FINAL
DRAFT

INTERNATIONAL
STANDARD

**ISO/FDIS
21872**

ISO/TC **34**/SC **9**

Secretariat: AFNOR

Voting begins on:
2017-02-15

Voting terminates on:
2017-04-12

Microbiology of the food chain — Horizontal method for the detection of potentially enteropathogenic *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio vulnificus*

Microbiologie de la chaîne alimentaire — Méthode horizontale pour la recherche des espèces potentiellement entéropathogènes Vibrio parahaemolyticus, Vibrio cholerae *et* Vibrio vulnificus

ISO/CEN PARALLEL PROCESSING

Reference number
ISO/FDIS 21872:2017(E)

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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 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 21872-1:2007 and ISO/TS 21872-2:2007, which have been technically revised. It also incorporates ISO/TS 21872-1:2007/Cor.1:2008.

The main changes are as follows:

— introduction of optional molecular identification methods for major food borne *Vibrio* spp.(*V. parahaemoltycus*, including potentially enteropathogenic strains, *V. vulnificus* and *V. cholerae*);

— performance characteristics of the method have been added in Annex E.

Introduction

Because of the large variety of food and feed products, the horizontal method described in this document 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.

The main changes, listed in the foreword, introduced in this document compared to ISO/TS 21872-1:2007 and ISO/TS 21872-2:2007 are considered as major (see ISO 17468).

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 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 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 the detection of potentially enteropathogenic *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio vulnificus*

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for detection of *Vibrio* spp., and 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 is exercised in the disposal of contaminated material.

1 Scope

This document specifies a horizontal method for the detection of enteropathogenic *Vibrio* spp., which causes human illness in or via the intestinal tract. The species detectable by the methods specified include *Vibrio parahaemolyticus, Vibrio cholerae* and *Vibrio vulnificus*.

It is applicable to the following:

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

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

NOTE 1 This method may not be appropriate in every detail for certain products (see Introduction).

NOTE 2 The World Health Organization (WHO) have identified that *V. parahaemolyticus, V. cholerae* and *V. vulnificus* are the major food-borne *Vibrio* spp. However, this document can also be appropriate for the identification of other *Vibrio* spp. causing illness in humans.[1]

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 6887-1:2017, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions*

ISO 6887-3, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 3: Specific rules for the preparation of fish and fishery products*

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*

ISO 22118, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection and quantification of food-borne pathogens — Performance characteristics*

ISO 22119, *Microbiology of food and animal feeding stuffs — Real-time polymerase chain reaction (PCR) for the detection of food-borne pathogens — General requirements and definitions*

ISO 22174, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — General requirements and definitions*

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
potentially enteropathogenic *Vibrio* spp.
microorganism which forms typical colonies on solid selective media and which possesses the described biochemical or molecular characteristics when the test is performed in accordance with this document

Note 1 to entry: This document describes specific procedures for *V. parahaemolyticus, V. cholerae* and *V. vulnificus.*

3.2
detection of potentially enteropathogenic *Vibrio* spp.
determination of the presence or absence of potentially enteropathogenic *Vibrio* spp. (3.1) *(V. parahaemolyticus, V. cholerae* and *V. vulnificus)* in a determined quantity of product, when the test is performed in accordance with this document

4 Principle

4.1 General

The detection of potentially enteropathogenic *Vibrio* spp. *(V. parahaemolyticus, V. cholerae* and *V. vulnificus)* requires four successive phases, as shown in the procedure diagram in Annex A.

Recovery of certain *Vibrio* spp. from foodstuffs may be improved by the use of different incubation temperatures depending upon the target species or state of the food matrix. For example, recovery of *V. parahaemolyticus* and *V. cholerae* in fresh products is enhanced by enrichment at 41,5 °C whereas for *V. vulnificus,* and for *V. parahaemolyticus* and *V. cholerae* in deep frozen (<−18 °C),[2] dried or salted products, recovery is enhanced by enrichment at 37 °C. If detection of *V. parahaemolyticus, V. cholerae* and *V. vulnificus* is required, all specified incubation temperatures should be used. If detection of *V. parahaemolyticus, V. cholerae* and *V. vulnificus* together is not required, the specific procedure(s) may be selected according to the species they are required to detect. Such a selection should be clearly specified in the test report.

NOTE *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* may 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.

4.2 Primary enrichment in a liquid selective medium

Inoculation of the test portion in the primary enrichment medium alkaline saline peptone water (ASPW) (5.1) at ambient temperature, followed by incubation at 41,5 °C ± 1°C for 6 h ± 1 h and/or 37 °C ± 1°C for 6 h ± 1 h.

The incubation conditions are determined by the target species and food product state.

For deep frozen, dried or salted products, primary enrichment should be at 37 °C for all target species.

For detection of *V. vulnificus* in all products, primary enrichment should be at 37 °C.

For detection of *V. parahaemolyticus* and/or *V. cholerae* only, in fresh products, primary enrichment should be at 41,5 °C.

4.3 Secondary enrichment in a liquid selective medium

Inoculation of the second enrichment medium (ASPW) with the cultures obtained in 4.2.

Incubation of inoculated enrichment medium at 41,5 °C ± 1 °C for 18 h ± 1 h and/or 37 °C ± 1 °C for 18 h ± 1 h.

For detection of *V. vulnificus* in all products, secondary enrichment should be at 37 °C.

For detection of *V. parahaemolyticus* and/or *V. cholerae* only, secondary enrichment should be at 41,5 °C.

4.4 Isolation and identification

From the cultures obtained in 4.2 and in 4.3, inoculation of two solid selective media:

— thiosulfate citrate bile and sucrose agar (TCBS) medium (5.2.1);

— another appropriate solid selective medium (left to the choice of the laboratory), such as chromogenic agar, complementary to the TCBS medium (5.2.2).

Incubation of the TCBS medium at 37 °C ± 1 °C, then examination after 24 h ± 3 h. Incubation of the second selective medium according to the manufacturer's recommendations.

4.5 Confirmation

Presumptive colonies of *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus* isolated in 4.4 are subcultured and confirmed by means of appropriate biochemical and/or polymerase chain reaction (PCR) tests.

Biochemical and/or PCR confirmation of isolates may be used for species identification, however, reliable detection of enteropathogenic *V. parahaemolyticus* as determined by presence of the direct thermostable haemolysin (*tdh*) and/or direct related haemolysin (*trh*) genes can only be carried out using PCR tests.

It has been demonstrated that in the screening of ASPW broths using conventional PCR absence of amplification of Vp-*toxR* can indicate no detection of *V. parahaemolyticus*.[3] To reduce the amount of downstream testing and, if shown to be reliable by the user laboratory, screening of incubated enrichment broths may be used. This approach is only recommended after secondary enrichment and does not apply to other *Vibrio* spp. molecular targets.

NOTE 1 Validation data generated in the preparation of this document demonstrated that PCR based identification can be achieved by conventional PCR for *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* or real-time PCR for *V. parahaemolyticus* and *V. vulnificus*. The PCR methods used in the development of this document are given in Annexes C and D.

NOTE 2 Validation data for screening of secondary enrichment broths for amplification of Vp-*toxR* was not generated in the preparation of this document.

5 Culture media and reagents

For general laboratory practice, refer to ISO 7218.

For clarity of the text, details of the composition of culture media and reagents and their preparation are described in Annex B.

For performance testing of culture media, refer to ISO 11133.

NOTE Primers, probes and PCR running conditions used in the development of this document are given in Annexes C and D.

5.1 Enrichment medium: alkaline saline peptone water (ASPW)

As specified in B.1.

5.2 Solid selective isolation media

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

As specified in B.2. See Table 1 for performance testing data.

Table 1 — Performance testing of thiosulphate, citrate, bile and sucrose agar medium (TCBS)

Function	Incubation	Control strains	WDCM [a]	Criteria	Characteristic reactions
Productivity	37 °C ± 1 °C for 24 h ± 3 h	*Vibrio parahaemolyticus*	00185 [b]	Good growth	Green (sucrose negative)
	37 °C ± 1 °C for 24 h ± 3 h	*Vibrio furnissii*	00186 [b]	Good growth	Yellow (sucrose positive)
Selectivity	37 °C ± 1 °C for 24 h ± 3 h	*Escherichia coli* [c d]	00012, 00013 or 00090	Total inhibition	—

[a] World Data Centre for Microorganisms (WDCM) strain catalogue available at http://refs.wdcm.org

[b] Strain to be used as a minimum (see ISO 11133)

[c] Some national restrictions and directions may require the use of a different *E. coli* serovar. Make reference to national requirements relating to the choice of *E. coli* serovars.

[d] Strain free of choice; one of the strains shall be used as a minimum (see ISO 11133).

5.2.2 Second medium

The selection of the second medium is left to the choice of the test laboratory. Preparation of the medium should be strictly according to the manufacturer's instructions.

5.3 Saline nutrient agar (SNA)

As specified in B.3.

5.4 Reagent for detection of oxidase

As specified in B.4.

5.5 Biochemical tests

5.5.1 Lysine decarboxylase saline medium (LDC)

As specified in B.5.

5.5.2 Arginine dihydrolase saline medium (ADH)

As specified in B.6.

5.5.3 Reagent for detection of β-galactosidase

As specified in B.7.

5.5.4 Saline medium for detection of indole

As specified in B.8.

5.5.5 Saline peptone waters

As specified in B.9.

5.5.6 Sodium chloride solution

As specified in B.10.

5.6 PCR

5.6.1 Tris acetate EDTA buffer (TAE) (or a buffer allowing similar performance for the purpose)

As specified in B.11.

5.6.2 Mastermix

Reagents shall be added in quantities as specified by the manufacturer's instructions. See Annexes C and D for example details of master mixes used in the development of this document.

5.6.3 Primers and probes

Primer (and hydrolysis probe) sequences if required shall be published in a peer-reviewed journal and be verified for use against a broad range of target *Vibrio* spp. and non-target strains. With advances in whole genome sequencing of bacterial strains, more appropriate species-specific markers may be identified in the future.

For *V. parahaemolyticus* the target region should be *toxR.*

For determination of pathogenic strains of *V. parahaemolyticus* genes encoding the thermostable direct (TDH) and the thermostable direct related (TRH) haemolysins should be targeted.

For *V. cholerae* the target region for conventional PCR should be the 16S-23S rRNA intergenic spacer region *prVC*.

For *V. vulnificus* the target region should be the *V. vulnificus* haemolysin region.

Target regions other than those specified above for the identification of *V. parahaemolyticus, V. vulnificus* and *V. cholerae* can be used if they have been shown to demonstrate equivalent performance to those used in the development of this document and described in Annexes C or D, are published in a peer-reviewed journal and are verified against a broad range of target *Vibrio* spp. and non-target strains.

See Annex C for example details of primers used for conventional PCR and Annex D for primers and hydrolysis probes for real-time PCR used in the development of this document.

5.6.4 Positive control material

Separate control material shall be used for each target *Vibrio* spp. See Annexes C and D for example details of control strains used in the development of this document.

5.6.5 Negative extraction control

Nuclease free water or sterile NaCl 0,85 % extracted according to 9.5.6.

6 Equipment and consumables

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

Ordinary microbiology laboratory equipment as specified in ISO 7218, and in particular the following.

6.1 Refrigerator, adjustable to 5 °C ± 3,0 °C.

6.2 Incubator, adjustable to 37 °C ± 1,0 °C.

6.3 Incubator, adjustable to 41,5 °C ± 1,0 °C.

6.4 Freezer, adjustable to <−15 °C.

6.5 Micro-centrifuge tubes, with a capacity of 1,5 ml and 2,0 ml.

6.6 Micro-centrifuge, for reaction tubes with a capacity of 1,5 ml and 2,0 ml and capable of running at 10 000*g.*

6.7 Heating block capable of operating at 95 °C ± 2,0 °C or equivalent.

6.8 Vortex.

6.9 Graduated pipettes and pipette filter tips, for volumes between 1 µl and 1 000 µl.

6.10 Associated consumables for conventional or real-time PCR, e.g. optical plates and caps, optical plate holder, suitable for use with the selected PCR machine.

6.11 Conventional or real-time PCR machine, gel electrophoresis and UV visualization equipment as appropriate.

7 Sampling

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

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

8 Preparation of the test sample

Prepare the test sample in accordance with ISO 6887-1 and ISO 6887-3 and/or the document concerning the product to be examined. If a specific document does not exist, it is recommended that the relevant parties reach agreement on this subject.

9 Procedure (See Figure A.1)

9.1 Test portion and initial suspension

This document has been validated for test portions of up to 25 g or 25 ml. A smaller test portion may be used without the need for additional validation/verification provided that the same ratio between (pre-)

enrichment broth and test portion is maintained. A larger test portion than that initially validated may be used if a validation/verification study has shown that there are no adverse effects on the detection of *Vibrio* spp.

NOTE Validation can be conducted accordance with ISO 16140-2. Verification for pooling samples can be conducted in accordance with the protocol described in ISO 6887-1:2017, Annex D.

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

Take test portions (25 g or 25 ml) and homogenize in 225 ml of enrichment medium.

In the case of large quantities (greater than 25 g or 25 ml), the ASPW should be warmed to 37 °C ± 1 °C and/or 41,5 °C ± 1 °C (4.2) before inoculation with the test portion.

In order to reduce the amount of examination work, where more than one 25 g or 25 ml test portion stemming from a determined 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. For example, if 10 test portions of 25 g or 25 ml are to be examined, it is possible to combine these 10 units in order to obtain a composite sample of 250 g or 250 ml and to add 2,25 l of enrichment medium.

Cell counts of *Vibrio* spp. potentially 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 Primary selective enrichment

Incubate the initial suspensions (9.1) at 41,5 °C ± 1 °C and/or 37 °C ± 1 °C for 6 h ± 1 h according to Table 2.

Table 2 — Primary incubation and target species/product state

Target *Vibrio* spp. in fresh product			
Incubation temperature [a] (6 h ± 1 h)	***Vibrio parahaemolyticus***	***Vibrio cholerae***	***Vibrio vulnificus***
41,5 °C ± 1 °C	✓	✓	X
37 °C ± 1 °C	X	X	✓
Target *Vibrio* spp. in deep frozen, dried or salted product (such as bacalhau, stockfish, boknafisk, katsuo-bushi, obambo)			
Incubation temperature [a] (6 h ± 1 h)	***Vibrio parahaemolyticus***	***Vibrio cholerae***	***Vibrio vulnificus***
41,5 °C ± 1 °C	X	X	X
37 °C ± 1 °C	✓	✓	✓
[a] *Vibrio* spp. other than those listed in Reference [1] may be recovered at these incubation temperatures.			

9.3 Secondary selective enrichment

Transfer 1 ml of the culture obtained in 9.2 taken from the surface into a tube containing 10 ml of ASPW (5.1). It is recommended that the sample is not agitated before taking the aliquot.

Incubate the ASPW according to Table 3.

NOTE Cultures obtained in 9.3 can be screened using PCR (see Annexes C and D).