TECHNICAL SPECIFICATION

ISO/TS 21872-2

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Microbiology of food and animal feeding stuffs — Horizontal method for the detection of potentially enteropathogenic *Vibrio* spp. —

Part 2: Detection of species other than *Vibrio parahaemolyticus* and *Vibrio cholerae*

Microbiologie des aliments — Méthode horizontale pour la recherche des Vibrio *spp. potentiellement entéropathogènes —*

Partie 2: Recherche des espèces autres que Vibrio parahaemolyticus *et* Vibrio cholerae

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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order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn.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 21872-2 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

ISO/TS 21872 consists of the following parts, under the general title *Microbiology of food and animal feeding stuffs — Horizontal method for the detection of potentially enteropathogenic* Vibrio *spp.*:

— *Part 1: Detection of* Vibrio parahaemolyticus *and* Vibrio cholerae

— *Part 2: Detection of species other than* Vibrio parahaemolyticus *and* Vibrio cholerae

Introduction

Because of the large variety of food and feed products, this horizontal method may not be appropriate in every detail for certain products. In this case, different methods, which are specific to these products may be used if absolutely necessary for justified technical reasons. Nevertheless, every attempt will be made to apply this horizontal method as far as possible.

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 method in the case of particular products.

The harmonization of test methods cannot be immediate, and for certain groups 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 Technical Specification 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 the detection of potentially enteropathogenic *Vibrio* spp. —

Part 2: Detection of species other than *Vibrio parahaemolyticus* and *Vibrio cholerae*

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for detection of *Vibrio* spp., and the particularly toxigenic *Vibrio cholerae*, be conducted only in laboratories equipped for this purpose and under the supervision of an experienced microbiologist, and that great care be exercised in the disposal of contaminated material.

1 Scope

This part of ISO/TS 21872 specifies a horizontal method for detection of the enteropathogenic *Vibrio* species, causing illness in or via the intestinal tract, other than *Vibrio parahaemolyticus and Vibrio cholerae.* The species detectable by the methods specified include *Vibrio fluvialis*, *Vibrio mimicus* and *Vibrio vulnificus* [1]. It is not suitable for the isolation of *Vibrio hollisae*. Strains of *V. parahaemolyticus* and *V. cholerae* may also be detected during the application of this method.

This part of ISO/TS 21872 is applicable to

— products intended for human consumption and the feeding of animals, and

— environmental samples in the area of food production and food handling.

This method is not appropriate for the detection of *Vibrio metschnikovii* as this is oxidase negative.

NOTE 1 *Vibrio metschnikovii* has been occasionally isolated from human faecal samples and can be a cause of diarrhoeal diseases.

NOTE 2 The identification of *Vibrio* species other than *V. parahaemolyticus* and *V. cholerae* is difficult, and needs further development. The biochemical tests given in this part of ISO/TS 21872 enable only a presumptive confirmation of these species.

NOTE 3 Reasons for not applying this method are discussed 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.

1) See 9.4.4.

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 requirements and guidance for microbiological examinations*

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

3 Terms and definitions

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

3.1
potentially enteropathogenic *Vibrio*
microorganisms which form typical colonies on solid selective media and which possess the described biochemical characteristics when the test is performed in accordance with this part of ISO/TS 21872

3.2
detection of potentially enteropathogenic *Vibrio*
determination of the presence or absence of presumptive, enteropathogenic *Vibrio*, in a specified quantity of product, when the test is performed in accordance with this part of ISO/TS 21872

4 Principle

4.1 General

The detection of potentially enteropathogenic *Vibrio* spp. requires four successive phases (see also Annex A).

NOTE *Vibrio* can, indeed, be present in small numbers and are often accompanied by a much larger number of other microorganisms belonging to the Vibrionaceae family or to other families. Consequently, two successive selective enrichments are necessary.

4.2 First enrichment in a liquid selective medium

The enrichment medium (alkaline saline peptone water, ASPW) (5.1) is inoculated with the test portion at ambient temperature. It is incubated at 37 °C for 6 h ± 1 h.

In the case of large quantities, the ASPW should be warmed to 37 °C before inoculation with the test portion.

4.3 Second enrichment in a liquid selective medium

The enrichment medium (ASPW) is then inoculated with the culture obtained in 4.2.

It is incubated at 37 °C for 18 h ± 1 h.

4.4 Isolation and identification

The following two solid selective media are inoculated with the cultures obtained in 4.2 and in 4.3:

— thiosulfate citrate bile and sucrose agar (TCBS);

— another appropriate solid selective medium (left to the choice of the laboratory) such as colistin polymixin β-cellobiose agar (CPC), sodium dodecyl sulfate polymixin B sucrose agar (SDS) or modified colistin polymixin cellobiose agar (mCPC) media.

The two isolation media are incubated at 37 °C, then examined after 24 h ± 3 h.

4.5 Confirmation

The characteristic colonies of enteropathogenic *Vibrio* spp. isolated in 4.4 are subcultured, then confirmed by means of appropriate biochemical tests.

5 Culture media, reagents

For general laboratory practice, see ISO 7218.

NOTE On account of the large number of culture media and reagents, for clarity of the text, their composition and preparation are given in Annex B.

5.1 Enrichment medium: Alkaline saline peptone water (ASPW)

See B.1.

5.2 Solid selective isolation media

5.2.1 First medium: Thiosulfate, citrate, bile and sucrose (TCBS) agar

See B.2.

5.2.2 Second medium

Choose between:

a) sodium dodecyl sulfate polymixin sucrose agar (SDS), see B.3.

b) cellobiose polymixin colistin agar (CPC), see B.4;

c) modified cellobiose polymixin colistin agar (mCPC), see B.5.

5.3 Saline nutrient agar (SNA)

See B.6.

5.4 Reagent for detection of oxidase

See B.7.

5.5 Saline triple sugar iron (TSI) agar

See B.8.

5.6 Saline medium for detection of ornithine decarboxylase (ODC)

See B.9.

5.7 Saline medium for detection of lysine decarboxylase (LDC)

See B.10.

5.8 Saline medium for detection of arginine dihydrolase (ADH)

See B.11.

5.9 Reagent for detection of β-galactosidase

See B.12.

5.10 Saline medium for detection of indole

See B.13.

5.11 Saline peptone waters

See B.14.

5.12 Sodium chloride solution

See B.15.

6 Apparatus and glassware

NOTE Disposable equipment is acceptable in the same way as reusable glassware, if the specifications are similar.

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

6.1 **Incubator**, adjustable to 37 °C ± 1 °C.

6.2 **Incubator** or **water bath**, adjustable to 41,5 °C ± 1 °C.

6.3 **Water bath**, adjustable from 44 °C to 47 °C.

6.4 **Water bath**, adjustable to 37 °C ± 1 °C.

It is recommended to use water baths (6.2, 6.3 and 6.4) containing an antibacterial agent.

7 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this part of ISO/TS 21872. See the International Standard specific to the relevant product. If a specific International Standard does not exist, it is recommended that the relevant parties reach agreement on this subject.

8 Preparation of test sample

Prepare the test sample in accordance with the relevant part of ISO 6887, and/or ISO 8261, and an International Standard concerning the product to be examined. If a specific International Standard does not exist, it is recommended that the relevant parties reach agreement on this subject.

9 Procedure (see Annex A)

9.1 Test portion and initial suspension

For the preparation of the initial suspension, use the first enrichment medium (ASPW) specified in 5.1.

Take a test portion (x g or x ml), according to the sensitivity required, and homogenize it in $9x$ ml (or $9x$ g) of enrichment medium.

In the case of large quantities, the ASPW should be warmed to 37 °C before inoculation with the test portion.

If the dilution and the incubation cannot be carried out the same day, store the initial suspension until the next day at a temperature of 5 °C ± 3 °C.

In order to reduce the amount of examination work, where more than one 25 g test portion stemming from the same batch of food is to be examined, and where proof is available indicating that a mixture (gathering together the test portions) does not modify the results concerning this product in particular, the test portions may be mixed.

EXAMPLE If 10 test portions of 25 g are to be examined, it is possible to combine these 10 units in order to obtain a composite sample of 250 g and to add 2,25 l of enrichment medium.

Cell counts of potentially enteropathogenic *Vibrio* spp. decline significantly on storage at refrigeration temperatures. Storage of samples and, to a lesser extent, of suspensions at such temperatures should be avoided where possible and should otherwise be kept to a minimum.

9.2 First selective enrichment

Incubate the initial suspension (9.1) at 37 °C for 6 h ± 1 h.

Care should be taken to apply the whole method to products with a high salt content, as the final salt concentration in the medium might alter the characteristics (see ISO 6887-4).

9.3 Second selective enrichment

9.3.1 Transfer 1 ml of the culture obtained in 9.2 taken from the surface into a tube containing 10 ml of ASPW (5.1).

9.3.2 Incubate the ASPW at 37 °C for 18 h ± 1 h.

9.4 Isolation and identification

9.4.1 From the culture obtained in the ASPW (9.2 and 9.3.2), inoculate with a sampling loop the surface of a TCBS agar plate (5.2.1), so as to permit the development of well-isolated colonies.

Proceed likewise with the chosen second selective isolation medium (5.2.2) using a new sampling loop.

9.4.2 Invert the agar plates (9.4.1) and place them in an incubator (6.1) set at 37 °C.

9.4.3 After 24 h ± 3 h of incubation, examine the dishes (9.4.1 and 9.4.2) for the presence of typical colonies of *Vibrio* spp. Mark their positions on the bottom of the dish.

There are two typical morphologies for colonies of *Vibrio* spp. on TCBS agar (5.2.1) as follows:

— typical colonies of *V. mimicus* and *V. vulnificus* are smooth, green (sucrose negative) and 2 mm to 3 mm in diameter;

— typical colonies of *V. fluvialis* are smooth, yellow (sucrose positive) and 2 mm to 3 mm in diameter.

NOTE *V. parahaemolyticus* and *V. cholerae*, covered by ISO/TS 21872-1, form green and yellow colonies respectively on TCBS.

There are two typical morphologies for colonies of *Vibrio* spp. on SDS medium [5.2.2 a)]:

— typical colonies of *V. mimicus* and *V. vulnificus* are purple and 2 mm or greater in diameter with an opaque halo;

— typical colonies of *V. cholerae* O1 are yellow, 2 mm or greater in diameter, with an opaque halo; *V. cholerae* non-O1 strains may or may not produce a halo.

Other *Vibrio* spp. will either not grow on SDS agar or will produce colonies without a halo.

There are two typical morphologies for colonies of *Vibrio* spp. on CPC and mCPC [5.2.2 b) and c)]:

— typical colonies of *V. vulnificus* are yellow, 2 mm or greater in diameter, and surrounded by a yellow zone;

— typical colonies of *V. cholerae* are purple, 2 mm or greater in diameter, and surrounded by a blue zone.

Some strains of other *Vibrio* spp. can grow on CPC or mCPC agars, producing colonies similar to those described above.

9.4.4 To recover *V. vulnificus*, attention shall be paid to the performance of CPC or mCPC media.

9.5 Confirmation

9.5.1 General

Current commercially available biochemical identification kits may be used to identify *Vibrio* to a species level, provided they are inoculated with a suspension of the bacteria to be identified in a sufficiently saline medium or dilution fluid, and provided the database or identification table for the product has been based on reactions obtained using similar media to those described in this part of ISO/TS 21872. These kits shall be used in accordance with the manufacturer's instructions.

NOTE Recognition of colonies of *Vibrio* is largely a question of experience and their appearance can sometimes vary not only from one species to another, but also from one batch of culture medium to another.

9.5.2 Selection of colonies for confirmation and preparation of pure cultures

For confirmation, subculture from each selective medium (see 9.4), at least five colonies considered to be typical or similar to each of the potentially pathogenic *Vibrio* spp. sought. If there are less than five colonies of the target type on a plate, subculture all of these colonies.

NOTE Foods, especially seafoods, can contain large numbers of bacteria, including non-pathogenic *Vibrio* spp. which may grow through the selective culture process. The subculture of small numbers of colonies may result in potentially pathogenic species being missed.

Inoculate the selected colonies onto the surface of plates of saline nutrient agar or inclined saline nutrient agar (5.3), to obtain isolated colonies. Incubate the inoculated plates (9.4.2) at 37 °C for 24 h ± 3 h.

Use pure cultures for biochemical confirmations.

9.5.3 Tests for presumptive identification

9.5.3.1 Oxidase test

Using a sampling loop, platinum iridium straight wire or a glass rod, take a portion of the pure culture from the saline nutrient agar (9.5.2) and streak onto the filter paper moistened with oxidase reagent (5.4), or use a