भारतीय मानक

Indian Standard

IS 18564 (Part 2) : 2024
ISO 10272-2 : 2017

खाद्य श्रृंखला का सूक्ष्म जीव विज्ञान — *कैम्पाइलोबैक्टर* स्पीशीज का पता लगाने और गणना के लिए क्षैतिज विधि

भाग 2 कॉलोनी-गणना तकनीक

Microbiology of the Food Chain — Horizontal Method for Detection and Enumeration of *Campylobacter* spp.

Part 2 Colony-Count Technique

ICS 07.100.30

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NATIONAL FOREWORD

This Indian Standard (Part 2) which is identical to ISO 10272-2 : 2017 'Microbiology of the food chain — Horizontal method for detection and enumeration of *Campylobacter* spp. — Part 2: Colony-count technique' issued by the International Organization for Standardization (ISO) was adopted by the Bureau of Indian Standards on the recommendation of the Food Microbiology Sectional Committee and approval of the Food and Agriculture Division Council.

This Indian Standard is published in two parts. The other part in this series is:

Part 1 Detection method

The text of ISO standard has been approved as suitable for publication as an Indian Standard without deviations. Certain conventions are, however, not identical to those used in Indian Standards. Attention is particularly drawn to the following:

a) Wherever the words 'International Standard' appear referring to this standard, they should be read as 'Indian Standard'; and

b) Comma (,) has been used as a decimal marker while in Indian Standards, the current practice is to use a point (.) as the decimal marker.

In this adopted standard, reference appears to the following International Standard for which Indian Standard also exists. The corresponding Indian Standard which is to be substituted in its place is listed below along with its degree of equivalence for the edition indicated.

International Standard	*Corresponding Indian Standard*	*Degree of Equivalence*
ISO 6887-1 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of initial suspension and decimal dilutions	IS 10232 : 2020/ISO 6887-1 : 2017 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — General rules for the preparation of initial suspension and decimal dilutions (*second revision*)	Identical
ISO 6887-2 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 2: Specific rules for the preparation of meat and meat products	IS 15990 : 2023/ISO 6887-2 : 2017 Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Specific rules for the preparation of meat and meat products (*first revision*)	Identical
ISO 6887-3 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 3: Specific rules for the preparation of fish and fishery products	IS 17448 : 2020/ISO 6887-3 : 2017 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Specific rules for the preparation of fish and fishery products	Identical

(*Continued on third cover*)

Contents

Page

Introduction

The main changes, listed in the foreword, introduced in this document compared to ISO/TS 10272-2:2006 are considered as minor (see ISO 17468).

Because of the large variety of food and feed products, this horizontal method may not be appropriate in every detail for certain products, and for some other products, it may be necessary to use different methods. Nevertheless, it is hoped that in all cases, every attempt will be made to apply this horizontal method as far as possible and that deviations from this will only be made if absolutely necessary for technical reasons.

When this document is next reviewed, account will be taken of all information then available regarding the extent to which this horizontal method has been followed and the reasons for deviations from this in the case of particular products. The harmonization of test methods cannot be immediate and, for certain group of products, International Standards and/or national standards may already exist that do not comply with this horizontal method. It is hoped that when such standards are reviewed, they will be changed to comply with this document, so that eventually, the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

Indian Standard

MICROBIOLOGY OF THE FOOD CHAIN — HORIZONTAL METHOD FOR DETECTION AND ENUMERATION OF *Campylobacter* spp.

PART 2 COLONY-COUNT TECHNIQUE

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for enumeration of *Campylobacter* are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist, and that great care is taken in the disposal of all incubated materials. Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety aspects, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices.

1 Scope

This document specifies a horizontal method for the enumeration of *Campylobacter* spp. It is applicable to

— products intended for human consumption,

— products intended for animal feeding,

— environmental samples in the area of food and feed production, handling, and

— samples from the primary production stage such as animal faeces, dust, and swabs.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 6887 (all parts), *Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination*

ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

ISO 11133, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at http://www.iso.org/obp

3.1
Campylobacter
microorganism forming characteristic colonies on solid selective media when incubated in a microaerobic atmosphere at 41,5 °C, and which possesses the characteristic morphology and motility and biochemical and growth properties described when the tests are conducted in accordance with this document

Note 1 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).

3.2
enumeration of *Campylobacter*
determination of the number of colony-forming units (cfu) of *Campylobacter* (3.1) found per gram, per millilitre, per square centimetre or per sampling device when the test is conducted in accordance with this document

4 Principle

4.1 General

The enumeration of *Campylobacter* requires three successive stages as specified in Annex A.

4.2 Preparation of dilutions

For the preparation of decimal dilutions from the test portion, see ISO 6887.

4.3 Enumeration

The solid selective medium modified Charcoal Cefoperozone Deoxycholate agar (mCCD agar) is inoculated with a specified quantity of the test portion if the product is liquid or of the initial suspension in the case of other products.

Other plates are prepared under the same conditions, using decimal dilutions of the test portion or of the initial suspension.

The plates are incubated at 41,5 °C in a microaerobic atmosphere and examined after 44 h to record the number of suspect *Campylobacter* colonies.

4.4 Confirmation

The suspect *Campylobacter* colonies are examined for morphology and motility using a microscope and sub-cultured on a non-selective blood agar, and then confirmed by detection of oxidase activity and an aerobic growth test at 25 °C. Optionally, the *Campylobacter* species are identified by specific biochemical tests and/or molecular methods.

The number of colony-forming units (cfu) of *Campylobacter* per unit of the test portion is calculated from the number of confirmed typical colonies per plate.

5 Culture media and reagents

For current laboratory practice, see ISO 7218 and ISO 11133.

Composition of culture media and reagents and their preparation are described in Annex B.

For performance testing of culture media, see Annex B.

6 Equipment and consumables

Disposable equipment is an acceptable alternative to reusable glassware if it has suitable specifications.

Usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following.

6.1 Incubators, capable of operating at 25 °C ± 1 °C, 37 °C ± 1 °C and 41,5 °C ± 1 °C.

6.2 Water bath, capable of operating at 37 °C ± 1 °C.

6.3 Sterile loops, of 10 µl volume and of 1 µl volume, and **inoculation needle** or **wire**.

A nickel/chromium loop is not suitable for use in the oxidase test (see 9.4.5).

6.4 Microscope, preferably with phase contrast (for observing the characteristic morphology and motility of *Campylobacter*).

6.5 Appropriate apparatus for achieving a microaerobic atmosphere with oxygen content of 5 % ± 2 %, carbon dioxide 10 % ± 3 %, optional hydrogen ≤10 %, with the balance nitrogen.

The appropriate microaerobic atmosphere can be obtained using gastight jars and gas-generating kits, following precisely the manufacturer's instructions. Alternatively, the jar or incubator may be filled with an appropriate gas mixture prior to incubation.

6.6 Sterile Petri dishes, with a diameter of approximately 90 mm and (optional) large size (diameter approximately 140 mm), preferably with vents to facilitate microaerobic incubation.

6.7 Refrigerators, capable of operating at 3 °C ± 2 °C and at 5 °C ± 3 °C.

7 Sampling

Sampling is not part of the method specified in this document. See the specific International Standard dealing with the product concerned. If there is no specific International Standard dealing with the sampling of the product concerned, it is recommended that the parties concerned come to an agreement on this subject.

A recommended sampling method is given in ISO/TS 17728 for food and animal feed, in ISO 13307 for sampling at the primary production stage, in ISO 17604 for sampling of carcasses, and in ISO 18593 for sampling of surfaces.

It is important that the laboratory receives a sample that is representative and the sample should not have been damaged or changed during transport or storage.

Since *Campylobacter* is very sensitive to freezing but survives best at low temperatures, samples to be tested should not be frozen, but stored at 3 °C (6.7) and subjected to analysis as rapidly as possible. Also, take care to prevent the samples from drying.

8 Preparation of test sample

Prepare the test sample from the laboratory sample in accordance with the specific International Standard dealing with the product concerned; see ISO 6887 (all parts). If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure

9.1 Test portion, initial suspension and dilutions

See ISO 6887 and the specific International Standard dealing with the product concerned.

Prepare a single decimal dilution series from the test portion if the product is liquid or from the initial suspension in the case of other products.

9.2 Inoculation and incubation

9.2.1 Using a sterile pipette, transfer 0,1 ml of the initial suspension (or sample if liquid) (9.1) to the mCCD agar plate (B.3). Repeat the procedure using further decimal dilutions if necessary. If only the initial suspension is used, prepare duplicate plates using an additional agar plate.

When, for certain products, it is necessary to estimate low numbers of *Campylobacter*, the limit of enumeration may be lowered by a factor of 10 by examining 1,0 ml of the initial suspension. Distribute the 1,0 ml of inoculum either on the surface of the agar medium in a large Petri dish (140 mm) or three regular plates (90 mm). In both cases, prepare duplicates by using two large plates or six regular plates.

9.2.2 Evenly spread the inoculum, as quickly as possible, over the surface of the agar plate, using a sterile spreader. Avoid touching the sides of the Petri dish with the spreader.

NOTE Drying of the plates is critical to produce countable plates. Each laboratory has to use its own standardised way to dry the plates in a proper way.

9.2.3 Incubate the plates (9.2.2) at 41,5 °C (6.1) in a microaerobic atmosphere (6.5).

9.3 Enumeration of characteristic colonies

9.3.1 After 44 h ± 4 h of incubation, examine the plates (9.2.3) for typical and/or suspect colonies of *Campylobacter*.

Typical colonies are greyish on mCCD agar, often with a metallic sheen, and are flat and moist, with a tendency to spread. Colonies tend to spread less on drier agar surfaces. Other forms of colonies may occur.

NOTE The recognition of colonies of *Campylobacter* is to a large extent a matter of experience and their appearance can vary somewhat, not only from strain to strain, but also from batch to batch of the selective culture medium used.

9.3.2 Select the plates (9.3.1) containing less than 150 typical or suspect colonies; count these colonies and record their number as presumptive colonies per dish. Then choose at random five such colonies for subculturing for the confirmation tests (9.4).

9.4 Confirmation of *Campylobacter*

9.4.1 General

As *Campylobacter* rapidly loses culturability in air, follow the procedure described in 9.4.2 to 9.4.5 without delay.

For a clear distinction between positive and negative confirmation reactions, it is helpful to verify this with well-characterized positive and negative control strains. Examples of suitable control strains are *Campylobacter jejuni* WDCM 00005 (positive control)[10] and *Escherichia coli* WDCM 00013 (negative control).

As an alternative, or in addition, to the confirmation and identification tests described in this document, other tests (PCR tests, serological methods, matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS) analysis, etc.) can be used, providing the suitability of the alternative procedure is verified (see ISO 7218).

9.4.2 Selection of colonies for confirmation

9.4.2.1 For confirmation, take five presumptive colonies from each dish retained for enumeration (9.3.2).

9.4.2.2 Streak each of the selected colonies onto a non-selective blood agar plate, e.g. Columbia blood agar (B.4) in order to allow the development of well-isolated colonies. Incubate the plates in a microaerobic atmosphere (6.5) at 41,5 °C (6.1) for 24 h to 48 h. Use well-isolated freshly grown colonies for examination of morphology and motility (9.4.3), absence of aerobic growth at 25 °C (9.4.4) and the presence of oxidase activity (9.4.5).

NOTE The suspect colony could be previewed for characteristic morphology and motility before streaking on blood agar.

9.4.3 Examination of morphology and motility

9.4.3.1 Examine a freshly grown colony from each individual plate (9.4.2.2) for morphology and motility using a microscope (6.4).

9.4.3.2 Retain for further examination all cultures (9.4.2.2) in which curved bacilli with a spiralling "corkscrew" motility are found (9.4.3.1).

9.4.4 Study of aerobic growth at 25 °C

Using the colonies isolated in 9.4.2.2, inoculate with the aid of a loop (6.3) the surface of a non-selective blood agar plate, e.g. Columbia blood agar (B.4).

Incubate the plate at 25 °C (6.1) aerobically for 44 h ± 4 h.

Examine the plate for absence of growth of colonies.

9.4.5 Detection of oxidase activity

Using a loop (6.3), take a portion of a well-isolated colony from each individual plate (9.4.2.2) and streak it onto a filter paper moistened with the oxidase reagent (B.5); the appearance of a mauve, violet or deep blue colour within 10 s indicates a positive reaction. If a commercially available oxidase test kit is used, follow the manufacturer's instructions.

9.4.6 Interpretation

Campylobacter gives results in accordance with Table 1.

Table 1 — Characteristics of *Campylobacter*

Morphology (9.4.3)	Small curved bacilli[a]
Motility (9.4.3)	Characteristic corkscrew darting[a]
Aerobic growth at 25 °C (9.4.4)	–
Oxidase activity (9.4.5)	+

\+ Positive.

– Negative.

[a] Older cultures may rapidly lose their characteristic shape and motility and turn into less motile coccoid- forms.

9.5 Identification of *Campylobacter* species (optional)

9.5.1 General

Among the *Campylobacter* spp. growing at 41,5 °C, the most frequently encountered species are *Campylobacter jejuni* and *Campylobacter coli*. Other species have, however, been described *(Campylobacter lari*, *Campylobacter upsaliensis* and others); the characteristics given in Table 2 permit their differentiation.

9.5.2 Detection of catalase activity

For each colony selected in 9.4.2.2, deposit a loop of culture into a drop of hydrogen peroxide solution (B.6) on a clean microscope slide.

The test is positive if bubbles appear within 30 s.

Confirm the results using positive and negative controls. Examples of suitable control strains are *Campylobacter jejuni* WDCM 00005 (positive control) and *Enterococcus faecalis* WDCM 00087 (negative control).

9.5.3 Detection of hippurate hydrolysis

For each colony selected in 9.4.2.2, use a 10 µl loop (6.3) with a heavy inoculum to prepare a suspension in a tube of appropriate size containing 0,4 ml of a sodium hippurate solution (B.7.1), taking care not to incorporate any agar.

Shake in order to mix thoroughly and incubate for 2 h ± 5 min in a water bath at 37 °C (6.2) or 4 h ± 5 min in an incubator at 37 °C (6.1).

Carefully add 0,2 ml of a ninhydrin solution (B.7.2) on top of the sodium hippurate solution. Do not shake.

Interpret after incubation of 5 min to 10 min at 37 °C (6.2 or 6.1).

A dark violet colour indicates a positive reaction.

A pale violet colour or no colour change indicates a negative reaction.

Confirm the results using positive and negative controls. Examples of suitable control strains are *Campylobacter jejuni* WDCM 00005 (positive control) and *Campylobacter coli* WDCM 00004 (negative control).

9.5.4 Detection of indoxyl acetate hydrolysis

Place a 1 µl loopful of colony material (9.4.2.2) on an indoxyl acetate disc (B.8) and add a drop of sterile distilled water.

If the indoxyl acetate is hydrolysed, a colour change to dark blue occurs within 5 min to 10 min. No colour change indicates hydrolysis has not taken place.

Confirm the results using positive and negative controls. Examples of suitable control strains are *Campylobacter jejuni* WDCM 00005 (positive control) and *Campylobacter lari* WDCM 00204 (negative control).

If commercially available indoxyl acetate discs are used, follow the manufacturer's instructions.

9.5.5 Interpretation

Campylobacter species growing at 41,5 °C may be identified at species level according to Table 2.

Table 2 — Characteristics of *Campylobacter* species

Characteristic	*C. jejuni*	*C. coli*	*C. lari*	*C. upsaliensis*
Catalase activity (9.5.2)	+	+	+	– or weak
Hippurate hydrolysis (9.5.3)	+[a]	–	–	–
Indoxyl acetate hydrolysis (9.5.4)	+	+	–	+

\+ Positive.

– Negative.

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

10 Expression of results

See ISO 7218. Calculate and report the result as the number of *Campylobacter* in cfu per gram, per millilitre, per square centimeter or per sampling device.

11 Performance characteristics of the method

11.1 Interlaboratory study

Results of the interlaboratory study to determine the precision of the method are summarized in Annex C. Repeatability and reproducibility limits were determined using 5 sample types (broiler caecal material, frozen spinach, frozen minced meat, raw milk, chicken skin) contaminated at various levels. The values derived from the interlaboratory study may not be applicable to concentration ranges and sample types other than those given in Annex C.

11.2 Repeatability limit

The absolute difference between two independent single ($\log_{10}$-transformed) test results (number of cfu per gram or per millilitre) or the ratio of the higher to the lower of the two test results on the normal scale, obtained using the same method on identical test material in the same laboratory by the same operator using the same apparatus within the shortest feasible time interval, will, in not more than 5 % of cases, exceed the repeatability limit, *r*.

As a general indication of repeatability limit (*r*), the following overall values (derived from the mean of the variance estimates for all levels per matrix tested in the ILS, see data in Annex C) may be used when testing **broiler caecal material** samples:

r = 0,38 (expressed as a difference between $\log_{10}$-transformed test results), or

r = 2,38 (expressed as a ratio between test results).

The following overall values may be used when testing **chicken skin** samples:

r = 0,98 (expressed as a difference between $\log_{10}$-transformed test results), or

r = 9,52 (expressed as a ratio between test results).

EXAMPLE A test result of 1 000 000 or $1,0 \times 10^6$ or $\log_{10}$ 6,0 cfu per gram of broiler caecal material was observed in a given laboratory. Under repeatability conditions, the difference between $\log_{10}$-transformed results should not be greater than ±0,38 $\log_{10}$ units. So the result from a second test of the same sample should be between 5,62 (6,0 – 0,38) and 6,38 (6,0 + 0,38) $\log_{10}$ units.

For non-log-transformed results, the ratio between the first test result and the second test result from the same sample should not be greater than 2,38. So the second test result should be between 420 000 (= 1 000 000/2,38) and 2 400 000 (1 000 000 × 2,38) cfu per gram.

11.3 Reproducibility limit

The absolute difference between two single ($\log_{10}$-transformed) test results (number of cfu per gram or per millilitre) or the ratio of the higher to the lower of the two test results on the normal scale, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will, in not more than 5 % of cases, exceed the reproducibility limit, R.

As a general indication of reproducibility limit (R), the following overall values (derived from the mean of the variance estimates for all levels per matrix tested in the ILS, see data in Annex C) may be used when testing **broiler caecal material** samples in general:

R = 0,91 (expressed as a difference between $\log_{10}$-transformed test results), or

R = 8,14 (expressed as a ratio between test results).

The following overall values may be used when testing **chicken skin** samples:

R = 1,31 (expressed as a difference between $\log_{10}$-transformed test results), or

R = 20,43 (expressed as a ratio between test results).

EXAMPLE 1 A test result of 1 000 000 or $1,0 \times 10^6$ or $\log_{10}$ 6,0 cfu per gram of broiler caecal material was observed in a first laboratory. Under reproducibility conditions, the difference between $\log_{10}$-transformed results should not be greater than ±0,91 $\log_{10}$ units. So the result from a second laboratory should be between 5,09 (6,0 – 0,91) and 6,91 (6,0 + 0,91) $\log_{10}$ units.

For non-log-transformed results, the ratio between the test result from this first laboratory and a second laboratory should not be greater than 8,14. So the result from the second laboratory should be between 120 000 (= 1 000 000/8,14) and 8 100 000 (1 000 000 × 8,14) cfu per gram.

EXAMPLE 2 A laboratory wants to know the maximum value it may find for a poultry skin sample, which is still in compliance with a pre-set limit (e.g. a limit of 1 000 or $\log_{10}$ 3). For this, the R value (on the log scale) has to be multiplied by a factor of 0,59.

The factor 0,59 reflects the fact that a test with a one-sided 95 % interval is used to test whether the limit is exceeded; it is obtained from the following formula: $0,59 = \frac{1,64}{1,96 \times \sqrt{2}}$.

The maximum value is 0,77 (1,31 × 0,59) as a difference between $\log_{10}$-transformed test results or 5,93 ($10^{0,77}$) as a ratio between test results. So results up to $\log_{10}$ 3,77 ($\log_{10}$ 3 + $\log_{10}$ 0,77) or 5 900 (1 000 × 5,93) do not indicate non-compliance with the limit.

12 Test report

The test report shall specify the following:

— the test method used, with a reference to this document, i.e. ISO 10272-2;

— the sampling method used, if known;

— the nature of the objects examined;

— all operating details not specified in document, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

— any deviation in the media or the incubation conditions used;

— all information necessary for the complete identification of the sample;

— the test result(s) obtained.

Annex A
(normative)

Diagram of procedure

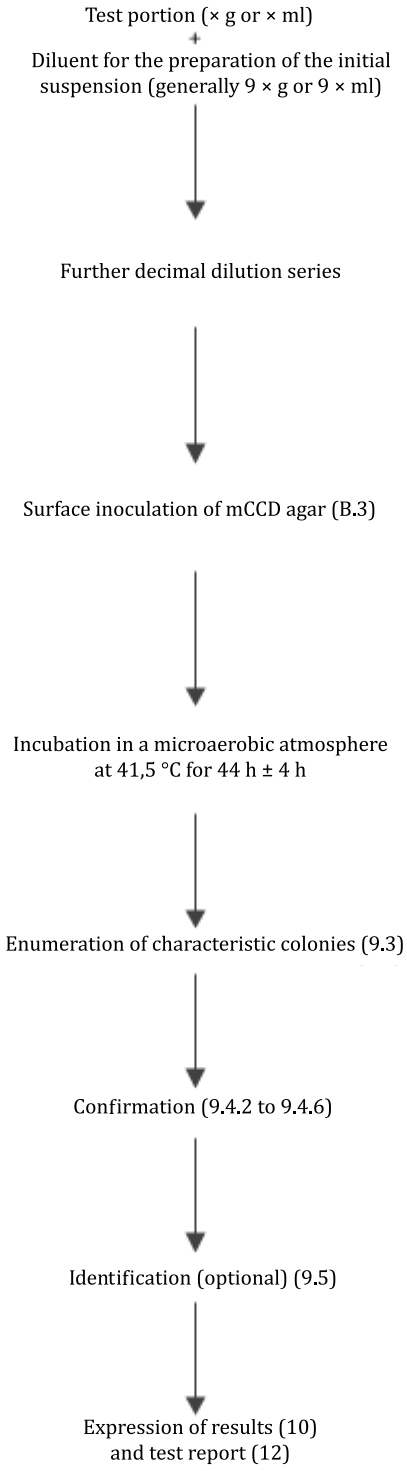

Figure A.1 — Diagram of the procedure for enumeration of *Campylobacter* in the food chain

Annex B
(normative)

Culture media and reagents

B.1 General

The general specifications of ISO 11133 are applicable to the preparation and performance testing of the culture media described in this annex. If culture media or reagents are prepared from dehydrated complete media/reagents or if ready-to-use media/reagents are used, follow the manufacturer's instructions regarding preparation, storage conditions, expiry date and use.

The shelf lives of the media indicated in this annex have been determined in some studies. The user should verify these under their own storage conditions (as specified in ISO 11133).

Performance testing of culture media is described in B.8.

B.2 Diluent

See ISO 6788.

B.3 Modified charcoal cefoperazone deoxycholate agar (mCCD agar)

B.3.1 Basic medium

B.3.1.1 Composition

Meat extract		10,0 g
Enzymatic digest of animal tissues		10,0 g
Sodium chloride	(CAS No. 7647-14-5)	5,0 g
Activated charcoal	(CAS No. 7440-44-0)	4,0 g
Enzymatic digest of casein		3,0 g
Sodium deoxycholate	(CAS No. 302-95-4)	1,0 g
Iron(II) sulfate hydrate	(CAS No. 13463-43-9)	0,25 g
Sodium pyruvate	(CAS No. 113-24-6)	0,25 g
Agar		8,0 g to 18,0 g[a]
Water		1 000 ml

[a] Depending on the gel strength of the agar.

B.3.1.2 Preparation

Dissolve the basic components or the dehydrated complete basic medium in the water, by bringing to the boil. Adjust the pH, if necessary, so that after sterilization it is 7,4 ± 0,2 at 25 °C. Dispense the basic medium into flasks of suitable capacity. Sterilize in the autoclave set at 121 °C for 15 min.

B.3.2 Antibiotic solution

B.3.2.1 Composition

Cefoperazone sodium salt	(CAS No. 62893-20-3)	0,032 g
Amphotericin B	(CAS No. 1397-89-3)	0,01 g
Water		5 ml

B.3.2.2 Preparation

Dissolve the components in the water. Sterilize by filtration.

B.3.3 Complete medium

B.3.3.1 Composition

Basic medium (B.3.1)	1 000 ml
Antibiotic solution (B.3.2)	5 ml

B.3.3.2 Preparation

Add the antibiotic solution to the basic medium, cooled down to 44 °C to 47 °C, then mix carefully. Pour 18 ml to 20 ml of the complete medium into sterile Petri dishes (6.6). Allow to solidify. Immediately before use, carefully dry the agar plates, preferably with the lids off and the agar surface downwards, in a drying cabinet for 30 min or until the agar surface is free of visible moisture. If they have been prepared in advance, store the undried agar plates in the dark at 5 °C (6.7) for up to 1 month.

B.4 Columbia blood agar

B.4.1 Basic medium

B.4.1.1 Composition

Enzymatic digest of animal tissues		23,0 g
Starch soluble	(CAS No. 9005-84-9)	1,0 g
Sodium chloride	(CAS No. 7647-14-5)	5,0 g
Agar		8,0 g to 18,0 g[a]
Water		1 000 ml

[a] Depending on the gel strength of the agar.

B.4.1.2 Preparation

Dissolve the basic components or the dehydrated complete medium in the water, by heating. Adjust the pH, if necessary, so that after sterilization it is 7,4 ± 0,2 at 25 °C. Dispense the basic medium into flasks of suitable capacity. Sterilize in the autoclave set at 121 °C for 15 min.

B.4.2 Sterile sheep or horse blood

B.4.3 Complete medium

B.4.3.1 Composition

Basic medium (B.4.1)	1 000 ml
Sterile blood (B.4.2)	50 ml

B.4.3.2 Preparation

Add the blood aseptically to the basic medium, cooled down to 44 °C to 47 °C, then mix. Pour 18 ml to 20 ml of the complete medium into sterile Petri dishes (6.6). Allow to solidify. Immediately before use, carefully dry the agar plates, preferably with the lids off and the agar surface downwards, in a drying cabinet for 30 min or until the agar surface is free of visible moisture. If they have been prepared in advance, store the undried agar plates in the dark at 5 °C (6.7) for up to 1 month.

B.5 Reagent for the detection of oxidase activity

B.5.1 Composition

N,N,N',N'-Tetramethyl-1,4-phenylenediamine dihydrochloride	(CAS No. 637-01-4)	1,0 g
Water		100 ml

B.5.2 Preparation

Dissolve the component in the water immediately prior to use.

B.6 Reagent for the detection of catalase activity

B.6.1 Composition

Hydrogen peroxide solution, volume fraction of 30 % in water	(CAS No. 7722-84-1)	1 ml
Water		9 ml

B.6.2 Preparation

Dissolve the component in the water immediately prior to use.

B.7 Reagents for the detection of hydrolysis of hippurate

B.7.1 Sodium hippurate solution

B.7.1.1 Composition

Sodium hippurate hydrate	(CAS No. 532-94-5)	10 g
Phosphate-buffered saline (PBS) consisting of:		
Sodium chloride	(CAS No. 7647-14-5)	8,5 g
Disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$)	(CAS No. 10028-24-7)	8,98 g
Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$)	(CAS No. 10049-21-5)	2,71 g
Water, to a final volume of		1 000 ml

B.7.1.2 Preparation

Dissolve the sodium hippurate in the PBS solution. Sterilize by filtration. Dispense the reagent aseptically in quantities of 0,4 ml into small tubes of suitable capacity. Store at about –20 °C.

B.7.2 Ninhydrin solution, mass fraction of 3,5 %

B.7.2.1 Composition

Ninhydrin	(CAS No. 485-47-2)	1,75 g
Acetone	(CAS No. 67-64-1)	25 ml
2-Butanol	(CAS No. 78-92-2)	25 ml

B.7.2.2 Preparation

Dissolve the ninhydrin in the acetone/butanol mixture. Store the solution in the dark.

The solution shall not be kept for more than 4 h at ambient temperature, or more than 7 days at 5 °C (6.7).

B.8 Indoxyl acetate discs

B.8.1 Composition

Indoxyl acetate	(CAS No. 608-08-2)	0,1 g
Acetone	(CAS No. 67-64-1)	1 ml

B.8.2 Preparation

Dissolve the indoxyl acetate in the acetone. Add 25 µl to 50 µl of this solution to blank paper discs (diameter 0,6 cm to 1,2 cm). After drying at room temperature, store the discs at 5 °C (6.7) in a brown tube or bottle in the presence of silica gel.

B.9 Performance testing for the quality assurance of the culture media

Performance testing of the culture media shall be carried out according to ISO 11133, which includes definitions for productivity and selectivity. Table B.1 gives details of control strains to be used for performance testing of culture media specified in this document. Where more than one strain is listed for each aspect of performance testing (productivity, selectivity), one of the strains which species is indicated by the letter d has to be used as a minimum. Commercial or non-commercial suppliers are expected to use additional strains, e.g. those shown in Table B.1 to further ensure the quality of the culture media they supply.

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

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

[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] Strain to be used as a minimum.

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

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

Annex C
(informative)

Method validation studies and performance characteristics

An interlaboratory study involving 15 laboratories in 12 countries was carried out. The following samples types were involved in the study: broiler caecal material, frozen spinach, frozen minced meat (pork/beef), raw milk and chicken skin. The samples were each tested at three different levels of contamination, plus a negative control. The study was organized in 2013 by the National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands, as part of the CEN Mandate M381 from the European Commission.

The broiler caecal material samples were contaminated artificially before sending to the participants. The samples of the other matrices were contaminated artificially by each lab according to the detailed standard operation procedure (SOP).

The performance characteristics shown in Tables C.1 to C.5 were calculated in accordance with ISO 5725-2:1994. Data obtained by some collaborators have been excluded from the calculations only on the basis of clearly identified technical reasons (deviations to the protocol, or all counts below 10 cfu per plate per sample).

Table C.1 — Results of data analysis obtained with broiler caecal material

Parameter	Sample type: **Broiler caecal material**		
	Low level[a]	Medium level[a]	High level[a]
Number of participating collaborators	15	15	15
Number of collaborators retained after evaluation of the data	13	8	11
Number of samples tested	30	30	30
Number of sample results retained after evaluation of the data	26	16	22
Mean value Σa ($\log_{10}$ cfu/g)	5,1	5,4	6,7
Repeatability standard deviation s_r ($\log_{10}$ cfu/g)	0,13	0,15	0,13
Repeatability limit r:			
as difference on $\log_{10}$ scale ($\log_{10}$ cfu/g)	0,36	0,42	0,35
as ratio on normal scale (cfu/g)	2,3	2,6	2,2
Reproducibility standard deviation s_R ($\log_{10}$ cfu/g)	0,38	0,31	0,28
Reproducibility limit R:			
as difference on $\log_{10}$ scale ($\log_{10}$ cfu/g)	1,07	0,86	0,78
as ratio on normal scale (cfu/g)	11,7	7,2	6,0

[a] Inoculation strain: *C. jejuni* (DSM 24306/CNET 076).

Table C.2 — Results of data analysis obtained with frozen spinach

Parameter	Sample type: **Frozen spinach**		
	Low level[a]	Medium level[a]	High level[a]
Number of participating collaborators	15	15	15
Number of collaborators retained after evaluation of the data	11	10	14
Number of samples tested	30	30	30
Number of sample results retained after evaluation of the data	22	20	28
Mean value Σa ($\log_{10}$ cfu/g)	3,7	4,6	5,3
Repeatability standard deviation s_r ($\log_{10}$ cfu/g)	0,17	0,10	0,17
Repeatability limit r:			
as difference on $\log_{10}$ scale ($\log_{10}$ cfu/g)	0,47	0,28	0,48
as ratio on normal scale (cfu/g)	3,0	1,9	3,0
Reproducibility standard deviation s_R ($\log_{10}$ cfu/g)	0,32	0,40	0,50
Reproducibility limit R:			
as difference on $\log_{10}$ scale ($\log_{10}$ cfu/g)	0,89	1,13	1,40
as ratio on normal scale (cfu/g)	7,7	13,4	25,0

[a] Inoculation strain: *C. jejuni* (WDCM 00005).

Table C.3 — Results of data analysis obtained with frozen minced meat (pork/beef)

Parameter	Sample type: **Frozen minced meat (pork/beef)**		
	Low level[a]	Medium level[a]	High level[a]
Number of participating collaborators	15	15	15
Number of collaborators retained after evaluation of the data	8	9	12
Number of samples tested	30	30	30
Number of sample results retained after evaluation of the data	16	18	24
Mean value Σa ($\log_{10}$ cfu/g)	3,6	4,7	5,1
Repeatability standard deviation s_r ($\log_{10}$ cfu/g)	0,17	0,11	0,43
Repeatability limit r:			
as difference on $\log_{10}$ scale ($\log_{10}$ cfu/g)	0,49	0,32	1,20
as ratio on normal scale (cfu/g)	3,1	2,1	15,7
Reproducibility standard deviation s_R ($\log_{10}$ cfu/g)	0,24	0,41	0,52
Reproducibility limit R:			
as difference on $\log_{10}$ scale ($\log_{10}$ cfu/g)	0,68	1,16	1,44
as ratio on normal scale (cfu/g)	4,8	14,4	27,8

[a] Inoculation strain: *C. coli* (WDCM 00072).

Table C.4 — Results of data analysis obtained with raw milk

Parameter	Sample type: **Raw milk**		
	Low level[a]	Medium level[a]	High level[a]
Number of participating collaborators	15	15	15
Number of collaborators retained after evaluation of the data	11	10	13
Number of samples tested	30	30	30
Number of sample results retained after evaluation of the data	22	20	26
Mean value Σa ($\log_{10}$ cfu/g)	3,7	4,8	5,9
Repeatability standard deviation s_r ($\log_{10}$ cfu/g)	0,19	0,22	0,12
Repeatability limit r:			
as difference on $\log_{10}$ scale ($\log_{10}$ cfu/g)	0,53	0,62	0,34
as ratio on normal scale (cfu/g)	3,4	4,2	2,2
Reproducibility standard deviation s_R ($\log_{10}$ cfu/g)	0,33	0,47	0,37
Reproducibility limit R:			
as difference on $\log_{10}$ scale ($\log_{10}$ cfu/g)	0,92	1,32	1,03
as ratio on normal scale (cfu/g)	8,4	21,0	10,6
[a] Inoculation strain: *C. jejuni* (WDCM 00156).			

Table C.5 — Results of data analysis obtained with chicken skin

Parameter	Sample type: **Chicken skin**		
	Low level[a]	Medium level[a]	High level[a]
Number of participating collaborators	15	15	15
Number of collaborators retained after evaluation of the data	9	11	13
Number of samples tested	30	30	30
Number of sample results retained after evaluation of the data	18	22	26
Mean value Σa ($\log_{10}$ cfu/g)	2,8	3,7	4,9
Repeatability standard deviation s_r ($\log_{10}$ cfu/g)	0,17	0,38	0,44
Repeatability limit r:			
as difference on $\log_{10}$ scale ($\log_{10}$ cfu/g)	0,48	1,06	1,23
as ratio on normal scale (cfu/g)	3,0	11,5	17,1
Reproducibility standard deviation s_R ($\log_{10}$ cfu/g)	0,45	0,41	0,54
Reproducibility limit R:			
as difference on $\log_{10}$ scale ($\log_{10}$ cfu/g)	1,26	1,15	1,50
as ratio on normal scale (cfu/g)	18,0	14,0	31,9
[a] Inoculation strain: *C. coli* (WDCM 00004).			

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[1] ISO 5725-2:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*

[2] ISO 13307, *Microbiology of food and animal feed — Primary production stage — Sampling techniques*

[3] ISO/TS 17728, *Microbiology of the food chain — Sampling techniques for microbiological analysis of food and feed samples*

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[10] World Data Centre for Microorganisms available at: http://www.wfcc.info

[11] ISO 17468, *Microbiology of the food chain — Technical requirements and guidance on establishment or revision of a standardized reference method*

(*Continued from second cover*)

International Standard	*Corresponding Indian Standard*	*Degree of Equivalence*
ISO 6887-4 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 4: Specific rules for the preparation of miscellaneous products	IS 17447 : 2020/ISO 6887-4 : 2017 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Specific rules for the preparation of miscellaneous products	Identical
ISO 6887-5 Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 5: Specific rules for the preparation of milk and milk products	IS 17779 : 2021/ISO 6887-5 : 2020 Microbiology of the food Chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Specific rules for the preparation of milk and milk products	Identical
ISO 6887-6 Microbiology of food and animal feed — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 6: Specific rules for the preparation of samples taken at the primary production stage	IS 16980 : 2018/ISO 6887-6 : 2013 Microbiology of food and animal feed — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Specific rules for the preparation of samples taken at the primary production stage	Identical
ISO 7218 Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations	IS 16122 : 2013/ISO 7218 : 2007 Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations	Identical
ISO 11133 Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media	IS 17383 : 2020/ ISO 11133 : 2014 Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media	Identical

In reporting the results of a test or analysis made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with IS 2 : 2022 ‘Rules for rounding off numerical values (*second revision*)’.

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