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

Part 2: Enumeration method

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

Partie 2: Méthode de dénombrement

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ISO copyright office
Ch. de Blandonnet 8 • CP 401
CH-1214 Vernier, Geneva, Switzerland
Tel. +41 22 749 01 11
Fax +41 22 749 09 47
copyright@iso.org
www.iso.org

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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).

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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

This document was prepared by the European Committee for Standardization (CEN) Technical Committee CEN/TC 275, *Food analysis — Horizontal methods*, in collaboration with ISO Technical Committee 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 11290-2:1998), which has been technically revised. It also incorporates the amendment ISO 11290-2:1998/Amd.1:2004.

The main changes, compared to ISO 11290-2:1998, are the following.

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

— Primary suspension with buffered peptone water, half-Fraser broth with or without supplements, and all appropriate diluents referred to in ISO 6887 (all parts).

— Resuscitation step deleted.

— Microscopic aspect, catalase and CAMP test for confirmation are optional.

— Inclusion of new performance characteristics.

— Moreover, enumeration 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 11290-2:1998 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 2: Enumeration 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 enumeration of *L. monocytogenes*, and

— the enumeration 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 enumerated or confirmed by this method.[3],[6],[9],[11]

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*

ISO 11290-1, *Microbiology of the food chain — Horizontal method for the detection and enumeration of Listeria monocytogenes* and of *Listeria* spp. — Part 1: Detection method

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 described and which display the morphological, physiological and biochemical characteristics described when the analysis is carried out in accordance with this document

3.2
enumeration of *Listeria monocytogenes*
determination of the number of colony-forming units (cfu) of *Listeria monocytogenes*, per gram, per millilitre, per square centimetre, or per sampling device when the analysis is 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
enumeration of *Listeria* spp.
determination of the number of colony-forming units (cfu) of *Listeria* spp per gram, per millilitre, per square centimetre, or per sampling device, when the analysis is carried out in accordance with this document

4 Principle

4.1 General

Within the limits of this document, the enumeration of *L. monocytogenes* and of *Listeria* spp. requires five successive steps, as described in the flowchart in Annex A.

4.2 Initial suspension

Preparation of the initial suspension in an appropriate diluent according to the sample type.

4.3 Surface plating

Surface plating on Agar *Listeria* according to Ottaviani and Agosti[13],[14] of a specified quantity of the test sample for liquid products or of the initial suspension for other products and/or decimal dilutions if required.

4.4 Incubation

Incubation of the Petri dishes at 37 °C and examination after 24 h and after a further 24 h.

4.5 Confirmation

Confirmation of presumptive colonies of *L. monocytogenes* and/or of presumptive *Listeria* spp. by means of appropriate morphological and/or biochemical tests.

4.6 Enumeration

From the number of confirmed colonies, calculation of the number of *L. monocytogenes* and/or of *Listeria* spp. per gram, per millilitre, per square centimetre, or per sampling device.

5 Culture media and reagents

For current laboratory practices, refer to ISO 11133.

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

6 Equipment and consumables

Usual microbiological laboratory apparatus (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 37 °C ± 1 °C and 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 Glass or **plastic spreaders,** sterile.

6.7 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.

6.8 Total-delivery graduated pipettes or automatic pipettes, of nominal capacities 1 ml and 10 ml.

6.9 Petri dishes, of diameter 90 mm and/or 140 mm.

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

6.11 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. For environmental samples, use ISO 18593 and see Reference [23].

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]. 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, initial suspension and dilutions

Buffered peptone water, as well as other appropriate diluents referred to in ISO 6887 (all parts) and any specific International Standard appropriate to the product concerned, may be used as diluent for the initial suspension.

Half-Fraser broth (as specified in ISO 11290-1), supplemented with selective agents or not, may be used as a diluent for the initial suspension when both the detection method (as specified in ISO 11290-1) and this enumeration method are carried out on the same test sample. The selective agents should be added (if required) to the suspension preferentially after enumeration, prior to the detection method.

If supplemented half-Fraser is used, inoculate the plates as soon as possible, up to 45 min.

9.2 Inoculation and incubation

9.2.1 Distribute, by means of a sterile pipette (6.8), 0,1 ml of the initial suspension (or sample if liquid) and 0,1 ml of further decimal dilutions each inoculated onto the surface of a small dish (90 mm) of Agar *Listeria* according to Ottaviani and Agosti (see B.2).

When, for certain products, it is desirable to estimate low numbers of *L. monocytogenes* and/or *Listeria* spp., the limits of detection may be raised by a factor of 10 by examining 1 ml of the test sample if the initial product is liquid, or 1 ml of the initial suspension for the other products. Distribute the 1 ml of inoculum either on the surface of a large Petri dish (140 mm) or over the surface of three small dishes (90 mm), dried beforehand if necessary in the incubator (6.2). If only the initial suspension is used, also prepare duplicate plates using an additional three small Petri dishes or one large dish of medium (see ISO 7218).

Repeat the procedure using 0,1 ml of the initial suspension (or sample if liquid) and 0,1 ml of further decimal dilutions if necessary each inoculated onto the surface of a small dish (90 mm) of agar medium.

9.2.2 Carefully spread the inoculum as quickly as possible over the surface of the agar plate without touching the sides of the dish with the spreader (6.6). Use a fresh sterile spreader for each dilution. Leave the plates closed and upright for about 15 min at ambient temperature for the inoculum to be absorbed into the agar.

It is possible to use the same spreader for all the dishes of a given sample, by beginning with the higher dilution.

9.2.3 Incubate the Agar *Listeria* according to Ottaviani and Agosti plates prepared in 9.2.2 inverted at 37 °C (6.3) for 24 h ± 2 h and for an additional incubation at 37°C for 24 ± 2h.

9.3 Enumeration of characteristic colonies

9.3.1 After incubation for 24 h ± 2 h (before excessive development of colonies with large and overlapping opaque halos, which may make reading difficult), and for an additional 24 h ± 2 h (which may allow better development of colonies and of an opaque halo), examine the Petri dishes (9.2.3) for the presence of presumptive colonies of *L. monocytogenes* (see 9.3.2) and/or *Listeria* spp. (see 9.3.3).

9.3.2 Consider as *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.

NOTE 1 Some strains of *L. monocytogenes* exposed to stress conditions, particularly acid stress, may 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, 4 days. Some of these strains could be pathogenic.[10] No *L. monocytogenes* strains have been described as PIPLC negative.

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

NOTE Some organisms other than *Listeria* spp. may produce blue colonies on this medium. See Annex C.

9.3.4 Count all the colonies presumed to be *L. monocytogenes* (9.3.2) on each Petri dish containing less than 150 *L. monocytogenes* characteristic colonies, or less than 360 *L. monocytogenes* characteristic colonies if 140 mm Petri dishes are used.

9.3.5 Count all the colonies presumed to be *Listeria* spp. (9.3.3.) on each Petri dish containing less than 150 *Listeria* spp. characteristic colonies, or less than 360 *Listeria* spp. characteristic colonies if 140 mm Petri dishes are used.

In case of mixed cultures of blue-green colonies with or without opaque halo, or in case of blue-green colonies with large and overlapping opaque halos, it is preferable to count the colonies on each Petri dish containing less than 100 *Listeria* spp. characteristic colonies, or less than 240 *Listeria* spp. characteristic colonies if 140 mm Petri dishes are used.

9.4 Confirmation of *L. monocytogenes* or *Listeria* spp.

9.4.1 Selection of colonies for confirmation

9.4.1.1 Consider each group of three 90 mm Petri dishes used for the initial suspension as one dish.

If on one dish there are fewer than five presumptive colonies, take all of them for confirmation.

9.4.1.2 For confirmation of presumptive *L. monocytogenes*, take from each Petri dish, representing each dilution, five colonies in total, representative for each colony type (e.g. with large halo and small halo).

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.1) 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 9.4.2.5 and Annex D). If streaking on blood agar does not show haemolysis, then the haemolysis test shall be done by stabbing (9.4.2.5.2) or in liquid medium (9.4.2.5.3).

Place the Petri dishes in the incubator set at 37°C 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 dish.

Carry out the following tests (9.4.2) from colonies of a pure culture on the non-selective agar.

9.4.1.3 For confirmation of presumptive *Listeria* spp. take, from each Petri dish, representing each dilution, five colonies in total, representative for each colony type (e.g. large and small colonies, with or without halo).

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

Streak the selected colonies onto the surface of pre-dried plates of TSYEA (B.1), in a manner which will allow isolated colonies to develop.

Place the Petri dishes in the incubator set at 37°C for 18 h to 24 h or until growth is satisfactory.

Typical colonies on TSYEA of *Listeria* spp. 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 dish.

Carry out the following tests (9.4.3) from typical colonies of a pure culture on TSYEA.

9.4.2 Confirmation of *L. monocytogenes*

9.4.2.1 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	**Beta-haemolysis (9.4.2.4)**	**+**
	L-Rhamnose (9.4.2.7)	**+**
	D-Xylose (9.4.2.7)	**-**
Optional	Microscopic aspect (9.4.3.4)	Slim short rods or coccobacilli
	Catalase (9.4.2.2)	+
	Motility at 25 °C (9.4.2.3)	+
	CAMP test (9.4.2.6)	+

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.*

9.4.2.2 Catalase reaction (optional)

Take an isolated colony obtained in 9.4.1 and suspend it in a drop of hydrogen peroxide solution (B.3) 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.

