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

Part 2: Enumeration of total and potentially enteropathogenic *Vibrio parahaemolyticus* in seafood using nucleic acid hybridization

Microbiologie de la chaîne alimentaire — Méthode horizontale pour la détermination des Vibrio *spp. —*
Partie 2: Dénombrement de Vibrio parahaemolyticus *total et potentiellement entéropathogène dans les fruits de mer, par hybridation des acides nucléiques*

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Foreword

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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).

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For an explanation of the voluntary nature of standards, 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 www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Potentially enteropathogenic strains of *Vibrio parahaemolyticus* possess thermostable direct haemolysin (TDH) and/or thermostable direct hemolysin-related hemolysin (TRH). TDH positive strains manifest Kanagawa phenomenon (KP)[1]. This characteristic is traditionally utilized in the identification of enterotoxigenic strains of *V. parahaemolyticus*. Strains possessing TRH do not share the haemolytic characteristics of TDH positive isolates and no conventional identification assay has been reported for TRH identification. Pathogenic strains in the environment are a minority[2] and differentiation between enteropathogenic and total *V. parahaemolyticus* presence is therefore useful.

This document enables the enumeration of potentially enteropathogenic *V. parahaemolyticus* and/or of total *V. parahaemolyticus*.

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

Part 2: Enumeration of total and potentially enteropathogenic *Vibrio parahaemolyticus* in seafood using nucleic acid hybridization

1 Scope

This document specifies a method for the direct enumeration of potentially enteropathogenic *V. parahaemolyticus* (*tdh* and/or *trh* positive) and/or the enumeration of total *V. parahaemolyticus* in seafood.

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 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

ISO 6887-1, *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 11133, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

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:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
oligoprobe
labelled, artificially produced segment of DNA with a defined sequence used to detect target DNA by hybridization

3.2
process control
set of characterized strains analysed together with the sample throughout all the stages of the procedure

3.3
hybridization control
DNA segment with a defined sequence used to monitor the hybridization step

3.4
detection control
labelled DNA segment used to monitor the detection step

4 Principle

4.1 General

This method is based upon direct plating of sample material on a nutrient medium. The resulting colonies are transferred onto nylon membranes and hybridized with DNA digoxigenin-labelled oligoprobes to detect genes associated to pathogenicity (*tdh*[3] and *trh*[4]) and/or with a DNA digoxigenin-labelled oligoprobe detecting the *toxR* gene of *V. parahaemolyticus*[5].

As genes associated with *V. parahaemolyticus* pathogenicity have been sporadically detected in other *Vibrio* species (i.e. *V. alginolyticus*[6]), definitive confirmation of the presence of *tdh* and/or *trh* positive *V. parahaemolyticus* strains may be reached by performing isolation procedures on these strains.

The method comprises the following consecutive steps:

a) plating (see 4.2);

b) preparation of membranes (see 4.3);

c) hybridization (see 4.4);

d) detection (see 4.5).

4.2 Plating

Appropriate dilutions of the sample are plated on non-selective nutrient medium in order to allow the growth of *V. parahaemolyticus*.

4.3 Preparation of membranes

Colonies are transferred onto hybridization membranes, bacterial cells are lysed and DNA is covalently linked to the membranes.

4.4 Hybridization

Membranes are hybridized with probes specific to the target genes: *tdh* and *trh* genes for the detection and enumeration of potentially enteropathogenic *V. parahaemolyticus*, and *toxR* gene for the detection and enumeration of total *V. parahaemolyticus*.

4.5 Detection

The presence of hybridized oligoprobes is detected by colorimetric detection with a specific antibody conjugated with an enzyme.

NOTE Enzymatic activity induces the precipitation of substrate in the presence of target colonies resulting in the development of coloured dots which are counted to provide colony enumeration.

5 Culture media and reagents

5.1 General

For current laboratory practice, see ISO 7218. The composition of culture media and reagents and their preparation shall be as described in Annex B. For the preparation, production and performance testing of culture media, follow the procedures in accordance with ISO 11133.

Commercially available reagents may be used if they fulfil the requirements of this document. Follow the manufacturer's instructions for storage and preparation.

5.2 Hybridization reagents

5.2.1 Hybridization buffer

Hybridization buffer composition may vary according to the probe sequences and hybridization temperature. See Annex C for the buffer composition adopted in this procedure.

5.2.2 Digoxigenin-labelled oligoprobes

The 5′-digoxigenin-labelled oligoprobes for the determination of total and potentially enteropathogenic *Vibrio parahaemolyticus* are listed in the Annex C.

Probe concentration can vary depending on synthesis scale. Refer to the data sheet supplied by the manufacturer to calculate the concentration and for storage conditions.

Alternatives to 5′-digoxigenin-labelled oligoprobes such as DIG-labelled amplicon sequences[7] may be used to obtain higher intensity of the signal if they can be shown to provide equivalent results.

5.2.3 Control materials

The control materials for the determination of total and potentially enteropathogenic *Vibrio parahaemolyticus* are listed in the Annex C.

6 Equipment and consumables

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

6.1 Rotary blender.

6.2 Dispenser and graduated pipettes.

6.3 Pipette and tips, capable of dispensing volumes of 100 µl.

6.4 Incubator, capable of operating at 37 °C ± 1 °C.

6.5 UV cross-linker or equivalent UV source (transilluminator) for DNA cross-linking.

6.6 Hybridization oven or water bath with shaking ability, capable of operating at the hybridization temperature.

6.7 Hybridization tubes or bags.

6.8 Hybridization meshes (optional).

6.9 Rocking platform, capable of operating at 120 oscillation min^{-1}.

6.10 Washing containers, preferably circular.

7 Sampling

Sampling is not part of the method specified in this document. Follow the specific International Standard dealing with the product concerned. If there is no specific International Standard dealing with sampling of the product concerned, it is recommended that the parties concerned come to an agreement on this subject.

8 Procedure

8.1 General

Perform the procedure in accordance with the diagrams given in Annex A. For current laboratory practice, see ISO 7218.

8.2 Preparation of the sample

Collect a test portion of the sample in accordance with ISO 6887-3 and prepare an initial 1:1 suspension in an appropriate diluent (B.1.1). Blend in a rotary homogenizer for approximately 90 s to 120 s and prepare sequential decimal dilutions in accordance with ISO 6887-1.

8.3 Plating

Weigh 0,2 g of the 1:1 diluted sample onto a well-dried TSA-S (B.1.2) plate; this dilution represents 0,1 g of sample. For further decimal dilutions, pipette 100 µl onto the surface of separate well-dried TSA-S plates. Immediately spread thoroughly with a sterile spreading rod.

Incubate the TSA-S plates at 37 °C ± 1 °C for 18 h to 24 h.

8.4 Preparation of process control

See Annex C for the preparation of the process control.

8.5 Preparation of membranes

8.5.1 Colony lift

Following incubation, cool down the plates of TSA-S by placing them at 5 °C ± 3 °C for at least 30 min. Record the sample information on the surface edge of a nylon membrane (B.2.1). The use of a sharpened graphite pencil to mark the membranes is recommended as ink can dissolve during the hybridization and washing steps.

Use one membrane for each plate, including the plate with the process control.

NOTE Laboratories wishing to perform isolation of *V. parahaemolyticus* strains detected by this method can mark the edge of the membranes and the corresponding plates with a reference point to allow orientation and, after colony lift, can store the plates at room temperature (up to 72 h) or at 5 °C ± 3 °C until completion of the hybridization procedure.

Place the membrane, label side upwards, onto the surface of the plate. Using a spreading rod, press the membrane onto the surface of the agar ensuring that the entire membrane is in contact with the plate surface to allow transfer of colonies.

Carefully remove the membranes from the plates and place them (colony side upwards) on absorbent paper soaked in Maas I lysis solution (B.2.2) for 30 min at room temperature.

Remove the membranes using forceps and place them (colony side upwards) on absorbent paper soaked in Maas II neutralizing solution (B.2.3) for 30 min at room temperature.

During lysis and neutralization steps, the downwards surface of the membrane should be in complete contact with the solution but the solution should not run over the upper surface and come into contact with the bacterial colonies.

Place the membranes (colony side upwards) on absorbent paper soaked in standard saline citrate 2× (SSC 2×) solution (B.2.6) and equilibrate them for 5 min at room temperature.

Move the membranes onto dry adsorbent paper to remove excess moisture and place them again on absorbent paper soaked with fresh SSC 2× solution (B.2.6) for 10 min.

Remove excess of SSC 2× buffer (B.2.6) placing the membranes on dry absorbent paper.

8.5.2 Cross-linking of DNA to the membrane

DNA adsorbed to the membranes should be covalently cross-linked by exposure to UV (120 mJ/cm^2 of membrane surface).

For 254 nm UV sources, DNA cross-linkage will require an exposure time of 1 min or less. UV sources with long wave emission could require longer exposure times (3 min to 5 min).

8.5.3 Proteinase K treatment

Fully immerse each membrane in at least 1 ml of proteinase K solution (B.2.9). Incubate at 37 °C ± 1 °C for 1 h. Avoid excessive evaporation during incubation.

Remove the membranes from the container and place them (colony side up) on absorbent paper.

Cover the membranes with absorbent paper moistened with distilled water and press gently to allow the adhesion of cell debris to the paper. Lift the absorbent paper to remove the cell debris and discard the paper. Repeat the step if cell debris are visibly still present on the membrane.

Membranes can be left to fully dry in the air and can be stored, avoiding direct light, at room temperature until performing hybridization, but no longer than 30 days. If storing membranes, care should be taken to prevent individual membranes from coming into direct contact with each other.

NOTE Separator discs provided together with nylon membranes or filter paper can be used to avoid contact between membranes during storage.

8.6 Hybridization

8.6.1 General

For the direct enumeration of potentially enteropathogenic *V. parahaemolyticus* (*tdh* and/or *trh* positive strains), follow 8.6.2. For the enumeration of total *V. parahaemolyticus*, follow 8.6.6. Enumeration of potentially enteropathogenic and of total *V. parahaemolyticus* can be performed on the same membrane by sequentially following the procedures described in 8.6.2 and 8.6.6.

Under the provisions of this document, hybridization can be carried out in a hybridization oven or shaking water bath. Buffer volumes should be adjusted to take account of the different sizes of hybridization tubes or sealable plastic bags. In each step, ensure that a sufficient buffer is used to completely cover the membranes during rotation, shaking or stationary phases.

It is advisable to use hybridization meshes to prevent membranes adhering to each other and to facilitate circulation of buffers. This can be required if hybridization is carried out in hybridization bags.

8.6.2 Hybridization of *tdh* and *trh* probes

8.6.2.1 Pre-hybridization

Pre-warm the hybridization buffer (5.2.1) to the temperature required for hybridization. See Annex C for the temperature conditions and hybridization buffer used in this procedure.

Distribute the membranes, including the membrane with the process control, into hybridization tubes or sealable plastic bags so that the surfaces of each membrane do not adhere to each other. Add to the hybridization vessels a sufficient amount of hybridization buffer to completely cover and saturate the membranes throughout the following rotation or shaking steps.

For each tube or bag, include a hybridization control. See Annex C for the preparation of the controls used in this procedure.

Incubate the membranes under hybridization conditions for at least 60 min.

NOTE This step will assist in the removal from the membranes of non-covalently linked residual material.

8.6.2.2 Hybridization with *tdh* and *trh* probes

Decant the buffer from the pre-hybridization step (see 8.6.2.1) and add the fresh pre-warmed hybridization buffer (5.2.1), ensuring that the membranes are completely covered during rotation or shaking. Add the DIG-labelled *tdh* and *trh* probes (5.2.2) in the required concentration. See Annex C for the conditions used in this procedure.

Allow the probes to hybridize at the required temperature with gentle rotation or shaking for at least 2 hours. Membranes may be left to hybridize overnight.

8.6.2.3 Stringency washes for *tdh* and *trh* detection

Transfer the membranes to vessels containing washing buffer A (B.2.10) and wash the membranes on a rocking platform at approximately 120 oscillations min^{-1} for 5 min at room temperature.

NOTE Membranes can be combined in one vessel, provided they freely float in the buffer and do not stick together. Hybridization meshes can be used to improve separation of membranes during washing steps.

Discard the buffer and repeat the washing step using fresh washing buffer A (B.2.10).

Pre-warm washing buffer B (B.2.11) to the temperature required for stringency washes. See Annex C for the conditions used in this procedure.

Transfer the membranes to vessels containing pre-warmed washing buffer B (B.2.11) and wash the membranes at the required temperature with rotation or shaking for 15 min ± 1 min.

Discard the buffer and repeat the washing step using pre-warmed washing buffer B (B.2.11).

8.6.3 Colorimetric detection

Transfer the membranes to vessels containing washing buffer C (B.2.13), add the detection control and wash the membranes on a rocking platform at approximately 120 oscillations min^{-1} for 1 min at room temperature.

Transfer each membrane colony side upwards to a separate container (e.g. Petri dishes) and add sufficient blocking solution (B.2.15) to fully cover the membrane. Incubate without shaking at room temperature for 30 min.

NOTE 1 If 90 mm Petri dishes are used, the use of at least 40 ml of blocking solution is appropriate.

