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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 1: Detection method

AMENDMENT 1: Inclusion of methods for molecular confirmation and identification of thermotolerant *Campylobacter* spp., the use of growth supplement in Preston broth 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 1: Méthode de recherche

AMENDEMENT 1: Ajout de méthodes pour la confirmation et l'identification moléculaires de Campylobacter spp. thermotolérants, de l'utilisation d'un supplément de croissance dans le bouillon de Preston, 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).

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

Part 1: Detection method

AMENDMENT 1: Inclusion of methods for molecular confirmation and identification of thermotolerant *Campylobacter* spp., the use of growth supplement in Preston broth 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 9.5.

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.

4.1

Add the following note after the last paragraph:

NOTE The enrichment broth used in Detection procedure B (Preston broth) can be too selective to allow the recovery of strains of *Campylobacter* species (particularly *C. coli*), see ISO 17995[18]. The addition of growth supplement to Preston broth enhances the recovery of *Campylobacter* spp. and some strains will not grow without it. This issue arose after the publication of ISO 10272-1:2017 and is due to properties of the antibiotic solution. Supporting data are available at: https://standards. iso. org/iso/10272/-1/ed-1/en/amd/1/.

9.5.1

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.

9.6.1, 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*.

9.6.1

Add the following text as the third sentence:

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 9.6.2 to 9.6.5.

9.6.4, 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.

9.6.5, Table 2

Replace the table with the following:

Characteristic	*C. jejuni*	*C. coli*	*C. lari*[b]	*C. upsaliensis*[b]
Catalase (9.6.2)	+	+	+	– or weak
Hydrolysis of hippurate (9.6.3)	+[a]	–	–	–
Indoxyl acetate (9.6.4)	+	+	–	+[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 [19].

Clause B.3

Replace the clause with the following:

B.3 Preston broth

B.3.1 Basic medium

B.3.1.1 Composition

Enzymatic digest of animal tissues		10,0 g
Peptone		10,0 g
Sodium chloride	(CAS Registry Number®[a] 7647-14-5)	5,0 g
Water		940 ml

[a] CAS Registry Number® is a trademark of CAS corporation. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

B.3.1.2 Preparation

Dissolve the basic components or the dehydrated complete basic medium in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization, the pH of the complete medium is 7,4 ± 0,2 at 25 °C. Dispense the basic medium into containers of suitable capacity. Sterilize in the autoclave set at 121 °C for 15 min.

B.3.2 Sterile lysed horse blood

Use horse blood saponin-lysed or lysed by freezing then thawing.

B.3.3 Antibiotic solution

B.3.3.1 Composition

Polymyxin B sulfate	(CAS No. 1405-20-5)	5 000 IU
Rifampicin	(CAS No. 13292-46-1)	0,01 g
Trimethoprim lactate salt	(CAS No. 23256-42-0)	0,01 g
Amphotericin B	(CAS No. 1397-89-3)	0,01 g
Ethanol, 95 % (volume fraction)		5 ml

B.3.3.2 Preparation

Dissolve the components in the ethanol.

B.3.4 Growth supplement

B.3.4.1 Composition

Sodium pyruvate	(CAS No. 113-24-6)	0,25 g
Sodium metabisulfite	(CAS No. 7681-57-4)	0,25 g
Iron(II) sulfate hydrate	(CAS No. 13463-43-9)	0,25 g
Water		5 ml

B.3.4.2 Preparation

Dissolve the components in the water and sterilize by filtration. Aliquots of 5 ml should be stored at (–20 ± 5) °C for not more than 12 months.

B.3.5 Complete medium

B.3.5.1 Composition

Basic medium (B.3.1)	940 ml
Sterile lysed horse blood (B.3.2)	50 ml
Antibiotic solution (B.3.3)	5 ml
Growth supplement (B.3.4)	5 ml

B.3.5.2 Preparation

To the basic medium, cooled down to below 47 °C, add the antibiotic solution and then the growth supplement and finally the lysed blood aseptically, and mix. Dispense the medium aseptically into tubes, bottles or flasks of suitable capacity to obtain the portions necessary for the test. If the enrichment medium has been prepared in advance, store it in the dark at 5 °C (6.7) for up to seven days.

Clause B.11, Table B.1

Replace Table B.1 with the following:

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

Medium	Function	Incubation	Control strains	WDCM numbers[a]	Method of control	Criteria[b]	Characteristic reactions of target microorganism
Bolton broth	Productivity	(5 ± 1) h / (37 ± 1) °C then (44 ± 4) h / (41,5 ± 1) °C microaerobic atmosphere	*Campylobacter jejuni*[c] + *Escherichia coli*[c] + *Staphylococcus aureus*[c]	00156 or 00005 + 00012 or 00013 + 00032 or 00034	Qualitative	> 10 characteristic colonies on mCCD agar	Greyish, flat and moist, sometimes with metallic sheen
			Campylobacter coli[c] + *Escherichia coli*[c] + *Staphylococcus aureus*[c]	00004 or 00072 + 00012 or 00013 + 00032 or 00034			
	Selectivity		*Escherichia coli*[c] *Staphylococcus aureus*[c]	00012 or 00013 00032 or 00034	Qualitative	< 10 colonies on TSA	—

[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.[17]

[b] Growth/turbidity is categorized as 0: no growth/turbidity; 1: weak growth/turbidity; 2: good growth/turbidity (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.

Table B.1 *(continued)*

Medium	Function	Incubation	Control strains	WDCM numbers[a]	Method of control	Criteria[b]	Characteristic reactions of target microorganism
Preston broth	Productivity	(24 ± 2) h / (41,5 ± 1) °C microaerobic atmosphere	*Campylobacter jejuni*[c] + *Escherichia coli*[c] + *Staphylococcus aureus*[c]	00156 or 00005 00012 or 00013 00032 or 00034	Qualitative	> 10 characteristic colonies on mCCD agar	Greyish, flat and moist, sometimes with metallic sheen
			Campylobacter coli[c] + *Escherichia coli*[c] + *Staphylococcus aureus*[c]	00004 or 00072 00012 or 00013 00032 or 00034			
	Selectivity		*Escherichia coli*[c] *Staphylococcus aureus*[c]	00012 or 00013 00032 or 00034	Qualitative	Total inhibition (0) on TSA	—
mCCD agar	Productivity	(44 ± 4) h / (41,5 ± 1) °C microaerobic atmosphere	*Campylobacter jejuni*[c] *Campylobacter coli*[c]	00156 or 00005 00004 or 00072	Qualitative	Good growth (2)	Greyish, flat and moist colonies, sometimes with metallic sheen
	Selectivity		*Escherichia coli*[c]	00012 or 00013	Qualitative	Total or partial inhibition (0–1)	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.[17]

[b] Growth/turbidity is categorized as 0: no growth/turbidity; 1: weak growth/turbidity; 2: good growth/turbidity (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.

Annex C

Add the following text after the fourth sentence:

NOTE Validation data were collected from participants using Preston broth without growth supplement as well as from participants using Preston broth with growth supplement (see 4.1). Therefore, the performance characteristics are considered to be still valid and thus re-verification is not required.[19]

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 method 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[20]. 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*.[21] 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 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-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[22] 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.