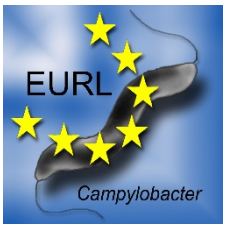

PROTOCOL FOR IDENTIFICATION OF *C. JEJUNI*, *C. COLI* AND *C. LARI* BY REAL-TIME PCR

April 2021

Version 1

History of changes			
Version	Sections changed	Description of the change	Date
Version 1		Text edits. This protocol is aligned with the SOP for the interlaboratory study performed 2021 to validate the method.	2021-04-21
Draft	New draft document	-	2020-11-02

Content

1. SCOPE OF THE METHOD

This protocol describes a probe-based multiplex real-time PCR assay for confirmation and identification of thermotolerant *Campylobacter* spp. *C. jejuni*, *C. coli* and *C. lari*.

2. REFERENCES

This protocol is based on ISO/DIS 10272:2017/Amd1 [1], Mayr et al. 2010 [2] and Pacholewicz et al. 2019 [3].

3. PROTOCOL

3.1. DNA extraction

Transfer one sterile loop with approximately 1 µl colony material into 1 ml of 0.1xTE buffer (10 mM Tris and 0.1 mM EDTA, pH 8.0) and try to make a homogeneous suspension. Extract DNA with a thermal lysis step (15 min at 95 °C). After centrifugation for 3 min at 10 000 x g, the supernatant is used as DNA template. If the PCR analysis is not run on the same day, the template shall be stored at -20 °C.

3.2. PCR set-up

Prepare the master mix as described in Table 2 using the primers described in Table 1. The DNA template should be diluted 1:100 in sterile water.

3.3. Controls

Suitable positive and negative controls shall be included in each test, besides the internal amplification control. It is advisable to have a positive control generating a Cq in the range 20–35 and in negative controls, the internal amplification control should generate Cq values between 32–38.

Table 1. Description of oligonucleotides.

Species (gene)	**Primer/probe**	**Sequence (5´ — 3´)**
C. jejuni (mapA)	Primer mapA-fw	CTG GTG GTT TTG AAG CAA AGA TT
	Primer mapA-re	CAA TAC CAG TGT CTA AAG TGC GTT TAT
	Probe mapA	FAM[a]-TTG AAT TCC AAC ATC GCT AAT GTA TAA AAG CCC TTT-NFQ[b]
C. coli (ceuE)	Primer ceuE-fw	AAG CTC TTA TTG TTC TAA CCA ATT CTA ACA
	Primer ceuE-re	TCA TCC ACA GCA TTG ATT CCT AA
	Probe ceuE	Cy5[a]-TTG GAC CTC AAT CTC GCT TTG GAA TCA TT-NFQ[b]
C. lari (gyrA)	Primer gyrA1-fw1	GAT AAA GAT ACG GTT GAT TTT GTA CC
	Primer gyrA1-fw2[c]	GAT AAA GAT ACA GTT GAT TTT ATA CC
	Primer gyrA1-re1	CAG CTA TAC CAC TTG ATC CAT TAA G
	Primer gyrA1-re2	TGC AAT ACC ACT TGA ACC ATT A
	Probe gyrA1	TAMRA[a]-TTA TGA TGA TTC TAT GAG TGA GCC TGA TG-NFQ[b]
Internal Amplification Control (IAC)[d]	IPC-ntb2-fw	ACC ACA ATG CCA GAG TGA CAA C
	IPC-ntb2-re	TAC CTG GTC TCC AGC TTT CAG TT
	IPC-ntb2-probe	JOE[a]-CAC GCG CAT GAA GTT AGG GGA CCA-NFQ[b]

[a] FAM, Cy5, ROX and HEX or FAM, Cy5, TAMRA and JOE were used in the method comparison study. The alternative combination FAM, TEXAS-RED, Cy5 and HEX have been used with equivalent results. Other reporter dyes and/or quencher dyes may be used if appropriate.

[b] NFQ: Non-fluorescence quencher (dark quencher).

[c] There is one nucleotide change in the primer compared to reference [4].

[d] Other internal amplification control systems can be used.

Table 2. Reagents.

Reagent	**Final concentration**	**Volume per sample (µl)**
Template DNA (1:100 dilution)	Maximum 250 ng	2,5 µl
PCR grade water	---	As required
PCR-buffer without $MgCl_2$[a,b]	1 x	As required
$MgCl_2$ solution[a]	3 mM	As required
dNTP solution[a]	0,25 mM of each dNTP	As required
PCR primers	300 nM of each primer	As required
PCR probes	100 nM of each probe	As required
IPC-ntb2-plasmid[c]	25 copies per reaction	As required
Hot Start *Taq* DNA Polymerase[a]	1 U	As required
Total volume	----	25

[a] TaqMan Universal PCR Master Mix (Thermo Fisher Scientific), PerfeCTa Multiplex qPCR ToughMix (Quantabio) and Platinum *Taq* DNA Polymerase (Thermo Fisher Scientific) have been used with similar results.

[b] If the PCR buffer solution already contains $MgCl_2$, the final concentration of $MgCl_2$ in the reaction mixture is adjusted to 3mM.

[c] Or use another internal amplification control system if implemented in the laboratory.

3.4. Amplification

Any well-maintained and calibrated cycler instrument can be used as long as it is appropriate for the method. Use the amplification program described in Table 3.

Table 3. Temperature-time program

Activation/initial denaturation[a]	3 min/95 °C
Number of cycles (amplification)	45
Amplification	30 s/94 °C
	45 s/60 °C
	30 s/72 °C
[a] Use an initial denaturation time appropriate for the enzyme	

3.5. Results

The threshold value to determine the cycle quantification value (Cq) can be defined by the analyst or by the cycler-specific software. A positive sample generates an amplification plot with at least the exponential phase of a typical amplification curve, see ISO 22119 [4]. The amplification curve of these samples crosses the defined threshold setting after a certain number of cycles. In this setting, DNA from isolates is used. Therefore, a sample with a fluorescence signal above the threshold and with a Cq value below 40 is considered positive. The internal amplification control might not generate a Cq value when the Cq of target is very low due to inevitable cross-inhibition of PCR amplification.

[1] ISO/CD 10272:2017/Amd1 "Amendment 1 of Microbiology of food and animal feeding stuffs – Horizontal method for detection and enumeration of *Campylobacter* spp. Part 1: detection method and Part 2: Colony-count technique".

[2] Mayr AM, Lick S, Bauer J, Thärigen D, Busch U, Huber I. 2010. Rapid detection and differentiation of Campylobacter jejuni, Campylobacter coli, and Campylobacter lari in food, using multiplex real-time PCR. J Food Prot. 73(2):241-250

[3] Pacholewicz, E., Buhler, C., Wulsten, I., Kraushaar, B., Luu, Q. H., Iwobi, A., Huber, I., Stingl, K. 2019. Internal sample process control improves cultivation-independent quantification of thermotolerant Campylobacter. Food Microbiology https://doi.org/10.1016/j.fm.2018.09.017

[4] EN ISO 22119: Microbiology of food and animal feeding stuffs — Real-time polymerase chain reaction (PCR) for the detection of food-borne pathogens — General requirements and definitions. International Organization for Standardization.