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Microbiology of the food chain — Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria* spp. —

Part 1: Detection method

Microbiologie de la chaîne alimentaire — Méthode horizontale pour la recherche et le dénombrement de Listeria monocytogenes *et de* Listeria *spp.* —

Partie 1: Méthode de recherche

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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 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 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 ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*, in accordance with the agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This second edition cancels and replaces the first edition (ISO 11290-1:1996), which has been technically revised. It also incorporates the amendment ISO 11290-1:1996/Amd.1:2004.

The main changes, compared to ISO 11290-1:1996, are the following.

— The detection of *Listeria monocytogenes* has been modified as listed below.

— Primary enrichment in half-Fraser broth: incubation for 25 h ± 1 h.

— Secondary enrichment in Fraser broth: incubation for 24 h ± 2 h.[29]

— Half-Fraser and Fraser broths may be refrigerated before transfer or isolation on selective agar for a maximum of 72 h.

— Storage of isolation plates: incubated plates can be refrigerated for a maximum of two days before reading.

— Microscopic aspect for confirmation is optional if the isolation agar allows distinction between pathogenic and non-pathogenic *Listeria* spp.

— CAMP test and catalase test are optional.

— Inclusion of new performance characteristics.

— Moreover, detection of *Listeria* spp. has been included in the scope and the title changed accordingly.

A list of parts in the ISO 11290 series can be found on the ISO website.

Introduction

The main changes, listed in the foreword, introduced in this document compared to ISO 11290-1:1996 are considered as major (see ISO 17468[28]). The technical changes were assessed and were considered to have no significant effect on the method performance characteristics or test results.

Because of the large variety of food and feed products, this horizontal method may not be appropriate in every detail for certain products for which it may be necessary to use different or specific methods. Nevertheless, in all cases, this horizontal method is intended to be applied as far as possible and deviations from this only be made for justified technical reasons.

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 it in the case of particular products.

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

Microbiology of the food chain — Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria* spp. —

Part 1: Detection method

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *L. monocytogenes* and *Listeria* spp. are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist, and that great care is taken in the disposal of all incubated materials. Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety aspects, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices. In particular, it is strongly recommended that tests for detecting *L. monocytogenes* are undertaken in laboratories providing biosafety level 2 conditions. It is strongly recommended that female laboratory staff are made aware of the particular risk to the developing foetus presented by infection of the mother through exposure to *L. monocytogenes* and *Listeria* spp., and that pregnant personnel and persons with recognized underlying conditions or diseases that impair cell-mediated immunity do not manipulate cultures of *L. monocytogenes* and *Listeria* spp.

1 Scope

This document specifies a horizontal method for

— the detection of *L. monocytogenes*, and

— the detection of *Listeria* spp. (including *L. monocytogenes*).

This document is applicable to

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

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

It is possible that certain additionally described *Listeria* species may not be detected or confirmed by this method.[5],[10],[12],[14],[25],[26],[27]

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 (all parts), *Microbiology of the food chain — 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 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:

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

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

3.1
Listeria monocytogenes
microorganisms which form typical colonies on solid selective media and which display the morphological, physiological and biochemical characteristics described when tests are carried out in accordance with this document

3.2
detection of *Listeria monocytogenes*
determination of the detection/non detection of *Listeria monocytogenes* (3.1), in a given mass or volume of product or a specified surface, when tests are carried out in accordance with this document

3.3
***Listeria* spp.**
microorganisms which form typical colonies on solid selective media and which display the morphological, physiological and biochemical characteristics described when tests are carried out in accordance with this document

3.4
detection of *Listeria* spp.
determination of the detection/non detection of *Listeria* spp. (3.3), in a given mass or volume of product or a specified surface, when tests are carried out in accordance with this document

4 Principle

4.1 General

Listeria spp. may be present in small numbers and are often accompanied by considerably larger numbers of other microorganisms; therefore selective enrichment is necessary. It is also necessary to detect injured and stressed *Listeria* spp. and the primary selective enrichment medium, with reduced inhibitor concentration, fulfils at least part of this function.

NOTE Presence of *L. monocytogenes* can be masked by the presence of other *Listeria* species, in particular *L. innocua* or *L. ivanovii*.

Within the limits of this document, the detection of *L. monocytogenes* and of *Listeria* spp. necessitates four successive stages, as described in the flowchart in Annex A.

4.2 Primary enrichment in a selective liquid enrichment medium with reduced concentration of selective agents (half-Fraser broth)

Inoculation of a selective primary enrichment medium containing half the concentrations of acriflavine and nalidixic acid (half-Fraser broth, see B.1), which is also used as a dilution fluid for the test portion (9.1).

Incubation of the initial suspension at 30 °C for 24 h to 26 h.

4.3 Secondary enrichment with a selective liquid enrichment medium with full concentration of selective agents (Fraser broth)

Inoculation of full-strength secondary liquid enrichment medium (Fraser broth) with a culture obtained from 4.2.

Incubation of the Fraser broth at 37 °C for 24 h.[29]

4.4 Plating out and identification

From the cultures obtained in 4.2 and 4.3, plating out on the two selective solid media:

— Agar *Listeria* according to Ottaviani and Agosti (see References [16] and [17] and B.3);

— any other solid selective medium at the choice of the laboratory complementary to Agar *Listeria* according to Ottaviani and Agosti, using a different substrate and/or principle than the one used in *Listeria* agar according to Ottaviani and Agosti (see B.4). See Annex E for information about media.

Incubation of the Agar *Listeria* according to Ottaviani and Agosti at 37 °C for a total of 48 h. If colonies of presumptive *L. monocytogenes* or *Listeria* spp. are evident at 24 h the incubation may be stopped at this stage. Incubation of the second selective medium at the appropriate temperature and examination after the appropriate time.

4.5 Confirmation

Subculturing of the colonies of presumptive *L. monocytogenes* or *Listeria* spp., plated out as described in 4.4, and confirmation by means of appropriate morphological and/or biochemical tests.

5 Culture media and reagents

For current laboratory practices, refer to ISO 11133.

Composition and performance testing of culture media and reagents and their preparation are described in Annex B.

6 Equipment and consumables

Usual microbiological equipment (as specified in ISO 7218) and, in particular, the following.

6.1 Apparatus for dry sterilization (oven) **or wet sterilization** (autoclave).

As specified in ISO 7218.

6.2 Drying cabinet or incubator, capable of being maintained between 25 °C and 50 °C.

6.3 Incubators, capable of operating at 30 °C ± 1 °C, 37 °C ± 1 °C and at 25 °C ± 1 °C (optional).

6.4 Water bath, capable of operating at 47 °C to 50 °C.

6.5 Sterile loops approximately 3 mm in diameter or 10 µl, and inoculating **needle** or **wire.**

6.6 pH meter, capable of being read to the nearest 0,01 pH unit at 25 °C, enabling measurements to be made which are accurate to ± 0,1 pH unit.

6.7 Graduated pipettes or automatic pipettes, of nominal capacities 1 ml and 10 ml.

6.8 Petri dishes, for example of diameter 90 mm.

6.9 Microscope, preferably with phase-contrast, and with slides and cover slips.

6.10 Refrigerator, capable of operating at 5 °C ± 3 °C.

7 Sampling

Sampling is not part of the method specified in this document. 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. For food and feed samples, refer to ISO/TS 17728[3]. For environmental samples, use ISO 18593[2] and see Reference [24].

It is important that the laboratory receives a sample which is truly representative and has not been damaged or changed during transport or storage (see ISO 7218).

8 Preparation of test sample

Prepare the test sample in accordance with the specific International Standard dealing with the product concerned [see ISO 6887 (all parts) and ISO 18593[2]]. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure

9.1 Test portion and initial suspension

Refer to ISO 6887 (all parts) and any specific International Standard appropriate to the product concerned.

For preparation of the initial suspension, use as dilution fluid the selective primary enrichment medium specified in B.1 (half-Fraser broth).

In general, to prepare the initial suspension, add a test portion of 25 g or 25 ml to 225 g or 225 ml of the selective primary enrichment medium (B.1), to obtain a tenfold dilution, and homogenize. Pre-warm the selective primary enrichment medium to room temperature before use.

This document has been validated for test portions of 25 g or ml. A smaller test portion may be used, without the need for additional validation/verification, providing that the same ratio between primary 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 *L. monocytogenes* or *Listeria* spp.

NOTE 1 Validation can be conducted in accordance with the appropriate document of ISO 16140 (all parts)[1]. Verification for pooling samples can be conducted in accordance with the protocol described in ISO 6887-1:2017, Annex D (verification protocol for pooling samples for qualitative tests). See References [21] and [22] as they provide information on the particular case of *Listeria* pooling samples.

NOTE 2 For large quantities, it is recommended to pre-warm the selective primary enrichment medium to 30 °C ± 1 °C before mixing it with the test portion.

9.2 Primary enrichment

Incubate the primary enrichment medium (half-Fraser broth, see B.1), prepared in accordance with 9.1, at 30 °C (6.3) for 25 h ± 1 h.

NOTE 1 A black coloration can develop during the incubation.

NOTE 2 It is possible to store at 5 °C (6.10) the pre-enriched sample after incubation before transfer to Fraser broth for a maximum of 72 h.

(See Reference [20].)

9.3 Secondary enrichment

9.3.1 After incubation of the initial suspension (primary enrichment) for 25 h ± 1 h (9.2), transfer 0,1 ml of the culture obtained in 9.2 (regardless of its colour) to a tube or bottle containing 10 ml of secondary enrichment medium (Fraser broth) (B.2).

9.3.2 Incubate the inoculated medium (9.3.1) for 24 h ± 2 h at 37 °C (6.3).

NOTE In the case of *Listeria* spp. other than *Listeria monocytogenes* detection, additional 24 h incubation can allow for recovery of more species.

9.4 Plating out and identification

9.4.1 General

9.4.1.1 From the primary enrichment culture (9.2) incubated for 25 h ± 1 h at 30 °C (6.3), inoculate, by means of a loop (6.5), the surface of the first selective plating medium, Agar *Listeria* according to Ottaviani and Agosti (B.3), to obtain well-separated colonies.

Proceed in the same way with the second selective plating-out medium of choice (B.4).

NOTE Half-Fraser broth and Fraser broth can be refrigerated at 5 °C (6.10) before isolation on selective agar for a maximum of 72 h.[20]

9.4.1.2 From the secondary enrichment medium incubated for 24 h ± 2 h at 37 °C (6.3) (9.3.2), repeat the procedure described in 9.4.1.1 with the two selective plating-out media.

9.4.1.3 Invert the Petri dishes obtained in 9.4.1.1 and 9.4.1.2 and place them in an incubator set at 37 °C (6.3) for Agar *Listeria* according to Ottaviani and Agosti (B.3). For the second selective medium (B.4), follow the manufacturer's instructions.

9.4.1.4 For Agar *Listeria* according to Ottaviani and Agosti incubate for a total of 48 h ± 2 h. If colonies of presumptive *L. monocytogenes* or *Listeria* spp. are evident at 24 h ± 2 h the incubation may be stopped at this stage. For second selective agar incubate for the appropriate time. Examine the dishes (9.4.1.3) for the presence of presumptive colonies of *L. monocytogenes* or *Listeria* spp.

NOTE After incubation plates can be refrigerated at 5 °C (6.10) for a maximum of 48 h before reading.

9.4.2 Agar *Listeria* according to Ottaviani and Agosti

Consider as presumptive *L. monocytogenes* the blue-green colonies surrounded by an opaque halo (typical colonies). Colonies of *L. ivanovii* are also blue-green and surrounded by an opaque halo.

Consider as presumptive *Listeria* spp. the blue-green colonies with or without opaque halo.

NOTE 1 Some strains of *L. monocytogenes* exposed to stress conditions, particularly acid stress, can show a very weak halo (or even no halo).

NOTE 2 Some rare *L. monocytogenes* are characterized by a slow PIPLC (phosphatidyl inositol phospholipase C) activity. Such bacteria are detected when the total duration of incubation is more than, for example, four days. Some of these strains could be pathogenic.[13] No *L. monocytogenes* strains have been described as PIPLC negative.

NOTE 3 Some organisms other than *Listeria* spp. can produce blue colonies on this medium. See Annex C and Reference [23].

9.4.3 Second selective medium

After the appropriate time, examine the plates for the presence of colonies which are considered to be presumptive *Listeria* spp. or *L. monocytogenes*, based on their characteristics for the type of medium used (B.4). See Annex E for more information.

9.5 Confirmation of *Listeria monocytogenes* or *Listeria* spp.

9.5.1 Selection of colonies for confirmation

9.5.1.1 For confirmation of presumptive *L. monocytogenes*, take at least one colony presumed to be *L. monocytogenes* (see 9.4.2 and 9.4.3). One confirmed isolate per sample is sufficient. If the first colony is negative take further colonies presumed to be *L. monocytogenes* from selective medium (up to a maximum of five colonies from each plate of each selective medium).

Streak the selected colonies onto the surface of pre-dried plates of a non-selective agar, for example blood agar, nutrient agar, tryptone soya yeast extract agar (TSYEA) (B.14), in a manner which will allow isolated colonies to develop.

Use of blood agar for pure culture enables interpretation of haemolysis, when positive, already at that stage (see 9.5.2.5.2 and Annex D). If streaking on blood agar does not show haemolysis, then the haemolysis test shall be done by stabbing (9.5.2.5.2) or in liquid medium (9.5.2.5.3).

Place the plates in the incubator set at 37 °C (6.3) for 18 h to 24 h or until growth is satisfactory.

If the colonies are not isolated, pick a typical *L. monocytogenes* colony onto another non-selective agar plate. Carry out the following tests (9.5.2) from colonies of a pure culture on the non-selective agar.

9.5.1.2 For confirmation of presumptive *Listeria* spp., take at least one colony presumed to be *Listeria* spp. (see 9.4.2 and 9.4.3). One confirmed isolate per sample is sufficient. If the first colony is negative take further colonies presumed to be *Listeria* spp. from selective medium (up to a maximum of five colonies from each plate of each selective medium).

For confirmation of *Listeria* spp. use plates of TSYEA.

Streak the selected colonies onto the surface of pre-dried plates of TSYEA (B.14), in a manner which will allow isolated colonies to develop.

Place the plates in the incubator set at 37 °C (6.3) for 18 h to 24 h or until growth is satisfactory.

Typical colonies of *Listeria* spp. on TSYEA are 1 mm to 2 mm in diameter, convex, colourless and opaque with an entire edge. When the plates are held to the light (artificial or natural) at about 45 degree angle, colonies exhibit a blue-grey colour and a granular surface.

If the colonies are not isolated, pick a typical *Listeria* spp. colony onto another non-selective agar plate.

Carry out the following tests (9.5.3) from typical colonies of a pure culture on TSYEA.

9.5.2 Confirmation of *L. monocytogenes*

9.5.2.1 General

Carry out the confirmation tests for *L. monocytogenes*. Appropriate positive and negative control strains for each of the confirmation tests shall be used.

Perform at minimum the mandatory tests as listed (in bold) in Table 1.

Table 1 — Confirmation tests for *L. monocytogenes*

Tests	*L. monocytogenes* confirmation tests	Results
Mandatory	**Microscopic aspect[a] (9.5.2.4)**	**Slim short rods or coccobacilli**
	Beta-haemolysis (9.5.2.5)	**+**
	L-Rhamnose (9.5.2.7)	**+**
	D-Xylose (9.5.2.7)	**-**
Optional	Catalase (9.5.2.2)	+
	Motility at 25°C (9.5.2.3)	+
	CAMP test (9.5.2.6)	+
[a] Microscopic aspect is optional for Agar *Listeria* according to Ottaviani and Agosti and for the second medium if it allows distinction between pathogenic and non-pathogenic *Listeria* spp.		

Details on results for confirmation tests can be found in Annex D.

NOTE An alternative procedure as mentioned in ISO 7218 can be used to confirm the isolate as *Listeria monocytogenes*, providing the suitability of the relevant procedure is verified.

If shown to be reliable, miniaturized galleries for the biochemical identification of *Listeria monocytogenes* may be used (see ISO 7218).

Rare strains of *L. monocytogenes* do not show beta-haemolysis or a positive reaction to the CAMP test under the conditions described in this document. If typical colonies on Agar *Listeria* according to Ottaviani and Agosti with PIPLC activity even if it is low, are negative for haemolysis, it is recommended to perform additional tests (e.g. Gram stain, catalase, motility, CAMP test, PCR), in order to determine whether this isolate is a non-haemolytic *L. monocytogenes.*

9.5.2.2 Catalase reaction (optional)

Take an isolated colony obtained in 9.5.1.1 and suspend it in a drop of hydrogen peroxide solution (B.6) on a slide. The immediate formation of gas bubbles indicates a positive reaction.

NOTE A catalase reaction performed from a colony originating from a blood agar can sometimes lead to false-positive results.

9.5.2.3 Motility test (optional)

Take an isolated colony obtained in 9.5.1.1 and suspend it in a tube containing a non-selective nutrient liquid medium.

Incubate in the incubator (6.3) set at 25 °C for 8 h to 24 h until the medium turns cloudy.

Take a drop of the above culture using a loop (6.5) onto a clean glass microscope slide. Place a cover slip on top and examine it under a microscope (6.9).

Listeria spp. (including *L. monocytogenes*) appear as slim, short rods with tumbling motility.

Cultures grown at temperatures above 25 °C may fail to exhibit this motion. Always compare them to a known *Listeria* culture. Cocci, large rods, or rods with rapid swimming motility are not *Listeria* spp.

As an alternative test for motility, using an inoculating needle (6.5), dilute in sterile water (or other appropriate diluent) a fragment of isolated colony obtained on non-selective agar. *Listeria* spp. (including *L. monocytogenes*) appear as slim, short rods with tumbling motility.

As another alternative test for motility, using an inoculating needle (6.5), stab the motility agar (B.7) with a culture taken from a typical colony obtained in 9.5.1.1. Incubate at 25 °C for 48 h ± 2 h.

Examine for growth around the stab. *Listeria* spp. are motile, giving a typical umbrella-like growth pattern. If growth is not sufficient, incubate for up to an additional five days and observe the stab again.

NOTE Some new *Listeria* species have been recently isolated.[5],[10],[12],[14],[25],[26],[27] Most of them are not mobile in the motility agar.

9.5.2.4 Microscopic aspect (optional in the case of use of agar specific for pathogenic *Listeria* spp.)

Make a microscopic preparation (e.g. the Gram stain, wet microscopy) on a well-separated colony obtained in 9.5.1.1. *Listeria* spp. (including *L. monocytogenes*) appear as Gram positive (if this stain is performed), slim, short rods or coccobacilli, with tumbling motility when originating from a fresh culture.

For Gram stain microscopic preparation see ISO 7218.

9.5.2.5 Haemolysis tests

9.5.2.5.1 General

Choose one of the haemolysis tests (9.5.2.5.2 or 9.5.2.5.3).

NOTE There exist rare strains of *L. monocytogenes* which do not show b-haemolysis or a positive reaction to the CAMP test under the conditions described in this document.

9.5.2.5.2 Haemolysis on blood agar

If the morphological and physiological characteristics are indicative of *Listeria* spp., inoculate blood agar plates (B.8) to determine the haemolytic reaction.

Dry the agar surface well before use. Take an isolated colony obtained in 9.5.1.1 using a wire (6.5), then stab a section of agar. Repeat for each culture. On the same plate, if possible, stab positive (*L. monocytogenes*) and negative (*L. innocua*) control cultures. For example, *L. monocytogenes* 4b WDCM 00021 or *L. monocytogenes* 1/2a WDCM 00109 and *L. innocua* WDCM 00017 may be used.

After incubation at 37 °C (6.3) for 24 h ± 2 h, examine the test strains and controls. *L. monocytogenes* show narrow, clear, light zones of haemolysis; *L. innocua* show no clear zone around the stab. *L. seeligeri* show mostly a weak zone of haemolysis. *L. ivanovii* usually show wide, clearly delineated zones of haemolysis. Examine the plates in a bright light to compare test cultures with controls.

NOTE 1 The haemolysis reaction is more readily seen by removing any colony growth on the surface of the agar around the inoculum mark.

NOTE 2 The haemolysis test can be performed by stabbing the blood agar plate used for the CAMP test.

9.5.2.5.3 Haemolysis reaction using red blood corpuscles

The haemolytic reaction may also be carried out using red blood corpuscles as follows.

Disperse the colony in 150 µl of a non-selective liquid nutrient medium, incubate at 37 °C (6.3) for 2 h. Add 150 µl of a suspension of red blood corpuscles (B.10). Incubate at 37 °C (6.3) for 15 min to 60 min, then refrigerate at 5 °C (6.10) for approximately 2 h. Examine for haemolytic activity. If the reaction is doubtful, leave at 5 °C (6.10) for up to 24 h ± 2 h. A sedimentation of red blood corpuscles (formation of a red point at the bottom of the tubes) indicates a negative reaction.

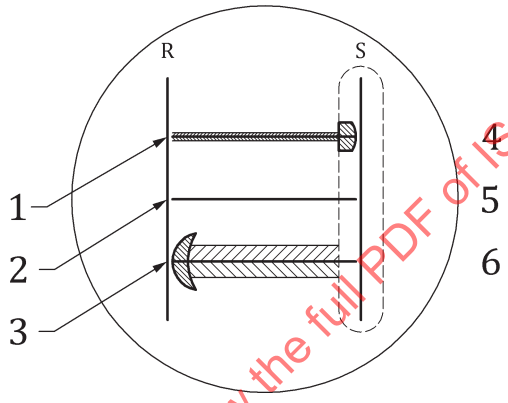
Include positive and negative controls. For control strains see 9.5.2.5.2.

9.5.2.6 CAMP test (optional)

If the result of the haemolysis test is difficult to interpret, the CAMP test is recommended to demonstrate clearly that haemolysis is due to listeriolysin activity.

A *β*-haemolytic strain of *Staphylococcus aureus* (e.g. WDCM 00034) and a strain of *Rhodococcus equi* (e.g. WDCM 0028) are required to undertake the CAMP test. Not all strains of *S. aureus* are suitable for the CAMP test. Streak each of the *S. aureus* and *R. equi* cultures in single lines across the blood agar plate (B.8.3 or B.11.4) so that the two cultures are parallel and diametrically opposite (see Figure 1). A thin, even inoculum is required. This can be obtained by using an inoculation loop or a wire (6.5) held at right angles to the agar.

Streak a well isolated colony of the strain under test (9.5.1.1) in a similar fashion at right angles to these cultures so that the test culture and *S. aureus* and *R. equi* cultures do not touch but at their closest are about 1 mm to 2 mm apart. Several test strains may be streaked on the same plate.



Key

1 narrow band of ß-haemolysis
2 no haemolysis
3 wide band of ß-haemolysis
4 *L. monocytogenes*
5 *L. innocua*
6 *L. ivanovii*

Figure 1 — Inoculation and interpretation of CAMP test plates

The vertical lines in Figure 1 represent streaks of *S. aureus* (S) and *R. equi* (R). Horizontal lines represent streaks of the test cultures. Hatched areas indicate the locations of enhanced haemolysis. The dotted area indicates the zone of influence of the *S. aureus* culture.

Simultaneously, streak control cultures of *L. monocytogenes, L. innocua* and *L. ivanovii*. For example, *L. monocytogenes* 4b WDCM 00021, *L. monocytogenes* 1/2a WDCM 00109, *L. innocua* WDCM 00017 and *L. ivanovii* WDCM 00018 may be used. Maintain stock cultures as specified in ISO 11133.

If blood agar (B.8.3) is used, incubate the plates at 37 °C for 18 h to 24 h. If double-layer plates (B.11.4) are used, incubate at 37 °C for 12 h to 18 h.

The positive reaction with *R. equi* is seen as a wide (5 mm to 10 mm) “arrow-head” of haemolysis. The reaction is considered as negative if a small zone of weak haemolysis extends only about 1 mm at the intersection of the test strain with the diffusion zone of the *R. equi* culture.

A positive reaction with *S. aureus* appears as a small zone of enhanced haemolysis extending only about 2 mm from the test strain and within the weakly haemolytic zone due to growth of the *S. aureus* culture. Large zones of haemolysis do not occur in the area of *S. aureus* and *L. monocytogenes*.

9.5.2.7 Carbohydrate utilization

Using a loop (6.5), inoculate each of the carbohydrate utilization broths (B.12) with the cultures obtained from the non-selective agar (9.5.1.1). Incubate at 37 °C. Positive reactions (acid formation) are indicated by a yellow colour and occur mostly within 24 h to 48 h for microvolumes tubes, and up to 5 days for macrovolumes tubes. L-Rhamnose and D-Xylose are used for the confirmation of *L. monocytogenes*, which is L-Rhamnose positive and D-Xylose negative (see Annex D).

NOTE There exist rare strains of *L. monocytogenes* which do not ferment L-Rhamnose.[15],[18]

For microvolumes, reactions are more rapid. The level of inoculation compared to the total volume is an important factor. For each chosen protocol, it is important to verify the time taken to obtain a yellow coloration. It is advised to use controls. For example *L. monocytogenes* 4b WDCM 00021, *L. innocua* WDCM 00017 and *L. ivanovii* WDCM 00018 may be used. Maintain stock cultures as specified in ISO 11133.

9.5.3 Confirmation of *Listeria* spp.

9.5.3.1 General

Carry out the confirmation tests for *Listeria* spp. from a typical colony (9.5.1.2). Appropriate positive and negative control strains for each of the confirmation tests shall be used.

Perform at minimum the mandatory tests as listed (in bold) in Table 2.

For further tests, if identification of species of *Listeria* is required, see Annex D.

Table 2 — Confirmation tests for *Listeria* spp.

Tests	*Listeria* spp.	Results
Mandatory	**Microscopic aspect (9.5.2.4)**	**Slim short rods or coccobacilli**
	Catalase (9.5.2.2)	**+**
Optional	VP test (9.5.3.5)	+
	Motility at 25°C (9.5.2.3)	+

NOTE 1 An alternative procedure as mentioned in ISO 7218 can be used to confirm the isolate as *Listeria* spp., providing the suitability of the relevant procedure is verified.

NOTE 2 It is possible that some new *Listeria* species recently isolated may not correspond to this scheme in particular for motility, VP test and growth at 37°C (see, for example, References [4], [10], [25], [26] and [27]).

9.5.3.2 Catalase reaction

Take an isolated colony obtained in 9.5.1.2 and perform the test as described in 9.5.2.2.

9.5.3.3 Motility test (optional)

Take an isolated colony obtained in 9.5.1.2 and perform the test as described in 9.5.2.3.

9.5.3.4 Microscopic aspect

Take an isolated colony obtained in 9.5.1.2 and perform the test as described in 9.5.2.4.

9.5.3.5 Voges – Proskauer (VP) reaction (optional)

Using a loop (6.5), inoculate a tube containing 3 ml of the VP medium (B.13.1). Incubate at 37 °C for 24 h ± 2 h. After incubation add 0,6 ml of 5 % α-naphthol solution (B.13.2) and 0,2 ml of 40 % potassium hydroxide solution (B.13.3). Shake well, slope the tube (to increase the area of the air-

liquid interface). Examine after 15 min and 1 h. A positive reaction is indicated by a strong red colour. *Listeria* spp. are VP positive.

NOTE Some new *Listeria* species have been recently isolated.[5],[10],[12],[14],[25],[26],[27] Most of them are VP negative.

9.6 Interpretation of morphological and physiological properties and of the biochemical reactions

All *Listeria* spp. are small, Gram-positive rods or coccobacilli that give a positive reaction in catalase test.

L. monocytogenes are confirmed according to tests listed in Table 1 and *Listeria* spp. are confirmed according tests listed in Table 2.

9.7 Additional characterization of isolated strains (optional)

Isolates which are considered to be *L. monocytogenes* (9.6) may be sent for further characterization to a recognized national or regional *Listeria* Reference Laboratory, or (if not available) to the World Health Organization Collaborating Centres for *Listeria*. The dispatch shall be accompanied by all possible information concerning the strain(s).

For further tests, if identification of species of *Listeria* is required, see Annex D.

10 Expression of results

In accordance with the interpretation of the results (see 9.6), report if *L. monocytogenes* is detected or not detected and/or if *Listeria* spp. is detected or not detected in the test portion by specifying the mass in grams, the volume in millilitres of the sample tested, the surface in squared centimetres or per sample device.

11 Performance characteristics of the method

11.1 Method validation studies

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

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 Level of detection (LOD_{50})

The level of detection at 50 % (LOD_{50}) is the concentration (cfu/test portion) for which the probability of detection is 50 %.

12 Test report

The test report shall specify the method used and the results obtained. It shall also mention any operating details not specified in this document, or regarded as optional, together with details of any incidents likely to have influenced the results (see Clause 10).

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

13 Quality assurance

The laboratory should have a clearly defined quality control system to ensure that the equipment, reagents and techniques are suitable for the test. The use of positive controls, negative controls and blanks are part of the test. Performance testing of culture media is specified in B.5 and described in ISO 11133.

Annex A
(normative)

Diagram of procedure

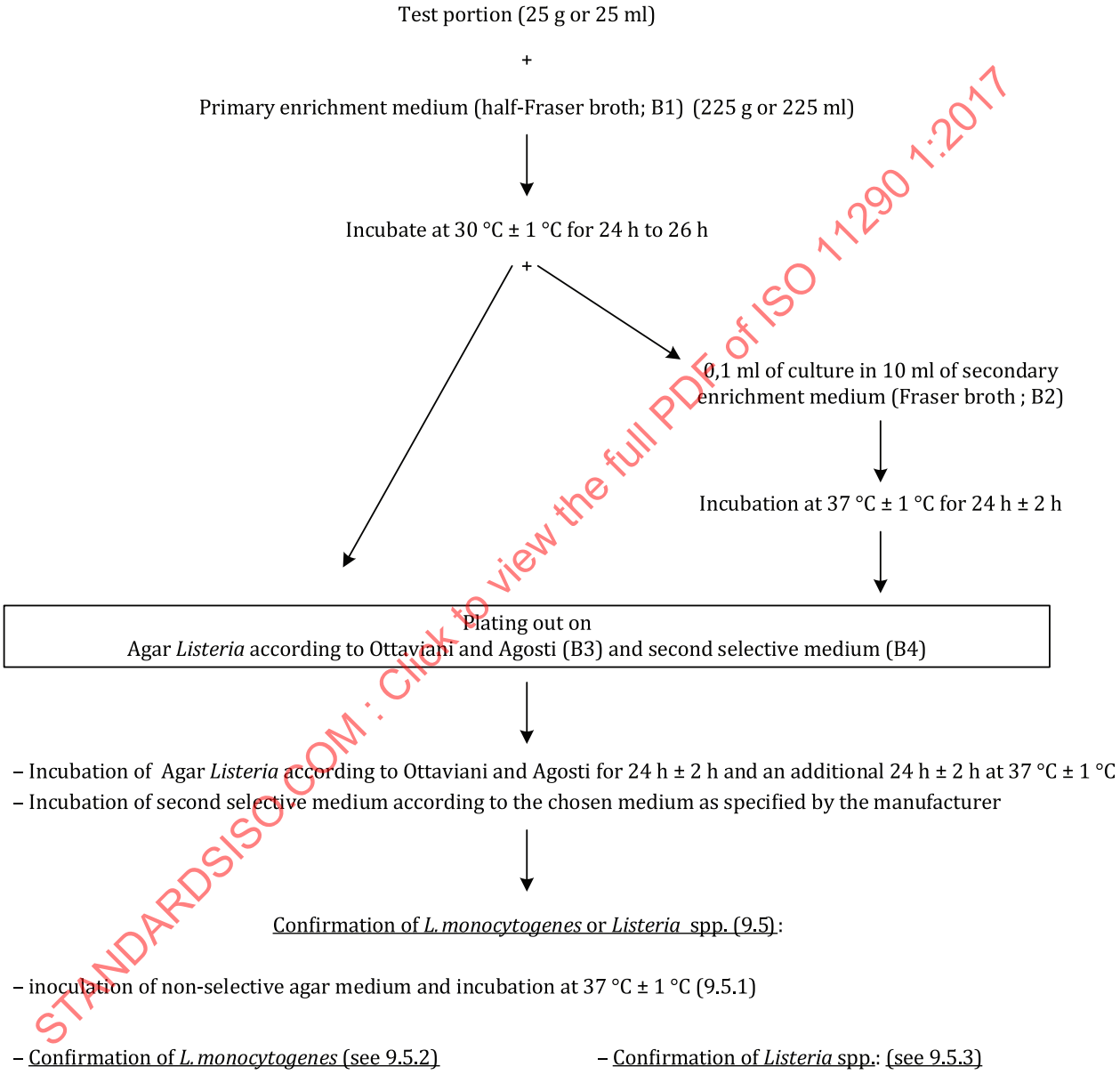


Figure A.1 — Diagram of procedure

Annex B
(normative)

Composition and preparation of culture media and reagents

B.1 Selective primary enrichment medium: half-Fraser broth

B.1.1 Base

B.1.1.1 Composition

Enzymatic digest of animal tissues	5,0 g
Enzymatic digest of casein	5,0 g
Meat extract	5,0 g
Yeast extract	5,0 g
Sodium chloride	20,0 g
Disodium hydrogen phosphate dihydrate	12,0 g
Potassium dihydrogen phosphate	1,35 g
Aesculin	1,0 g
Water	1 000 ml

B.1.1.2 Preparation

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

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

Dispense the base in flasks of suitable capacity to obtain portions appropriate for the test (see 9.1).

Sterilize for 15 min in the autoclave (6.1) at 121 °C.

The lithium chloride solution (B.1.2) and nalidixic acid solution (B.1.3) may be added to the base (B.1.1) before autoclaving.

B.1.2 Lithium chloride solution

B.1.2.1 Composition

Lithium chloride	3 g
Water	10 ml

B.1.2.2 Preparation

Add the lithium chloride to the water.

Sterilize by filtration through a 0,45 µm membrane.

WARNING — Take all necessary precautions when dissolving the lithium chloride in the water as the reaction is strongly exothermic. Also this solution irritates the mucous membranes.

B.1.3 Solution of sodium salt of nalidixic acid

B.1.3.1 Composition

Sodium salt of nalidixic acid	0,1 g
Sodium hydroxide, 0,05 mol/l solution	10 ml

B.1.3.2 Preparation

Dissolve the nalidixic acid salt in the sodium hydroxide.

Sterilize by filtration through a 0,45 µm membrane.

B.1.4 Acriflavine hydrochloride solution

B.1.4.1 Composition

Acriflavine hydrochloride	0,25 g
Water	100 ml

B.1.4.2 Preparation

Dissolve the acriflavine hydrochloride in the water.

Sterilize by filtration through a 0,45 µm membrane.

B.1.5 Ammonium iron(III) citrate solution

B.1.5.1 Composition

Ammonium iron(III) citrate	5,0 g
Water	100 ml

B.1.5.2 Preparation

Dissolve the ammonium iron(III) citrate in the water.

Sterilize by filtration through a 0,45 µm membrane.

B.1.6 Complete medium

B.1.6.1 Composition

Base (B.1.1)	100 ml
Lithium chloride solution (B.1.2)	1,0 ml
Sodium salt of nalidixic acid solution (B.1.3)	0,1 ml
Acriflavine hydrochloride solution (B.1.4)	0,5 ml
Ammonium iron(III) citrate solution (B.1.5)	1,0 ml

B.1.6.2 Preparation

Immediately before use, add the four solutions (B.1.2 to B.1.5) to each 100 ml portion of the base (B.1.1).

B.2 Selective secondary enrichment medium: Fraser broth

B.2.1 Base

B.2.1.1 Composition

Enzymatic digest of animal tissues	5,0 g
Enzymatic digest of casein	5,0 g
Meat extract	5,0 g
Yeast extract	5,0 g
Sodium chloride	20,0 g
Disodium hydrogen phosphate dihydrate	12,0 g
Potassium dihydrogen phosphate	1,35 g
Aesculin	1,0 g
Lithium chloride	3,0 g
Sodium salt of nalidixic acid	0,02 g
Water	1 000 ml

B.2.1.2 Preparation

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

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

Dispense the medium in test tubes of suitable capacity to obtain portions appropriate for the test.

Sterilize for 15 min in the autoclave at 121 °C.

B.2.2 Acriflavine hydrochloride solution

See B.1.4.

B.2.3 Ammonium iron(III) citrate solution

See B.1.5.

B.2.4 Complete medium

Immediately before use, to each tube (10 ml volumes) of base (B.2.1) add 0,1 ml portions of solutions B.2.2 and B.2.3. Mix gently.

B.3 Agar *Listeria* according to Ottaviani and Agosti[16],[17]

B.3.1 Base medium

B.3.1.1 Composition

Enzymatic digest of animal tissues	18 g
Enzymatic digest of casein	6 g
Yeast extract	10 g
Sodium pyruvate	2 g
Glucose	2 g
Magnesium glycerophosphate	1 g
Magnesium sulfate (anhydrous)	0,5 g
Sodium chloride	5 g
Lithium chloride	10 g
Disodium hydrogen phosphate (anhydrous)	2,5 g
5-Bromo-4-chloro-3-indolyl-ß-D-glucopyranoside	0,05 g
Agar	12 g to 18 g [a]
Water	930 ml [b]

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

[b] 925 ml if Amphotericin B solution is used (see B.3.5.2).

B.3.1.2 Preparation

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

Sterilize for 15 min in the autoclave at 121 °C.

Adjust the pH, if necessary, so that after sterilization it is 7,2 ± 0,2.

B.3.2 Nalidixic acid solution

Nalidixic acid sodium salt	0,02 g
Sodium hydroxide (0,05 mol/l)	5 ml

Dissolve the nalidixic acid sodium salt in 5 ml of sodium hydroxide and sterilize by filtration through a 0,45 µm membrane.

B.3.3 Ceftazidime solution

Ceftazidime	0,02 g
Water	5 ml

Dissolve the ceftazidime in 5 ml of water and sterilize by filtration through a 0,45 µm membrane.

B.3.4 Polymyxin B solution

Polymyxin B sulfate	76 700 IU
Water	5 ml

Dissolve the polymyxin B in 5 ml of water. Sterilize by filtration through a 0,45 µm membrane.

B.3.5 Antibiotic supplement

B.3.5.1 Cycloheximide solution

Cycloheximide	0,05 g
Ethanol	2,5 ml
Water	2,5 ml

Dissolve the cycloheximide in 2,5 ml of ethanol then add 2,5 ml of water. Sterilize by filtration through a 0,45 µm membrane.

B.3.5.2 **Amphotericin B solution** (as an alternative to cycloheximide solution)

Amphotericin B	0,01 g
HCl (1 mol/l)	2,5 ml
Dimethylformamide (DMF)	7,5 ml

Dissolve the amphotericin in the HCl/DMF solution. Sterilize by filtration through a 0,45 µm membrane. Other techniques of dissolution (e.g. in water or in hot medium base) may be performed according to media suppliers.

WARNING — The HCl/DMF solution is toxic, handle with care.

B.3.6 Supplement

Dissolve 2 g of L-α-phosphatidylinositol [1)] in 50 ml of water.

2 g of soy lecithin containing at least 9 % to 15 % unfractionated phosphatidylinositol may be used instead of L-α-phosphatidylinositol.[6],[9],[19]

Stir for about 30 min until a homogeneous suspension is obtained. Autoclave at 121 °C for 15 min and cool to 47 °C to 50 °C.

1) P 6636® supplied by Sigma is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

B.3.7 Complete medium

B.3.7.1 Composition

Base medium (B.3.1)	930 ml [a]
Nalidixic acid solution (B.3.2)	5 ml
Ceftazidime solution (B.3.3)	5 ml
Polymyxin B solution (B.3.4)	5 ml
Cycloheximide solution (B.3.5.1)	5 ml
or Amphotericin B solution (B.3.5.2)	10 ml
Supplement (B.3.6)	50 ml

[a] 925 ml if Amphotericin B solution is used.

B.3.7.2 Preparation

Add the solutions to the molten base at 47 °C to 50 °C (6.4), mixing thoroughly between each addition.

The pH of the complete medium shall be 7,2 ± 0,2 at 25 °C.

The medium shall be homogeneously slightly opalescent.

B.3.7.3 Preparation of agar plates

Place in each Petri dish 18 ml to 20 ml of the freshly prepared complete medium, then allow to solidify.

B.4 Second selective solid plating-out medium

The choice of the second medium is left to the discretion of the testing laboratory. If a commercial medium is used, follow the manufacturer's instructions regarding its preparation and use.

B.5 Performance testing for the quality assurance of the culture media

Performance testing of the culture media shall be carried out in accordance with ISO 11133, which includes definitions for productivity, selectivity and specificity. Table B.1 gives details of control strains to be used for performance testing of culture media specified in this document.

For the second isolation medium, use performance testing given in ISO 11133 or according to the manufacturer's specifications.

NOTE For productivity testing, in the case of detection of *Listeria* spp., it could be informative to add another control strain (from another *Listeria* species).

Table B.1 — Performance testing of culture media for *Listeria monocytogenes*

Media[a]	Function	Incubation	Control strains	WDCM numbers[c]	Reference media	Method of control	Criteria	Characteristic reactions of target micro-organism
Half-Fraser	Productivity	(25 ± 1) h/ (30 ± 1) °C	*Listeria monocytogenes* 4b	00021[b]	—	Qualitative	> 10 colonies on Agar *Listeria* according to Ottaviani and Agosti	Blue green colonies with opaque halo
			+ *Escherichia coli*[d]	00012 or 00013				
			+ *Enterococcus faecalis*[d]	00009 or 00087				
			Listeria monocytogenes 1/2a	00109				
			+ *Escherichia coli*[d]	00012 or 00013				
			+ *Enterococcus faecalis*[d]	00009 or 00087				
	Selectivity		*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Total inhibition (0) on TSA	—
			Enterococcus faecalis[d]	00009 or 00087	—	Qualitative	< 100 colonies on TSA	—
Fraser	Productivity	(24 ± 2) h/ (37 ± 1) °C	*Listeria monocytogenes* 4b	00021[b]	—	Qualitative	> 10 colonies on Agar *Listeria* according to Ottaviani and Agosti	Blue green colonies with opaque halo
			+ *Escherichia coli*[d]	00012 or 00013				
			+ *Enterococcus faecalis*[d]	00009 or 00087				
			Listeria monocytogenes 1/2a	00109				
			+ *Escherichia coli*[d]	00012 or 00013				
			+ *Enterococcus faecalis*[d]	00009 or 00087				
	Selectivity		*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Total inhibition (0) on TSA	—
			Enterococcus faecalis[d]	00009 or 00087	—	Qualitative	< 100 colonies on TSA	—

[a] Full names of media abbreviated terms.

[b] Strains to be used as a minimum.

[c] Refer to the reference strain catalogue available at www.wfcc.info for information on culture collection strain numbers and contact details.

[d] Strain free of choice; one of the strains has to be used as a minimum.

Table B.1 *(continued)*

Media[a]	Function	Incubation	Control strains	WDCM numbers[c]	Reference media	Method of control	Criteria	Characteristic reactions of target micro-organism
Agar *Listeria* according to Ottaviani and Agosti	Productivity	(48 ± 4) h/ (37 ± 1) °C	*Listeria monocytogenes* 4b	00021[b]	—	Qualitative	Good growth (2)	Blue green colonies with opaque halo
			Listeria monocytogenes 1/2a	00109				
	Selectivity		*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Total inhibition (0)	—
			Enterococcus faecalis[d]	00009 or 00087				
	Specificity		*Listeria innocua*	00017	—	Qualitative	—	Blue green colonies without opaque halo

[a] Full names of media abbreviated terms.

[b] Strains to be used as a minimum.

[c] Refer to the reference strain catalogue available at www.wfcc.info for information on culture collection strain numbers and contact details.

[d] Strain free of choice; one of the strains has to be used as a minimum.

B.6 Hydrogen peroxide solution

Use a mass fraction of 3 %, i.e. 10 volume solution.

B.7 Motility agar

B.7.1 Composition

Enzymatic digest of casein	20,0 g
Enzymatic digest of animal tissues	6,1 g
Agar	3,5 g
Water	1 000 ml

B.7.2 Preparation

Dissolve the components in the water by boiling.

Adjust the pH, if necessary, so that after sterilization it is 7,3 ± 0,2 at 25 °C.

Dispense the medium into tubes in quantities of about 5 ml.

Sterilize for 15 min in the autoclave at 121 °C.

B.8 Blood agar

B.8.1 Base

B.8.1.1 Composition

Enzymatic digest of animal tissues	15 g
Liver digest	2,5 g
Yeast extract	5 g
Sodium chloride	5 g
Agar	9 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 in the water by boiling.

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

Dispense the medium into flasks of suitable capacity to obtain portions appropriate for the test.

Sterilize for 15 min in the autoclave at 121 °C.

B.8.2 Defibrinated blood (sheep, calf or bovine)

B.8.3 Complete medium

B.8.3.1 Composition

Base (B.8.1)	100 ml
Defibrinated blood (B.8.2)	5 ml to 7 ml

B.8.3.2 Preparation

Add the blood to the base previously cooled at 47 °C to 50 °C (6.4). Mix well.

Dispense the medium into sterile Petri dishes in portions appropriate for the test. Allow to solidify.

B.9 Phosphate-buffered saline (PBS)

B.9.1 Composition

Disodium hydrogen phosphate dihydrate	8,98 g
Sodium dihydrogen phosphate	2,71 g
Sodium chloride	8,5 g
Water	1 000 ml

B.9.2 Preparation

Dissolve the components in the water.

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

Sterilize in the autoclave for 15 min at 121 °C.

B.10 Red blood corpuscle suspension

Maintain the red blood corpuscles at 5 °C ± 2 °C before use.

Before use, examine for signs of haemolysis (reddening) in the top layer of the serum. If no haemolysis has occurred, introduce 2 ml of the blood corpuscles from the bottom layer into 98 ml of PBS buffer (B.9).

If haemolysis has occurred, suspend approximately 4 ml of the blood corpuscles layer into 10 ml of PBS buffer and mix gently, then centrifuge. If the supernatant liquid clearly becomes red, due to significant haemolysis, do not use the stock suspension and discard it. Otherwise, decant the supernatant liquid and add 2 ml of this blood corpuscle solution to 98 ml of PBS buffer. Keep the suspension for five days at 5 °C ± 2 °C. Discard it if haemolysis occurs.

B.11 CAMP (Christie, Atkins, Munch-Petersen) medium and test strains

B.11.1 General

Blood agar plates (B.8) may be used for this test, but it is preferable to use double-layered agar plates with a very thin blood layer (B.11.4).

B.11.2 Base

See B.8.1.

B.11.3 Blood medium

See B.8.3.

B.11.4 Complete medium

Dispense the base (B.11.2) into sterile Petri dishes in quantities of about 10 ml and allow to solidify. Pour a very thin layer of the blood medium (B.11.3) using amounts not greater than 3 ml per plate.

Allow to solidify. If the blood medium is added to dishes containing the base which have been prepared in advance, it may be necessary to warm the dishes for 20 min by placing them in an incubator set at 37 °C before pouring the blood medium layer.

B.12 Carbohydrate utilization broth (L-Rhamnose and D-Xylose)

B.12.1 Base

B.12.1.1 Composition

Enzymatic digest of animal tissues	10 g
Meat extract	1 g
Sodium chloride	5 g
Bromocresol purple	0,02 g
Water	1 000 ml

B.12.1.2 Preparation

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

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

Dispense the medium into tubes of suitable capacity to obtain portions appropriate for the test.

Sterilize for 15 min in the autoclave at 121 °C.

B.12.2 Carbohydrate solutions

B.12.2.1 Composition

Carbohydrate [a]	5 g
Water	100 ml

[a] L-Rhamnose or D-Xylose.

B.12.2.2 Preparation

Dissolve the components in the water, and sterilize by filtration through a 0,45 µm membrane.

B.12.3 Complete medium

For each carbohydrate, add aseptically *x* ml of solution B.12.2 to 9*x* ml of the base (B.12.1).

B.13 Reagents for Voges-Proskauer (VP) Reaction

B.13.1 VP medium

B.13.1.1 Composition

Enzymatic digest of animal tissues	7 g
Sodium chloride	5 g
Glucose	5 g
Water	1 000 ml

B.13.1.2 Preparation

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

Adjust the pH, if necessary, so that after sterilization it is 6,9 ± 0,2 at 25 °C.

Dispense the medium into tubes in quantities of 3 ml.

Sterilize for 15 min in the autoclave at 121 °C.

NOTE Commercially available pre-prepared formulations with a pH within 0,5 pH unit of 6,9 are acceptable.

B.13.2 α-Naphthol, ethanolic solution

B.13.2.1 Composition

α-Naphthol	5 g
Ethanol, 96 % (volume fraction)	100 ml

B.13.2.2 Preparation

Dissolve the α-naphthol in the ethanol.

WARNING — The α-naphthol is toxic, handle with care.

B.13.3 Potassium hydroxide solution

B.13.3.1 Composition

Potassium hydroxide	40 g
Water	100 ml

B.13.3.2 Preparation

Dissolve the potassium hydroxide in the water

B.14 Tryptone soya yeast extract agar (TSYEA)

B.14.1 Composition

Enzymatic digest of casein	17 g
Papaic digest of soyabean meal	3 g
Yeast extract	6 g
Glucose	2,5 g
Sodium chloride	5 g
Dipotassium hydrogen phosphate	2,5 g

Agar	12 g to 18 g [a]
Water	1 000 ml

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

B.14.2 Preparation

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

Adjust the pH, if necessary, so that after sterilization it is 7,3 ± 0,2 at 25 °C.

Sterilize for 15 min in the autoclave at 121 °C.

B.14.2.1 Preparation of agar plates

Place in each Petri dish 18 ml to 20 ml of the freshly prepared complete medium, then allow to solidify.

Annex C
(informative)

Distinction of *Listeria* spp. from other genera

Table C.1 — Distinction of *Listeria* spp. from other bacterial genera or species

	Gram appearance	Catalase	Motility (20°C to 25°C)	VP test	Growth at 37°C
Listeria spp.	Gpb/Gpcb	+	+	+	+
Bacillus spp. [a]	Large Gpb[b] spore bearer (young culture)	+	V	V	+
Carnobacterium spp. [a]	Gpb	-	-	-	+
Staphylococcus spp.[a]	Gpc	+	-	V	+
Streptococcus spp. [a]	Gpc	-	-	V	+
Lactobacillus spp.	Gpb	- (occasional +)	- (occasional +)	-	+
Brochothrix spp.	Gpb	+	-	+	-
Kurthia spp.	Gpb	+	+	-	+
Erysipelothrix spp.	Gpb	-	-	-	+
Corynebacterium spp.	Gpb	+	-	- (occasional +)	+
Enterococcus spp. [a]	Gpc	-	-	+	+
Cellulosimicrobium funkei [a]	Gpcb	+	+	-	+
Kocuria kristinae [a]	Gpc	+	-	+ (occasional -)	+
Marinilactibacillus psychrotolerans [a,c]	Gpb	+	+	+	+
Rothia terrae [a]	Gpc	+	-	+	+

[a] Known to grow on Agar *Listeria* according to Ottaviani and Agosti and other chromogenic media and sometimes forming blue or bluish colonies.

[b] *Bacillus oleronius* displays a variable reaction to the Gram stain; most cells appear as Gram-negative.[23]

[c] Distinction of *M. psychrotolerans* from *Listeria* spp. can be aided by its different colony characteristics on TSYEA (opaque, pale-yellowish, lenticular colonies, 2 mm to 3 mm in diameter after 24 h of incubation at 37 °C).[23]

Gpb: Gram positive bacillus

Gpcb: Gram positive coccobacillus

Gpc: Gram positive coccus or coccoid

V: variable reaction

NOTE 1 Some new *Listeria* species recently isolated may not correspond to this scheme in particular for motility, VP test and growth at 37 °C (see, for example, References [5], [10], [12], [14], [25], [26] and [27]).

NOTE 2 Some rare *Listeria* strains are slow or not catalase positive.

Annex D
(informative)

Reactions for the identification of *Listeria* species

Table D.1 — Main tests prescribed in this document (see 9.5)

Species	PI-PLC	β-haemolysis	Production of acid			CAMP test	
			L-Rhamnose	D-Xylose	Mannitol	*S. aureus*	*R. equi*
L. monocytogenes	+ (24 h)	+	+	-	-	+	-
L. innocua	-	-	V	-	-	-	-
L. ivanovii	+ (24 h to 48 h)	+	-	+	-	-	+
L. seeligeri	-	(+)	-	+	-	(+)	-
L. welshimeri	-	–	V	+	-	-	-
L. grayi	-	-	V	-	+	-	-
L. fleischmanii	-	_	+	+	+	-	-
L. marthii	-	-	-	-	+	-	-
L. rocourtiae	-	-	+	+	+	-	-
L. weihenstephanensis	-	-	+	+	-	-	-

PI-PLC: phosphatidylinositol phospholipase C

V: variable reaction

(+): weak reaction

+ : more than 90 % of positive reactions

-: no reaction

NOTE 1 There exist rare strains of *L. monocytogenes* which do not show *β*-haemolysis or a positive reaction to the CAMP test under the conditions described in this document.

NOTE 2 Some strains of *L. seeligeri* may give the same haemolysis results as *L. monocytogenes*.

NOTE 3 Some new *Listeria* species have been recently isolated. Additional tests may be necessary to identify them accurately.[5],[10],[12],[14],[25],[26],[27]

NOTE 4 Some atypical *L. innocua* strains showed phenotypic characteristics of *L. monocytogenes* and could be only distinguished by a Reference Laboratory.[11]

NOTE 5 There exist rare strains of *L. monocytogenes* which do not ferment L-Rhamnose.[15],[18]

Table D.2 — Additional reactions for the identification of *Listeria* species[e]

Genus Species	Catalase	Motility (22–30 °C)	Esculin (β-glucosidase)	PI-PLC [a]	Mannitol	β-haemol-ysis	CAMP test		DIM [a]	MAN	DAR	XYL	RHA	MDG	RIB	G1P	TAG
							Rhodococcus equi	*Staphylococcus aureus*									
L. fleischmannii subsp. *coloradensis*	+	-	+	-	+	-	-	-	-	-	+	+	+	+	-	-	-
L. fleischmannii subsp. *fleischmannii*	+	-	+	-	+	-	-	-	-	-	+	+	+	+	-	-	-
L. grayi biovar *grayi* [c]	+	+	+	-	+	-	-	-	+	+	+	-	(-)	V [b]	+	-	-
L. grayi biovar *murrayi* [c]	+	+	+	-	+	-	-	-	+	+	+	-	+	V	+	-	-
L. innocua	+	+	+	-	-	-	-	-	+	+	+	-	V	+	-	-	-
L. ivanovii subsp. *ivanovii*	+	+	+	+ (24 h to 48 h)	-	+	+	-	V	-	+	+	-	+	+	V	-
L. ivanovii subsp. *londoniensis*	+	+	+	+ (24 h to 48 h)	-	+	+	-	V	-	+	+	-	+	-	V	-
L. marthii	+	+	+	-	-	-	-	-	-	+	+	-	-	+	-	-	-
L. monocytogenes	+	+	+	+ (24 h)	-	+ (24 h to 72 h)	-	+	-	+	+	-	+ [d]	+	-	-	-
L. rocourtiae	+	+ weak	+	-	+	-	-	-	-	+	-	+	+	+	+	-	-
L. seeligeri	+	+	+	-	-	+	-	+	+	-	+	+	-	+	-	-	-
L. weihenstephanensis	+	+ weak	+	-	-	-	-	-	-	-	+	+	+	+	+	-	-
L. welshimeri	+	+	+	-	-	-	-	-	(+)	+	+	+	V	+	-	-	+

[a] PI-PLC: phosphatidylinositol phospholipase C; DIM: D-arylamidase; MAN: alpha-mannosidase; DAR: D-arabitol; XYL: D-xylose; RHA: L-rhamnose; MDG: alpha-méthyl-D-glucoside; RIB: D-ribose; GIP: glucose-I-phosphate; TAG: D-Tagatose.

[b] V: variable; + or - : majority of strains are positive or negative; (+) or (-) : 90 % of positive or negative reactions.

[c] *grayi* biovar is negative for Nitrate and Turanose though *murrayi* biovar is positive for Nitrate and Turanose.

[d] There exist rare rhamnose negative strains, that belong to lineage IIIB/IIIC.

[e] Source: National Reference Centre/WHO Collaborative Centre for *Listeria,* Institut Pasteur, Paris.