भारतीय मानक

Indian Standard

IS 18564 (Part 1) : 2024
ISO 10272-1 : 2017

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

भाग 1 पता लगाने की विधि

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

Part 1 Detection Method

ICS 07.100.30

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Food Microbiology Sectional Committee, FAD 31

NATIONAL FOREWORD

This Indian Standard (Part 1) which is identical to ISO 10272-1 : 2017 'Microbiology of the food chain — Horizontal method for detection and enumeration of *Campylobacter* spp. — Part 1: Detection method' 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 2 Colony-count technique

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 food and animal feeding stuffs — 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

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Introduction

The main changes, listed in the foreword, introduced in this document compared to ISO 10272-1:2006 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 *Compylobacter* spp.

PART 1 DETECTION METHOD

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *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 detection by enrichment or direct plating 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 thermotolerant species are *Campylobacter jejuni* and *Campylobacter coli*. However, other species have been described *(Campylobacter lari*, *Campylobacter upsaliensis* and others).

3.2
detection of *Campylobacter*
determination of the presence or absence of *Campylobacter* (3.1) in a defined quantity of product, when the test is conducted in accordance with this document

4 Principle

4.1 General

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

Depending on the type of sample and the purpose of the test, three different detection procedures can be used:

— **detection procedure A**: Detection of *Campylobacter* by enrichment, in samples with low numbers of campylobacters and low level of background microflora and/or with stressed campylobacters;

— **detection procedure B:** Detection of *Campylobacter* by enrichment, in samples with low numbers of campylobacters and high level of background microflora;

— **detection procedure C**: Detection of *Campylobacter* by direct plating, in samples with high numbers of campylobacters.

4.2 Enrichment in selective liquid medium

4.2.1 Detection procedure A

The test portion is added to the liquid enrichment medium (Bolton broth).

It is incubated in a microaerobic atmosphere at 37 °C for 4 h to 6 h and then at 41,5 °C for 44 h.

4.2.2 Detection procedure B

The test portion is added to the liquid enrichment medium (Preston broth).

It is incubated in a microaerobic atmosphere at 41,5 °C for 24 h.

4.2.3 Detection procedure C

Enrichment technique is not used.

4.3 Isolation on selective solid medium

4.3.1 Detection procedure A

From the enrichment culture obtained in 4.2, two selective solid media are inoculated:

— modified Charcoal Cefoperozone Deoxycholate agar (mCCD agar);

— any other solid selective *Campylobacter* medium using different selective principles from those in mCCD agar.

4.3.2 Detection procedure B

From the enrichment culture obtained in 4.2, the selective mCCD agar is inoculated.

4.3.3 Detection procedure C

The test portion is plated directly or after suspending in an appropriate amount of liquid onto the selective mCCD agar.

4.3.4 Detection procedure A, B and C

The selective solid media are incubated at 41,5 °C in a microaerobic atmosphere and examined after 44 h to detect the presence 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.

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 9.5.5).

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

6.5 Apparatus suitable 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, 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 General

Depending on the type of sample and the purpose of the test, one or more of three different detection procedures is/are used:

— **detection procedure A:** Detection of *Campylobacter* by enrichment, in samples with low numbers of campylobacters and low level of background microflora and/or with stressed campylobacters, e.g. cooked or frozen products;

— **detection procedure B:** Detection of *Campylobacter* by enrichment, in samples with low numbers of campylobacters and high level of background microflora, e.g. raw meats (including poultry) or raw milk;

— **detection procedure C:** Detection of *Campylobacter* by direct plating, in samples with high numbers of campylobacters, e.g. faeces, poultry caecal contents or raw poultry meat. This can be used in combination with ISO 10272-2 in order to count numbers of *Campylobacter* per g, per ml, or per cm^2 in the test material.

If little information is available concerning the best method for the particular type of sample to be tested, then use detection procedure C, in parallel with detection procedure(s) A and/or B.

In general, detection procedure B is useful for products (including cooked or frozen) that contain significant numbers of microflora resistant to third generation ß-lactams like cefoperazone. Cefoperazone is used in Bolton broth (detection procedure A) as well as in mCCD agar. Preston broth (detection procedure B) uses different selective principles and is therefore more suitable to suppress this type of resistant microflora.

9.2 Test portion and initial suspension

9.2.1 General

For preparation of the initial suspension, in the general case, use as diluent the enrichment medium specified in 9.2.2 or 9.2.3. Pre-warm the enrichment medium to room temperature before use.

In general, an amount of test portion (mass or volume) is mixed with a quantity of enrichment medium (mass or volume) to yield a tenfold dilution. However, for some types of samples (e.g. boot socks, swabs), it may be necessary to use another ratio.

This document has been validated for test portions of 10 g (or ml), except for the caecal samples. A smaller size of test portion may be used, without the need for additional validation/verification, providing that the same ratio between enrichment broth and test portion is maintained. A larger test portion than that initially validated may be used, if a validation/verification study has shown that there are no adverse effects on the detection of *Campylobacter*.

NOTE Validation can be conducted in accordance with the appropriate documents of ISO 16140 (all parts). Verification for pooling samples can be conducted in accordance with the protocol described in ISO 6887-1:2017, Annex D (verification protocol for pooling samples for qualitative tests).

9.2.2 Detection procedure A

In general, for preparing the initial suspension, combine a quantity of 10 g or 10 ml of the test portion with 90 ml of the enrichment medium Bolton broth (B.2), so as to obtain a 1 in 10 dilution, and homogenize (see ISO 7218).

9.2.3 Detection procedure B

In general, for preparing the initial suspension, combine a quantity of 10 g or 10 ml of the test portion with 90 ml of the enrichment medium Preston broth (B.3), so as to obtain a 1 in 10 dilution, and homogenize (see ISO 7218).

9.2.4 Detection procedure C

9.2.4.1 For caecal or faecal samples, use a loop (6.3) or a sterile swab to bring some of the well-mixed sample material onto the first half of a mCCD agar plate (B.4). Use another loop to streak out on the second half of the plate.

9.2.4.2 For all other samples, add an appropriate amount of liquid (e.g. peptone salt solution or Preston broth), for example, 1 in 2 (volume fraction), mix well, and either streak the plate using a loop (6.3), or dispense a suitable volume and spread it over the mCCD agar plate (B.4).

NOTE Using a second plating medium (B.5) with selective agents different from those in mCCD agar could improve *Campylobacter* detection, especially in the presence of background flora resistant to 3rd generation ß-lactams like cefoperazone.

9.3 Enrichment

9.3.1 Detection procedure A

Incubate the initial suspension (9.2.2) in a microaerobic atmosphere (6.5) at 37 °C (6.1) for 4 h to 6 h, then at 41,5 °C (6.1) for 44 h ± 4 h.

9.3.2 Detection procedure B

Incubate the initial suspension (9.2.3) in a microaerobic atmosphere (6.5) at 41,5 °C (6.1) for 24 h ± 2 h.

9.4 Isolation

9.4.1 Detection procedure A

Using the culture obtained in the enrichment medium (9.3.1), inoculate with a sterile 10 µl loop (6.3) the surface of the first selective isolation medium, mCCD agar (B.4).

Proceed in the same manner with the second *Campylobacter* selective isolation medium chosen (B.5).

9.4.2 Detection procedure B

Using the culture obtained in the enrichment medium (9.3.2), inoculate with a sterile 10 µl loop (6.3) the surface of the isolation medium, mCCD agar (B.4).

9.4.3 Detection procedures A, B and C

Incubate the plates (9.2.4, 9.4.1 and 9.4.2) at 41,5 °C (6.1) in a microaerobic atmosphere (6.5).

After 44 h ± 4 h of incubation, examine the plates 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.

9.5 Confirmation of *Campylobacter*

9.5.1 General

As *Campylobacter* rapidly loses culturability in air, follow the procedure described in 9.5.2 to 9.5.5 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)[17] 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).

9.5.2 Selection of colonies for confirmation

9.5.2.1 Select at least one typical or suspect *Campylobacter* colony (9.4.3) for purification and confirmation. One confirmed isolate per sample is sufficient. If the first colony is negative, select up to four more suspect colonies.

If needed, store the original isolation plates (9.4.3) preferably under microaerobic conditions at 5 °C (6.7) for use in further confirmation and/or identification.

9.5.2.2 Streak each of the selected colonies onto a non-selective blood agar plate, e.g. Columbia blood agar (B.6) 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 (9.5.3), absence of aerobic growth at 25 °C (9.5.4) and the presence of oxidase activity (9.5.5).

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

9.5.3 Examination of morphology and motility

9.5.3.1 Examine a freshly grown colony from the blood agar plate (9.5.2.2) for morphology and motility using a microscope (6.4).

9.5.3.2 Retain for further examination all cultures (9.5.2.2) in which curved bacilli with a spiralling "corkscrew" motility are found (9.5.3.1).

9.5.4 Study of aerobic growth at 25 °C

Using the colonies isolated in 9.5.2.2, inoculate with the aid of a loop (6.3) the surface of a non-selective blood agar plate, e.g. Columbia blood agar (B.6).

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

Examine the plate for absence of growth of colonies.

9.5.5 Detection of oxidase activity

Using a loop (6.3), take a portion of a well-isolated colony from the blood agar plate (9.5.2.2) and streak it onto a filter paper moistened with the oxidase reagent (B.7); 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.

9.5.6 Interpretation

Campylobacter gives results in accordance with Table 1.

Campylobacter is present if at least one colony presents the characteristics.

Table 1 — Characteristics of *Campylobacter*

Morphology (9.5.3)	Small curved bacilli[a]
Motility (9.5.3)	Characteristic corkscrew darting[a]
Aerobic growth at 25 °C (9.5.4)	–
Oxidase activity (9.5.5)	+

\+ Positive.

– Negative.

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

9.6 Identification of *Campylobacter* species (optional)

9.6.1 General

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

9.6.2 Detection of catalase activity

For each colony selected in 9.5.2.2, deposit a loop of culture into a drop of hydrogen peroxide solution (B.8) 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).

9.6.3 Detection of hippurate hydrolysis

For each colony selected in 9.5.2.2, 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.9.1), 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.9.2) 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).

9.6.4 Detection of indoxyl acetate hydrolysis

Place a 1 µl loopful of colony material (9.5.2.2) on an indoxyl acetate disc (B.10) 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.

9.6.5 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 (9.6.2)	+	+	+	– or weak
Hippurate hydrolysis (9.6.3)	+[a]	–	–	–
Indoxyl acetate hydrolysis (9.6.4)	+	+	–	+

\+ Positive.

– Negative.

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

10 Expression of results

In accordance with the interpretation of the results, indicate *Campylobacter* detected or not detected in the test portion examined.

11 Performance characteristics of the method

11.1 Interlaboratory study

The performance characteristics of the method were determined in an interlaboratory study to determine the specificity, sensitivity and the LOD_{50} of the method. The data are summarized in Annex C. The values derived from the interlaboratory study may not be applicable to food types or strains other than those given in Annex C[13].

11.2 Sensitivity

The sensitivity is defined as the number of samples found positive divided by the number of true positive samples tested at a given level of contamination. The results are thus dependent on the level of contamination of the sample.

11.3 Specificity

The specificity is defined as the number of samples found negative divided by the number of true negative (or blank) samples tested.

11.4 LOD_{50}

The LOD_{50} (level of detection at 50 %), is the concentration (cfu/test portion) for which the probability of detection is 50 %.

12 Test report

The test report shall specify the following:

— the test method used, with reference to this document, i.e. ISO 10272-1;

— the sampling method used, if known;

— the size of the test portion and/or the nature of the objects examined;

— all operating details not specified in this 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 procedures

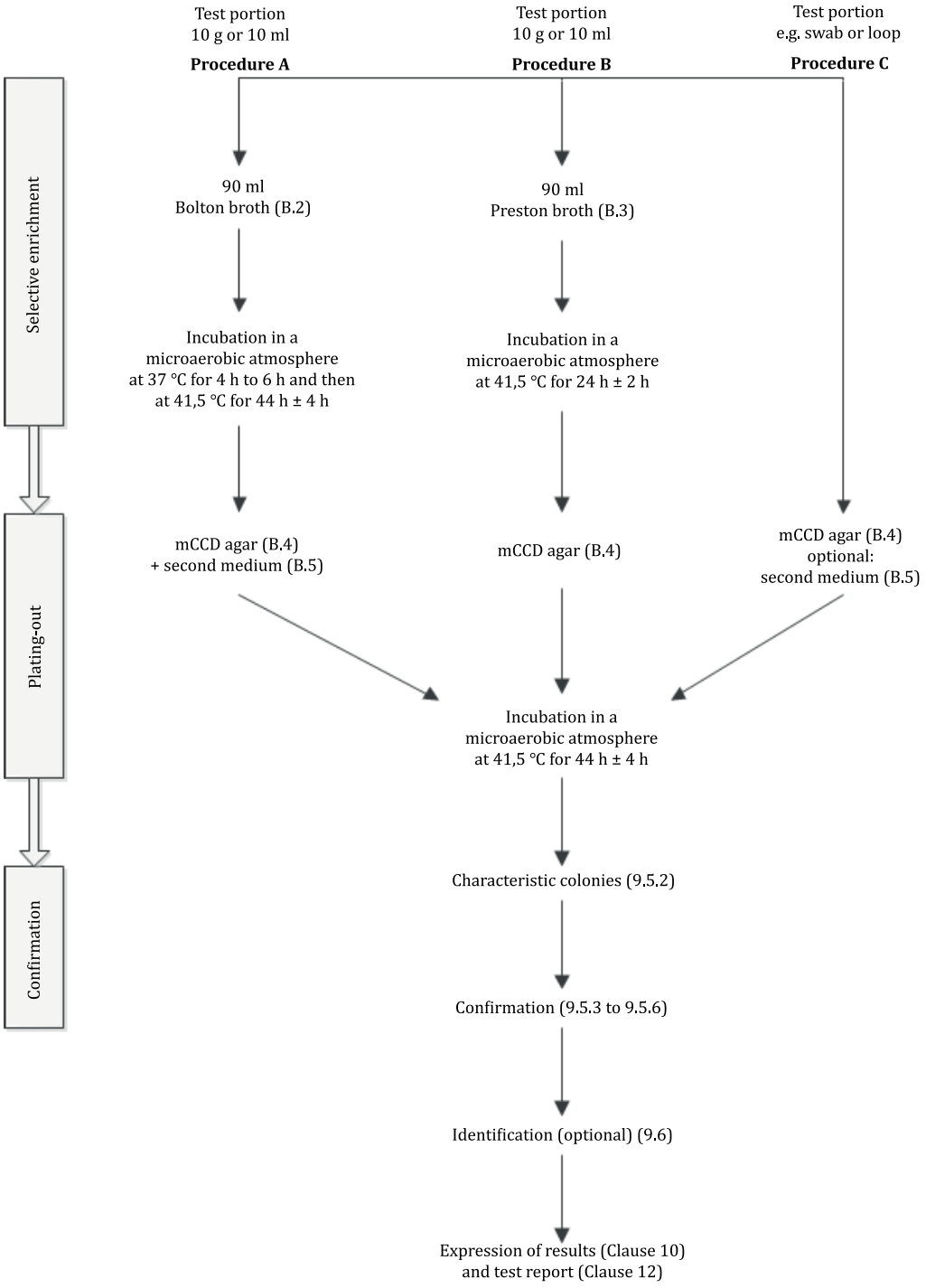

Figure A.1 — Diagram of the procedures for detection of *Campylobacter* in the food chain

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.11.

B.2 Bolton broth

B.2.1 Basic medium

B.2.1.1 Composition

Enzymatic digest of animal tissues		10,0 g
Lactalbumin hydrolysate		5,0 g
Yeast extract		5,0 g
Sodium chloride	(CAS No. 7647-14-5)	5,0 g
Sodium pyruvate	(CAS No. 113-24-6)	0,5 g
Sodium metabisulfite	(CAS No. 7681-57-4)	0,5 g
Sodium carbonate anhydrous	(CAS No. 497-19-8)	0,6 g
α-Ketoglutaric acid, monopotassium salt	(CAS No. 58485-42-0)	1,0 g
Haemin (dissolved in 0,1 % sodium hydroxide)		0,01 g
Water		945 ml

B.2.1.2 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.2.2 Sterile lysed horse blood

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

B.2.3 Antibiotic solution

B.2.3.1 Composition

Cefoperazone sodium salt	(CAS No. 62893-20-3)	0,02 g
Vancomycin hydrochloride	(CAS No. 1404-93-9)	0,02 g
Trimethoprim lactate salt	(CAS No. 23256-42-0)	0,02 g
Amphotericin B	(CAS No. 1397-89-3)	0,01 g
Ethanol/sterile distilled water 50/50 (volume fraction)		5 ml

B.2.3.2 Preparation

Dissolve the components in the 50/50 mixture of ethanol and sterile distilled water.

B.2.4 Complete medium

B.2.4.1 Composition

Basic medium (B.2.1)	945 ml
Sterile lysed horse blood (B.2.2)	50 ml
Antibiotic solution (B.2.3)	5 ml

B.2.4.2 Preparation

To the basic medium, cooled down to below 47 °C, add the blood aseptically, then the antibiotic solution and mix. Dispense the medium aseptically into tubes 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 7 days.

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 No. 7647-14-5)	5,0 g
Water		945 ml

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 Complete medium

B.3.4.1 Composition

Basic medium (B.3.1)	945 ml
Sterile lysed horse blood (B.3.2)	50 ml
Antibiotic solution (B.3.3)	5 ml

B.3.4.2 Preparation

To the basic medium, cooled down to below 47 °C, add the blood aseptically, then the antibiotic solution 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 7 days.

B.4 Modified charcoal cefoperazone deoxycholate agar (mCCD agar)

B.4.1 Basic medium

B.4.1.1 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.4.1.2 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 or bottles of suitable capacity. Sterilize in the autoclave set at 121 °C for 15 min.

B.4.2 Antibiotic solution

B.4.2.1 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.4.2.2 Preparation

Dissolve the components in the water. Sterilize by filtration.

B.4.3 Complete medium

B.4.3.1 Composition

Basic medium (B.4.1)	1 000 ml
Antibiotic solution (B.4.2)	5 ml

B.4.3.2 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.5 Selective plating medium using different selective principles from those in mCCD agar

Selective principles in mCCD agar in this case mainly refer to the use of third generation ß-lactams. Examples are Preston agar[9], Butzler agar[15], etc. More information on media can be found in Reference [12].

B.6 Colombia blood agar

B.6.1 Basic medium

B.6.1.1 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.6.1.2 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.6.2 Sterile sheep or horse blood

B.6.3 Complete medium

B.6.3.1 Composition

Basic medium (B.6.1)	1 000 ml
Sterile blood (B.6.2)	50 ml

B.6.3.2 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.7 Reagent for the detection of oxidase activity

B.7.1 Composition

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

B.7.2 Preparation

Dissolve the component in the water immediately prior to use.

B.8 Reagent for the detection of catalase activity

B.8.1 Composition

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

B.8.2 Preparation

Dissolve the component in the water immediately prior to use.

B.9 Reagents for the detection of hydrolysis of hippurate

B.9.1 Sodium hippurate solution

B.9.1.1 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.9.1.2 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.9.2 Ninhydrin solution, mass fraction of 3,5 %

B.9.2.1 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.9.2.2 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.10 Indoxyl acetate discs

B.10.1 Composition

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

B.10.2 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.11 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]	Method of control	Criteria[c]	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*[d] + *Escherichia coli*[d] + *Proteus mirabilis*	00156 or 00005 00012 or 00013 00023	Qualitative	>10 characteristic colonies on mCCD agar	Greyish, flat and moist, sometimes with metallic sheen
			Campylobacter coli + *Escherichia coli*[d] + *Proteus mirabilis*	00004 00012 or 00013 00023			
	Selectivity		*Escherichia coli*[d] *Proteus mirabilis*	00012 or 00013 00023	Qualitative	Total inhibition (0) on TSA	—
Preston broth	Productivity	(24 ± 2) h / (41,5 ± 1) °C microaerobic atmosphere	*Campylobacter jejuni*[d] + *Escherichia coli*[d] + *Proteus mirabilis*	00156 or 00005 00012 or 00013 00023	Qualitative	>10 characteristic colonies on mCCD agar	Greyish, flat and moist, sometimes with metallic sheen
			Campylobacter coli + *Escherichia coli*[d] + *Proteus mirabilis*	00004 00012 or 00013 00023			
	Selectivity		*Escherichia coli*[d] *Proteus mirabilis*	00012 or 00013 00023	Qualitative	Total inhibition 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] Strain to be used as a minimum.

[c] Growth/turbidity is categorized as 0: no growth/turbidity; 1: weak growth/turbidity; 2: good growth/turbidity (see ISO 11133).

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

Table B.1 *(continued)*

Medium	Function	Incubation	Control strains	WDCM numbers[a]	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	Qualitative	Good growth (2)	Greyish, flat and moist colonies, 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)	—
Colombia blood agar	Productivity	24 h to 48 h / (41,5 ± 1) °C microaerobic atmosphere	*Campylobacter jejuni*[d] *Campylobacter coli*[d]	00156 or 00005 00004	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] Strain to be used as a minimum.

[c] Growth/turbidity is categorized as 0: no growth/turbidity; 1: weak growth/turbidity; 2: good growth/turbidity (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 17 collaborators in 13 countries was carried out. The following sample 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 two different levels of contamination, plus a negative control. Broiler caecal material was tested according to detection procedure C (direct plating), frozen spinach and frozen minced meat according to detection procedure A (Bolton broth), and raw milk and chicken skin according to detection procedure B (Preston broth). The study was organized in 2013 by the Netherlands Food and Consumer Product Safety Authority (NVWA) 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 values of the performance characteristics derived from this interlaboratory study are shown per type of sample in Table C.1 to Table C.5. Data obtained by some collaborators have been excluded from the calculations only on the basis of clearly identified technical reasons (deviations to the protocol).

The LOD_{50} has been determined according to Reference [16].

Table C.1 — Results of data analysis obtained with broiler caecal material (procedure C, direct plating)

Parameter	Broiler caecal material		
	Blank	0,55 cfu/test portion (low level contamination[a])	136 cfu/test portion (high level contamination[a])
Number of participating collaborators	17	17	17
Number of samples per collaborator	8	8	8
Number of collaborators retained after evaluation of the data	17	17	17
Number of sample results retained after evaluation of the data	135	136	136
Test portion size in ml (10 µl-loop)	0,01	0,01	0,01
Sensitivity in %	—	28	99
Specificity in %	100	—	—
LOD_{50} (95 % confidence interval) in cfu/test portion	—	6,1 (3,1 to 12)	

NOTE Generally, the expected level of *Campylobacter* in naturally contaminated broiler caecal material is higher than 10^6 cfu/g.

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

Table C.2 — Results of data analysis obtained with frozen spinach (procedure A, Bolton broth)

Parameter	Frozen spinach		
	Blank	1,6 cfu/test portion (low level contamination[a])	16 cfu/test portion (high level contamination[a])
Number of participating collaborators	17	17	17
Number of samples per collaborator	8	8	8
Number of collaborators retained after evaluation of the data	16	16	16
Number of sample results retained after evaluation of the data	128	128	128
Test portion size in g	10	10	10
Sensitivity in %	—	73	100
Specificity in %	98	—	—
LOD_{50} (95 % confidence interval) in cfu/test portion	—	0,84 (0,67 to 1,0)	
[a] Inoculation strain: *C. jejuni* (WDCM 00005).			

Table C.3 — Results of data analysis obtained with frozen minced meat (pork/beef) (procedure A, Bolton broth)

Parameter	Frozen minced meat		
	Blank	1,3 cfu/test portion (low level contamination[a])	9,6 cfu/test portion (high level contamination[a])
Number of participating collaborators	17	17	17
Number of samples per collaborator	8	8	8
Number of collaborators retained after evaluation of the data	16	16	16
Number of sample results retained after evaluation of the data	128	128	128
Test portion size in g	10	10	10
Sensitivity in %	—	45	91
Specificity in %	100	—	—
LOD_{50} (95 % confidence interval) in cfu/test portion	—	2,2 (1,8 to 2,6)	
[a] Inoculation strain: *C. coli* (WDCM 00072).			

Table C.4 — Results of data analysis obtained with raw milk (procedure B, Preston broth)

Parameter	Raw milk		
	Blank	17 cfu/test portion (low level contamination[a])	206 cfu/test portion (high level contamination[a])
Number of participating collaborators	17	17	17
Number of samples per collaborator	8	8	8
Number of collaborators retained after evaluation of the data	16	16	16
Number of sample results retained after evaluation of the data	128	128	128
Test portion size in g	10	10	10
Sensitivity in %	—	53	78
Specificity in %	100	—	—
LOD_{50} (95 % confidence interval) in cfu/test portion	—	57 (46 to 70)	

[a] Inoculation strain: *C. jejuni* (WDCM 00156).

Table C.5 — Results of data analysis obtained with chicken skin (procedure B, Preston broth)

Parameter	Chicken skin		
	Blank	7,5 cfu/test portion (low level contamination[a])	105 cfu/test portion (high level contamination[a])
Number of participating collaborators	17	17	17
Number of samples per collaborator	8	8	8
Number of collaborators retained after evaluation of the data	15	15	15
Number of sample results retained after evaluation of the data	120	120	120
Test portion size in g	10	10	10
Sensitivity in %	—	64	92
Specificity in %	99	—	—
LOD_{50} (95 % confidence interval) in cfu/test portion	—	14 (11 to 19)	

[a] Inoculation strain: *C. coli* (WDCM 00004).

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

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(*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

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