

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

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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)^[47] 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.

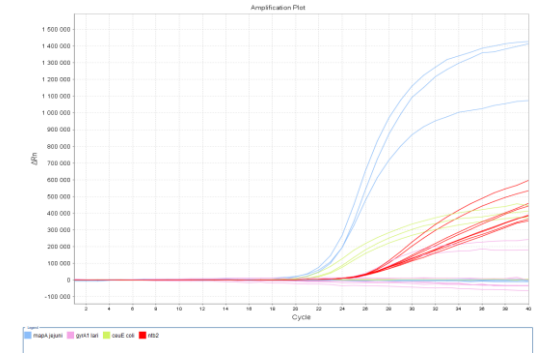
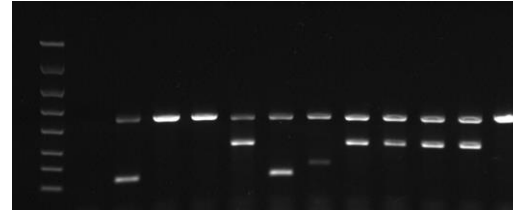
Molecular methods as alternative to biochemical tests for confirmation and species identification of *Campylobacter* spp.

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

<u>confirmation method:</u>	16 target strains (jejuni/coli/lari)	}	PCR 1
	8 non-target strains		
	= 24 strains		

Species level

<u>identification methods:</u>	16 <i>C. jejuni</i> , 16 <i>C. coli</i> , 10 <i>C. lari</i> ,	}	PCR 2 PCR 3
	2 <i>C. upsaliensis</i> (PCR 2)		
	8 non-target strains		
	= 50 strains		

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

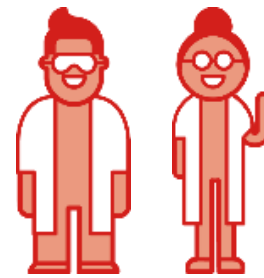
PREPARATIONS OF STRAINS



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	reagent	comments	Primers	Probes	IAC	jejun 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 generu	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	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

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	SHIPPING ITEM LIST												
2	Country: - Laboratory: - Contact:												
3									Primers and probes - Dilution table				
4		Label	Material	Concentration	Volume	PCR no.	Box no.	Storage temp.	Dilution	H2O volume	Working conc.	Volume Per 25 µl reaction	Conc. per reaction
5	Primers	Jos-F1	Josefsen Forward Primer	100 µM	15 µl	1	1	-20°C	1:10	135 µl	10 µM	1.25 µl	0.5 µM
6		Jos-R1	Josefsen Reverse Primer	100 µM	15 µl	1	1	-20°C	1:10	135 µl	10 µM	1.25 µl	0.5 µM
7		IPC-ntb2-fw	IAC forward primer	100 µM	15 µl	1 and 3	1	-20°C	1:10	135 µl	10 µM	0.75 µl	0.3 µM
8		IPC-ntb2-re	IAC forward primer	100 µM	15 µl	1 and 3	1	-20°C	1:10	135 µl	10 µM	0.75 µl	0.3 µM
9													
10	Probes	Jos-P-FAM	Josefsen Probe - FAM reporter	100 µM	10 µl	1	1	-20°C	1:10	90 µl	10 µM	0.25µl	0.1 µM
11		IPC-ntb2-p-HEX	IAC probe - HEX reporter	100 µM	10 µl	1 and 3	1	-20°C	1:10	90 µl	10 µM	0.25µl	0.1 µM
12													
13	Other components	IPC-ntb2 plasmid	IPC-ntb2 plasmid, stabilized and air-dried	2x10 ⁸ copies	1 tube	1 and 3	1	RT					
14		Salmon Sperm DNA	UltraPure Salmon Sperm DNA solution (Thermo Sci. 15632011)	10 ng/ml	1.4 ml	1 and 3	1	4°C					
15		Water, Nuclease free	Thermo Nuclease free water (Thermo Sci. R0581)		2x 1.25 ml	All	1	-20°C					
16		dNTPs mix	dNTPs mix (Thermo Sci. R0192)	10 mM each	135 µl	All	1	-20°C					
17		MgCl ₂	MgCl ₂ (Thermo Sci. R0971)	25 mM	1.25 ml	All	1	-20°C					
18		PCR buffer	Invitrogen Platinum Taq PCR buffer (Thermo Sci. 10966034)	10x	125 µl	All	1	-20°C					
19		Platinum Taq Polymerase	Invitrogen Platinum Taq polymerase (Thermo Sci. 10966034)	10 U/µl	5 µl	All	1	-20°C					
20		Strains 1-25	Strains 1-25, freeze-dried in vials		25 vials	All	Jar 1	-20°C					
21													
22	<i>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.</i>												
23													
24													
25													

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, randomly 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 (<i>C. jejuni</i> , <i>C. coli</i> and <i>C. lari</i>)	<i>C.</i> Morphology, motility, aerobic growth at 25°C and oxidase activity (Table 1)
PCR 2: Wang et al. 2002 Conventional PCR	Confirmation and species id (<i>C. jejuni</i> , <i>C. coli</i> , <i>C. lari</i> and <i>C. upsaliensis</i>)	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 (<i>C. jejuni</i> , <i>C. coli</i> , <i>C. lari</i>)	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.	

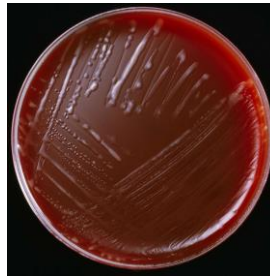
Table 2 — Characteristics of *Campylobacter* species

Characteristic	<i>C. jejuni</i>	<i>C. coli</i>	<i>C. lari</i>	<i>C. upsaliensis</i>
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 <i>C. jejuni</i> 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 $C_q < 40$ = 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
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