ANALYSIS AND INTERPRETATION OF DATA

Indoxyl acetate test – weak and late color change (after 10 min) = pos

pPCR (PCR 1 and PCR 3) – with typical amplification curve and Cq <40 = pos

PCR 2 – both 23S band and species specific band = pos

SUMMARY OF THE RESULTS

IA : inclusivity agreement
ID : inclusivity deviation
EA : exclusivity agreement
ED : exclusivity deviation

PCR 1

	Number of strains	IA	ID	EA	ED
Inclusivity	200	199	1	Not applicable	Not applicable
Exclusivity	82	Not applicable	Not applicable	82	0

PCR 2

Target		Number of strains	IA	ID	EA	ED
C. jejuni	Inclusivity	147	145	2	Not applicable	Not applicable
C. jejuni	Exclusivity	295	Not applicable	Not applicable	295	0
C. coli	Inclusivity	149	149	0	Not applicable	Not applicable
C. coli	Exclusivity	293	Not applicable	Not applicable	293	0
C. lari	Inclusivity	64	64	0	Not applicable	Not applicable
C. lari	Exclusivity	330	Not applicable	Not applicable	330	0
C. upsaliensis	Inclusivity	9	9	0	Not applicable	Not applicable
C. upsaliensis	Exclusivity	433	Not applicable	Not applicable	433	0

PCR 3

Target		Number of strains	IA	ID	EA	ED
C. jejuni	Inclusivity	150	150	0	Not applicable	Not applicable
C. jejuni	Exclusivity	291	Not applicable	Not applicable	290	1
C. coli	Inclusivity	149	149	0	Not applicable	Not applicable
C. coli	Exclusivity	292	Not applicable	Not applicable	292	0
C. lari	Inclusivity	73	73	0	Not applicable	Not applicable
C. lari	Exclusivity	368	Not applicable	Not applicable	368	4

AL – Acceptability Limit = 2 (Maximum positive or negative acceptable difference)

On behalf of WG3 – thank you for your participation!

Acknowledgement: Experts in CEN/TC 463/WG3 *Campylobacter* and Ute Messelhäuser at Bavarian Health and Food Safety Authority in Germany