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Microbiology — General guidance for the detection of *Vibrio parahaemolyticus*

Microbiologie — Directives générales pour la recherche de Vibrio parahaemolyticus

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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.

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.

International Standard ISO 8914 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*.

Annexes A and B form an integral part of this International Standard. Annex C is for information only.

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Introduction

This International Standard is intended to provide general guidance for the examination of products not dealt with by existing International Standards and for reference by bodies preparing microbiological methods of test for application to foods or to animal feeding stuffs. Because of the large variety of products within this field of application, these guidelines may not be appropriate for some products in every detail, and, for some other products, it may be necessary to use different methods. Nevertheless, it is hoped that, in all cases, every attempt will be made to apply these guidelines as far as possible and that deviations from them will only be made if absolutely necessary for technical reasons.

When this International Standard is next reviewed, account will be taken of all information then available regarding the extent to which the guidelines have been followed and the reasons for deviation from them 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 these guidelines. In cases where International Standards already exist for the product to be tested, they should be followed. However, it is hoped that when such standards are reviewed they will be changed to comply with this International Standard so that, eventually, the only remaining departures from these guidelines will be those necessary for well-established technical reasons.

To accommodate national practice, this method may be carried out at an incubation temperature of either 35 °C or 37 °C.

The method to be used for a particular product will be specified in the International Standard specific to that product.

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Microbiology — General guidance for the detection of *Vibrio parahaemolyticus*

1 Scope

This International Standard gives general guidance for the detection of *Vibrio parahaemolyticus* present in products intended for human consumption or animal feeding stuffs.

The incubation temperature used may be 35 °C or 37 °C, this temperature forming the subject of agreement between the parties concerned.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 6887:1983, *Microbiology — General guidance for the preparation of dilutions for microbiological examination.*

ISO 7218:1985, *Microbiology — General guidance for microbiological examinations.*

3 Definitions

For the purposes of this International Standard, the following definitions apply.

3.1 *Vibrio parahaemolyticus*: Halophilic microorganisms which form characteristic colonies on solid selective media, and which display the biochemical characteristics described when tests are carried out in accordance with this International Standard.

3.2 detection of *Vibrio parahaemolyticus*: Determination of the presence or absence of these microorganisms in a particular mass of product when tests are carried out in accordance with this International Standard.

4 Principle

In general, the detection of *Vibrio parahaemolyticus* is carried out in three successive phases as follows.

4.1 Enrichment in selective media

Inoculation of the test portion into the following two selective enrichment media:

a) salt polymyxin B broth (SPB);

b) alkaline saline peptone water or saline glucose culture medium with sodium dodecyl sulfate (known previously as sodium lauryl sulfate) (GST).

Incubation at 35 °C or 37 °C (as agreed) for 7 h to 8 h.[1)]

4.2 Plating out and identification

From the cultures obtained in (4.1), inoculation of the following two selective solid media:

a) thiosulfate citrate bile sucrose agar (TCBS);

b) triphenyltetrazolium chloride soya tryptone agar (TSAT).

Incubation at 35 °C or 37 °C, and then examination after 18 h, 24 h or 48 h (depending on the medium)

1) It is recommended for deep-frozen products that two plating out operations as described in 4.2 be carried out, one after incubation for 7 h to 8 h and the other after incubation for 18 h.

to check for the presence of colonies presumed to be *Vibrio parahaemolyticus* by their visible characteristics. The temperature forms the subject of agreement between the parties concerned and is indicated in the test report.

4.3 Confirmation

Testing of the presumptive *Vibrio parahaemolyticus* colonies by confirmatory tests.

5 Culture media and reagents

5.1 General

For general guidance, see ISO 7218.

5.2 Enrichment media

NOTE 1 Because of the large number of culture media and reagents, it was considered preferable, for the clarity of the text, to give their composition and preparation in annex B, which also includes details of dispensing, storage, etc.

5.2.1 Salt polymyxin B broth (SPB)

See B.1.1.

5.2.2 Alkaline saline peptone water

See B.1.2.

5.2.3 Saline glucose culture medium with sodium dodecyl sulfate (GST)

See B.1.3.

5.3 Plating-out media

5.3.1 Thiosulfate citrate bile sucrose agar (TCBS)

See B.2.1.

5.3.2 Triphenyltetrazolium chloride soya tryptone agar (TSAT) [1]

See B.2.2.

5.3.3 Saline nutrient agar

See B.2.3.

5.4 Identification media

5.4.1 Triple sugar saline iron agar (TSI saline agar)

See B.3.1.

5.4.2 Saline meat-yeast agar

See B.3.2.

5.4.3 Saline medium for the detection of lysine decarboxylase

See B.3.3.

5.4.4 Medium and reagents for indole detection

5.4.4.1 Saline tryptone/tryptophane medium

See B.3.4.1.

5.4.4.2 Kovacs reagent

See B.3.4.2.

5.4.5 Reagent for oxidase detection

See B.3.5.

5.4.6 Reagent for β-galactosidase detection

5.4.6.1 ONPG solution

See B.3.6.1.

5.4.6.2 Buffer solution

See B.3.6.2.

5.4.6.3 Complete reagent

See B.3.6.3.

5.5 Sodium chloride solution

See clause B.4.

5.6 Zinc powder

6 Apparatus and glassware

NOTE 2 Disposable apparatus is an acceptable alternative to reusable glassware if it has suitable specifications.

Usual microbiological laboratory apparatus and, in particular, the following.

6.1 Apparatus for dry sterilization (oven) or **wet sterilization (autoclave)**, see ISO 7218.

6.2 Incubator, capable of operating at 35 °C ± 1 °C or 37 °C ± 1 °C.

6.3 Drying cabinet or **oven**, ventilated by convection, capable of operating at between 37 °C ± 1 °C and 55 °C ± 1 °C.

6.4 Water-bath, capable of operating at 37 °C ± 1 °C.

6.5 Containers, especially **test tubes** of dimensions 18 mm × 180 mm and 9 mm × 180 mm, and **flasks** or **bottles** of appropriate capacity.

6.6 Petri dishes, made of glass or plastic, of 90 mm to 100 mm diameter.

6.7 Total delivery pipettes (blow-out pipettes), with wide openings, having nominal capacities of 10 ml and 1 ml, graduated in 0,1 ml divisions.

6.8 Rubber bulbs, or any other type of safety device suitable for use with the pipettes (6.7).

6.9 Loop, approximately 3 mm in diameter, and **straight wire**, both made of platinum-iridium or nickel-chromium, and/or a **glass rod**.

NOTE 3 A nickel-chromium loop or wire is not suitable for use in the oxidase test (see 9.4.3.1).

6.10 pH-meter, accurate to ± 0,1 pH unit at 25 °C.

6.11 Microscope, with an immersion objective.

7 Sampling

Sampling shall have been carried out in accordance with the specific International Standard appropriate to the product concerned.

If there is no specific International Standard, it is recommended that agreement be reached on this subject by the parties concerned.

8 Preparation of the test sample

Prepare the test sample in accordance with the specific International Standard appropriate to the product concerned.

If there is no specific International Standard, it is recommended that agreement be reached on this subject by the parties concerned.

9 Procedure

(See the diagram in annex A.)

9.1 Test portion and initial suspensions

9.1.1 See ISO 6887 and the specific International Standard concerning the product to be tested. Use the enrichment medium as the diluent.

9.1.2 In general, to prepare the first initial suspension, dispense a 25 g test portion in 225 ml of the enrichment medium, salt polymyxin B broth (5.2.1). If a test portion of 25 g is not available, use a smaller amount and add the quantity of enrichment medium necessary to obtain a 10^{-1} dilution.

9.1.3 Prepare the second initial suspension in a manner similar to that used in 9.1.2 but using the second enrichment medium chosen, either the alkaline saline peptone water (5.2.2) or the GST broth (5.2.3).

9.2 Enrichment

Incubate the two initial suspensions in the incubator set at 35 °C or 37 °C (as agreed) for 7 h to 8 h. If the dilution and incubation cannot be completed in one working day, the diluted sample shall be stored overnight at between 0 °C and + 5 °C.

NOTE 4 For deep-frozen products, it is recommended that a second plating out be carried out after incubation for 18 h.

9.3 Plating out and identification

9.3.1 After incubation, using a loop (6.9), inoculate the culture obtained in the first enrichment medium (9.1.2) on to the surface of a plate containing the first selective plating out medium, TCBS (5.3.1), to allow the development of clearly separated colonies.

Proceed in the same way with the second selective plating out medium, TSAT (5.3.2).

9.3.2 Using the culture obtained in the second enrichment medium (9.1.3), repeat the procedure described in 9.3.1 with the two selective plating out media.

9.3.3 Invert the plates (9.3.1 and 9.3.2) and place them in an incubator (6.2) set at 35 °C or 37 °C (as agreed).

Incubate for 18 h for the TCBS medium and for 20 h to 24 h for the TSAT medium.

9.3.4 Examine the plates to identify the presence of characteristic colonies of *Vibrio parahaemolyticus* as follows:

a) on TCBS medium, the characteristic colonies of *Vibrio parahaemolyticus* are smooth, green (sucrose negative) and 2 mm to 3 mm in diameter;

b) on TSAT medium, the characteristic colonies of *Vibrio parahaemolyticus* are smooth, flat, dark red (reduction of triphenyltetrazolium chloride) and 2 mm to 3 mm in diameter.

9.3.5 If the development of colonies is slow, if the coloration is weak, or in the absence of a characteristic colony, continue the incubation for up to a total of 24 h for the TCBS medium and for a total of 48 h for the TSAT medium, and then re-examine them.

9.4 Confirmation

9.4.1 Choice of colonies

Select at least five characteristic colonies from the plates (9.3.4 or 9.3.5).

If there are fewer than five characteristic colonies use them all.

9.4.2 Preparation of pure cultures

Inoculate separately each of the selected characteristic colonies on to the surface of a saline nutrient agar (5.3.3) to allow well-separated colonies to develop. Incubate the inoculated plates in the incubator set at 35 °C or 37 °C (as agreed) for 18 h to 24 h. Pick up a single well-isolated colony from each of the plates and inoculate slants of saline nutrient agar (5.3.3). Incubate in the incubator set at 35 °C or 37 °C (as agreed) for 18 h to 24 h. Use these pure cultures for the microscopical examination and biochemical tests.

9.4.3 Preliminary tests

9.4.3.1 Oxidase test

Using the platinum-iridium loop or straight wire, or a glass rod (6.9), take a portion of the pure culture from the saline nutrient agar slant (9.4.2) and streak on to a filter paper moistened with the oxidase reagent (5.4.5) or use a commercially available test, following the manufacturer's instructions. A nickel-chromium loop or wire shall not be used.

The test is positive if the colour changes to mauve, violet or deep blue within 10 s.

9.4.3.2 Microscopical examination

Take separate fractions of each of the pure cultures (9.4.2), and test as in a) and b) below.

a) Place a drop of the sodium chloride solution (5.5) on to a clean microscope slide, add a fraction of a pure culture and emulsify to give a thin suspension. Allow the slide to dry in air and fix by passing through a Bunsen burner flame. Carry out a Gram stain, and note the morphology and the Gram reaction of the micro-organisms.

b) Inoculate a tube of the alkaline saline peptone water (5.2.2). Incubate in the incubator set at 35 °C or 37 °C (as agreed) for 1 h to 6 h. Place a drop of the culture on to a clean microscope slide, cover with a cover slip and examine the micro-organisms microscopically for motility. Note cultures showing motility.

9.4.3.3 Selection of colonies for biochemical tests

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

9.4.4 Biochemical tests

Using a straight wire (6.9), inoculate the identification and reagent media (9.4.4.1 to 9.4.4.5) with each of the pure cultures prepared in 9.4.2 and selected in 9.4.3.3, incubate and carry out the biochemical tests.

9.4.4.1 Saline TSI agar (5.4.1)

Inoculate the slant surface (slope) with a longitudinal streak and stab the butt to the bottom of the agar.

Incubate in the incubator set at 35 °C or 37 °C (as agreed) for 24 h.

Interpret the changes in the medium as shown in table 1.

Table 1

Position	Colour	Interpretation
Butt	Yellow	Glucose positive (fermentation of glucose)
	Red or unchanged	Glucose negative (no fermentation of glucose)
	Black	Formation of hydrogen sulfide (H_2S)
	Bubbles or cracks	Gas formation from glucose
Slant surface	Yellow	Lactose and/or sucrose positive (one or both sugars utilized)
	Red or unchanged	Lactose and sucrose negative (neither sugar utilized)

Characteristic cultures of *Vibrio parahaemolyticus* show an alkaline red slant (lactose negative and sucrose negative) and an acid yellow butt (glucose positive) without gas formation (gas negative) or blackening (H_2S negative).

9.4.4.2 Saline meat-yeast agar (5.4.2)

Using a loop, inoculate the molten regenerated agar cooled to 45 °C throughout its depth without introducing air bubbles. Immediately immerse the tubes in cold water to solidify the medium.

Incubate in the incubator set at 35 °C or 37 °C (as agreed) for 24 h.

Examine the bacterial growth and determine the breathing type.

9.4.4.3 Saline for the detection of lysine decarboxylase (5.4.3)

Inoculate just below the surface of the liquid medium.

Incubate in the incubator set at 35 °C or 37 °C (as agreed) for 24 h.

A purple colour and turbidity after incubation indicates a positive reaction (bacterial growth and lysine decarboxylation).

A yellow colour indicates a negative reaction.

9.4.4.4 Saline medium for indole detection (5.4.4)

Inoculate a tube containing tryptone/tryptophane medium (5.4.4.1).

Incubate in the incubator set at 35 °C or 37 °C (as agreed) for 24 h.

After incubation, add 1 ml of Kovacs reagent (5.4.4.2).

The formation of a red ring indicates a positive reaction (indole formation).

A yellow-brown ring indicates a negative reaction.

9.4.4.5 Reagent for β-galactosidase detection (5.4.6)

Suspend a loopful of the pure culture in a tube containing 0,25 ml of the saline solution (5.5).

Add one drop of toluene and shake the tube.

Put the tube in a water-bath (6.4) set at 37 °C for a few minutes.

Add 0,25 ml of the β-galactosidase reagent (5.4.6.3) and mix, or use a commercially available test and follow the manufacturer's instructions.

Replace the tube in the water-bath (6.4) set at 37 °C for 24 h and examine at intervals during the 24 h.

A positive reaction (presence of β-galactosidase) is indicated by a yellow colour that usually appears in less than 30 min. The absence of coloration after 24 h indicates a negative reaction.

9.5 Interpretation of results

Vibrio parahaemolyticus generally shows the following reactions:

Sucrose (9.4.4.1)	–
Oxidase (9.4.3.1)	+
Motility (9.4.3.2)	+
Glucose (9.4.4.1)	+
Gas formation from glucose (9.4.4.1)	–
Lactose (9.4.4.1)	–
H_2S (9.4.4.1)	–
Aerobic and anaerobic growth (9.4.4.2)	+
Lysine decarboxylation (9.4.4.3)	+
Indole detection (9.4.4.4)	+
β-galactosidase detection (9.4.4.5)	–

Conclude that *Vibrio parahaemolyticus* is present if at least one colony is confirmed as *Vibrio parahaemolyticus*.

Identification kits currently available commercially, and permitting the identification of *Vibrio parahaemolyticus*, may be used. In this case, the emulsion of the culture shall be prepared with the saline solution recommended by the manufacturer.

10 Expression of results

In accordance with the results of the interpretation, indicate the presence or absence of *Vibrio parahaemolyticus* in a test portion of x g of product.

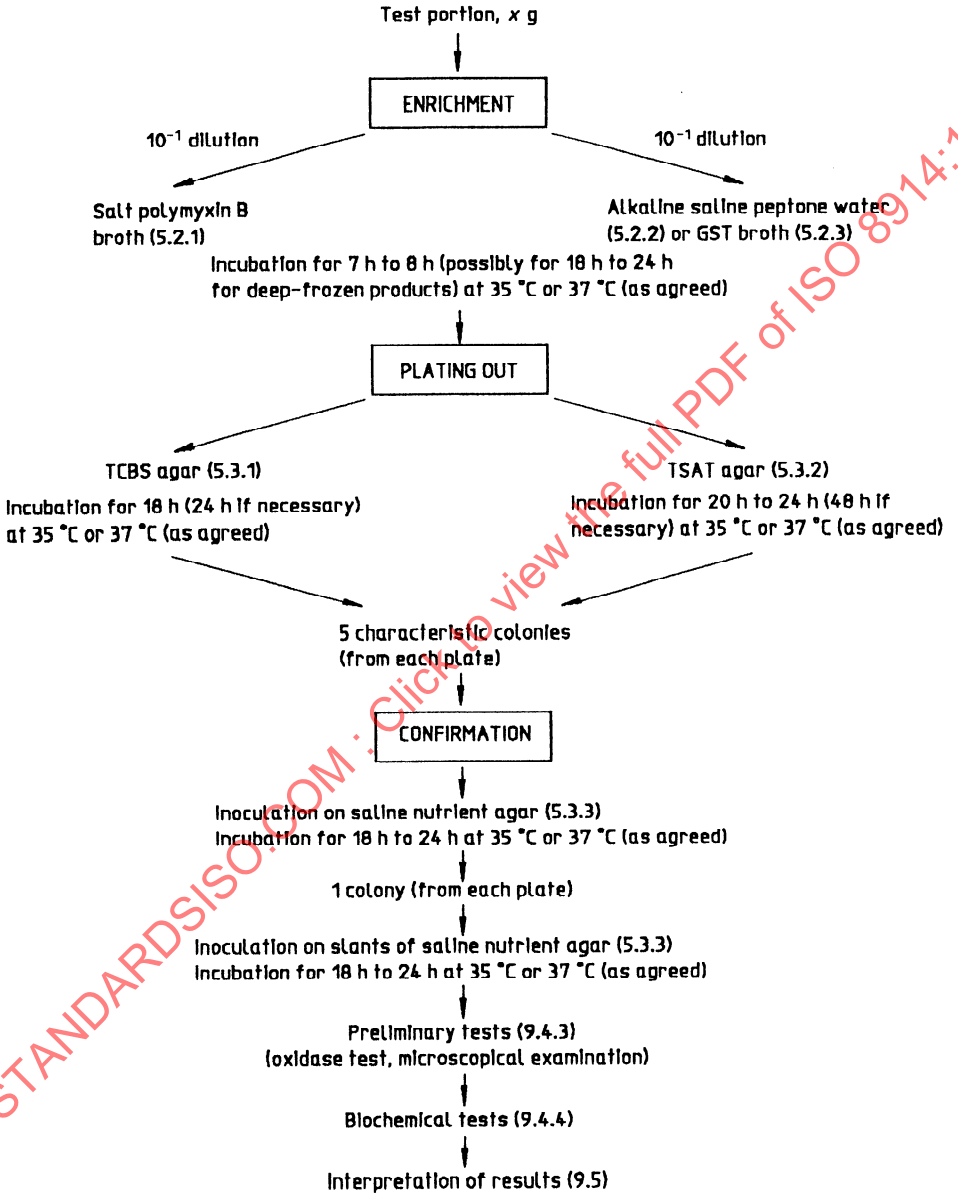
11 Test report

The test report shall specify the method used, the incubation temperature used and the result obtained. It shall also mention all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the result.

The test report shall include all information necessary for the complete identification of the sample.

Annex A
(normative)

Diagram of procedure



Annex B
(normative)

Composition and preparation of culture media and reagents

B.1 Enrichment media

B.1.1 Salt polymyxin B broth (SPB)

B.1.1.1 Base

Composition

Peptone	10,0 g
Yeast extract	3,0 g
Sodium chloride	20,0 g
Water	1 000 ml

Preparation

Dissolve all the components or the dehydrated base in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 7,4 at 25 °C.

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

B.1.1.2 Polymyxin B solution

Composition

Polymyxin B	100 000 IU
Water	100 ml

Preparation

Dissolve the polymyxin B in the water.

Sterilize by filtration.

B.1.1.3 Complete medium

Composition

Base (B.1.1.1)	900 ml
Polymyxin B solution (B.1.1.2)	100 ml

Preparation

Add aseptically the freshly prepared polymyxin B solution to the previously cooled base.

Distribute aseptically the medium in bottles or flasks of appropriate capacity (see 9.1.2).

By preference use the same day. Otherwise, store the broth overnight at 0 °C to + 5 °C.

B.1.2 Alkaline saline peptone water

Composition

Peptone	20,0 g
Sodium chloride	30,0 g
Water	1 000 ml

Preparation

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

Adjust the pH, if necessary, so that after sterilization it is 8,6 at 25 °C.

Distribute the medium in bottles or flasks of appropriate capacity (see 9.1.3).

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

B.1.3 Saline glucose culture medium with sodium dodecyl sulfate (GST)

Composition

Peptone	10,0 g
Meat extract	3,0 g
Sodium chloride	30,0 g
Glucose	5,0 g
Methyl violet	0,002 g
Sodium dodecyl sulfate	1,36 g
Water	1 000 ml

Preparation

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

Adjust the pH, if necessary, so that after sterilization it is 8,6 at 25 °C.

Distribute the medium in bottles or flasks of appropriate capacity (see 9.1.3).

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

B.2 Plating-out media

B.2.1 Thiosulfate citrate bile sucrose agar (TCBS)

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
Ox 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[2]
Water	1 000 ml

Preparation

Dissolve the components or the dehydrated complete medium in the water by boiling.

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

Do not sterilize.

Preparation of plates

Pour 15 ml to 20 ml of the complete medium thus prepared, and cooled to about 45 °C, into Petri dishes (6.6) and allow to set.

Just before use, thoroughly dry the agar plates (preferably after having removed the covers and turned the plates upside down) in an oven (6.3) until the agar surface is dry.

If they have been prepared in advance, the undried agar plates shall not be stored for more than 4 h at laboratory temperature or more than 1 day between 0 °C and + 5 °C.

B.2.2 Triphenyltetrazolium chloride soya tryptone agar (TSAT) [1]

B.2.2.1 Basic medium

Composition

Tryptone	15,0 g
Soya peptone	5,0 g
Sodium chloride	30,0 g
Sucrose	20,0 g
Bile salt	0,5 g
Agar	8,0 g to 18,0 g[2]
Water	1 000 ml

Preparation

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

Adjust the pH, if necessary, so that after sterilization it is 7,1 at 25 °C.

Distribute the medium in bottles or flasks of appropriate capacity.

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

B.2.2.2 1 % triphenyltetrazolium chloride solution

Composition

Triphenyltetrazolium chloride	0,1 g
Water	10 ml

Preparation

Dissolve the component in the water.

Sterilize by filtration.

B.2.2.3 Complete medium

Composition

Basic medium (B.2.2.1)	1 000 ml
1 % triphenyltetrazolium chloride solution (B.2.2.2)	3 ml

2) According to the gel strength of the agar.