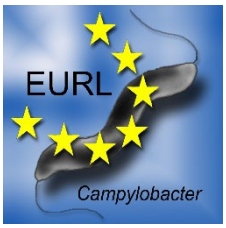

PROTOCOLS FOR IDENTIFICATION OF *C. JEJUNI*, *C. COLI*, *C. LARI* AND *C. UPSALIENSIS* BY GEL-BASED PCR

December 2022

Version 2

History of changes			
Version	Sections changed	Description of the change	Date
Version 2		Addition of a PCR protocol for *C. upsaliensis* and *C. helveticus* and performance evaluation of both protocols	2022.12.19
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

The protocol in section 3 describes a gel-based multiplex PCR assay for confirmation and identification of *Campylobacter jejuni*, *C. coli* and *C. lari*. It also targets *C. upsaliensis*, but it is not as specific for this species. The method both detects the 23S rRNA of *Campylobacterales* and specific species targets.

The protocol in section 4 describes a gel-based multiplex PCR assay for confirmation and identification of *C. upsaliensis* and *C. helveticus*. The method detects the 16S rRNA of *C. upsaliensis and C. helveticus*.

2. REFERENCES

The protocol in section 3 is from ISO 10272:2017/Amd1 [1] and based on Wang et al. 2002 [2] and Chaban et al. 2009 [3]. The protocol in section 4 is based on Lawson et al. 1997 [4].

3. PROTOCOL - MULTIPLEX PCR ASSAY FOR CONFIRMATION AND IDENTIFICATION OF *CAMPYLOBACTER JEJUNI, C. COLI* AND *C. LARI.*

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 is used undiluted for this PCR, but in case of only one band detected (species target or 23S target), dilute it and repeat the test.

Table 1. Description of oligonucleotides and amplicons.

Species (gene)	**Primer**	**Sequence** (5´ — 3´)	**Amplicon size (bp)**
C. jejuni (*hipO*)	CJF	ACT TCT TTA TTG CTT GCT GC	323
	CJR	GCC ACA ACA AGT AAA GAA GC	
C. coli (glyA)	CCF	GTA AAA CCA AAG CTT ATC GTG	126
	CCR	TCC AGC AAT GTG TGC AAT G	
C. lari (*cpn60*)[a]	JH0015	TCT GCA AAT TCA GAT GAG AAA A	180
	JH0016	TTT TTC AGT ATT TGT AAT GAA ATA TGG	
C. upsaliensis (glyA)	CUF	AAT TGA AAC TCT TGC TAT CC	204
	CUR	TCA TAC ATT TTA CCC GAG CT	

Campylobacterales (23S rRNA)	23SF	TAT ACC GGT AAG GAG TGC TGG AG	650
	23SR	ATC AAT TAA CCT TCG AGC ACC G	
[a] These primers, from reference [3], have replaced those in reference [2] since they detect both *C. lari* subsp. *lari and C. lari* subsp. *concheus*.			

Table 2. Reagents.

Reagent	**Final concentration**	**Volume per sample (µl)**
Template DNA	Maximum 250 ng	2,5
PCR grade water	---	As required
PCR-buffer (without $MgCl_2$)[a]	1 x	As required
$MgCl_2$ solution	2 mM	As required
dNTP solution	0,2 mM of each dNTP	As required
PCR primers *C. jejuni* and *C. lari*	0,5 µM of each primer	As required
PCR primers *C. coli*	1 µM of each primer	As required
PCR primers *C. upsaliensis*	2 µM of each primer	As required
PCR primers 23S rRNA	0,2 µM of each primer	As required
Taq DNA polymerase	1,25 U	As required
Total volume	----	25
[a] If the PCR buffer solution already contains $MgCl_2$, the final concentration of $MgCl_2$ in the reaction mixture is adjusted to 2 mM.		

3.3. 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
Amplification	30 s/95 °C
	30 s/59 °C
	30 s/72 °C
Number of cycles (amplification)	30
Final extension	7 min/72 °C
[a] Use an initial denaturation time appropriate for the enzyme.	

3.4. Electrophoresis

The amplified PCR products are detected using a 1,5 % agarose gel. For example, the GeneRuler 100 bp DNA ladder can be used for determination of size.

3.5. Results

The target sequences are detected if the sizes of the PCR product correspond to the expected length of the target DNA sequences (see Table 1). This should be determined using an appropriate DNA ladder and positive controls for each target. The detection of both species target and *Campylobacterales* 23S target are required for a positive result. If only one of the bands is detected, the analysis should be re-run with a dilution of the template DNA. If still not detecting both targets, the sample is considered negative for *C. jejuni*, *C. coli*, *C. lari* and *C. upsalinensis*.

3.6. Evaluation of performance

The protocol has been validated according to ISO 16140-6 by comparison to the phenotypical tests for confirmation and identification described in ISO 10272.

4. PROTOCOL - MULTIPLEX PCR ASSAY FOR CONFIRMATION AND IDENTIFICATION OF *C. UPSALIENSIS* AND *C. HELVETICUS*

4.1. 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.

4.2. PCR setup

Prepare the master mix as described in Table 5 using the primers described in Table 4. The DNA template is used undiluted for this PCR.

Table 4. Description of oligonucleotides and amplicons.

Species (gene)	**Primer**	**Sequence** (5´ — 3´)	**Amplicon size (bp)**
C. upsaliensis 16S rRNA	CHCU146F [a]	GGG ACA ACA CTT AGA AAT GAG	878
	CU1024R	CAC TTC CGT ATC TCT ACA GA	
C. helveticus 16S rRNA	CHCU146F [a]	GGG ACA ACA CTT AGA AAT GAG	1225 or 1375[b]
	CH1371R	CCG TGA CAT GGC TGA TTC AC	

[a]The same forward primer is used för both *C. upsaliensis* and *C. helveticus.*

[b]Two different sizes of the product can be obtained for *C. helveticus* due to presence of an atypical intervening sequence of 150 bp within the gene.

Table 5. Reagents.

Reagent	**Final concentration**	**Volume per sample (µl)**
Template DNA	Maximum 250 ng	2,5
PCR grade water	---	As required
PCR-buffer (without $MgCl_2$)[a]	1 x	As required
$MgCl_2$ solution	2,5 mM	As required
dNTP solution	0,2 mM of each dNTP	As required
PCR forward primer *C. upsaliensis* and *C. helveticus*	0,4 µM	As required
PCR reverse primer *C. upsaliensis*	0,4 µM	As required
PCR reverse primer *C. helveticus*	0,4 µM	As required
Taq DNA polymerase	1,25 U	As required
Total volume	----	25

[a] If the PCR buffer solution already contains $MgCl_2$, the final concentration of $MgCl_2$ in the reaction mixture is adjusted to 2,5 mM.

4.3. 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 6.

Table 6. Temperature-time program.

Activation/initial denaturation[a]	3 min/95 °C
Amplification	30 s/94 °C
	30 s/58 °C
	30 s/72 °C
Number of cycles (amplification)	30
Final extension	7 min/72 °C
[a] Use an initial denaturation time appropriate for the enzyme.	

4.4. Electrophoresis

The amplified PCR products are detected using a 1-1,5 % agarose gel. For example, the GeneRuler 100bp plus or GeneRuler 1kb DNA ladder can be used for determination of size.

4.5. Results

The target sequences are detected if the sizes of the PCR product correspond to the expected length of the target DNA sequences (see Table 4). This should be determined using an appropriate DNA ladder and positive controls for each target.

4.6. Performance evaluation

The same *C. upsaliensis* (33) and *C. helveticus* (4) strains used in the validation study of the protocol in section 3, were used to test the performance of the Lawson et al., 1997 protocol. The protocol correctly identified all strains with no false positives.

5. REFERENCES

[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] Wang G, Clark CG, Taylor TM, Pucknell C, Barton C, Price L, Woodward DL, Rodgers FG. 2002. Colony multiplex PCR assay for identification and differentiation of *Campylobacter jejuni*, *C. coli, C. lari*, *C. upsaliensis*, and *C. fetus* subsp. *fetus*. J Clin Microbiol. 40(12):4744-4747

[3] Chaban B, Musil KM, Himsworth CG, Hill JE. 2009. Development of cpn60-based real-time quantitative PCR assays for the detection of 14 *Campylobacter* species and application to screening of canine fecal samples. Appl Environ Microbiol. 75(10):3055-61. doi: 10.1128/AEM.00101-09.

[4] Lawson AJ, Linton D, Stanley J, Owen RJ. 1997. Polymerase chain reaction detection and speciation of *Campylobacter upsaliensis* and *C. helveticus* in human faeces and comparison with culture techniques. Journal of Applied Microbiology. 83: 375-380.