INTERNATIONAL STANDARD

ISO 10272-2

First edition
2017-06

AMENDMENT 1
2023-01

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

Reference number
ISO 10272-2:2017/Amd.1:2023(E)

ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

Published in Switzerland

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

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.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

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 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[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.