INTERNATIONAL STANDARD

ISO 10272-1

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Microbiology of food and animal feeding stuffs — Horizontal method for detection and enumeration of *Campylobacter* spp. —

Part 1: Detection method

Microbiologie des aliments — Méthode horizontale pour la recherche et le dénombrement de Campylobacter *spp. —*

Partie 1: Méthode de recherche

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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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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.

ISO 10272-1 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

This first edition of ISO 10272-1, together with ISO/TS 10272-2:2006, cancels and replaces ISO 10272:1995, which has been technically revised.

ISO 10272 consists of the following parts, under the general title *Microbiology of food and animal feeding stuffs — Horizontal method for detection and enumeration of* Campylobacter *spp.*:

— *Part 1: Detection method*

— *Part 2: Colony-count technique* (Technical Specification)

Introduction

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 International Standard 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 International Standard so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

Microbiology of food and animal feeding stuffs — Horizontal method for detection and enumeration of *Campylobacter* spp. —

Part 1: Detection method

1 Scope

This part of ISO 10272 describes a horizontal method for the detection of *Campylobacter* spp.

It is applicable to products intended for human consumption or for the feeding of animals, and to environmental samples in the area of food production and food handling, subject to the limitations stated in the Introduction.

2 Normative references

The following referenced documents are indispensable for the application 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 food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination*

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

ISO 8261, *Milk and milk products — General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examinations*

ISO/TS 11133-1, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 1: General guidelines on quality assurance for the preparation of culture media in the laboratory*

ISO/TS 11133-2:2003, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 2: Practical guidelines on performance testing of culture media*

3 Terms and definitions

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

3.1
Campylobacter
microorganisms forming characteristic colonies on solid selective media when incubated microaerobically at 41,5 °C but not at 25 °C, and which possess the characteristic motility and biochemical and growth properties described when the tests are conducted in accordance with this part of ISO 10272

NOTE The most frequently encountered species are *Campylobacter jejuni* and *Campylobacter coli*. Other species have, however, been described *(Campylobacter lari*, *Campylobacter upsaliensis* and some others).

3.2
detection of *Campylobacter*
determination of the presence or absence of these microorganisms in a defined quantity of product, when the test is conducted in accordance with this part of ISO 10272

4 Principle

4.1 General

In general, the detection of *Campylobacter* requires the following stages (see Annex A for a diagram of the procedure).

4.2 Enrichment in selective liquid medium

The test portion is inoculated into the liquid enrichment medium (Bolton broth) and homogenized.

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 h.

4.3 Isolation and selection for confirmation

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

— modified charcoal cefoperazone deoxycholate agar (mCCD agar);

— any other solid selective medium based on a principle different from that of mCCD agar.

They are then incubated at 41,5 °C in a microaerobic atmosphere and inspected after 44 h ± 4 h to detect the presence of colonies presumed because of their characteristics to be *Campylobacter*.

4.4 Confirmation

The colonies presumed to be *Campylobacter* are subcultured on the non-selective Columbia blood agar, then confirmed by means of microscopic examination and appropriate biochemical and growth tests. Optionally, the *Campylobacter* species are identified by specific biochemical tests and antibiotic sensitivity tests.

5 Culture media and reagents

5.1 General

For current laboratory practice, see ISO 7218, ISO/TS 11133-1 and ISO/TS 11133-2.

NOTE Because of the large number of culture media and reagents and for the clarity of the text, their compositions and preparations are given in Annex B.

5.2 Liquid enrichment medium: Bolton broth

See B.1.

5.3 Selective plating medium: Modified charcoal cefoperazone deoxycholate agar (mCCD agar)

See B.2.

5.4 Confirmation and identification media and reagents

5.4.1 Columbia blood agar

See B.3.

5.4.2 Brucella broth

See B.4.

5.4.3 Reagent for the detection of oxidase

See B.5.

5.4.4 Hydrogen peroxide solution, 3 % (volume fraction)

5.4.5 Reagents for the detection of hydrolysis of hippurate

See B.6.

5.4.6 Mueller Hinton blood agar

See B.7.

5.4.7 **Nalidixic acid** (30 µg) **and cephalothin** (30 µg) **discs**

5.4.8 Indoxyl acetate discs

See B.8.

6 Apparatus and glassware

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

6.1 Apparatus for dry sterilization (oven) or wet sterilization (autoclave)

See ISO 7218.

6.2 **Oven, laminar flow cabinet or incubator**, capable of operating between 37 °C and 55 °C.

6.3 **Incubator**, capable of operating at 41,5 °C ± 1 °C.

6.4 **Water baths**, capable of operating at 25 °C ± 1 °C and 37 °C ± 1 °C, or incubators capable of operating at 25 °C ± 1 °C and 37 °C ± 1 °C.

6.5 **Water bath**, capable of operating between 47 °C and 50 °C.

6.6 **pH-meter**, accurate to within 0,1 pH units at 25 °C.

6.7 Containers, in particular culture tubes of dimensions 18 mm × 180 mm and 9 mm × 180 mm, haemolysis tubes of dimensions 13 mm × 75 mm, bottles with non-toxic metal closures and/or flasks of appropriate capacity with appropriate covers.

6.8 Petri dishes, in glass or plastic, with diameters 90 mm to 100 mm.

6.9 Total-delivery graduated pipettes, with a wide opening, and a nominal capacity of 1 ml and 10 ml, graduated in 0,1 ml divisions, and **Pasteur pipettes**.

6.10 Rubber teats, or any other safety system capable of being adapted to the graduated pipettes.

6.11 Sterile loops, of platinum/iridium, nickel/chromium or plastic, approximately 3 mm in diameter, and **wires** of the same material, or a **glass or plastic rod**.

A nickel/chromium loop is not suitable for use in the oxidase test (see 9.4.6).

6.12 Forceps, fine, round-ended, of stainless steel.

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

6.14 Apparatus suitable for achieving a microaerobic atmosphere with oxygen content of 5 % ± 2 %, carbon dioxide 10 % ± 3 %, optional hydrogen ⩽ 10 %, with the balance nitrogen. Appropriate gastight containers are used to hold Petri dishes and/or flasks or bottles of about 350 ml capacity used for the enrichment broth, e.g. bacteriological anaerobic jars.

NOTE 1 The appropriate microaerobic atmosphere can be obtained using commercially available gas-generating kits, following precisely the manufacturer's instructions, particularly those relating to the volume of the jar and the capacity of the gas-generating kit. Alternatively, the jar may be filled with an appropriate gas mixture prior to incubation.

NOTE 2 As an alternative to incubation in a microaerobic atmosphere, the enrichment broth can be incubated in screw-capped bottles or flasks filled with enrichment broth, leaving a headspace of less than 2 cm, and tightly closing the caps.

7 Sampling

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

Sampling is not part of the method specified in this part of ISO 10272. See the specific International Standard dealing with the product concerned. If there is no specific International Standard dealing with sampling of the product concerned, it is recommended that the parties concerned come to an agreement on this subject.

Since *Campylobacter s*pp. are very sensitive to freezing but survive best at low temperatures, it is recommended that samples to be tested should not be frozen, but stored at +3 °C ± 2 °C 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 in accordance with the specific International Standard dealing with the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure (see diagram in Annex A)

9.1 Test portion, initial suspension and dilutions

9.1.1 See the suitable part of ISO 6887, or ISO 8261.

9.1.2 In general, for preparing the initial suspension, introduce a quantity x of the test portion (mass or volume) into nine times its volume of the enrichment medium Bolton broth (5.2), so as to obtain a test portion/enrichment medium ratio of 1:10 (mass/volume or volume/volume), and homogenize.

9.2 Enrichment

Incubate the initial suspension (9.1.2) in a microaerobic atmosphere (6.14) at 37 °C for 4 h to 6 h, then at 41,5 °C for 44 h $\pm$ 4 h.

9.3 Isolation

9.3.1 Using the culture obtained in the enrichment medium (9.2), inoculate with a sterile loop (6.11) the surface of the first selective isolation medium, mCCD agar (5.3).

Proceed in the same manner with the second *Campylobacter* selective isolation medium chosen.

NOTE It is preferable to take a second isolation medium that is based on a principle different from mCCD agar. Examples of isolation media to be used are Skirrow agar, Karmali agar and Preston agar (see Bibliography).

9.3.2 Incubate the plates (9.3.1) at 41,5 °C in a microaerobic atmosphere (6.14).

9.3.3 After 44 h $\pm$ 4 h of incubation, examine the plates for typical and/or suspect colonies of *Campylobacter.*

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

9.4 Confirmation of *Campylobacter* species

9.4.1 General

As the bacteria rapidly deteriorate in air, follow the procedure described in 9.4.2 to 9.4.6 without any delay.

9.4.2 Selection of colonies for confirmation

9.4.2.1 For confirmation, take from each plate of each selective medium (9.3.1) at least one colony considered to be typical or suspected as being *Campylobacter* and a further four colonies if the first is negative.

9.4.2.2 Streak each of the selected colonies onto a Columbia blood agar plate (5.4.1) in order to allow the development of well-isolated colonies. Incubate the plates in a microaerobic atmosphere at 41,5 °C for 24 h to 48 h. Use the pure cultures for examination of morphology, motility, microaerobic growth at 25 °C, aerobic growth at 41,5 °C and the presence of oxidase.

9.4.3 Examination of morphology and motility

9.4.3.1 Suspend one colony from the Columbia blood agar plate (9.4.2.2) in 1 ml of Brucella broth (5.4.2) and examine for morphology and motility using a microscope (6.13).

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 growth at 25 °C (microaerobic)

Using the colonies isolated in 9.4.2.2, inoculate with the aid of a loop (6.11) the surface of a Columbia blood agar plate (5.4.1).

Incubate the plate at 25 °C in a microaerobic atmosphere (6.14) for 44 h ± 4 h.

Examine the plate for visible growth of *Campylobacter* colonies.

9.4.5 Study of growth at 41,5 °C (aerobic)

Using the colonies isolated in 9.4.2.2, inoculate with the aid of a loop (6.11) the surface of a Columbia blood agar plate (5.4.1).

Incubate the plate at 41,5 °C in an aerobic atmosphere (6.14) for 44 h ± 4 h.

Examine the plate for visible growth of *Campylobacter* colonies.

9.4.6 Detection of oxidase

Using a platinum/iridium loop or glass rod (6.11), 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 (5.4.3); 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.

Confirm the results using positive and negative controls. Examples of suitable control strains are *Pseudomonas aeruginosa* NCTC 10662 (positive control), *Escherichia coli* NCTC 9001 (negative control).

9.4.7 Interpretation

Campylobacter spp. give results in accordance with Table 1.

Table 1 — Characteristics of *Campylobacter* spp.

Morphology (9.4.3)	small curved bacilli
Motility (9.4.3)	characteristic
Microaerobic growth at 25 °C (9.4.4)	–
Aerobic growth at 41,5 °C (9.4.5)	–
Oxidase (9.4.6)	+

Campylobacter spp. are present if at least one colony presents the above characteristics.

9.5 Identification of *Campylobacter* species (optional)

9.5.1 General

Among the *Campylobacter s*pp. 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 some others); the characteristics given in Table 2 permit their differentiation.

9.5.2 Detection of catalase

For each colony selected in 9.4.2.2, deposit a loop of culture into a drop of hydrogen peroxide solution (5.4.4) 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 *Staphylococcus aureus* NCTC 8532 (positive control), *Enterococcus faecalis* NCTC 775 (negative control).

9.5.3 Detection of sensitivity to nalidixic acid and to cephalothin

For each colony selected in 9.4.2.2, use a loop (6.11) to prepare a suspension in Brucella broth (5.4.2) of density 0,5 on the McFarland scale.

Dilute this suspension 1/10 with the same broth.

Flood the surface of a Mueller Hinton 5 % blood agar plate (5.4.6) with the suspension.

Leave in contact for 5 min, then drain off excess suspension.

Dry the plates in a drying cabinet (6.2) set at 37 °C for 10 min.

On the surface of the agar, place a disc of nalidixic acid and a disc of cephalothin (5.4.7).

Incubate the plates, with lids uppermost, at 37 °C for 22 h ± 2 h in a microaerobic atmosphere (6.14).

Interpret the bacterial growth in the following manner:

— growth that is in contact with the disc is classified as **resistant**;

— the presence of a zone of any size due to inhibition of growth is classified as **susceptible**.

9.5.4 Detection of hippurate hydrolysis

For each colony selected in 9.4.2.2, use a loop (6.11) with a heavy inoculum to prepare a suspension in a haemolysis tube (6.7) containing 0,4 ml of a sodium hippurate solution (5.4.5), taking care not to incorporate any agar.

Shake in order to mix thoroughly and incubate for 2 h in a water bath (6.4) set at 37 °C or 4 h in an incubator set at 37 °C.

Carefully add 0,2 ml of a ninhydrin solution (5.4.5) on the top of the sodium hippurate solution. Do not shake.

Interpret after an additional incubation of 10 min in the water bath (6.4) set at 37 °C or in an incubator set at 37 °C.

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* NCTC 11351 (positive control), *Campylobacter coli* NCTC 11366 (negative control).

9.5.5 Detection of indoxyl acetate hydrolysis

Place a colony selected in 9.4.2.2 on an indoxyl acetate disc (5.4.8) and add a drop of sterile distilled water. A loopful of colony material is required for a clear reaction.

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* NCTC 11351 (positive control), *Campylobacter lari* NCTC 11352 (negative control).

9.5.6 Interpretation

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

Table 2 — Characteristics of *Campylobacter* species

Characteristic	*C. jejuni*	*C. coli*	*C. lari*	*C. upsaliensis*
Catalase (9.5.2)	+	+	+	– or slight
Nalidixic acid (9.5.3)	S [a]	S [a]	R/S [b]	S
Cephalothin (9.5.3)	R	R	R	S
Hydrolysis of hippurate (9.5.4)	+	–	–	–
Indoxyl acetate (9.5.5)	+	+	–	+

Key: + = positive; – = negative; S = sensitive; R = resistant.

[a] An increase in the resistance to nalidixic acid of *C. jejuni* and *C. coli* strains has been shown.

[b] Both sensitive and resistant *C. lari* strains exist.

10 Expression of results

According to the interpretation of the results, indicate the presence or absence of *Campylobacter* in a test portion of x g or x ml of product. (See ISO 7218.)

11 Test report

The test report shall indicate

a) all information necessary for the complete identification of the sample;

b) the sampling method used, if known

c) any deviation in the enrichment medium or first isolation medium or incubation conditions used;

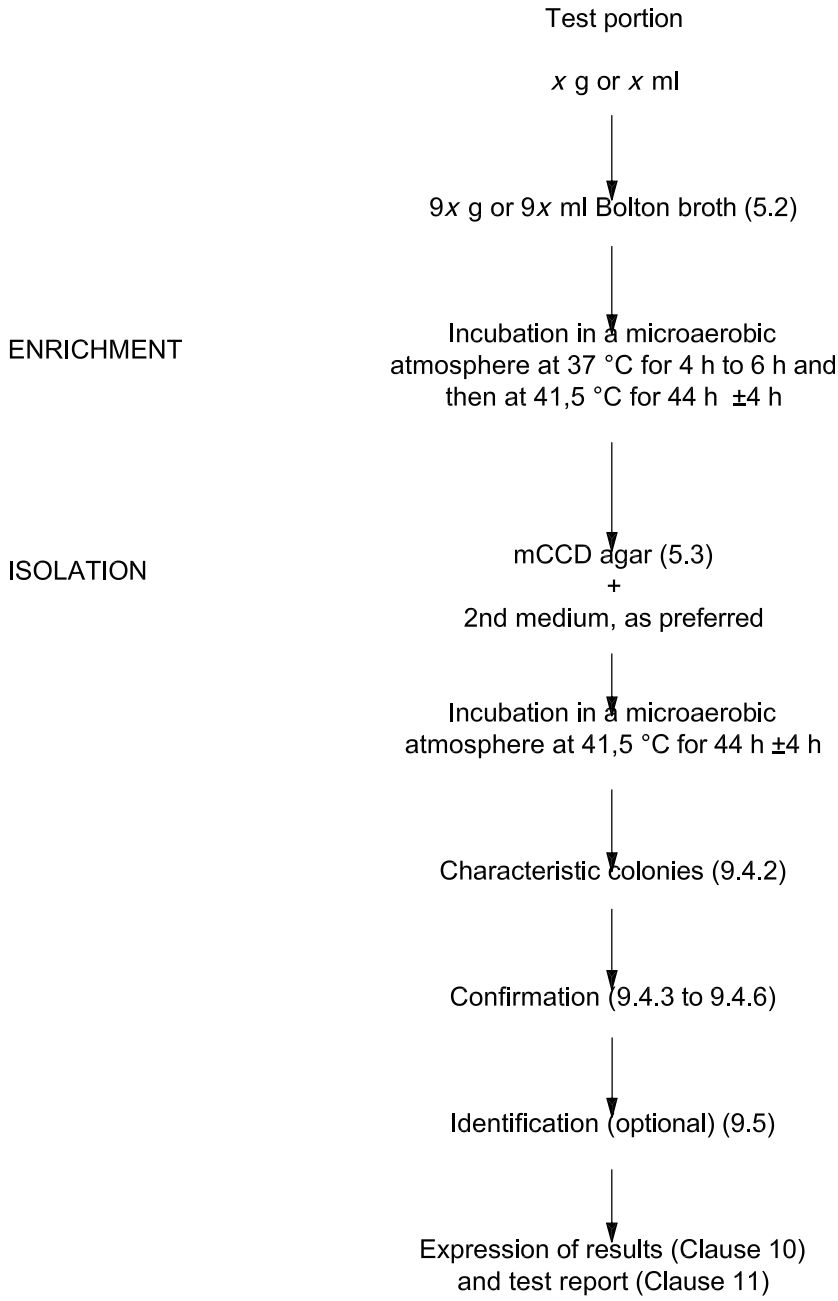
d) the second isolation medium used;

e) all operating details not specified in this part of ISO 10272, or regarded as optional, together with details of any incidents which may have influenced the test results;

f) the test results obtained.

Annex A
(normative)

Diagram of procedure



Annex B
(normative)

Composition and preparation of culture media and reagents

B.1 Bolton broth

B.1.1 Basic medium

B.1.1.1 Composition

Enzymatic digest of animal tissues	10,0 g
Lactalbumin hydrolysate	5,0 g
Yeast extract	5,0 g
Sodium chloride	5,0 g
Sodium pyruvate	0,5 g
Sodium metabisulphite	0,5 g
Sodium carbonate	0,6 g
α-Ketoglutaric acid	1,0 g
Haemin (dissolved in 0,1 % sodium hydroxide)	0,01 g
Water	1 000 ml

B.1.1.2 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 $7,4 \pm 0,2$ at 25 °C. Dispense the basic medium into flasks of suitable capacity. Sterilize in the autoclave (6.1) set at 121 °C for 15 min.

B.1.2 Sterile lysed defibrinated horse blood

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

B.1.3 Antibiotic solution

B.1.3.1 Composition

Cefoperazone	0,02 g
Vancomycin	0,02 g
Trimethoprim lactate	0,02 g
Amphotericin B	0,01 g
Ethanol/sterile distilled water 50/50 (volume fraction)	5 ml

B.1.3.2 Preparation

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

B.1.4 Complete medium

B.1.4.1 Composition

Basic medium (B.1.1)	1 000 ml
Sterile lysed defibrinated horse blood (B.1.2)	50 ml
Antibiotic solution (B.1.3)	5 ml

B.1.4.2 Preparation

To the basic medium, at a temperature of 47 °C to 50 °C, add the blood aseptically, then the antibiotic solution and mix. Dispense the medium aseptically into tubes or flasks of suitable capacity (see 9.1.2) to obtain the portions necessary for the test. If the enrichment medium has been prepared in advance, it shall not be kept for more than 4 h at ambient temperature, or in the dark at 3 °C ± 2 °C for not more than 7 days.

B.1.5 Performance testing

The performance of Bolton broth shall be tested according to the methods and criteria described in ISO/TS 11133-2. Examples of suitable control strains are *Campylobacter jejuni* NCTC 11351 or ATCC 33291 with the following criteria: > 10 colonies on modified charcoal cefoperazone deoxycholate (mCCD) agar after microaerobic incubation at 41,5 °C for 44 h ± 4 h.

B.2 Modified charcoal cefoperazone deoxycholate agar (mCCD agar)

B.2.1 Basic medium

B.2.1.1 Composition

Meat extract	10,0 g
Enzymatic digest of animal tissues	10,0 g
Sodium chloride	5,0 g
Charcoal	4,0 g

Enzymatic digest of casein	3,0 g
Sodium deoxycholate	1,0 g
Iron(II) sulfate	0,25 g
Sodium pyruvate	0,25 g
Agar	8,0 g to 18,0 g [1)]
Water	1 000 ml

B.2.1.2 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 (6.1) set at 121 °C for 15 min.

B.2.2 Antibiotic solution

B.2.2.1 Composition

Cefoperazone	0,032 g
Amphotericin B	0,01 g
Water	5 ml

B.2.2.2 Preparation

Dissolve the components in the water. Sterilize by filtration.

B.2.3 Complete medium

B.2.3.1 Composition

Basic medium (B.2.1)	1 000 ml
Antibiotic solution (B.2.2)	5 ml

B.2.3.2 Preparation

Add the antibiotic solution to the basic medium, cooled down to 47 °C to 50 °C, then mix carefully. Pour about 15 ml of the complete medium into sterile Petri dishes. 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 (6.2) for 30 min or until the agar surface is free of visible moisture. If they have been prepared in advance, the undried agar plates shall not be kept for more than 4 h at ambient temperature, or in the dark at 3 °C ± 2 °C for not more than 7 days.

B.2.4 Performance testing

For the definition of selectivity and productivity, refer to ISO/TS 11133-1. For the performance criteria, refer to ISO/TS 11133-2:2003, Table B.5.

1) Depending on the gel strength of the agar.

B.3 Columbia blood agar

B.3.1 Basic medium

B.3.1.1 Composition

Enzymatic digest of animal tissues	23,0 g
Starch	1,0 g
Sodium chloride	5,0 g
Agar	8,0 g to 18,0 g [1)]
Water	1 000 ml

B.3.1.2 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 7,3 ± 0,2 at 25 °C. Dispense the basic medium into flasks of suitable capacity. Sterilize in the autoclave (6.1) set at 121 °C for 15 min.

B.3.2 Sterile defibrinated sheep blood

B.3.3 Complete medium

B.3.3.1 Composition

Basic medium (B.3.1)	1 000 ml
Sterile defibrinated sheep blood (B.3.2)	50 ml

B.3.3.2 Preparation

Add the blood aseptically to the basic medium, cooled down to 47 °C to 50 °C, then mix. Pour about 15 ml of the complete medium into sterile Petri dishes. 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 (6.2) for 30 min or until the agar surface is free of visible moisture. If they have been prepared in advance, the undried agar plates shall not be kept for more than 4 h at ambient temperature, or more than 7 days at 3 °C ± 2 °C.

B.4 Brucella broth

B.4.1 Composition

Enzymatic digest of casein	10,0 g
Enzymatic digest of animal tissues	10,0 g
Glucose	1,0 g
Yeast extract	2,0 g
Sodium chloride	5,0 g
Sodium hydrogen sulfite	0,1 g
Water	1 000 ml

B.4.2 Preparation

Dissolve the basic components or the dehydrated complete medium in the water, by heating if necessary. Adjust the pH, if necessary, so that after sterilization it is 7,0 ± 0,2 at 25 °C. Dispense the medium in quantities of 10 ml into tubes of suitable capacity. Sterilize in the autoclave (6.1) set at 121 °C for 15 min.

B.5 Reagent for the detection of oxidase

B.5.1 Composition

N,*N*,*N*',*N*'-Tetramethyl-1,4-phenylenediamine dihydrochloride	1,0 g
Water	100 ml

B.5.2 Preparation

Dissolve the component in the water immediately prior to use.

B.6 Reagents for the detection of hydrolysis of hippurate

B.6.1 Sodium hippurate solution

B.6.1.1 Composition

Sodium hippurate	10 g
Phosphate-buffered saline (PBS) consisting of:	
sodium chloride	8,5 g
disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$)	8,98 g
sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$)	2,71 g
Water, to a final volume of	1 000 ml

B.6.1.2 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 (6.7). Store at about −20 °C.

B.6.2 Ninhydrin solution, 3,5 % (mass/volume)

B.6.2.1 Composition

Ninhydrin	1,75 g
Acetone	25 ml
Butanol	25 ml

B.6.2.2 Preparation

Dissolve the ninhydrin in the acetone/butanol mixture. Store the solution in the refrigerator for a maximum period of 1 week in the dark.

B.7 Mueller Hinton blood agar

B.7.1 Basic medium

B.7.1.1 Composition

Enzymatic digest of animal tissues	6,0 g
Enzymatic digest of casein	17,5 g
Starch, soluble	1,5 g
Agar	8,0 g to 18,0 g [1)]
Water	1 000 ml

B.7.1.2 Preparation

Dissolve the basic components or the dehydrated complete basic medium in the water, bringing to the boil. Adjust the pH, if necessary, so that after sterilization it is 7,3 ± 0,2 at 25 °C. Dispense the basic medium into flasks of suitable capacity. Sterilize in the autoclave (6.1) set at 121 °C for 15 min.

B.7.2 Sterile defibrinated sheep blood

B.7.3 Complete medium

B.7.3.1 Composition

Basic medium (B.7.1)	1 000 ml
Sterile defibrinated sheep blood (B.7.2)	50 ml

B.7.3.2 Preparation

Add the blood aseptically to the basic medium, cooled down to 47 °C to 50 °C, then mix. Pour about 15 ml of the complete medium into sterile Petri dishes. 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 (6.2) for 30 min or until the agar surface is free of visible moisture. If they have been prepared in advance, the undried agar plates shall not be kept for more than 4 h at ambient temperature, or more than 7 days at 3 °C ± 2 °C.

B.8 Indoxyl acetate discs

B.8.1 Composition

Indoxyl acetate	0,1 g
Acetone	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 4 °C in a brown tube or bottle in the presence of silica gel.

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