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**Microbiology of food and animal feeding  
stuffs — Horizontal method for detection  
of thermotolerant *Campylobacter***

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*Microbiologie des aliments — Méthode horizontale pour la recherche de  
Campylobacter thermotolérants*

ISO 10272:1995

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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.

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.

International Standard ISO 10272 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 9, *Microbiology*.

Annexes A and B form an integral part of this International Standard.

## 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.

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# Microbiology of food and animal feeding stuffs — Horizontal method for detection of thermotolerant *Campylobacter*

## 1 Scope

This International Standard describes a horizontal method for the detection of thermotolerant *Campylobacter*.

It is applicable to products intended for human consumption or for the feeding of animals, subject to the limitations stated in the introduction.

## 2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 6887:1983, *Microbiology — General guidance for the preparation of dilutions for microbiological examination*.

ISO 7218:—<sup>1)</sup>, *Microbiology of food and animal feeding stuffs — General rules for microbiological examinations*.

## 3 Definitions

For the purposes of this International Standard, the following definitions apply.

1) To be published. (Revision of ISO 7218:1985)

**3.1 thermotolerant *Campylobacter*:** Microorganisms forming characteristic colonies on solid selective media when incubated at 42 °C and which possess the characteristic motility and biochemical properties described when the test is conducted in accordance with this International Standard.

**3.2 detection of thermotolerant *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 International Standard.

## 4 Principle

In general, the detection of thermotolerant *Campylobacter* requires the following stages (see annex A for a diagram of the procedure):

### 4.1 Enrichment in the liquid medium

Inoculation of the test portion in one of two liquid enrichment media:

- Preston broth, or
- Park and Sanders broth when the sample or the product it comes from has received some physical treatment, for example freezing, which could entail bacterial stress.

Incubation in a microaerophilic atmosphere (5 % oxygen and, for example, 10 % carbon dioxide and 85 % nitrogen):

- at 42° C for 18 h for the Preston broth;

— at 32 °C for 4 h, then at 37 °C for 2 h and, finally, at 42 °C for 40 h to 42 h for the Park and Sanders broth.

NOTE 1 Since *Campylobacter* spp. are very sensitive, it is recommended that they should not be frozen, that they should be stored at + 3 °C ± 2 °C, and subjected to analysis as rapidly as possible.

## 4.2 Plating out and identification

From the cultures obtained in 4.1, inoculation of solid selective media:

— Karmali agar and another solid selective medium [modified Butzler agar, Skirrow agar, charcoal cefoperazone deoxycholate agar (CCDA) or Preston agar].

Incubation at 42 °C in a microaerophilic atmosphere (5 % oxygen and, for example, 10 % carbon dioxide and 85 % nitrogen) and inspection after 48 h, 72 h and, if necessary, 5 days, to detect the presence of colonies presumed to be thermotolerant *Campylobacter*.

## 4.3 Confirmation

Subculturing of the colonies presumed to be thermotolerant *Campylobacter* and which were plated out in 4.2, then confirmation by means of appropriate biochemical tests.

# 5 Culture media and reagents

## 5.1 General

For current laboratory practice, see ISO 7218.

NOTE 2 Because of the large number of culture media and reagents, it is considered preferable for the clarity of the text to give their compositions and preparations in annex B.

## 5.2 Liquid enrichment media

### 5.2.1 Preston broth

See B.1.

### 5.2.2 Park and Sanders broth

See B.2.

## 5.3 Selective plating-out media

### 5.3.1 Karmali agar

See B.3.

### 5.3.2 Modified Butzler agar

See B.4.

### 5.3.3 Skirrow agar

See B.5.

### 5.3.4 Charcoal cefoperazone deoxycholate agar (CCDA)

See B.6.

### 5.3.5 Preston agar

See B.7.

## 5.4 Identification media and reagents

### 5.4.1 *Brucella* broth

See B.8.

### 5.4.2 Columbia blood agar

See B.9.

### 5.4.3 Reagent for the detection of oxidase

See B.10.

### 5.4.4 Triple sugar/iron(III) agar (TSI agar)

See B.11.

### 5.4.5 Hydrogen peroxide solution, 3 %

### 5.4.6 Reagents for the detection of the hydrolysis of hippurate

See B.12.

### 5.4.7 Mueller Hinton blood agar

See B.13.

## 6 Apparatus and glassware

NOTE 3 Disposable apparatus is an acceptable alternative to re-useable glassware, if it has similar specifications.

Usual microbiological laboratory equipment and, in particular, the following.

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

See ISO 7218.

**6.2 Drying cabinet** or **oven**, ventilated by convection, capable of operating between  $37\text{ °C} \pm 1\text{ °C}$  and  $55\text{ °C} \pm 1\text{ °C}$ .

**6.3 Incubator**, capable of operating at  $42\text{ °C} \pm 1\text{ °C}$ .

**6.4 Water baths**, capable of operating at  $25\text{ °C} \pm 1\text{ °C}$ ,  $32\text{ °C} \pm 1\text{ °C}$ ,  $37\text{ °C} \pm 1\text{ °C}$  and  $42\text{ °C} \pm 1\text{ °C}$ , or **incubators** capable of operating at  $25\text{ °C} \pm 1\text{ °C}$ ,  $32\text{ °C} \pm 1\text{ °C}$  and  $37\text{ °C} \pm 1\text{ °C}$ .

**6.5 Water bath**, capable of operating at  $47\text{ °C} \pm 2\text{ °C}$ .

**6.6 pH-meter**, capable of being read to the nearest 0,01 pH unit at  $25\text{ °C}$ .

**6.7 Containers**, in particular **culture tubes** of dimensions  $18\text{ mm} \times 180\text{ mm}$  and  $9\text{ mm} \times 180\text{ mm}$ , **haemolysis tubes** of dimensions  $13\text{ mm} \times 75\text{ mm}$ , **bottles** with non-toxic metal closures and/or **flasks** of appropriate capacity with appropriate covers or plugged with cotton wool to allow gases to pass so that a microaerophilic atmosphere can be achieved when needed.

**6.8 Petri dishes**, in glass or plastic, with a diameter between 90 mm and 100 mm.

**6.9 Total-delivery graduated pipettes**, with a wide opening, and a nominal capacity of 1 ml and 10 ml, graduated respectively in 0,1 ml and 0,5 ml divisions, and **Pasteur pipettes**.

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

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

NOTE 4 A nickel/chromium loop is not suitable for use in the oxidase test (see 9.5.5.1).

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

**6.13 Microscope**, if possible with phase contrast (for observing the characteristic movement of *Campylobacter*).

**6.14 Appropriate apparatus** for growth in a microaerophilic atmosphere and for allowing a constant oxygen content of 5 % and, for example, a carbon dioxide content of 10 % and nitrogen content of 85 % to be maintained throughout the incubation.

NOTE 5 Use appropriate gastight containers able to hold Petri dishes and/or flasks or bottles of about 350 ml capacity used for the enrichment broth; e.g. bacteriological anaerobic jars. A microaerophilic atmosphere with an oxygen content of between 3 % and 6 % 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, sparging of the jar prior to incubation with a gas mixture containing 5 % oxygen in 10 % carbon dioxide and 85 % nitrogen 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 International Standard. 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.

## 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

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

**9.1.2** In general, for preparing the initial suspension, introduce a quantity  $x$  of the test portion (mass or volume) into a  $9x$  volume of the enrichment medium, Preston broth (5.2.1) or Park and Sanders broth

(5.2.2), so as to obtain a test portion/enrichment medium ratio of 1/10 (mass/volume or volume/volume ratio).

## 9.2 Direct plating out

For those products suspected of containing a large quantity of thermotolerant *Campylobacter*, use a loop (6.11) to plate the non-incubated initial suspension (9.1.2) onto the surface of the Karmali agar (5.3.1) and another medium (5.3.2, 5.3.3, 5.3.4 or 5.3.5).

Incubate at 42 °C in a microaerophilic atmosphere (6.14) with inspection after 48 h, 72 h and, if necessary, 5 days to detect the presence of colonies presumed to be thermotolerant *Campylobacter*.

Then proceed as described in 9.4.3 and 9.5.

## 9.3 Enrichment

**9.3.1** If the Preston broth is used, incubate the initial suspension (9.1.2) at 42 °C in a microaerophilic atmosphere (6.14) for 18 h.

**9.3.2** If the Park and Sanders broth is used, incubate in a microaerophilic atmosphere (6.14) the initial suspension (9.1.2) at 32 °C for 4 h, then add the antibiotic solution B (B.2.4) at a concentration of 5 % (V/V). Incubate at 37 °C for 2 h, then at 42 °C for 40 h to 42 h.

## 9.4 Plating out and identification

**9.4.1** Using the culture obtained in the enrichment medium (9.3.1 or 9.3.2), inoculate with a loop (6.11) the surface of the first selective isolation medium, the Karmali agar (5.3.1).

Proceed in the same manner with the second selective isolation medium chosen (5.3.2, 5.3.3, 5.3.4 or 5.3.5).

**9.4.2** Incubate the dishes (9.4.1) at 42 °C in a microaerophilic atmosphere (6.14).

**9.4.3** After 24 h or, more generally, 48 h and even 3 to 5 days of incubation, examine the dishes to detect the presence of characteristic colonies of thermotolerant *Campylobacter*.

On the Karmali agar, the characteristic colonies are greyish, flat and moistened, with a tendency to

spread. On the Butzler and Skirrow agars, the characteristic colonies have a greyish to brownish colour, and may be of different sizes on the two media.

## 9.5 Confirmation

As the bacteria need a microaerophilic atmosphere to survive, follow the procedure described in 9.5.1 to 9.5.2.4 without any delay.

### 9.5.1 Selection of colonies for confirmation

Select, for confirmation tests, a total of five typical and/or suspect colonies from all the inoculated dishes (9.2, 9.4.3). If there are less than five, select all the colonies.

### 9.5.2 Examination of morphology and motility

**9.5.2.1** Using a well-isolated colony, conduct a Gram's stain on each of the dishes (9.4.3).

**9.5.2.2** Emulsify separately each of the colonies selected in 9.5.1 in 1 ml of *Brucella* broth (5.4.1).

**9.5.2.3** Examine each of the selected colonies for morphology and motility using the microscope (6.13).

**9.5.2.4** Retain for further examination all suspensions (9.5.2.2) in which curved Gram-negative bacilli (9.5.2.1) with a spiralling "corkscrew" motility are found (9.5.2.3).

### 9.5.3 Study of morphology in a Gram's strain

Using each selected suspension (9.5.2.4), inoculate with a loop (6.11) the surface of a Columbia blood agar plate (5.4.2) in order to allow the development of well-isolated colonies.

Incubate the inoculated dishes in a microaerophilic atmosphere (6.14) at 42 °C for 24 h and use the pure cultures for the biochemical tests.

### 9.5.4 Study of growth at 25 °C

Using the colonies isolated in 9.5.3, inoculate, using a loop (6.11), a tube of *Brucella* broth (5.4.1).

Incubate at 25 °C in a microaerophilic atmosphere (6.14) for 2 to 5 days.

Examine to check whether or not there is growth.



## 9.5.5 Biochemical tests

### 9.5.5.1 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 (9.5.3) and streak 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 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.

### 9.5.5.2 TSI agar (5.4.4)

Using the wire (6.11), inoculate the agar with each of the colonies selected in 9.5.3.

Inoculate the agar slant in longitudinal streaks and stab the butt.

Incubate at 42 °C for 24 h and extend the incubation to 5 days if necessary in a microaerophilic atmosphere (6.14).

Interpret the reactions in the following manner:

<b>Butt</b>	
Yellow	glucose positive (fermentation of glucose)
Red or unchanged	glucose negative (no fermentation of glucose)
Black	formation of hydrogen sulfide (H <sub>2</sub> S)
Bubbles or cracks	gas formation from glucose
<b>Slant surface</b>	
Yellow	lactose and/or sucrose positive (one or both sugars used)
Red or unchanged	lactose and sucrose negative (no sugar used)

### 9.5.5.3 Detection of catalase

Deposit a loop of culture from the TSI agar (9.5.5.2) into a drop of hydrogen peroxide solution (5.4.5) on a clean microscope slide.

The test is positive if bubbles appear in 30 s.

### 9.5.5.4 Detection of sensitivity to nalidixic acid and to cephalothin<sup>2)</sup>

Inoculate, using a loop (6.11), the colonies selected in 9.5.2.4, in a suspension of density 0,5 on the MacFarland scale, into the *Brucella* broth.

Then dilute this suspension 1:10 with the same broth.

Flood the surface of a Mueller Hinton 5 % blood agar plate (5.4.7) with the suspension.

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

Dry the dishes in the oven (6.2) set at 37 °C for 10 min.

On the surface of the agar, place a disc of nalidixic acid (30 µg) and a disc of cephalothin (30 µg).

Incubate at 37 °C for 24 h in a microaerophilic 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 **sensitive**.

### 9.5.5.5 Detection of hydrolysis of hippurate<sup>2)</sup>

In a haemolysis tube (6.7) containing 0,4 ml of the sodium hippurate solution (5.4.6), inoculate, using a loop (6.11), the colonies selected in 9.5.3 taking care not to incorporate any agar.

Shake in order to mix thoroughly and incubate for 2 h in the water bath (6.4) set at 37 °C.

Carefully add 0,2 ml of the ninhydrin solution (B.12.2) 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.

The reaction is positive when the colour is dark violet.

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

2) These tests are useful for differentiating the species of thermotolerant *Campylobacter*.

## 9.6 Interpretation of results

Thermotolerant *Campylobacter* give the following results:

<i>Campylobacter</i> spp.	
Morphology (9.5.2.3)	small curved bacilli
Motility (9.5.2.3)	characteristic
Gram's strain (9.5.3)	—
Oxidase (9.5.5.1)	+
Glucose (9.5.5.2)	—
Lactose (9.5.5.2)	—
Sucrose (9.5.5.2)	—
Gas (9.5.5.2)	—

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

Among the *Campylobacter* spp. growing at 42 °C, certain species constitute the thermotolerant group, of which the most frequently encountered species are *Campylobacter jejuni* and *Campylobacter coli*. Other species have, however, been described (*Campylobacter lari* and *Campylobacter upsaliensis*); the characteristics given in table 1 allow differentiation of them.

## 10 Expression of results

According to the interpretation of the results, indicate the presence or absence of thermotolerant *Campylobacter* in a test portion of  $x$  g or  $x$  ml of product.

## 11 Test report

The test report shall indicate

- the method in accordance with which sampling was carried out, if known;
- the method used;
- the chosen incubation temperature, and
- the test results obtained.

It shall also mention all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test results.

The test report shall include all information necessary for the complete identification of the sample.

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Table 1

Characteristics	<i>C. jejuni</i>	<i>C. coli</i>	<i>C. lari</i>	<i>C. upsaliensis</i>
Growth at 25 °C (9.5.4)	—	—	—	—
H <sub>2</sub> S (TSI) (9.5.5.2) <sup>1)</sup>	—	(+)	—	—
Nalidixic acid (9.5.5.4)	S	S	R	S
Hydrolysis of hippurate (9.5.5.5)	+	—	—	—
Catalase (9.5.5.3)	+	+	+	— or slight
Cephalothin (9.5.5.4)	R	R	R	S

**Key:** + = positive; — = negative; (+) = slightly positive; S = sensitive; R = resistant.

1) Slight development of H<sub>2</sub>S in the condensed water after 5 days.

# Annex A (normative)

## Diagram of procedure

