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Microbiology of the food chain — Horizontal method for the determination of *Vibrio* spp. —

Part 1: Detection of potentially enteropathogenic *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio vulnificus*

Microbiologie de la chaîne alimentaire — Méthode horizontale pour la détermination des Vibrio *spp.* —

Partie 1: Recherche des espèces de Vibrio parahaemolyticus, Vibrio cholerae *et* Vibrio vulnificus *potentiellement entéropathogènes*

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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, which has 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. parahaemolyticus*, including potentially enteropathogenic strains, *V. vulnificus* and *V. cholerae*);

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

A list of all parts in the ISO 21872 series can be found on the ISO website.

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 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 determination of *Vibrio* spp. —

Part 1: 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) has identified that *V. parahaemolyticus, V. cholerae* and *V. vulnificus* are the major food-borne *Vibrio* spp. However, the method in 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 the food chain — 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 the food chain — 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 being sought. 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 for 6 h and/or 37 °C for 6 h.

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

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

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 for 18 h and/or 37 °C for 18 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, in all products, 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, then examination after 24 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 by screening ASPW broths using conventional PCR, the 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 molecular targets for other *Vibrio* spp.

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* were 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]	Method of control	Criteria [e]	Characteristic reactions
Productivity	37 °C ± 1 °C for 24 h ± 3 h	*Vibrio parahaemolyticus*	00185 [b]	Qualitative	Good growth (2)	Green colonies (sucrose negative)
	37 °C ± 1 °C for 24 h ± 3 h	*Vibrio furnissii*	00186 [b]	Qualitative	Good growth (2)	Yellow colonies (sucrose positive)
Selectivity	37 °C ± 1 °C for 24 h ± 3 h	*Escherichia coli* [c d]	00012, 00013 or 00090	Qualitative	Total inhibition (0)	—

[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).

[e] Growth is categorized as 0: no growth, 1: weak growth (partial inhibition), and 2: good growth (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 L-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 (*vvha*).

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 in accordance with the appropriate document of ISO 16140 (all parts). 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]	***Vibrio parahaemolyticus***	***Vibrio cholerae***	***Vibrio vulnificus***
41,5 °C ± 1 °C	✓	✓	
37 °C ± 1 °C			✓
Target *Vibrio* spp. in deep frozen, dried or salted product (such as bacalhau, stockfish, boknafisk, katsuo-bushi, obambo)			
Incubation temperature [a]	***Vibrio parahaemolyticus***	***Vibrio cholerae***	***Vibrio vulnificus***
41,5 °C ± 1 °C			
37 °C ± 1 °C	✓	✓	✓

[a] *Vibrio* spp. other than those listed in Reference [1], such as *V. alginolyticus*, 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 at 41,5 °C ± 1 °C and/or 37 °C ± 1 °C for 18 h ± 1 h according to Table 3.

NOTE Cultures and/or boiled broths obtained in 9.3 can be screened using PCR

Table 3 — Secondary incubation and target species/product state

Target *Vibrio* spp. in all product states			
Incubation temperature [a]	***Vibrio parahaemolyticus***	***Vibrio cholerae***	***Vibrio vulnificus***
41,5 °C ± 1 °C	✓	✓	
37 °C ± 1 °C			✓

[a] *Vibrio* spp. other than those listed in Reference [1], such as *V. alginolyticus*, may be recovered at these incubation temperatures.

9.4 Isolation and identification

From the cultures obtained in the ASPW (9.2 and 9.3), inoculate with a 1 µl 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 fresh sampling loop.

Invert the agar plates:

— for TCBS agar plates, incubate at 37 °C ± 1 °C for 24 h ± 3 h;

— for the second isolation medium, incubate according to the manufacturer's instructions.

After incubation, examine the TCBS and second selective medium for the presence of typical colonies of presumptive pathogenic *Vibrio* spp. Mark their position on the bottom of the plates.

On TCBS agar *V. parahaemolyticus*, *V. vulnificus,* and *V. cholerae* exhibit different typical colony morphologies:

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

— typical colonies of *V. cholerae* are smooth, yellow (positive sucrose) and of 1 mm to 2 mm in diameter.

For the second selective medium, examine for the presence of colonies, which, according to their characteristics, may be considered as possible isolates of *V. parahaemolyticus*, *V. vulnificus,* and/or *V. cholerae.*

NOTE 1 *Vibrio* spp. other than those listed in Reference [1], such as *V. alginolyticus*, can be recovered on TCBS and the second selective medium.

NOTE 2 Recognition of colonies of *Vibrio* spp. 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.

NOTE 3 For enhanced recovery of *V. vulnificus*, medium containing derivatives of cellobiose-polymyxin B-colistin and cellobiose-colistin has been shown to be effective.[4]

9.5 Confirmation

9.5.1 General

Using this document, *V. parahaemolyticus*, *V. vulnificus,* and *V. cholerae* can be confirmed by molecular PCR and/or biochemical approaches. Confirmation may be carried out at the end user laboratory or by a specialist reference laboratory. Other *Vibrio* spp. may be isolated using this document.

Not all *V. parahaemolyticus* possess pathogenicity traits. In order to confirm the pathogenic character of the strains, it is preferable to detect the presence of thermostable direct haemolysin (*tdh*) or TDH-

related haemolysin (*trh*) genes. This should be carried out using PCR tests. PCR based confirmation also may reduce the subjective interpretation of biochemical identification tests and accelerate the identification process.

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

If shown to be reliable, commercially available molecular detection kits may be used to identify *Vibrio* to the species level. These kits shall be used in accordance with the manufacturer's instructions.

9.5.2 Selection of colonies for confirmation and preparation of pure cultures

For confirmation, subculture from each selective medium (9.4) at least one well isolated colony considered to be typical or similar to each of the potentially pathogenic *Vibrio* spp. sought. If the result of the first isolated colony tested is negative, a further four well isolated colonies should be tested.

In samples where it is considered important to optimize the detection of potentially pathogenic *V. parahaemolyticus* based upon the presence of thermostable direct and thermostable direct related haemolysins, it is recommended that at least five and, where possible, all colonies exhibiting typical *V. parahaemolyticus* colony morphology are sub-cultured for downstream testing.

Inoculate the colonies selected onto the surface of plates of saline nutrient agar (SNA) (5.3) or suitable medium of the laboratory's choice to obtain isolated colonies. Incubate at 37 °C ± 1 °C for 24 h ± 3 h.

NOTE 1 Food, especially seafood, may contain large numbers of bacteria, including non-pathogenic *Vibrio* spp. which may grow through the selective culture process. Subculture of small numbers of colonies may result in potentially pathogenic species being missed. This is particularly critical with the use of TCBS on which non *Vibrio* spp. or non-enteropathogenic *Vibrio* spp. colonies may be morphologically similar to enteropathogenic species.

NOTE 2 The proportion of pathogenic strains in environmental and food samples is usually low (for *V. parahaemolyticus* they usually represent less than 5 % of the total *V. parahaemolyticus* present in a sample) and thus the likelihood of detecting pathogenic strains by sub-culturing a small number of colonies for identification and confirmation is very low. Confirmation of as many colonies as possible and preferably all colonies increases the likelihood of detecting pathogenic strains.

9.5.3 Tests for presumptive identification

9.5.3.1 Oxidase test

Using a sampling loop, platinum iridium straight wire or 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 commercially available test, following the manufacturer's instructions. Neither a nickel-chromium sampling loop nor a metallic wire shall be used. Nickel or chrome wire sampling loops may give false-positive results, and should be avoided.

9.5.3.2 Microscopic examination (optional)

For each pure culture obtained in 9.4, test according to a) and b).

a) Prepare a film for Gram staining; see ISO 7218. After staining, examine for morphology and the Gram reaction using a microscope and record the results.

b) Inoculate a tube of ASPW (5.1). Incubate at 37 °C ± 1 °C for 1 h to 6 h. Deposit a drop of the culture onto a clean slide, cover with a cover slip and examine for the motility under the microscope. Note the cultures showing a positive result for motility.

9.5.3.3 Selection of the cultures

For confirmation, retain the oxidase-positive colonies.

For confirmation, also retain Gram-negative colonies which give a positive result in the motility test (if examined).

9.5.4 Biochemical confirmation

9.5.4.1 General

Using an inoculation loop, inoculate the media indicated in 9.5.4.2 to 9.5.4.6 with each of the cultures obtained from the colonies retained in 9.5.2.

9.5.4.2 L-lysine decarboxylase saline medium (5.5.1)

Inoculate the liquid medium just below the surface. Add about 1 ml of sterile mineral oil on the top of the medium.

Incubate at 37 °C ± 1 °C for 24 h ± 3 h.

Turbidity and a violet colour after incubation indicate a positive reaction (bacterial growth and decarboxylation of the L-lysine). A yellow colour indicates a negative reaction.

9.5.4.3 Arginine dihydrolase saline medium (5.5.2)

Inoculate the liquid medium just below the surface. Add about 1 ml of sterile mineral oil on the top of the medium.

Incubate at 37 °C ± 1 °C for 24 h ± 3 h.

Turbidity and a violet colour after incubation indicate a positive reaction (bacterial growth and dihydrolation of arginine). A yellow colour indicates a negative reaction.

9.5.4.4 Detection of *β*-galactosidase (5.5.3)

Inoculate the presumptive colony into a tube containing 0,25 ml of sodium chloride solution (5.5.6).

Add one drop of toluene and shake the tube.

Place the tube in the incubator (6.2) set at 37 °C ± 1 °C and leave to stand for approximately 5 min.

Add 0,25 ml of the reagent for the detection of *β*-galactosidase and mix.

Replace the tube in the water bath set at 37 °C ± 1 °C, leave to stand for 24 h ± 3 h, examining it from time to time.

A yellow colour indicates a positive reaction (presence of *β*-galactosidase). The reaction is often visible after 20 min. Absence of colouring after 24 h indicates a negative reaction.

If ready-to-use paper disks (5.5.3) are used, follow the manufacturer's instructions.

9.5.4.5 Detection of indole (5.5.4)

Inoculate a tube containing 5 ml of the tryptophan saline medium with the presumptive colony.

Incubate at 37 °C ± 1 °C for 24 h ± 3 h. After incubation, add 1 ml of Kovacs reagent (5.5.4).

The formation of a red ring indicates a positive reaction (formation of indole). A yellow-brown ring indicates a negative reaction.

9.5.4.6 Halotolerance test (5.5.5)

Produce a series of saline peptone waters with increasing salt (NaCl) concentration: 0 %, 6 % and 10 % (5.5.5).

Inoculate each of the tubes (5.5.5) with the presumptive colony.

Incubate at 37 °C ± 1 °C for 24 h ± 3 h.

Observation of turbidity indicates that the bacterium can grow at the concentration of sodium chloride present in the tube of saline peptone water.

9.5.4.7 Interpretation of biochemical tests

V. parahaemolyticus, V. cholerae and *V. vulnificus* generally give the reactions indicated in Table 4.

Table 4 — Interpretation of biochemical tests

Test	*Vibrio cholerae* [a]	*Vibrio parahaemolyticus* [a]	*Vibrio vulnificus* [a]	*Vibrio mimicus* [a] [b]	*Vibrio alginolyticus* [a] [b]
Oxidase	+	+	+	+	+
LDC	+	+	+	+	+
ADH	–	–	–	–	-
ONPG hydrolysis	+	–	+	+	-
Production of indole	+	+	+	+	+
Growth in peptone water with					
0 % NaCl	+	–	–	+	-
6 % NaCl	–	+	+	–	+
10 % NaCl	–	–	–	–	+

[a] The sign + denotes 76 % to 89 % positive reactions.

[b] Provided for reference purposes.

NOTE 1 The reactions given in Table 4 are a guide to the identification of the listed species. Additional phenotypic tests can be required to fully distinguish these species from each other and from non-pathogenic *Vibrio* spp.

NOTE 2 It is preferable to conduct serology for *V. cholerae* (at least to determine whether they are serogroups O1 or O139) or an appropriate PCR-based test to determine toxigenic strains, such as those that carry the cholera toxin gene *ctx*. This can be carried out by the end user laboratory or a specialist reference laboratory.

NOTE 3 There are some rare instances where *V. parahaemolyticus* strains are positive for ONPG hydrolysis. Additional phenotypic and/or molecular tests may be required to fully distinguish these strains from each other and from non-pathogenic *Vibrio* spp.

9.5.4.8 Step by step confirmation (optional)

Both *V. cholerae* and *V. parahaemolyticus* have different salinity tolerances. With the cultures that are selected in 9.5.3.3, undertake tests for growth in 10 % saline peptone water (5.5.5) and arginine dihydrolase (5.5.2). Continue with the other confirmation tests on any colonies that do not show growth in 10 % saline peptone water and which give a negative arginine dihydrolase reaction.

It is preferable to subculture onto saline nutrient agar (5.3) at the same time in order to make sure that the "no growth" in the 10 % saline peptone water is not due to a dead culture.

9.5.5 PCR confirmation

The minimum requirements for the amplification and detection of nucleic acid sequences by PCR are laid out in ISO 22118, ISO 22119 and ISO 22174.

PCR may be carried out by conventional or real-time PCR formats. See 9.5.7 and Annex C for example details of conventional PCR, and 9.5.8 and Annex D for real time PCR used in the development of this document.

— For PCR, use colonies retained in 9.5.3.3.

— For transport to a specialist laboratory, inoculate colonies onto saline nutrient agar slants (5.3) or other suitable media.

9.5.6 DNA extraction

Prepare a bacterial suspension using an inoculating loop. Take one well isolated colony from the saline nutrient agar (5.3) and inoculate into 500 µl of sterile 0,85 % NaCl (5.5.6) or nuclease free water in a 1,5 ml micro-centrifuge tube.

Heat the micro-centrifuge tubes in a heating block (6.7) set at 95 °C ± 2 °C for 5 min ± 1 min.

Centrifuge the micro-centrifuge tubes at 10 000*g* for 1 min. Retain the supernatant for PCR testing.

If shown to be reliable, commercially available DNA extraction kits may be used to extract bacterial DNA. These kits shall be used in accordance with the manufacturer's instructions.

For each batch of samples tested a negative extraction control shall be included. DNA extraction shall be carried out in parallel on 500 µl of nuclease free water or sterile 0,85 % NaCl.

For long term storage a temperature of <–15 °C is recommended. Short-term storage (<1 month) samples can be refrigerated at 5 °C ± 3 °C.

9.5.7 Conventional PCR

Prepare master mix using appropriate primers, adjusting volumes depending on the required number of reactions.

Place *x* µl of extracted DNA to a clearly labelled tube and add *y* µl of master mix.

Load PCR tubes into the thermocycler and set the appropriate running conditions depending on the primer set used.

Thermocycler running conditions should be carried out as single reactions and run for 20 to 30 cycles depending upon the target (see Annex C). The duration and temperature of each stage (denaturation, annealing and extension) depend on the reagents used. See Annex C for details of the PCR primers and running conditions used in the development of this document.

Prepare a 2 % agarose gel by mixing 2 g of agarose with 100 ml of 1X TAE buffer (or equivalent buffer) (5.6.1). Allow to cool before adding a few drops of ethidium bromide or alternative dye enabling visualization of the product.

Place an appropriate sized gel comb to the mould before pouring the gel and allow to solidify before removing the comb.

Load the gel with 10 µl of 100 bp DNA ladder and the following wells with 20 µl of PCR product prepared previously, using an appropriate loading dye as necessary. Run the gel at 130 V for 25 min to 30 min. Alternative volumes of PCR product and DNA ladder may be used according to the individual laboratory procedures and manufacturers' recommendations.

Following electrophoresis, visualize the gel using an ultraviolet transilluminator. See Annex C for details of expected product sizes for primers used in the development of this document.

PCR products should be confirmed by an appropriate method, following ISO 22174.

PCR may be used to screen for the presence of target bacterium in primary (9.1) and secondary enrichment broths (9.2) following incubation. This may enable downstream targeting of identification. It is recommended that for samples considered of importance, e.g. official controls and/or outbreak investigations, colony isolation and confirmation is carried out according to this document.

9.5.8 Real-time PCR

Add 20 µl of the relevant mastermix to each well. Alternative volumes of PCR reagents may be modified according the individual laboratory procedures or from published protocols.

For every batch of samples in a cycler run, prepare an optical plate with the following:

— at least 1 well with 5 µl of undiluted sample DNA;

— at least 1 well with 5 µl of negative extraction control;

— at least 1 well with 5 µl of control DNA for each target assay;

— at least 1 well with 5 µl of nuclease free water.

Subject the plate to at least 45 cycles. The duration and temperature of each stage (denaturation, annealing and extension) depend on the reagents used; they should be based upon the manufacturer's recommendations.

For real-time PCR machines where the user can set the point of fluorescence measurement, this shall be set at the end of the extension stage.

Analyse the amplification plots using the approach recommended by the manufacturer of the real-time PCR machine. The threshold should ideally be set so that it crosses the area where the amplification plots (logarithmic view) are parallel (the exponential phase).

Negative controls (nuclease-free water and negative extraction controls) shall always be negative; if positive results occur in these controls then any samples giving positive results shall be retested.

See Annex D for example details of an amplification method used in the development of this document.

It is recommended that for samples considered of importance, e.g. official controls and/or outbreak investigations, colony isolation and confirmation is carried out according to this document.

10 Expression of results

Depending on the interpretation of results, indicate that potentially enteropathogenic *Vibrio* spp. is detected or not detected in a test portion of *x* grams or *x* ml of product (see ISO 7218), specifying the name of the relevant species and any pathogenicity characteristics if they have been tested.

11 Performance characteristics of the method

11.1 Interlaboratory study

The performance characteristics of the method were determined in an interlaboratory study to determine the specificity, sensitivity and, when possible, the LOD_{50} of the method. The data are summarized in Annex E. The values derived from the interlaboratory study may not be applicable to food types other than those given in Annex E.

11.2 Sensitivity

The sensitivity is defined as the number of samples found positive divided by the number of samples tested at a given level of contamination. The results are thus dependent on the level of contamination of the sample.

11.3 Specificity

The specificity is defined as the number of samples found negative divided by the number of blank samples tested.

11.4 LOD_{50}

The LOD_{50} is the concentration (cfu/sample) for which the probability of detection is 50 %.

12 Test report

The test report shall specify:

— the reference of this document, i.e. ISO 21872-1;

— all information required for the complete identification of the sample;

— the sampling method used, if known;

— any deviation with respect to the enrichment medium or the incubation conditions used;

— all operating details not specified in this document, or regarded as optional, together with details of any incidents which may have influenced the results;

— the results obtained, and in particular if the pathogenicity markers of the isolated strains were confirmed;

— whether a positive result has been obtained when using an isolation medium (5.2.2) not specified in this document.

Annex A
(normative)

Diagram of procedure

Identification of *trh* positive *V. parahaemolyticus* is by conventional PCR only (refer to Annex C), identification of *V. cholerae* is by conventional PCR and/or biochemical tests (refer to Annexes B and C).

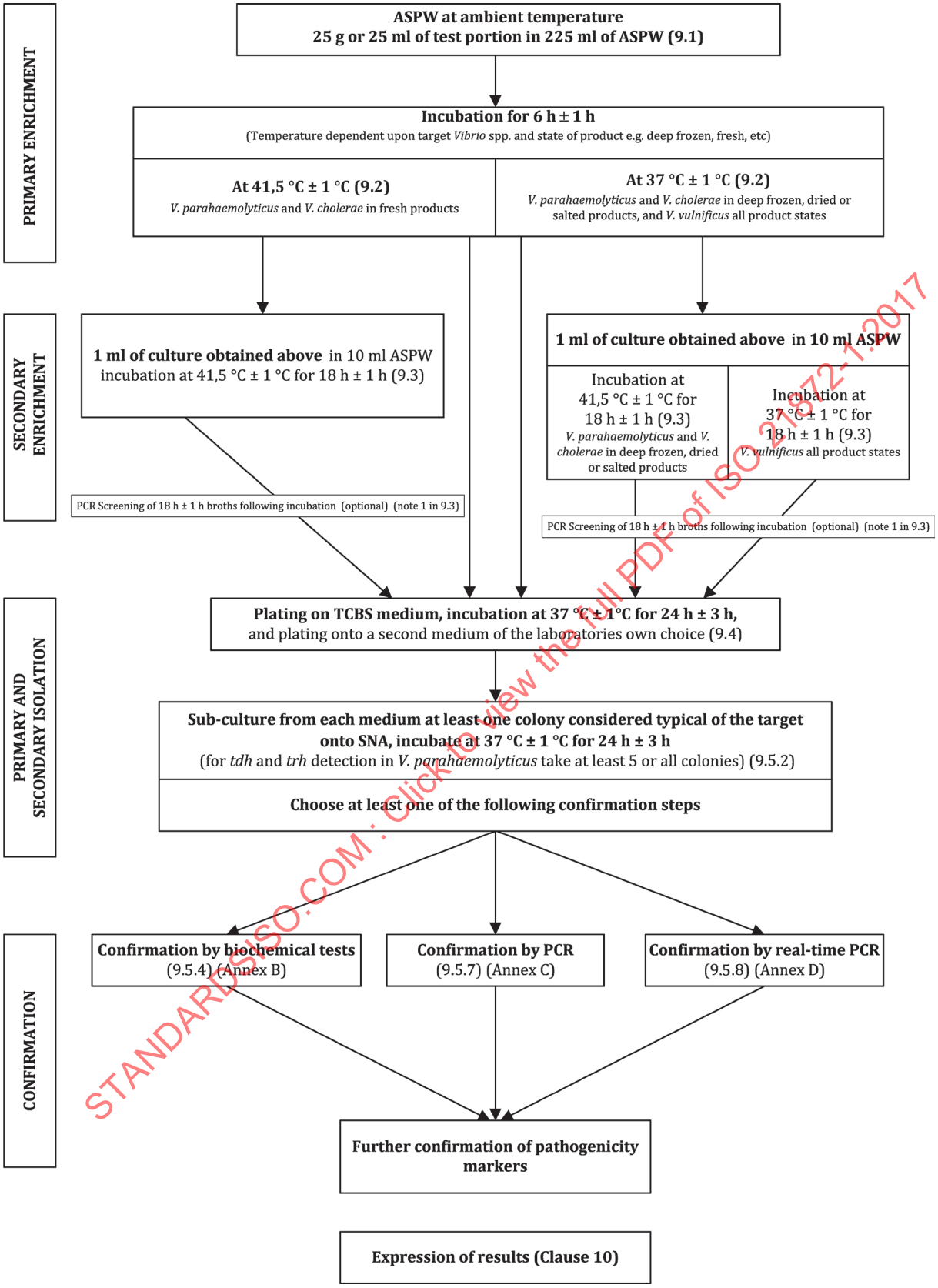

Figure A.1 — Diagram of procedure for the detection of enteropathogenic *V. parahaemolyticus*, *V. cholerae* and *V. vulnificus*

Annex B
(normative)

Composition and preparation of the culture media and reagents

B.1 Alkaline saline peptone water (ASPW)

B.1.1 Composition

Peptone	20,0 g
Sodium chloride	20,0 g
Water	1 000 ml

B.1.2 Preparation

Dissolve the components in the water, by heating if necessary.

If necessary, adjust the pH so that, after sterilization, it is 8,6 ± 0,2 at 25 °C.

Dispense the medium, in quantities required for the examination.

Sterilize in an autoclave set at 121 °C for 15 min.

B.2 Thiosulfate citrate bile and sucrose agar (TCBS)

B.2.1 Composition

Peptone	10,0 g
Yeast extract	5,0 g
Sodium citrate	10,0 g
Sodium thiosulfate	10,0 g
Iron (III) citrate	1,0 g
Sodium chloride	10,0 g
Dried bovine bile	8,0 g
Sucrose	20,0 g
Bromothymol blue	0,04 g
Thymol blue	0,04 g
Agar-agar	8,0 g to 18,0 g [a]
Water	1 000 ml

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

B.2.2 Preparation

Dissolve the components or the complete dehydrated medium in the water, by bringing to the boil.

Adjust the pH, if necessary, so that it is 8,6 ± 0,2 at 25 °C.

Do not autoclave.

B.2.3 Preparation of the agar dishes

Dispense 15 ml to 20 ml of the medium, cooled down to approximately 50 °C, into sterile Petri dishes and leave to solidify.

B.3 Saline nutrient agar (SNA)

B.3.1 Composition

Meat extract	5,0 g
Peptone	3,0 g
Sodium chloride	10,0 g
Agar-agar	8 g to 18 g [a]
Water	1 000 ml

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

B.3.2 Preparation

Dissolve the dehydrated components or the complete dehydrated medium in the water, by heating if necessary.

Adjust the pH so that, after sterilization, it is 7,2 ± 0,2 at 25 °C.

Transfer the medium into containers of appropriate capacity.

Sterilize in an autoclave set at 121 °C for 15 min.

B.3.3 Preparation of the agar dishes

Dispense 15 ml to 20 ml of the medium, cooled down to approximately 50 °C, into sterile Petri dishes and leave to solidify.

Just prior to use, carefully dry the dishes of agar medium (preferably after having removed the lids and inverted the dishes), in an incubator (6.2) until the agar surface is dry.

B.3.4 Preparation of slants of saline nutrient agar

Dispense approximately 10 ml of the medium, cooled down to approximately 50 °C, into tubes of appropriate capacity.

Leave to settle and solidify in an inclined position.

B.4 Reagent for detection of oxidase

B.4.1 Composition

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

B.4.2 Preparation

Dissolve the components in the cold water immediately before use.

B.5 L-lysine decarboxylase saline medium (LDC)

B.5.1 Composition

L-lysine monohydrochloride	5,0 g
Yeast extract	3,0 g
Glucose	1,0 g
Bromocresol purple	0,015 g
Sodium chloride	10,0 g
Water	1 000 ml

B.5.2 Preparation

Dissolve the components in the water, by heating if necessary.

If necessary, adjust the pH to 6,8 ± 0,2 at 25 °C after sterilization.

Dispense the medium, in volumes of 2 ml to 5 ml into narrow tubes.

Sterilize in an autoclave set at 121 °C for 15 min.

B.6 Arginine dihydrolase saline medium (ADH)

B.6.1 Composition

Arginine monohydrochloride	5,0 g
Yeast extract	3,0 g
Glucose	1,0 g
Bromocresol purple	0,015 g
Sodium chloride	10,0 g
Water	1 000 ml

B.6.2 Preparation

Dissolve the components in the water, by heating if necessary.

If necessary, adjust the pH to 6,8 ± 0,2 at 25 °C after sterilization.

Dispense the medium in volume of 2 ml to 5 ml, into narrow tubes.

Sterilize in an autoclave set at 121 °C for 15 min.

B.7 Detection of β-galactosidase

B.7.1 ONPG solution

B.7.1.1 Composition

2-orthonitrophenyl- β-D-galactosipyranoside (ONPG)	0,08 g
Water	15 ml

B.7.1.2 Preparation

Dissolve the ONPG in the water at 50 °C ± 1 °C.

Cool the solution.

B.7.2 Buffer solution

B.7.2.1 Composition

Sodium dihydrogen-orthophosphate (NaH_2PO_4)	6,9 g
Sodium hydroxide (0,1 mol/l solution)	approx. 3 ml
Water, quantity sufficient for a final volume of	50 ml

B.7.2.2 Preparation

Dissolve the sodium dihydrogen-orthophosphate in about 45 ml of water, in a volumetric flask.

Adjust the pH to 7,0 ± 0,2 at 25 °C with a 0,1 mol/l solution of sodium hydroxide.

Make up to 50 ml with water.

B.7.3 Complete reagent

B.7.3.1 Composition

Buffer solution (B.10.2)	5 ml
ONPG solution (B.10.1)	15 ml

B.7.3.2 Preparation

Add the buffer solution to the ONPG solution.

Store at 3 °C ± 2,0 °C.

B.8 Saline medium for detection of indole

B.8.1 Tryptophan saline medium

B.8.1.1 Composition

Enzymatic digest of casein	10,0 g
DL-trytophan	1,0 g
Sodium chloride	10,0 g
Water	1 000 ml

B.8.1.2 Preparation

Dissolve the components in the water, by heating if necessary, and filter.

If necessary, adjust the pH, so that, after sterilization, it is 7,0 ± 0,2 at 25 °C.

Dispense the medium in quantities of 5 ml into tubes of appropriate capacity.

Sterilize in an autoclave set at 121 °C for 15 min.

B.8.2 Kovacs reagent

B.8.2.1 Composition

Dimethylamino-4 benzaldehyde	5 g
Hydrochloric acid, ρ = 1,18 g/ml to 1,19 g/ml	25 ml
Methyl-2 butan-2-ol	75 ml

B.8.2.2 Preparation

Mix the components.

B.9 Saline peptone water

B.9.1 Composition

Peptone	10 g
NaCl	0 g or 60 g or 100 g
Water	1 000 ml

B.9.2 Preparation

Dissolve the component in the water, by heating if necessary.

If necessary, adjust the pH, so that, after sterilization, it is 7,5 ± 0,2 at 25 °C.

Dispense into tubes of appropriate capacity.

Sterilize in an autoclave set at 121 °C for 15 min.

B.10 Sodium chloride solution

B.10.1 Composition

Sodium chloride	8,5 g
Water	1 000 ml

B.10.2 Preparation

Dissolve the component in the water, by heating if necessary.

If necessary, adjust the pH, so that, after sterilization, it is 7,5 ± 0,2 at 25 °C.

Dispense into tubes of appropriate capacity.

Sterilize in an autoclave set at 121 °C for 15 min.

B.11 Tris acetate EDTA (TAE) buffer

B.11.1 Composition

50X TAE buffer	20 ml
Water	1 000 ml

B.11.2 Preparation

Add the buffer solution to the water.

Store at 3 °C ± 2,0 °C.

Annex C
(informative)

Conventional PCR for the detection of *Vibrio parahaemolyticus*, thermostable direct haemolysin (*tdh*) and thermostable direct related haemolysin (*trh*) genes, *Vibrio cholerae* and *Vibrio vulnificus*

C.1 Composition of mastermix

See Table C.1.

Table C.1 — Composition of mastermix

Reagent	Volume per reaction µl
Reaction buffer (5x) [a]	10
$MgCl_2$ (25 mM)	5
dNTPs (20 mM)	0,625
Forward primer (nM) (100 µM)	0,5
Reverse primer (nM) (100 µM)	0,5
Water	30,625
Taq polymerase	0,25
Total volume	47,5
[a] Dependent upon the initial concentration of the reaction buffer	

C.2 *Vibrio parahaemolyticus* primers (conventional PCR)

VpToxR (FW): GTC TTC TGA CGC AAT CGT TG[5]

VpToxR (REV): ATA CGA GTG GTT GCT GTC ATG[5]

The target region used for detection of *toxR* is based on previously published and validated data.[5] The *toxR* region of all publicly available and sequenced *V. parahaemolyticus* strains, as well as publicly deposited *toxR* sequences was used to ensure specificity. Sequence alignment using all sequences available in GenBank of the assay target region demonstrates that this primer/probe set is adequate for the quantification of all *toxR* sequences. The primer sequences do not align with any other sequences available in GenBank.

C.3 Thermostable direct haemolysin (*tdh*) and thermostable direct related haemolysin (*trh*) genes *Vibrio parahaemolyticus* primers (conventional PCR)

Thermostable direct haemolysin L-*tdh* (FW): GTA AAG GTC TCT GAC TTT TGG AC[6]

Thermostable direct haemolysin R-*tdh* (REV): TGG AAT AGA ACC TTC ATC TTC ACC[6]

Thermostable direct related haemolysin L-*trh* (FW): TTG GCT TCG ATA TTT TCA GTA TCT[6]

Thermostable direct related haemolysin R-*trh* (REV):CAT AAC AAA CAT ATG CCC ATT TCC G[6]

The target region used for detection of *tdh* and *trh* is based on previously published and validated data.[6] The primer sequences do not align with any other sequences available in GenBank.

C.4 *Vibrio cholerae* primers (conventional PCR)

prVC (FW): TTA AGC STT TTC RCT GAG AAT G[7]

prVC (REV): AGT CAC TTA ACC ATA CAA CCC G[7]

The target region used for detection of *prVC* is based on previously published and validated data.[7] The primer sequences do not align with any other sequences available in GenBank.

C.5 *Vibrio vulnificus* primers (conventional PCR)

VVH (FW): CCG GCG GTA CAG GTT GGC GC[8]

VVH (REV): CGC CAC CCA CTT TCG GGC C[8]

The target region used for detection of *vvH* is based on previously published and validated data.[8] The primer sequences do not align with any other sequences available in GenBank.

C.6 Cycling parameters — *VptoxR* and *VVH*

See Table C.2.

Table C.2 — Cycling parameters — *VptoxR* and *VVH*

Step description		Temperature and time	Number of cycles
Pre-heating		96 °C for 5 min	1
Amplification	Denaturation	94 °C for 1 min	30
	Annealing	63 °C for 1,5 min	
	Extension	72 °C for 1,5 min	
Post amplification		72 °C for 7 min	1

C.7 Cycling parameters — *prVC*

See Table C.3.

Table C.3 — Cycling parameters — *prVC*

Step description		Temperature and time	Number of cycles
Pre-heating		94 °C for 2 min	1
Amplification	Denaturation	94 °C for 1 min	30
	Annealing	50 °C for 1 min	
	Extension	72 °C for 1,5 min	
Post amplification		72 °C for 10 min	1

C.8 Cycling parameters — *tdh* and *trh*

See Table C.4.

Table C.4 — Cycling parameters — *tdh* and *trh*

Step description		Temperature and time	Number of cycles
Pre-heating		94 °C for 3 min	1
Amplification	Denaturation	94 °C for 1 min	30
	Annealing	58 °C for 1 min	
	Extension	72 °C for 1 min	
Post amplification		72 °C for 5 min	1

C.9 Control material — conventional PCR

For each target assay DNA was extracted from characterized reference strains (see Table C.5).

A bacterial suspension was prepared by taking one to three colonies from a saline nutrient agar (5.3) and inoculating 500 µl of sterile NaCl 0,85 % (5.5.6) in a 1,5 ml micro-centrifuge tube.

Each micro-centrifuge tube was placed in a heating block (6.7) set at 95 °C ± 1 °C for 5 min ± 1 min.

The micro-centrifuge tubes were centrifuged at 10 000*g* for 1 min and the supernatant removed for PCR testing.

For long term storage a temperature of <–15 °C is recommended.

Table C.5 — Reference strains

Function	Target	WDCM [a] control strains	Identification	Product size bp
Specificity	*toxR*	*Vibrio parahaemolyticus* WDCM 00185	*Vibrio parahaemolyticus* [b]	368
	tdh	*Vibrio parahaemolyticus* NCTC10884 (WDCM number claimed)	thermostable direct haemolysin [c]	269
	trh	*Vibrio parahaemolyticus* WDCM 00037	thermostable direct related haemolysin [c]	500
	prVC	*Vibrio cholerae* WDCM 00136	*Vibrio cholerae* [b]	295 to 310
	VVH	*Vibrio vulnificus* WDCM 00139	*Vibrio vulnificus* [b]	519

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

[b] Denotes species identification.

[c] Indicates pathogenic strain of *Vibrio parahaemolyticus*.

C.10 Interlaboratory study

The PCR methods were validated in an interlaboratory study organized by the European Union Reference Laboratory for monitoring bacteriological and viral contamination of bivalve molluscs in 2011. Twenty-three laboratories were recruited according to the requirements of ISO 5725-1 and ISO 5725-2; all followed a single protocol. Test samples were anonymized and distributed as semi-solid marine agar (MA) swabs inoculated with reference strains of target and non-target *Vibrio* spp. Swabs were inoculated into 225 ml ± 5 ml ASPW and subject to primary enrichment at 41,5 °C ± 1 °C and 37 °C ± 1 °C for 6 h ± 1 h. One millilitre aliquots of each enrichment were subject to secondary enrichment in 10 ml ± 0,5 ml fresh ASPW at 41,5 °C ± 1 °C and 37 °C ± 1 °C for 18 h ± 3 h. Following primary and secondary enrichment, 1 µl of each enrichment broth was streaked onto the surface of TCBS and a second plating medium. TCBS plates were incubated at 37 °C ± 1 °C for 24 h ± 3 h; second plating media were incubated according to the manufacturers' instructions. A minimum of two colonies showing typical phenotypic characteristics of *Vibrio* spp. were sub-cultured onto SNA and incubated at 37 °C ± 1 °C for 24 h ± 3 h. Subsequent cultures were checked visually for purity and subject to oxidase tests and microscopic examination (motility and Gram stain). Oxidase positive, Gram negative, motile isolates were subject to tests for glucose utilization, lactose and sucrose fermentation, L-lysine decarboxylation, β-galactosidase activity, presence of arginine dihydrolase, indole production, ornithine decarboxylase production and growth in 0 %, 2 %, 6 %, 8 %, and 10 % NaCl. In parallel, DNA was extracted from a single colony suspended in 500 µl of nuclease free water, the bacterial suspension was heated at 95 °C ± 1 °C for 5 min ± 1 min and centrifuged at 10 000*g*. The resultant supernatant was stored at <–15 °C for PCR analysis. Aliquots of 2,5 µl of extracted DNA were added to a mastermix containing 10 µl reaction buffer, 5 µl $MgCl_2$, 0,625 µl dNTPs (20 mM), 0,5 µl primer (forward and reverse), 0,25 µl Taq polymerase and 30,625 µl nuclease free water. All samples were subjected to PCR according to the cycling parameters described in C.6, C.7 and C.8 for *toxR*[5], *tdh*[6], *trh*[6], *prVC*[7] and *VVH*[8]. Products were visualized on 2 % agarose gels following electrophoresis at 130 V for 25 min to 30 min.

A summary of the results from the interlaboratory study is given in Table C.6.

Table C.6 — Interlaboratory study data

Parameters	Results			
Year	2011			
Maximum number of laboratories reporting PCR and/or biochemical results for both primary enrichment temperatures	23			
Number of samples per laboratory	5			
Maximum number of results per laboratory	20			
Number of accepted results	370			
	37 °C		41,5 °C	
	PCR	Bio	PCR	Bio
Laboratories assigning correct species identification (*V. vulnificus*) (%)	93,3	86,4	100,0	95,2
Laboratories assigning correct species identification (*V. parahaemolyticus*) (%)	100,0	82,6	100,0	85,7
Laboratories assigning correct species identification (*V. cholerae*) (%)	87,5	69,6	73,3	61,9
Laboratories assigning correct species identification (*V. mimicus*) (%)	100,0	73,9	100,0	71,4
Laboratories assigning correct species identification (*V. fluvialis*) (%)	100,0	77,3	100,0	76,2
	PCR		Bio	
Total correct, all samples (%)	94,8		78,3	
	PCR tests for *tdh* and *trh*			
	tdh		*trh*	
Laboratories correctly assigning presence of *tdh* and *trh*, all samples[6] (%)	94,6		88,04	
PCR = polymerase chain reaction Bio = biochemical tests				