INTERNATIONAL STANDARD

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

Part 2: Colony-count technique

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

Partie 2: Technique par comptage des colonies

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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 on 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 the following URL: www.iso.org/iso/foreword.html.

This document was prepared by the European Committee for Standardization (CEN), Technical Committee CEN/TC 275, *Food Analysis — Horizontal methods, in* collaboration with ISO Technical Committee ISO/TC 34, *Food products,* Subcommittee SC 9, *Microbiology* in accordance with the agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This first edition cancels and replaces ISO/TS 10272-2:2006, which has been technically revised with the following main changes:

— samples from the primary production stage have been added to the scope;

— serial dilutions are plated in single instead of in duplicate, to be in line with ISO 7218;

— the confirmation tests on study of microaerobic growth at 25 °C and aerobic growth at 41,5 °C were replaced by the study of aerobic growth at 25 °C;

— performance testing for the quality assurance of the culture media has been added to Annex B;

— performance characteristics have been added to Annex C.

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

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.

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

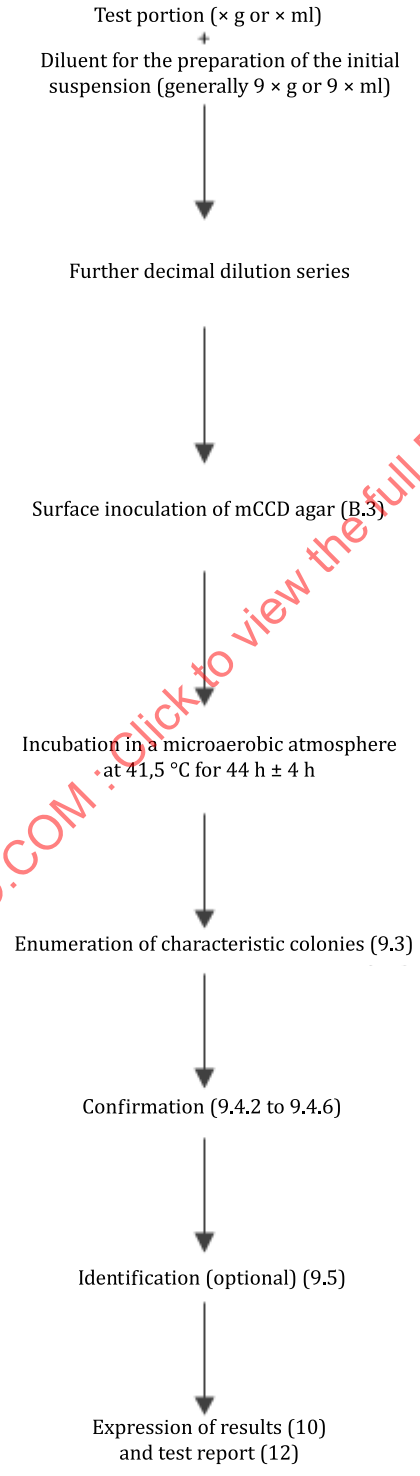

Figure A.1 — Diagram of the procedure for enumeration of *Campylobacter* in the food chain

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.