TECHNICAL SPECIFICATION

ISO/TS 10272-2

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

Part 2: Colony-count technique

Microbiologie des aliments — 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.

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.

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of normative document:

— an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;

— an ISO Technical Specification (ISO/TS) represents an agreement between the members of a technical committee and is accepted for publication if it is approved by 2/3 of the members of the committee casting a vote.

An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

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/TS 10272-2 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

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

ISO/TS 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 Technical Specification 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 2: Colony-count technique

1 Scope

This Technical Specification describes a horizontal method for the enumeration 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 micro-aerobically 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 Technical Specification

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
count of *Campylobacter*
number of *Campylobacter* found per millilitre or per gram of test sample when the test is conducted in accordance with this Technical Specification

4 Principle

4.1 Preparation of dilutions

For the preparation of decimal dilutions from the test sample, see ISO 6887 and ISO 8261.

4.2 Enumeration

The solid selective medium, modified charcoal cefoperazone deoxycholate agar (mCCD agar), is inoculated with a specified quantity of the test sample 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 sample or of the initial suspension.

The plates are incubated at 41,5 °C in a micro-aerobic atmosphere for 40 h to 48 h.

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

The number of *Campylobacter* per millilitre or per gram of the test sample is calculated from the number of confirmed typical colonies per plate.

5 Culture media and reagents

5.1 General

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

5.2 Diluent

See ISO 6887.

5.3 Modified charcoal cefoperazone desoxycholate agar (mCCD agar)

5.3.1 Basic medium

5.3.1.1 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 desoxycholate	1,0 g
Iron(II) sulfate	0,25 g
Sodium pyruvate	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.

5.3.1.2 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 (6.1) set at 121 °C for 15 min.

5.3.2 Antibiotic solution

5.3.2.1 Composition

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

5.3.2.2 Preparation

Dissolve the components in the water. Sterilize by filtration.

5.3.3 Complete medium

5.3.3.1 Composition

Basic medium (5.3.1)	1 000 ml
Antibiotic solution (5.3.2)	5 ml

5.3.3.2 Preparation

Add the antibiotic solution to the basic medium, cooled down to 47 °C ± 2 °C, then mix carefully. Pour about 15 ml of the complete medium into sterile Petri dishes (6.8). 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 be kept for not more than 4 h at ambient temperature, or in the dark at 5 °C ± 3 °C for not more than 7 days.

5.3.3.3 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.

5.4 Columbia blood agar

5.4.1 Basic medium

5.4.1.1 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[a]
Water	1 000 ml

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

5.4.1.2 Preparation

Dissolve the basic components or the dehydrated complete medium in the water, by 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.

5.4.2 Sterile defibrinated sheep blood

5.4.3 Complete medium

5.4.3.1 Composition

Basic medium (5.4.1)	1 000 ml
Sterile defibrinated sheep blood (5.4.2)	50 ml

5.4.3.2 Preparation

Add the blood aseptically to the basic medium, cooled down to 47 °C ± 2 °C, then mix. Pour about 15 ml of the complete medium into sterile Petri dishes (6.8). 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 be kept for not more than 4 h at ambient temperature, or not more than 7 days at 5 °C ± 3 °C.

5.4.3.3 Performance testing

For the definition of selectivity and productivity, refer to ISO/TS 11133-1.

For the performance testing, refer to ISO/TS 11133-2. Control strains *C. coli* ATCC 43478 or *C. jejuni* ATCC 33291 shall show good growth on Columbia blood agar after microaerobic incubation for 24 h at 37 °C.

5.5 Brucella broth

5.5.1 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

5.5.2 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.

5.5.3 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.4.

5.6 Reagent for the detection of oxidase

5.6.1 Composition

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

5.6.2 Preparation

Dissolve the component in the water immediately prior to use.

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 Incubator, capable of operating at 25 °C ± 1 °C.

6.5 Water bath, capable of operating between 44 °C and 47 °C.

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

6.7 Containers, for example bottles, tubes, flasks, suitable for the sterilization and storage of diluent and culture media.

6.8 Petri dishes, preferably with nocks, in glass or plastic, with a diameter of 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.

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**.

NOTE A nickel/chromium loop is not suitable for use in the oxidase test (see 9.4.3).

6.12 Spreader, of glass or plastic.

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

6.14 Appropriate apparatus for achieving a microaerobic atmosphere with oxygen content of 5 ± 2 %, carbon dioxide 10 ± 3 %, optional hydrogen ⩽ 10 %, with the balance nitrogen. Use appropriate gastight containers able to hold Petri dishes, for example bacteriological anaerobic jars. The appropriate micro-aerobic atmosphere may be obtained using commercially available gas-generating kits (follow precisely the manufacturers instructions, particularly those relating to the volume of the jar and the capacity of the gas-generating kit). Alternatively, flushing of the jar prior to incubation with an appropriate gas mixture may be used.

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 Technical Specification. 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* spp. 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

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 sample if the product is liquid, or from the initial suspension in the case of other products.

9.2 Inoculation and incubation

9.2.1 Transfer, using a sterile pipette (6.9), 0,1 ml of the initial suspensions (9.1) to each of two plates of the mCCD agar medium (5.3). Carefully spread the inoculum uniformly and as quickly as possible over the surface of the agar plate, without touching the sides of the dish, using a sterile spreader (6.12), until there is no longer any liquid visible on the agar surface.

Repeat the procedure using further decimal dilutions if necessary.

When, for certain products, it is necessary to estimate low numbers of *Campylobacter*, the limit of enumeration may be lowered by a factor 10 by examining 1,0 ml of the initial suspension. Distribute the 1 ml of inoculum either on the surface of the agar medium in a large Petri dish (140 mm) or over the surface of the agar medium in three small plates (90 mm) using a sterile spreader (6.12). In both cases, prepare duplicates by using two large plates or six small plates.

9.2.2 Incubate the plates (9.2.1) at 41,5 °C for 40 h to 48 h in a micro-aerobic atmosphere (6.14).

9.3 Counting and selection of colonies for confirmation

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

Select the plates (9.2.2) containing less than 150 typical or suspect colonies; count these colonies. Then choose at random five such colonies for subculturing for the confirmation tests (9.4).

9.3.2 Streak each of the colonies selected (9.3.1) onto a Columbia blood agar plate (5.4) in order to allow the development of well-isolated colonies. Incubate the plates in a micro-aerobic atmosphere at 41,5 °C for 24 h to 48 h. Use the pure cultures for examination of the morphology, motility, micro-aerobic growth at 25 °C, aerobic growth at 41,5 °C and presence of oxidase.

9.4 Confirmation of *Campylobacter* species

Since the bacteria rapidly deteriorate in air, follow the procedure described in 9.4.1 to 9.4.3 without any delay.

9.4.1 Examination of morphology and motility

9.4.1.1 Suspend one colony from the Colombia blood agar plate (9.3.2) in 1 ml of Brucella broth (5.5) and examine for morphology and motility using a microscope (6.13).

9.4.1.2 Retain for further examination all cultures (9.3.2) in which curved bacilli with a spiralling "corkscrew" motility are found (9.4.1.1).

9.4.2 Study of growth at 25 °C (micro-aerobic) and 41,5 °C (aerobic)

Using the colonies isolated in 9.4.1.2, inoculate, using a loop (6.11), the surface of two Columbia blood agar plates (5.4).

Incubate one plate at 25 °C in a micro-aerobic atmosphere (6.14) for 40 h to 48 h. Incubate the other plate at 41,5 °C in an aerobic atmosphere for 40 h to 48 h.

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

9.4.3 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 (9.4.1.2) and streak it onto a filter paper moistened with the oxidase reagent (5.6). The appearance of a mauve, violet or deep blue colour within 10 s is 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).

9.4.4 Interpretation

Campylobacter spp. give results in accordance with Table 1.

Table 1 — Characteristics of *Campylobacter* spp.

Morphology (9.4.1)	small curved bacilli
Motility (9.4.1)	characteristic
Micro-aerobic growth at 25 °C (9.4.2)	–
Aerobic growth at 41,5 °C (9.4.2)	–
Oxidase (9.4.3)	+

10 Expression of results

10.1 Count of *Campylobacter* colonies

10.1.1 If at least 80 % of the selected colonies are confirmed (9.4.4), take as the number of *Campylobacter* the number given by the count made as in 9.3.

10.1.2 In all other cases, calculate the number of *Campylobacter* obtained as in 9.3 which are confirmed (9.4.4). Round the result to a whole number of colonies.

10.2 Method of calculation

10.2.1 General case – Plates containing 15 to 150 colonies of presumptive *Campylobacter*

Calculate the number N of *Campylobacter* present in the test sample as a weighted mean from two successive dilutions using Equation (1):

$$N = \frac{\sum a}{V \times [n_1 + (0{,}1 \times n_2)] \times d} \quad (1)$$

where

$\sum a$ is the sum of colonies complying with the identification criteria, counted on all the plates retained from two successive dilutions, and where at least one plate contains a minimum of 15 colonies;

V is the volume of inoculum applied to each dish, in millilitres;

n_1 is the number of plates retained at the first dilution;

n_2 is the number of plates retained at the second dilution

d is the dilution factor corresponding to the first dilution retained (d = 1 when the undiluted liquid product (test sample) is used).

Round off the results to two significant figures. In order to do this, if the third figure is less than 5, do not modify the preceding figure; if the third figure is greater than or equal to 5, increase the preceding figure by one unit.

Take as the result a number preferably between 1,0 and 9,9 multiplied by the appropriate power of 10, or a whole number with two significant figures.

Express the result as follows:

number N of *Campylobacter* per millilitre (liquid products) or per gram (other products).

EXAMPLE Counting has produced the following results:

— at the first dilution (10^{-2}) retained: 66 and 80 colonies;

— at the second dilution (10^{-3}) retained: 4 and 7 colonies.

Testing of selected colonies was carried out:

— for 66 colonies: 5 colonies of which 4 agreed with the criteria, giving a = 66 (see 10.1.1)

— for 80 colonies: 5 colonies of which 3 agreed with the criteria, giving a = 48

— for 7 colonies: 5 colonies of which 4 agreed with the criteria, giving a = 7

— for 4 colonies: all 4 colonies were confirmed.

$$N = \frac{\sum a}{V \times [n_1 + (0{,}1 \times n_2)] \times d} = \frac{66 + 48 + 7 + 4}{0{,}1 \times [2 + (0{,}1 \times 2)] \times 10^{-2}} = \frac{125}{2{,}2 \times 10^{-3}} = 56\,818$$

By rounding off the result, the number of *Campylobacter* is 570 00 or 5,7 × 10^4 per millilitre or per gram of product.

10.2.2 Case of two plates containing less than 15 colonies

If the two plates, at the test sample (liquid products) or at the initial suspension (other products) or at the first dilution inoculated or retained, contain less than 15 typical colonies, calculate the estimated number N of *Campylobacter* present in the test sample as an arithmetical mean of the colonies counted on the two plates, using Equation (2):

$$N_E = \frac{\sum a}{V \times n \times d} \quad (2)$$

where

$\sum a$ is the sum of the colonies counted on the two plates;

V is the volume of inoculum applied to each dish, in millilitres;

n is the number of plates retained (in this case, n = 2);

d is the dilution factor of the initial suspension or of the first dilution inoculated or (d = 1 when the undiluted liquid product (test sample) is used).

Express the result as follows:

estimated number N_E of *Campylobacter* per millilitre (liquid products) or per gram (other products).

EXAMPLE Counting has produced the following results:

— at the first dilution (10^{-1}) retained, 12 and 13 colonies were counted:

$$N_E = \frac{12 + 13}{0{,}1 \times 2 \times 10^{-1}} = \frac{25}{0{,}02} = 1\,250$$

By rounding off the result as recommended in 10.2.1, the estimated number N_E of *Campylobacter* is 1 300 or $1{,}3 \times 10^3$ per millilitre or per gram of product.

10.2.3 Case of two plates containing no colonies

If the two plates, at the test sample (liquid products) or at the initial suspension (other products) or at the first dilution inoculated or retained, do not contain any colonies, express the result as follows:

less than $1/d \times V$ of *Campylobacter* per millilitre (liquid products) or per gram (other products);

where

d is the dilution factor of the initial suspension or of the first dilution inoculated or retained [$d = 10^0 = 1$ where the directly inoculated test sample (liquid products) is retained];

V is the volume of the inoculum applied to the plates, in millilitres.

10.2.4 Special cases

Special cases are described in ISO 7218.

10.3 Precision

For statistical reasons alone, in 95 % of cases the confidence limits of the colony-count technique vary by ±16 % to 52 %[3]. For colony counts of less than 15 per plate, the confidence limits are given in Annex A. In practice, even greater variation may be found, especially among results obtained by different workers.