TECHNICAL SPECIFICATION

ISO/TS 21872-2

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

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

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

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

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of normative document:

— an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;

— an ISO Technical Specification (ISO/TS) represents an agreement between the members of a technical committee and is accepted for publication if it is approved by 2/3 of the members of the committee casting a vote.

An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO/TS 21872-2 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

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

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

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

Introduction

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

When this Technical Specification is next reviewed, account will be taken of all information then available regarding the extent to which this horizontal method has been followed and the reasons for deviations from this method in the case of particular products.

The harmonization of test methods cannot be immediate, and for certain groups of products International Standards and/or national standards may already exist that do not comply with this horizontal method. It is hoped that when such standards are reviewed they will be changed to comply with this Technical Specification so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

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

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

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

1 Scope

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

This part of ISO/TS 21872 is applicable to

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

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

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

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

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

NOTE 3 Reasons for not applying this method are discussed in the Introduction.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

1) See 9.4.4.

ISO 6887 (all parts), *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination*

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

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

3 Terms and definitions

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

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

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

4 Principle

4.1 General

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

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

4.2 First enrichment in a liquid selective medium

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

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

4.3 Second enrichment in a liquid selective medium

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

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

4.4 Isolation and identification

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

— thiosulfate citrate bile and sucrose agar (TCBS);

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

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

4.5 Confirmation

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

5 Culture media, reagents

For general laboratory practice, see ISO 7218.

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

5.1 Enrichment medium: Alkaline saline peptone water (ASPW)

See B.1.

5.2 Solid selective isolation media

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

See B.2.

5.2.2 Second medium

Choose between:

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

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

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

5.3 Saline nutrient agar (SNA)

See B.6.

5.4 Reagent for detection of oxidase

See B.7.

5.5 Saline triple sugar iron (TSI) agar

See B.8.

5.6 Saline medium for detection of ornithine decarboxylase (ODC)

See B.9.

5.7 Saline medium for detection of lysine decarboxylase (LDC)

See B.10.

5.8 Saline medium for detection of arginine dihydrolase (ADH)

See B.11.

5.9 Reagent for detection of β-galactosidase

See B.12.

5.10 Saline medium for detection of indole

See B.13.

5.11 Saline peptone waters

See B.14.

5.12 Sodium chloride solution

See B.15.

6 Apparatus and glassware

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

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

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

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

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

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

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

7 Sampling

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

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

8 Preparation of test sample

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

9 Procedure (see Annex A)

9.1 Test portion and initial suspension

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

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

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

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

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

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

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

9.2 First selective enrichment

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

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

9.3 Second selective enrichment

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

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

9.4 Isolation and identification

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

9.5 Confirmation

9.5.1 General

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

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

9.5.2 Selection of colonies for confirmation and preparation of pure cultures

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

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

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

Use pure cultures for biochemical confirmations.

9.5.3 Tests for presumptive identification

9.5.3.1 Oxidase test

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

commercially available test following the manufacturer's instructions. Neither a nickel-chromium sampling loop nor a metallic wire shall be used. The test is positive if the colour turns to mauve, violet or deep purple within 10 s.

9.5.3.2 Microscopic examination

For each pure culture obtained in 9.4.2, test according to a) and b) as follows.

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

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

9.5.3.3 Selection of cultures for biochemical tests

Retain, for biochemical confirmation, the oxidase-positive and Gram-negative colonies which give a positive result in the motility test.

9.5.4 Biochemical confirmation

9.5.4.1 General

Using an inoculation loop, inoculate the media indicated in 9.5.4.2 to 9.5.4.8 with each of the cultures obtained from the colonies retained in 9.5.3.3.

9.5.4.2 Test with saline TSI agar (5.5)

Inoculate the agar slope by stabbing to the bottom of the agar butt and streaking longitudinally along the slope. Incubate at 37 °C for 24 h ± 3 h.

Interpret the reactions as follows.

a) Agar medium butt

- yellow: glucose positive (fermentation of the glucose);
- red or unchanged: glucose negative (no fermentation of the glucose);
- black: formation of hydrogen sulfide;
- bubbles or cracks: formation of gas from the glucose.

b) Agar medium slant

- yellow: lactose and/or sucrose positive (utilization of lactose and/or sucrose);
- red or unchanged: lactose and sucrose negative (no utilization of lactose or sucrose).

Typical reactions of *V. vulnificus* and *V. fluvialis* correspond to an acid slant (yellow) and an acid butt (yellow), without formation of gas or hydrogen sulfide.

Typical reactions of *V. mimicus* correspond to an alkaline slant (red) (occasionally acid: yellow) and an acid butt (yellow) without formation of gas or hydrogen sulfide.

9.5.4.3 Detection of ornithine decarboxylase

Inoculate the liquid saline medium (5.6) just below the surface. Add about 1 ml of sterile mineral oil to the top of the medium. Incubate at 37 °C for 24 h ± 3 h.

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

9.5.4.4 Detection of L-lysine decarboxylase

Inoculate the liquid saline medium (5.7) just below the surface. Add about 1 ml of sterile mineral oil to the top of the medium. Incubate at 37 °C for 24 h ± 3 h.

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

9.5.4.5 Detection of arginine dihydroxylase

Inoculate the liquid saline medium (5.8) just below the surface. Add about 1 ml of sterile mineral oil to the top of the medium. Incubate at 37 °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.

9.5.4.6 Detection of β-galactosidase

Inoculate the suspect colony into a tube containing 0,25 ml of the saline solution (5.12). Add 1 drop of toluene and shake the tube.

Place the tube in the water bath (6.4) set at 37 °C and leave it to stand for approximately 5 min.

Add 0,25 ml of the reagent for the detection of β-galactosidase(5.9) and mix. Replace the tube in the water bath set at 37 °C, leave it 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 are used, follow the manufacturer's instructions.

9.5.4.7 Detection of indole

Inoculate a tube containing 5 ml of the tryptone-tryptophan saline medium (5.10) with the suspect colony. Incubate at 37 °C for 24 h ± 3 h. After incubation, add 1 ml of Kovacs' reagent.

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

9.5.4.8 Halotolerance test

Produce a series of peptone waters with increasing salt (NaCl) concentration: 0 %, 2 %, 4 %, 6 %, 8 % and 10 % (5.11).

Prepare a suspension with the colony to be identified and lightly inoculate each of the tubes (with a loopful). Incubate at 37 °C for 24 h ± 3 h.

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

9.5.4.9 Interpretation of biochemical tests

Species of potentially enteropathogenic *Vibrio* spp. generally give the reactions indicated in Table 1. The reactions of *V. parahaemolyticus* and *V. cholerae*, covered by ISO/TS 21872-1, are also given as they may be isolated when applying the procedures given in this part of ISO/TS 21872. The reactions for *V. hollisae* are not given as it is unlikely that strains of this species will be detected by these procedures.

Table 1 — Interpretation of biochemical tests

Test	*V. cholerae* [a]	*V. mimicus* [a]	*V. parahaemolyticus* [a]	*V. vulnificus* [a]	*V. fluvialis* [a]
Oxidase	+	+	+	+	+
Production of gas (glucose)	–	–	–	–	–
Lactose	–	–	–	+	–
Sucrose	+	–	–	–	+
ODC	+	+	+	+	–
LDC	+	+	+	+	–
ADH	–	–	–	–	+
ONPG hydrolysis	+	+	–	+	+
Production of indole	+	+	+	+	[b]
Growth in peptone water with					
0 % NaCl	+	+	–	–	–
2 % NaCl	+	+	+	+	+
6 % NaCl	–	+	+	+	+
8 % NaCl	–	–	+	–	–
10 % NaCl	–	–	–	–	–

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

[b] Variable results.

NOTE The reactions given in Table 1 are a guide to the identification of the listed species. Additional phenotypic tests are required to fully distinguish these species from each other and from non-pathogenic *Vibrio* species and other fermentative Gram-negative organisms such as *Aeromonas* spp.

9.5.4.10 **Step-by-step confirmation** (if desired)

With the cultures that are selected in 9.5.3.3, perform a culture in 10 % saline peptone water (5.11). Then continue the confirmation (the other tests) on the colonies that do not grow in 10 % saline peptone water.

NOTE It is advisable to subculture either into 2 % saline peptone water or onto saline nutrient agar at the same time in order to be sure that the "no growth" in the 10 % saline peptone water is not because the culture has died.

9.5.5 Confirmation of biochemical identification

The biochemical identification of *Vibrio* is difficult, and it is preferable to obtain confirmation of identification of isolates thought to be potentially enteropathogenic *Vibrio* species by sending them to a specialist/reference laboratory.

For transport, inoculate onto saline nutrient agar slopes (5.3).

10 Expression of results

Depending on the interpretation results, indicate the presence or absence of potentially enteropathogenic *Vibrio* in a test portion of x g or x ml of product (see ISO 7218), specifying the name of the relevant bacterial species.

11 Test report

The test report shall specify:

— 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;

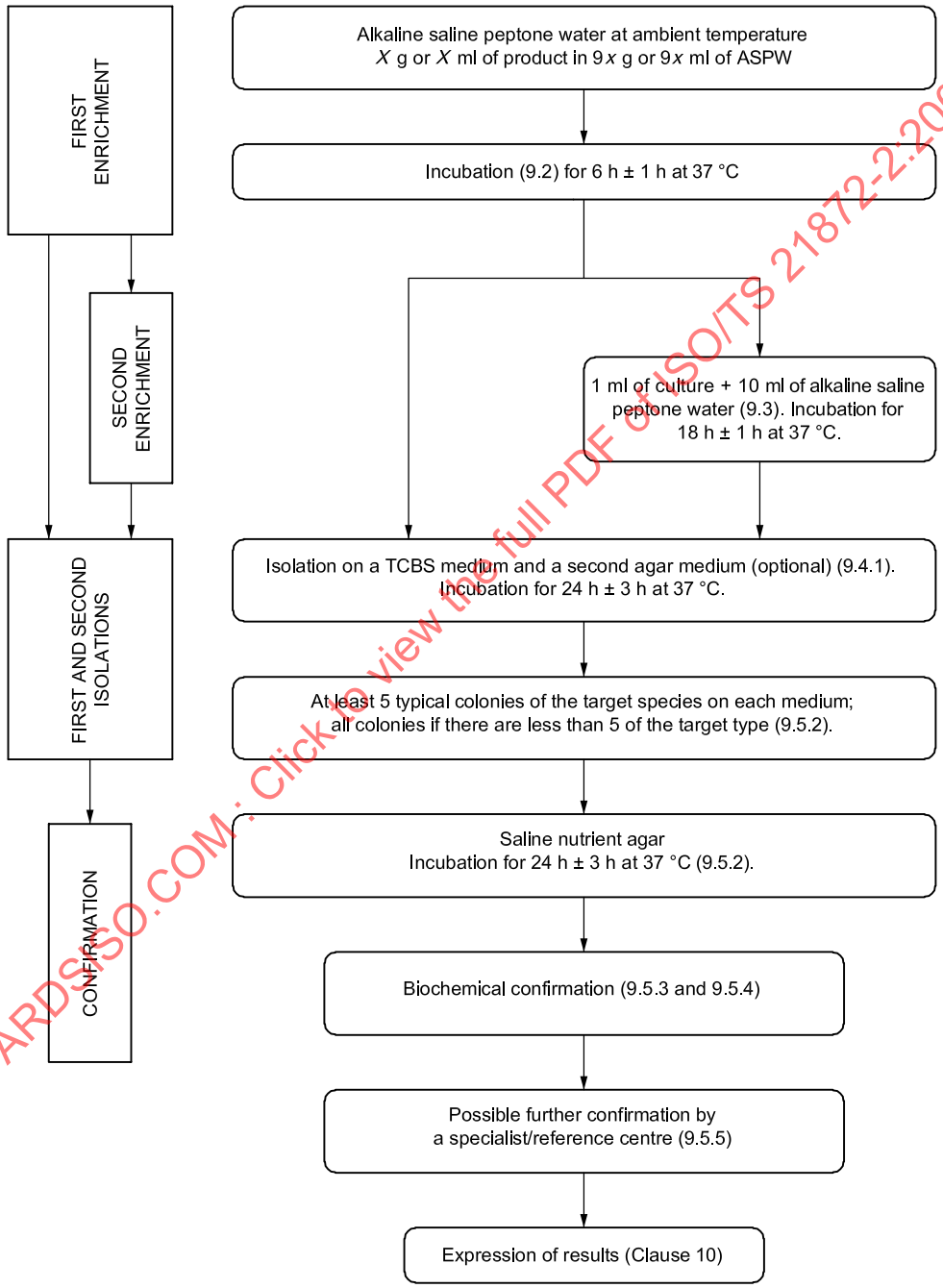
— all operating details not specified in this part of ISO/TS 21872, or regarded as optional, together with details of any incidents which may have influenced the results;

— the results obtained.

The test report shall also mention whether a positive result was obtained when using only an isolation medium (5.2) not specified in this part of ISO/TS 21872.

Annex A
(normative)

Diagram of procedure



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 (NaCl)	20,0 g
Water	1 000 ml

B.1.2 Preparation

Dissolve the components in the water, by heating if necessary. Adjust the pH if necessary, so that after sterilization it is 8,6 ± 0,2 at 25 °C.

Dispense the medium, in quantities required for the examination, into flasks or tubes of sufficient capacity (9.1 and 9.3.1). 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 (NaCl)	10,0 g
Dried bovine bile	8,0 g
Sucrose	20,0 g
Bromothymol blue	0,04 g
Thymol blue	0,04 g
Agar	8,0 g to 18,0 g [a]
Water	1 000 ml

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

B.2.2 Preparation

Dissolve the components or the dehydrated complete 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 thus-prepared medium, cooled down to approximately 50 °C, into 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) until the agar surface is dry.

B.2.4 Control of media

See ISO/TS 11133 for guidance.

Undertake a quantitative estimate of the plating efficiency for each batch of TCBS using saline nutrient agar (SNA) as comparison medium and the following strains:

— *V. parahaemolyticus*: NCTC 10885;

— *V. furnissii*: NCTC 11218;

— *Escherichia coli*: ATCC 25922, 8739 or 11775.

The plating efficiency is calculated from

$$\left(\frac{N_{\text{TCBS}}}{N_{\text{SNA}}} \times 100 \right)$$

where N is the number of colonies counted.

The plating efficiency should be at least 50 % for each of the *Vibrio* strains (positive control organisms) and less than 1 % for the *E. coli*. (negative control organisms). Colonies of *V. parahaemolyticus* NCTC 10885 should be green (sucrose negative), while those of *V. furnissii* and NCTC 11218 should be yellow (sucrose positive).

B.3 Sodium dodecyl sulfate polymixin sucrose (SDS) agar

B.3.1 Basic medium

B.3.1.1 Composition

Proteose peptone	10,0 g
Beef extract	5,0 g
Sucrose	15,0 g
Sodium chloride (NaCl)	20,0 g
Sodium dodecyl sulfate	1,0 g
Bromothymol blue	0,04 g
Cresol red	0,04 g
Agar	15,00 g [a]
Distilled water	1 000 ml

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

B.3.1.2 Preparation

Dissolve all the components in the water. Adjust the pH to 7,6 ± 0,2 at 25 °C. Autoclave at 121 °C for 15 min.

B.3.2 Polymyxin B solution

B.3.2.1 Composition

Polymyxin B sulfate	100 000 units
Water	5 ml

B.3.2.2 Preparation

Dissolve the component in the water. Sterilize by filtration.

B.3.3 Preparation of the complete medium

Cool the basic medium to approximately 50 °C and add 5 ml of the sterile polymyxin B solution. Mix well.

B.3.4 Preparation of the agar dishes

Pour into Petri dishes in 15 ml to 20 ml volumes. Leave until the agar surface is dry before use.

B.3.5 Control of media

See ISO/TS 11133 for guidance.

Undertake a quantitative estimate of the plating efficiency for each batch of SDS using saline nutrient agar (SNA) as comparison medium and the following strains:

— *V. vulnificus*: NCTC 11067 (ATCC 29307);

— *V. cholerae* non-O1/non-O139: NCTC 8042 (ATCC 14733);

— *Escherichia coli*: ATCC 25922, 8739, or 11775.

The plating efficiency is calculated from

$$\left(\frac{N_{\text{SDS}}}{N_{\text{SNA}}} \times 100 \right)$$

where N is the number of colonies counted.

The plating efficiency should be at least 50 % for each of the *Vibrio* strains and less than 1 % for the *E. coli*. Colonies of *V. vulnificus* NCTC 11067 should be purple/green with an opaque halo while those of *V. cholerae* NCTC 8042 should be yellow with an opaque halo.

B.4 Cellobiose polymyxin colistin (CPC) agar

B.4.1 Solution 1

B.4.1.1 Composition

Bacteriological peptone	10,0 g
Beef extract	5,0 g
Ferric citrate	0,10 g
Sodium chloride (NaCl)	20,0 g
Bromothymol blue	0,04 g
Cresol red	0,04 g
Agar	15,0 g
Distilled water	900 ml

B.4.1.2 Preparation

Dissolve the components in the water. Adjust the pH to 7,6 ± 0,2.

Autoclave at 121 °C for 15 min. Cool to approximately 50 °C.

B.4.2 Solution 2

B.4.2.1 Composition

Cellobiose	15,0 g
Colistin	1 360 000 units
Polymyxin B	100 000 units
Distilled water	100 ml

B.4.2.2 Preparation

Dissolve the cellobiose in the distilled water by heating gently. Cool, then add the antibiotics.

Sterilize by filtration.

B.4.3 Complete medium

Add Solution 2 (100 ml) to Solution 1 (900 ml) and mix.

B.4.4 Preparation of the agar dishes

Pour into Petri dishes in 20 ml volumes. Leave until the agar surface is dry before use.

B.4.5 Control of media

Undertake a quantitative estimate of the plating efficiency for each batch of CPC using saline nutrient agar (SNA) as comparison medium and the following strains:

— *Vibrio vulnificus:* NCTC 11067 (ATCC 29307);

— *Vibrio cholerae* non-O1/non-O139: NCTC 8042 (ATCC 14733);

— *Escherichia coli:* ATCC 25922, 8739 or 11775.

The plating efficiency is calculated from

$$\left(\frac{N_{\text{CPC}}}{N_{\text{SNA}}} \times 100 \right)$$

where N is the number of colonies counted.

The plating efficiency should be at least 50 % for each of the *Vibrio* strains and less than 1 % for the *E. coli*. Colonies of *V. vulnificus* NCTC 11067 should be yellow surrounded by a yellow coloration in the medium, while those of *V. cholerae* NCTC 8042 should be purple surrounded by a purple coloration in the medium.

B.5 Modified cellobiose polymixin colistin (mCPC) agar

B.5.1 1 000 X stock dye solution

B.5.1.1 Composition

Bromothymol blue	4,0 g
Cresol red	4,0 g
Ethanol, 95 %	100 ml

B.5.1.2 Preparation

Dissolve dyes in ethanol for 4 % (mass by volume) stock solution.

Add 1 ml of this solution per litre of mCPC agar to obtain a final concentration of 40 mg of bromothymol blue and 40 mg of cresol red per litre.

B.5.2 Solution 1

B.5.2.1 Composition

Peptone	10 g
Beef extract	5 g
Sodium chloride (NaCl)	20 g
1 000 X stock dye solution	1 ml
Agar	15 g
Distilled water	900 ml

B.5.2.2 Preparation

Mix the components and adjust the pH to 7,6. Boil to dissolve the agar. Cool to approximately 50 °C.

B.5.3 Solution 2

B.5.3.1 Composition

Cellobiose	10 g
Colistin	400 000 units
Polymyxin B	100 000 units
Distilled water	100 ml

B.5.3.2 Preparation

Dissolve the cellobiose in the distilled water by heating gently. Cool to approximately 50 °C.

Add the antibiotics and mix.

B.5.4 Complete medium

B.5.4.1 Composition

1 000 X stock dye solution	1 ml
Solution 1	900 ml
Solution 2	100 ml

B.5.4.2 Preparation

Add Solution 2 to Solution 1 and mix.

Add 1 ml of this solution per litre of mCPC agar to obtain a final concentration of 40 mg of bromothymol blue and 40 mg of cresol red per litre.

B.5.5 Preparation of agar dishes

Pour into Petri dishes in 20 ml volumes. Leave until the agar surface is dry before use.

B.5.6 Control of media

Undertake a quantitative estimate of the plating efficiency for each batch of mCPC using alkaline saline nutrient agar as comparison medium and the following strains:

— *V. vulnificus:* NCTC 11067 (ATCC 29307);

— *V. cholerae* non-O1/non-O139: NCTC 8042 (ATCC 14733);

— *Escherichia coli*: ATCC 25922, 8739, or 11775.

The plating efficiency is calculated from

$$\left(\frac{N_{\text{mCPC}}}{N_{\text{SNA}}} \times 100 \right)$$

where N is the number of colonies counted.

The plating efficiency should be at least 50 % for each of the *Vibrio* strains and less than 1 % for the *E. coli*. Colonies of *V. vulnificus* NCTC 11067 should be yellow surrounded by a yellow coloration in the medium, while those of *V. cholerae* NCTC 8042 should be purple surrounded by a purple coloration in the medium.

B.6 Saline nutrient agar (SNA)

B.6.1 Composition

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

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

B.6.2 Preparation

Dissolve the dehydrated components or the dehydrated complete 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.6.3 Preparation of the dishes of saline nutrient agar medium

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

Immediately before use, carefully dry the dishes of agar medium (preferably after having removed the lids and inverted the dishes), until the agar surface is dry.

B.6.4 Preparation of the inclined agar tubes

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.7 Reagent for detection of oxidase

B.7.1 Composition

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

B.7.2 Preparation

Dissolve the components in the cold water immediately before use.

B.8 Saline triple sugar iron (TSI) agar

B.8.1 Base medium

B.8.1.1 Composition

Peptone	20,0 g
Meat extract	3,0 g
Yeast extract	3,0 g
Sodium chloride (NaCl)	10,0 g
Lactose	10,0 g
Sucrose	10,0 g
Glucose	1,0 g
Iron(III) citrate	0,3 g
Phenol red	0,024 g
Agar	8 g to 18 g [a]
Water	1 000 ml

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

B.8.1.2 Preparation

Dissolve the components or the dehydrated complete base medium in the water, by heating if necessary. Adjust the pH, if necessary, so that after sterilization it is 7,4 ± 0,2 at 25 °C.

Dispense the medium in quantities of 10 ml in tubes of appropriate capacity. Sterilize in an autoclave set at 121 °C for 15 min.

Leave to solidify in an inclined position so as to obtain a butt of around 2,5 cm in depth.

If the medium is used more than 8 days after its preparation, regenerate it by melting in a boiling water bath or in free flowing steam for 10 min. Leave to solidify as above.