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AMENDMENT 1
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Microbiology of the food chain — Horizontal method for detection and enumeration of *Campylobacter* spp. —

Part 2: Colony-count technique

AMENDMENT 1: Inclusion of methods for molecular confirmation and identification of thermotolerant *Campylobacter* spp. and changes in the performance testing of culture media

Microbiologie de la chaîne alimentaire — Méthode horizontale pour la recherche et le dénombrement de Campylobacter spp. —

Partie 2: Technique par comptage des colonies

AMENDEMENT 1: Ajout de méthodes pour la confirmation et l'identification moléculaires de Campylobacter spp. thermotolérants, et modification des essais de performance des milieux de culture

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This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology,* in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 463, *Microbiology of the food chain*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

A list of all the parts in the ISO 10272 series can be found on the ISO website.

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Microbiology of the food chain — Horizontal method for detection and enumeration of *Campylobacter* spp. —

Part 2: Colony-count technique

AMENDMENT 1: Inclusion of methods for molecular confirmation and identification of thermotolerant *Campylobacter* spp. and changes in the performance testing of culture media

3.1

Replace the text with the following:

3.1
Campylobacter
genus of microorganisms of the family *Campylobacteraceae*, forming characteristic colonies on solid selective media, such as modified Charcoal Cefoperazone Deoxycholate (mCCD) agar, when incubated in a microaerobic atmosphere at 41,5 °C and displaying certain characteristics with biochemical confirmation tests and by microscopy

Note 1 to entry: Microscopy, the biochemical confirmation tests and the characteristics of *Campylobacter* are described in 9.4.

Note 2 to entry: This document targets the thermotolerant *Campylobacter* species relevant to human health. The most frequently encountered and relevant to human health are *Campylobacter jejuni* and *Campylobacter coli*. However, other species have been described (*Campylobacter lari, Campylobacter upsaliensis* and others).

Note 3 to entry: *Campylobacter* is usually capable of growth in the selective enrichment media Bolton broth and Preston broth.

9.4.1

Add the following text after the last paragraph:

NOTE PCR tests for confirmation and species identification are described in Annexes D and E. The results for the ILS study are described in Annex F.

9.5.1, second sentence

Replace the text with the following:

However, other species have been described *(Campylobacter lari*, *Campylobacter upsaliensis* and others); the characteristics given in Table 2 permit their differentiation from *Campylobacter jejuni* and *Campylobacter coli*.

9.5.1

Add the following text as the second paragraph:

Additionally, Annex E describes molecular methods for identification of thermotolerant *Campylobacter* species, which can be used as an alternative to the biochemical identification described in 9.5.2 to 9.5.5.

9.5.4, second paragraph

Replace the text with the following:

If the indoxyl acetate is hydrolysed, a colour change to blue occurs within 5 min to 10 min. If there is an unclear result after 10 min, a better result can be obtained after waiting for another 20 min. No colour change indicates hydrolysis has not taken place.

9.5.5, Table 2

Replace the table with the following:

Characteristic	*C. jejuni*	*C. coli*	*C. lari*[b]	*C. upsaliensis*[b]
Catalase (9.5.2)	+	+	+	– or weak
Hydrolysis of hippurate (9.5.3)	+[a]	–	–	–
Indoxyl acetate (9.5.4)	+	+	–	+[c]

Key

\+ = positive

– = negative

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

[b] The same characteristics can appear also for other *Campylobacter* spp.

[c] Indoxyl acetate negative *C. upsaliensis* strains have been reported.

11.1

Add the following text after the first sentence:

The results have been published, see Reference [12].

Clause B.2

Replace the text with the following:

See the ISO 6887 series.

Clause B.9, Table B.1

Replace the table with the following:

Table B.1 — Performance testing of culture media for *Campylobacter*

Medium	Function	Incubation	Control strains	WDCM numbers[a]	Reference media	Method of control	Criteria[b]	Characteristic reactions of target microorganism
mCCD agar	Productivity	(44 ± 4) h/ (41,5 ± 1) °C microaerobic atmosphere	*Campylobacter jejuni*[c] *Campylobacter coli*[c]	00156 or 00005 00004 or 00072	Blood agar	Quantitative	$P_R \geq 0,5$	Greyish, flat and moist, sometimes with metallic sheen
	Selectivity		*Escherichia coli*[c]	00012 or 00013	—	Qualitative	Total or partial inhibition (0 to 1)	No characteristic colonies
			Staphylococcus aureus[c]	00032 or 00034	—	Qualitative	Total inhibition (0)	—
Columbia blood agar	Confirmation	24 h to 48 h/ (41,5 ± 1) °C microaerobic atmosphere	*Campylobacter jejuni*[d] or *Campylobacter coli*[d]	00156 or 00005 or 00004 or 00072	—	Qualitative	Good growth (2)	—

[a] WDCM: World Data Centre for Microorganisms. Refer to the reference strain catalogue available at www.wfcc.info for information on culture strain numbers and contact details.[10]

[b] Growth is categorized as 0: no growth; 1: weak growth; 2: good growth, P_R = productivity ratio (see ISO 11133).

[c] Strain free of choice, one of the strains has to be used as a minimum.

[d] Strain free of choice, one of the *Campylobacter* strains has to be used as a minimum.

After Annex C

Add the following as Annexes D, E and F:

Annex D
(informative)

Multiplex real-time PCR assay for confirmation of thermotolerant *Campylobacter* spp.

D.1 General

This annex describes a probe-based multiplex real-time PCR for confirmation of thermotolerant *Campylobacter* spp. (C. *jejuni, C. coli, C. lari*).

D.2 Principle

A specific fragment of the 16S rRNA of thermotolerant *Campylobacter* spp. *C. jejuni, C. coli, C. lari* is amplified by multiplex real-time PCR. The PCR product is detected by measuring fluorescence of the hydrolysed probe.

D.3 Reagents

For quality of reagents used, see ISO 22174[13]. Ready-to-use reagents can be commercially available. The manufacturer's instructions for use should be considered.

D.3.1 Reagents for nucleic acid extraction

D.3.1.1 NaCl, 0,9 % (mass fraction).

D.3.1.2 PCR grade water.

D.3.1.3 TE-buffer.

D.3.2 Reagents for real-time PCR

D.3.2.1 PCR grade water.

D.3.2.2 PCR buffer solution, 10×.

NOTE 10× means 10-fold, i.e. the concentration of the PCR buffer.

The PCR buffer solution is usually delivered with the DNA polymerase, which may or may not include $MgCl_2$ in a concentration specified by the manufacturer. The final $MgCl_2$ concentration is method specific and therefore listed in Table D.2 (see D.5.2).

D.3.2.3 $MgCl_2$ solution.

D.3.2.4 Thermostable *Taq* DNA polymerase (for hot-start PCR).

D.3.2.5 dNTP solution.

D.3.2.6 Oligonucleotides.

Sequences of the oligonucleotides are listed in Table D.1.

D.3.2.7 IPC-ntb2 plasmid.

A vector plasmid carrying a 125-bp sequence of the gene *rbcMT-T* encoding Ribulose-1,5-bisphosphate carboxylase/oxygenase N-methyltransferase from *Nicotiana tabacum*.[14] The plasmid is used as an internal amplification control.[1)]

Table D.1 — Sequences of oligonucleotides

Gene	Primer/probe	Sequence (5′ — 3′)
16S rRNA	Jos-F1 (forward)	CCT GCT TAA CAC AAG TTG AGT AGG
	Jos-R1 (reverse)	TTC CTT AGG TAC CGT CAG AAT TC
	Jos-P (probe)	FAM[a]- TGT CAT CCT CCA CGC GGC GTT GCT GC-NFQ[b]
Internal amplification control (IAC)	IPC-ntb2-fw (forward)	ACC ACA ATG CCA GAG TGA CAA C
	IPC-ntb2-re (reverse)	TAC CTG GTC TCC AGC TTT CAG TT
	IPC-ntb2-probe (probe)	ROX[a]-CAC GCG CAT GAA GTT ACG GGA CCA-NFQ[b]

[a] Equivalent reporter dyes and/or quencher dyes may be used for the probes if they can be shown to yield similar or better results. The alternative combinations FAM-HEX, FAM-TAMRA, FAM-JOE and FAM-Cy5 have been used with equivalent result in the validation of the method.

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

D.4 Apparatus

Appropriate equipment according to the method and, in particular, the following shall be used.

D.4.1 Equipment used for nucleic acid extraction

D.4.1.1 Microcentrifuge tubes, of capacities of 1,5 ml and 2,0 ml.

D.4.1.2 Thermo block, obtaining a temperature of 95 °C.

D.4.1.3 Pipettes and pipette filter tips, for volumes between 1 µl and 1 000 µl.

D.4.1.4 Centrifuge, for microcentrifuge tubes having a capacity of 1,5 ml and 2,0 ml, e.g. microcentrifuge, capable of achieving an acceleration of up to 12 000*g*. In some steps a refrigerated centrifuge is required.

D.4.2 Equipment used for real-time PCR

D.4.2.1 Pipettes and pipette filter tips, having a capacity between 1 µl and 1 000 µl.

D.4.2.2 Microcentrifuge tubes, having a capacity of 1,5 ml and 2,0 ml.

D.4.2.3 Thin-walled PCR microtubes, 0,2 ml or 0,5 ml reaction tubes, multi-well PCR microplates or other suitable consumables.

D.4.2.4 Real-time PCR instrument.

1) The plasmid IPC-ntb2 was used as an internal amplification control in the validation study of the PCR system. This information is given for convenience of users of this document and does not constitute an endorsement by ISO of the product named. Alternative internal amplification control systems may be used if they can be shown to give equivalent or better results. If necessary, adapt the amounts of the reagents and the temperature-time programme.

D.5 Procedure

D.5.1 Nucleic acid extraction

One 1 µl-loop of suspected colonies (see 9.5.2) is suspended in 1 ml of 0,9 % NaCl solution and DNA is extracted with a thermal lysis step (15 min at 95 °C). After an additional centrifugation step for 3 min at 10 000*g*, 5 µl of the supernatant is used as DNA template. If the DNA will be stored, TE-buffer should be used instead of 0,9 % NaCl. Other methods for DNA extraction can be used if they have been shown to be suitable. Before addition to the PCR mastermix, the template should be 100-fold diluted in sterile water.

D.5.2 PCR set-up

The method is described for a total PCR volume of 25 µl per reaction with the reagents as listed in Table D.2. The PCR can also be carried out in a larger volume if the solutions are adjusted appropriately. The final concentrations of reagents as outlined in Table D.2 have proven to be suitable.

Table D.2 — Reagents

Reagent	Final concentration	Volume per sample µl
Template DNA (1:100 dilution)	maximum 250 ng	2,5 µl
Taq DNA Polymerase[a]	1 IU	as required
PCR-buffer (without $MgCl_2$)[b]	1×	as required
$MgCl_2$ solution	2,5 mM	as required
dNTP solution	0,2 mM of each dNTP	as required
PCR primers (according to Table D.1)	500 nM each primer	as required
PCR probes (according to Table D.1)	100 nM each probe	as required
PCR grade water	—	as required
IPC-ntb2 plasmid	25 copies per reaction	as required
Total volume	—	25

[a] Hot Start *Taq* DNA Polymerase was used in the validation of the method.

[b] 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.

D.5.3 PCR controls

In accordance with ISO 22174[13] the following controls are necessary:

a) Negative PCR control: PCR grade water is used as negative control.

b) Positive PCR control: DNA from *C. jejuni*, *C. coli* or *C. lari* is used as positive control.

c) Amplification control: The system contains an internal amplification control (see D.3.2.7).

D.5.4 Temperature-time programme

The temperature-time programme as outlined in Table D.3 has been used in the validation of the method using thermal cyclers Applied Biosystem 7500 Fast, Stratagene MX3000P, Biorad CFX 96 and iCycler iQ5[2]. The use of other thermal cyclers can make an adaptation necessary. The time for activation/ initial denaturation depends on the polymerase used.

2) Applied Biosystem 7500 Fast, Stratagene MX3000P, Biorad CFX 96 and iCycler iQ5 are examples of suitable products available commercially from ThermoFisher Scientific, Agilent Technologies and Bio-Rad. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to give the same results.

Table D.3 — Temperature-time programme

Steps	Temperature-time combination
Activation/initial denaturation	3 min/95 °C
Number of cycles (amplification)	45
Amplification	15 s/95 °C
	60 s/60 °C
	30 s/72 °C

D.6 Interpretation of the results

The threshold value to determine the cycle of threshold (Cq) shall 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[15]. The amplification curve of these samples crosses the defined threshold setting after a certain number of cycles. A sample with a fluorescence signal above the threshold is considered positive. In the validation of the method, all true positive samples generated Cq values below 38.

D.7 Performance characteristics of the method

D.7.1 General

The method (including inhouse validation data) has been published, see References [16] and [17]. Additionally, the performance characteristics of the method were determined in a method comparison study conducted in two different laboratories and in an interlaboratory study in accordance with ISO 16140-6[18], see Reference [19]. The data of the interlaboratory study are summarized in Annex F.

D.7.2 Theoretical evaluation of the method

In silico evaluation was done by performing a sequence similarity search against the GenBank/EMBL/DDBJ database (NCBI Blast® search[3], EMBL database, 22 September 2015). The result of the search confirmed a 100 % similarity only with the expected target sequences.

NOTE A 100 % similarity only with the expected target sequences does not exclude the presence of false-positive and/or false-negative results. These are addressed in the original publications and in Table D.4.

D.7.3 Inclusivity and exclusivity

The inclusivity of the method was tested in the method comparison study with 104 *C. jejuni*, 105 *C. coli* and 56 *C. lari* strains (in total 265 strains of thermotolerant *Campylobacter* spp.). The strains showed the expected results in comparison with the reference method (see also Table D.4).

The exclusivity of the method was tested in the method comparison study with 66 non-target *Campylobacter* spp., and 76 strains other than *Campylobacter* spp. (in total 142 strains). The strains showed the expected results in comparison with the reference method (see also Table D.4).

Table D.4 — Inclusivity and exclusivity

Inclusivity/ exclusivity	Number of strains	Inclusivity agreement	Inclusivity deviation	Exclusivity agreement	Exclusivity deviation
Inclusivity	265	265	0	Not applicable	Not applicable
Exclusivity	142	Not applicable	Not applicable	142	0

3) NCBI Blast® search is an example of a suitable product freely available under https://blast.ncbi.nlm.nih.gov/Blast.cgi. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to give the same results.

NOTE Table D.4 shows a comparison of the results of the reference method with the results of the PCR method described in Annex D. Considering the real identity of the strains, false-positive results were obtained with both the reference method and the Annex D PCR method for 2 *C. upsaliensis*, 1 *C. peloridis* and 1 *C. insulaenigrae* strains, but the latter did not grow on the selective media at 41,5 °C. The reference method was not able to distinguish between the target organisms of the Annex D PCR method (*C. jejuni, C. coli* and *C. lari*), and other *Campylobacte*r spp. able to grow on the selective media at 41,5 °C.

Annex E
(informative)

PCR methods for molecular confirmation and identification of thermotolerant *Campylobacter* spp.

E.1 General

This annex describes both a gel-based multiplex PCR assay and a probe-based multiplex real-time PCR assay for confirmation and identification of thermotolerant *Campylobacter* spp.

E.2 Gel-based multiplex PCR assay for confirmation and identification of thermotolerant *Campylobacter* spp.

E.2.1 General

This clause describes a method for the amplification and detection of genes specific for different species of thermotolerant *Campylobacter* (*C. jejuni, C. coli, C. lari, and C. upsaliensis*) using agarose gel electrophoresis.

E.2.2 Principle

Specific DNA fragments of the genes specific for the different *Campylobacter* spp. are amplified by multiplex PCR using five primer pairs. The detection of the PCR products is done using agarose gel-electrophoresis.

E.2.3 Reagents

For quality of reagents used, see ISO 22174[13]. Ready-to-use reagents may be commercially available. The manufacturer's instructions for use should be considered.

E.2.3.1 Reagents for nucleic acid extraction

E.2.3.1.1 NaCl, 0,9 % (mass fraction).

E.2.3.1.2 TE-buffer.

E.2.3.2 Reagents for PCR

E.2.3.2.1 PCR grade water.

E.2.3.2.2 PCR buffer solution, 10×.

The PCR buffer solution is usually delivered with the DNA polymerase, which may include $MgCl_2$ in a concentration specified by the manufacturer. The final $MgCl_2$ concentration is method specific and therefore listed in Table E.2 (see E.2.5.2).

E.2.3.2.3 $MgCl_2$ solution.

E.2.3.2.4 Thermostable *Taq* DNA polymerase.

E.2.3.2.5 dNTP solution.

E.2.3.2.6 Oligonucleotides.

Sequences of the oligonucleotides are listed in Table E.1.

Table E.1 — Sequences of oligonucleotides

Species (gene)	Primer	Sequence (5′ — 3′)	Amplicon size bp
C. jejuni (*hipO*)[20]	CJF (forward)	ACT TCT TTA TTG CTT GCT GC	323
	CJR (reverse)	GCC ACA ACA AGT AAA GAA GC	
C. coli (*glyA*)[20]	CCF (forward)	GTA AAA CCA AAG CTT ATC GTG	126
	CCR (reverse)	TCC AGC AAT GTG TGC AAT G	
C. lari (*cpn60*)[21]	JH0015 (forward)	TCT GCA AAT TCA GAT GAG AAA A	180
	JH0016 (reverse)	TTT TTC AGT ATT TGT AAT GAA ATA TGG	
C. upsaliensis (*glyA*)[20]	CUF (forward)	AAT TGA AAC TCT TGC TAT CC	204
	CUR (reverse)	TCA TAC ATT TTA CCC GAG CT	
Campylobacter spp. (23S rRNA)[20]	23SF (forward)	TAT ACC GGT AAG GAG TGC TGG AG	650
	23SR (reverse)	ATC AAT TAA CCT TCG AGC ACC G	

NOTE The system detecting *Campylobacter* spp. (23S rRNA) can also be used as an internal amplification control (IAC) for *Campylobacter*. The method comparison study showed that this system (23S rRNA) also targets *Arcobacter* and *Helicobacter* spp.

E.2.3.3 Reagents for gel electrophoresis

The agarose gel electrophoresis may be carried out with TAE buffer or TBE buffer. Solutions as described in this method do not usually need to be autoclaved.

E.2.3.3.1 Agarose, suitable for DNA electrophoresis and for the intended size separation of the DNA fragments.

E.2.3.3.2 Boric acid (H_3BO_3), for electrophoresis with TBE buffer system only.

E.2.3.3.3 Bromophenol blue ($C_{19}H_9Br_4O_5SNa$) and/or xylene cyanole FF ($C_{25}H_{27}N_2O_6S_2Na$).

E.2.3.3.4 DNA molecular mass standard, e.g. a commercial preparation containing DNA fragments from very high to very low molecular mass (but to be at least 100 bp).

E.2.3.3.5 Glacial acetic acid (CH_3COOH), for electrophoresis with the TAE buffer system only.

E.2.3.3.6 Ethylenediaminetetraacetic acid disodium salt (Na_2-EDTA) ($C_{10}H_{14}N_2O_8Na_2$).

E.2.3.3.7 Ethidium bromide (EtBr) ($C_{21}H_{20}N_3Br$) or other appropriate DNA intercalating dyes.

Take care when using ethidium bromide solution as it is mutagenic/teratogenic. Other intercalating dyes can be used, but refer to the manufacturer's material safety data sheet.

E.2.3.3.8 Glycerol ($C_3H_8O_3$).

E.2.3.3.9 Sodium acetate ($C_2H_3O_2Na$), for electrophoresis with TAE buffer only.

E.2.3.3.10 Hydrochloric acid, *w* (HCl) = 37 % (volume fraction).

E.2.3.3.11 Sodium hydroxide (NaOH).

E.2.3.3.12 Tris(hydroxymethyl)-aminomethane (Tris) ($C_4H_{11}NO_3$).

E.2.3.3.13 TAE buffer solution (1×), *c* (Tris) = 0,050 mol/l, *c* ($C_2H_3O_2Na$) = 20 mmol/l, *c* (Na_2-EDTA) = 0,001 mol/l.

Adjust the pH to 8,0 with glacial acetic acid or NaOH at 25 °C. It is advisable to prepare the TAE buffer solution as a concentrated stock solution (maximum 50-fold concentrated). Discard it if a precipitate is visible. Dilution of the concentrated electrophoresis buffer can be carried out, immediately before its use, with non-sterile, (mono)-distilled or deionized water.

E.2.3.3.14 TBE buffer solution (0,5×), c (Tris) = 0,055 mol/l, c (boric acid) = 0,055 mol/l, c (Na_2EDTA) = 0,001 mol/l.

Adjust the pH to 8,0 with HCl or NaOH at 25 °C. It is advisable to prepare the TBE buffer solution as a concentrated stock solution (maximum 10-fold concentrated). Discard it if precipitation is visible. Dilution of the concentrated electrophoresis buffer can be carried out, immediately before its use, with non-sterile, (mono)-distilled or deionized water.

When using TBE take care that it is toxic to reproduction and teratogenic. The TAE buffer is preferable.

E.2.3.3.15 Sample loading buffer solution (5×), c (glycerol) = 50 % (volume fraction), ρ (bromophenol blue) = 2,5 g/l and/or c (xylene cyanol) = 2,5 g/l, dissolved in electrophoresis buffer solution.

NOTE Other concentrations of loading buffer solution can also be used.

E.2.4 Apparatus

Appropriate equipment according to the method and, in particular, the following shall be used.

E.2.4.1 Equipment used for thermal lysis

E.2.4.1.1 Microcentrifuge tubes, of capacities of 1,5 ml and 2,0 ml.

E.2.4.1.2 Thermo block, with a temperature capacity up to +95 °C.

E.2.4.1.3 Graduated pipettes and pipette filter tips, for volumes between 1 µl and 1 000 µl.

E.2.4.1.4 Centrifuge, for microcentrifuge tubes having a capacity of 1,5 ml and 2,0 ml, e.g. microcentrifuge, capable of achieving an acceleration of up to 12 000*g*. In some steps a refrigerated centrifuge is required.

E.2.4.1.5 Mixer, e.g. type vortex.

E.2.4.2 Equipment used for PCR

E.2.4.2.1 Pipettes and pipette filter tips, having a capacity between 1 µl and 1 000 µl.

E.2.4.2.2 Microcentrifuge tubes, having a capacity of 1,5 ml and 2,0 ml.

E.2.4.2.3 Thin-walled PCR microtubes, 0,2 ml or 0,5 ml reaction tubes, multi-well PCR microplates or other suitable equipment.

E.2.4.2.4 Thermal cycler.

E.2.4.3 Equipment used for detection of the PCR product

E.2.4.3.1 Microwave oven or boiling water bath.

E.2.4.3.2 Horizontal gel system.

E.2.4.3.3 Power supply.

E.2.4.3.4 UV transilluminator or UV light box.

E.2.4.3.5 Gel documentation system.

E.2.5 Procedure

E.2.5.1 Nucleic acid (DNA) extraction

One 1 µl-loop of suspected colonies (see 9.5.2) is suspended in 1 ml of 0,9 % NaCl solution and DNA is extracted with a thermal lysis step (15 min at 95 °C). After an additional centrifugation step for 3 min at 10 000*g* 2,5 µl of the supernatant is used as DNA template. If the DNA will be stored, TE-buffer should be used instead of 0,9 % NaCl. Other methods for DNA extraction can be used if they have been shown to be suitable.

E.2.5.2 PCR set-up

The method is described for a total PCR volume of 25 µl per reaction, containing 2,5 µl of template DNA, with the reagents as listed in Table E.2. The PCR can also be carried out in a larger volume if the solutions are adjusted accordingly. The final concentrations of reagents as outlined in Table E.2 have proven to be suitable.

Table E.2 — Reagents

Reagent	Final concentration	Volume per sample µl
Template DNA	maximum 250 ng	2,5
Taq DNA polymerase	1,25 IU	as required
PCR-buffer (without $MgCl_2$)[a]	1×	as required
$MgCl_2$ solution	2 mM	as required
dNTP solution	0,2 mM of each	as required
PCR primers *C. jejuni* and *C. lari* (according to Table E.1)	0,5 µM each	as required
PCR primers *C. coli* (according to Table E.1)	1 µM each	as required
PCR primers *C. upsaliensis* (according to Table E.1)	2 µM each	as required
PCR primers 23S rRNA (according to Table E.1)	0,2 µM each	as required
PCR grade water	—	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.

E.2.5.3 PCR controls

In accordance with ISO 22174[13] the following controls are necessary:

a) Negative PCR control: PCR grade water is used as negative control.

b) Positive PCR control: DNA from *Campylobacter* spp., positive for all target sequences (*C. jejuni, C. coli, C. lari* and *C. upsaliensis*) is used as positive control.

c) Amplification control: The primer-system for detection of 23S rRNA of *Campylobacter* genus is used as internal amplification control.

E.2.5.4 Temperature-time programme

The temperature-time programme as outlined in Table E.3 has been used in the validation of the method using thermal cyclers Eppendorf Mastercycler® Gradient, Bio-Rad S1000 and T100, Analytic

Jena Biometra, Applied Biosystems Veriti and Simpli Amp[4]. The use of other thermal cyclers can make an adaptation necessary. The time for activation/initial denaturation depends on the polymerase used.

Table E.3 — Temperature-time programme

Steps	Temperature-time combination
Activation/initial denaturation	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

E.2.5.5 Detection of PCR products (gel electrophoresis)

E.2.5.5.1 General

The agarose gel electrophoresis may be carried out with TAE buffer or with TBE buffer. Use the same buffer to dissolve the agarose and to fill the electrophoresis tank.

E.2.5.5.2 Agarose gel preparation

The amplified PCR products should be detected using a 1,5 % (mass fraction) agarose gel. Weigh an appropriate amount of agarose (E.2.3.3.1) and add it to the electrophoresis buffer solution (E.2.3.3.13 or E.2.3.3.14). Allow the solution to boil in a microwave oven or in a water bath (E.2.4.3.1) until the agarose is completely dissolved, cool down the solution to about 60 °C and keep it at this temperature until use. Prepare a gel support (gel tray) with a suitable sample comb placed in the right position. Pour the agarose solution onto the gel tray and allow the gel to solidify at room temperature (1 h is usually recommended).

E.2.5.5.3 Agarose gel electrophoresis

Following the amplification step add the loading buffer to 10 µl PCR product in the ratio 1:4 (e.g. add 2,5 µl of loading buffer to 10 µl of PCR product) and mix.

NOTE Other concentrations of loading buffer solution can also be used.

Carefully remove the sample comb from the gel. Transfer the gel (with its gel tray) to the electrophoresis cell, so that the wells are close to the cathode (negative electrode). Fill the cell with the electrophoresis buffer (E.2.3.3.13 or E.2.3.3.14). Overlay the gel with approximately 2 mm of the same buffer and load the mixture to the sample slots (wells) using a micropipette. If the unknown samples are suspected to be too concentrated, also prepare some dilutions and load them onto the gel.

To determine the size of the PCR products, add the loading buffer (E.2.3.3.15) and DNA molecular mass standard (E.2.3.3.4) in the proportion of 1:4. The DNA molecular mass standard is loaded on the gel at least before the first and after the last sample well.

Carry out the electrophoresis at room temperature at the appropriate voltage and power intensity (generally a maximum constant voltage of 5 V/cm, with respect to the distance between the electrodes, is recommended). Under the described conditions, DNA is negatively charged, so it migrates from the cathode to the anode. The electrophoresis time depends on the migration distance required, on the current generated by the power supply, the buffer used, the electro-endosmosis and the concentration

4) Eppendorf Mastercycler® Gradient, Bio-Rad S1000 and T100, Analytic Jena Biometra, Applied Biosystems Veriti and Simpli Amp are examples of suitable products available commercially from Eppendorf, Bio-Rad, Analytic Jena and ThermoFisher Scientific. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to give the same results.

of the agarose in the gel. The optimal electrophoresis time should be determined in each laboratory in order to be able to correctly identify each product.

E.2.5.5.4 Staining

After completing the electrophoresis, incubate the gel for 15 min to 50 min in an appropriate dye solution (E.2.3.3.7) at room temperature, preferably in the dark with gentle shaking. If necessary, reduce the background staining by de-staining the gel in water for 10 min to 30 min. As an alternative to post-electrophoresis staining, the dye can be added to the gel before pouring it. In this case, the dye is added to the gel when it has been cooled to a temperature of about 60 °C.

To minimize the problems of EtBr movement in the gel, some EtBr can also be added to the electrophoresis (tank) buffer. After the gel electrophoresis, no de-staining step is usually required. When using Ethidium bromide in the tank buffer, take care that it is mutagenic/teratogenic.

E.2.5.5.5 Imaging

Transfer the gel to the transilluminator surface, switch on the UV light and record the DNA fluorescence by photography or video-documentation. The sizes of the different amplification products are given in Table E.4.

Table E.4 — Size of amplification products

Type	Primer	Product size bp
Campylobacter spp.	23SF/23SR	650
C. jejuni	CJF/CJR	323
C. upsaliensis	CUF/CUR	204
C. lari	JH0015/JH0016	180
C. coli	CCF/CCR	126

E.2.6 Interpretation of the results

The target sequences are considered to be detected if the size of the PCR product corresponds to the expected length of the target DNA sequences. For the interpretation of the results see ISO 22174[13].

The detection of both species target and genus target are required for a positive confirmation and identification of a thermotolerant *Campylobacter* spp. If only one of the targets is detected, the assay should be re-run using a dilution of the template. The detection of the genus target only is not a positive confirmation of a thermotolerant *Campylobacter* spp.

E.2.7 Performance characteristics

E.2.7.1 General

The method (including inhouse validation data) has been published, see References [20] and [21]. Additionally, the performance characteristics of the method were determined in a method comparison study conducted in two different laboratories and in an interlaboratory study following ISO 16140-6[18], see Reference [19]. The data from the interlaboratory study are summarized in Annex F.

E.2.7.2 Theoretical evaluation of the method

Theoretical evaluation was done by performing a sequence similarity search against the GenBank/EMBL/DDBJ database (NCBI Blast® search[5)], EMBL database, 22 September 2015). The result of the search confirmed a 100 % sequence similarity only with the expected target sequences.

NOTE A 100 % similarity only with the expected target sequences does not exclude the presence of false-positive and/or false-negative results. These are addressed in the original publications and in Table E.5.

E.2.7.3 Inclusivity and exclusivity

The inclusivity of the method was tested in the method comparison study with 104 *C. jejuni*, 105 *C. coli*, 56 *C. lari* and 33 *C. upsaliensis* strains. The results are shown in Table E.5.

The exclusivity of the method was tested in the method comparison study with both non-target *Campylobacter* spp. and 76 strains other than *Campylobacter* spp. The strains showed the expected results in comparison with the reference method (see also Table E.5).

Table E.5 — Inclusivity and exclusivity

Campylobacter species	Inclusivity/ exclusivity	Number of strains	Inclusivity agreement	Inclusivity deviation	Exclusivity agreement	Exclusivity deviation
C. jejuni	Inclusivity	104	102	2	Not applicable	Not applicable
	Exclusivity	303	Not applicable	Not applicable	303	0
C. coli	Inclusivity	105	99	6	Not applicable	Not applicable
	Exclusivity	302	Not applicable	Not applicable	302	0
C. lari	Inclusivity	56	56	0	Not applicable	Not applicable
	Exclusivity	351	Not applicable	Not applicable	351	0
C. upsaliensis	Inclusivity	33	25	8	Not applicable	Not applicable
	Exclusivity	374	Not applicable	Not applicable	374	0

NOTE Table E.5 shows a comparison of the results of the reference method with the results of the PCR method described in Clause E.2. Among the deviations, one strain was a *C. jejuni* subsp. *doylei*, and three of the *C. coli* strains were isolated from water. Considering the real identity of the strains, the inclusivity of the Clause E.2 PCR method showed equal results (for *C. jejuni* and *C. upsaliensis*) or better results (for *C. lari*) than with the reference method, and the exclusivity of the Clause E.2 PCR method showed equal results (for *C. upsaliensis*) or better results (all other targets) than with the reference method.

E.3 Multiplex real-time PCR assay for confirmation and identification of thermotolerant *Campylobacter* spp.

E.3.1 General

This clause describes a probe-based multiplex real-time PCR method based on 5′ exonuclease activity for the detection of genes specific for three different species of thermotolerant *Campylobacter* (*C. jejuni, C. coli* and *C. lari*).

5) NCBI Blast® search is an example of a suitable product freely available under https://blast.ncbi.nlm.nih.gov/Blast.cgi. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to give the same results.

E.3.2 Principle

Specific DNA fragments of the genes specific for the different thermotolerant *Campylobacter* spp. are amplified by multiplex real-time PCR. The PCR products are detected by measuring the fluorescence of the hydrolysed probes.

E.3.3 Reagents

For quality of reagents used, see ISO 22174[13]. Ready-to-use reagents may be commercially available. The manufacturer's instructions for use should be considered.

E.3.3.1 Reagents for nucleic acid extraction

E.3.3.1.1 NaCl, 0,9 % (mass fraction).

E.3.3.1.2 PCR grade water.

E.3.3.1.3 TE-buffer.

E.3.3.2 Reagents for real-time PCR

E.3.3.2.1 PCR grade water.

E.3.3.2.2 PCR buffer solution, 10×.

The PCR buffer solution is usually delivered with the DNA polymerase, which may or may not include $MgCl_2$ in a concentration specified by the manufacturer. The final $MgCl_2$ concentration is method specific and therefore listed in Table E.7 (see E.3.5.2).

E.3.3.2.3 $MgCl_2$ solution.

E.3.3.2.4 Thermostable *Taq* DNA polymerase (for hot-start PCR).

E.3.3.2.5 dNTP solution.

E.3.3.2.6 IPC-ntb2 plasmid.

A vector plasmid carrying a 125-bp sequence of the gene *rbcMT-T* encoding Ribulose-1,5-bisphosphate carboxylase/oxygenase N-methyltransferase from *Nicotiana tabacum*.[14] The plasmid is used as an internal amplification control[6].

E.3.3.3.7 Oligonucleotides.

Sequences of the oligonucleotides are listed in Table E.6.

6) The plasmid IPC-ntb2 was used as an internal amplification control in the validation study of the PCR system. This information is given for convenience of users of this document and does not constitute an endorsement by ISO of the product named. Alternative internal amplification control systems may be used if they can be shown to give equivalent or better results. If necessary, adapt the amounts of the reagents and the temperature-time programme.

Table E.6 — Sequences of oligonucleotides

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

[a] Equivalent reporter dyes and/or quencher dyes may be used for the probes if they can be shown to yield similar or better results. The alternative combinations FAM-Cy5-TAMRA-JOE, and FAM-TEXAS-RED-Cy5-HEX, have been used with equivalent result in the validation of the method.

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

[c] There is one nucleotide change in the primer compared with Reference [22], see Reference [23]. Both variants of the primer were used in the method comparison study, the primer sequence in this table was used in the ILS.

[d] Due to the genetic diversity of *C. lari*, two forward and two reverse primers have to be used to get reliable results.

E.3.4 Apparatus

Appropriate equipment according to the method and, in particular, the following shall be used.

E.3.4.1 Equipment used for nucleic acid extraction

E.3.4.1.1 Microcentrifuge tubes, of capacities of 1,5 ml and 2,0 ml.

E.3.4.1.2 Thermo block, with a temperature capacity up to +95 °C.

E.3.4.1.3 Pipettes and pipette filter tips, for volumes between 1 µl and 1 000 µl.

E.3.4.1.4 Centrifuge, for microcentrifuge tubes having a capacity of 1,5 ml and 2,0 ml, e.g. microcentrifuge, capable of achieving an acceleration of up to 12 000*g*. In some steps a refrigerated centrifuge is required.

E.3.4.2 Equipment used for real-time PCR

E.3.4.2.1 Pipettes and pipette filter tips, having a capacity between 1 µl and 1 000 µl.

E.3.4.2.2 Microcentrifuge tubes, having a capacity of 1,5 ml and 2,0 ml.

E.3.4.2.3 Thin-walled PCR microtubes, 0,2 ml or 0,5 ml reaction tubes, **multi-well PCR microplates or other suitable consumables**.

E.3.4.2.4 Real-time PCR instrument.

E.3.5 Procedure

E.3.5.1 Nucleic acid extraction

One 1 µl-loop of suspected colonies (see 9.5.2) is suspended in 1 ml of 0,9 % NaCl solution and DNA is extracted with a thermal lysis step (15 min at 95 °C). After an additional centrifugation step for 3 min at 10 000*g*, 5 µl of the supernatant is used as DNA template. If the DNA will be stored, TE-buffer should be used instead of 0,9 % NaCl. Other methods for DNA extraction can be used if they have been shown to be suitable. Before addition to the PCR mastermix, the template should be 100-fold diluted in sterile water.

E.3.5.2 PCR set-up

The method is described for a total PCR volume of 25 µl per reaction with the reagents as listed in Table E.6. The PCR can also be carried out in a larger volume if the solutions are adjusted appropriately. The final concentrations of reagents as outlined in Table E.7 have proven to be suitable.

Table E.7 — Reagents

Reagent	Final concentration	Volume per sample µl
Template DNA (1:100 dilution)	maximum 250 ng	2,5 µl
Taq DNA polymerase[a]	1 IU	as required
PCR-buffer (without $MgCl_2$)[b]	1×	as required
$MgCl_2$ solution	3 mM	as required
dNTP solution	0,25 mM of each dNTP	as required
PCR primers (according to Table E.6)	300 nM of each primer	as required
PCR probes (according to Table E.6)	100 nM of each probe	as required
PCR grade water	—	as required
IPC-ntb2 plasmid	25 copies per reaction	as required
Total volume	—	25

[a] TaqMan Universal PCR Master Mix (ThermoFisher Scientific) was used in the method comparison study. PerfeCTa Multiplex qPCR ToughMix (Quantabio) has been used with similar results. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to give the same results.

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

E.3.5.3 PCR controls

In accordance with ISO 22174[13] the following controls are necessary:

a) Negative PCR control: PCR grade water is used as negative control.

b) Positive PCR control: DNA from *Campylobacter* spp., positive for all target sequences (*C. jejuni, C. coli,* and *C. lari*) is used as positive control.

c) Amplification control: The system contains an internal amplification control (see E.3.3.2.6).

E.3.5.4 Temperature-time programme

The temperature-time programme as outlined in Table E.8 has been used in the validation of the method using thermal cyclers Applied Biosystem 7500 Fast, Stratagene MX3000P, Biorad CFX 96 and iCycler

iQ5[7]. The use of other thermal cyclers can make an adaptation necessary. The time for activation/initial denaturation depends on the polymerase used.

Table E.8 — Temperature-time programme

Steps	Temperature-time combination
Activation/initial denaturation	3 min/95 °C
Number of cycles (amplification)	45
Amplification	30 s/94 °C
	45 s/60 °C
	30 s/72 °C

E.3.6 Interpretation of the results

The threshold value to determine the cycle of threshold (Cq) shall 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[15]. The amplification curve of these samples crosses the defined threshold setting after a certain number of cycles. A sample with a fluorescence signal above the threshold is considered positive. In the validation of the method, all true positive samples generated Cq values below 38.

E.3.7 Performance characteristics

E.3.7.1 General

The method (including inhouse validation data and data of a national ILS) has been published in References [22] and [14]. Additionally, the performance characteristics of the method were determined in a method comparison study conducted in two different laboratories and in an interlaboratory study following ISO 16140-6[18], see Reference [19]. The data from the interlaboratory study are summarized in Annex F.

E.3.7.2 Theoretical evaluation of the method

Theoretical evaluation was done by performing a sequence similarity search against the GenBank/EMBL/DDBJ database (NCBI Blast® search[8], EMBL database, 22 September 2015). The result of the search confirmed a 100 % sequence similarity only with the expected target sequences.

NOTE A 100 % similarity only with the expected target sequences does not exclude the presence of false-positive and/or false-negative results. These are addressed in the original publications and in Table E.9.

E.3.7.3 Inclusivity and exclusivity

The inclusivity of the method was tested in the method comparison study with 104 *C. jejuni*, 105 *C. coli*, and 50 *C. lari* strains. The strains showed the expected results in comparison with the reference method (see also Table E.9).

The exclusivity of the method was tested in the method comparison study with both non-target *Campylobacter* spp. and 76 strains other than *Campylobacter* spp. (see also Table E.9).

7) Applied Biosystem 7500 Fast, Stratagene MX3000P, Biorad CFX 96 and iCycler iQ5 are examples of suitable products available commercially from ThermoFisher Scientific, Agilent Technologies and Bio-Rad. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to give the same results.

8) NCBI Blast® search is an example of a suitable product freely available under https://blast.ncbi.nlm.nih.gov/Blast.cgi. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to give the same results.

Table E.9 — Inclusivity and exclusivity

Campylobacter species	Inclusivity/ exclusivity	Number of strains	Inclusivity agreement	Inclusivity deviation	Exclusivity agreement	Exclusivity deviation
C. jejuni	Inclusivity	104	104	0	Not applicable	Not applicable
	Exclusivity	303	Not applicable	Not applicable	299	4
C. coli	Inclusivity	105	103	2	Not applicable	Not applicable
	Exclusivity	302	Not applicable	Not applicable	302	0
C. lari	Inclusivity	56	54	2	Not applicable	Not applicable
	Exclusivity	351	Not applicable	Not applicable	351	0

NOTE Table E.9 shows a comparison of the results of the reference method with the results of the PCR method described in Clause E.3. Among the deviations, one of the *C. coli* strains had a hybrid *C. jejuni*/*C. coli* target and three *C. coli* strains had both *C. jejuni* and *C. coli* targets, all resulting in false-positive *C. jejuni* signals. Considering the real identity of the strains, both inclusivity and exclusivity of the Clause E.3 PCR method showed better results compared with the reference method. *C. peloridis* resulted in a false-positive result with both methods.