INTERNATIONAL STANDARD

ISO 11290-1

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Microbiology of food and animal feeding stuffs — Horizontal method for the detection and enumeration of *Listeria monocytogenes* —

Part 1: Detection method

Microbiologie des aliments — Méthode horizontale pour la recherche et le dénombrement de Listeria monocytogenes —

Partie 1: Méthode de recherche

Reference number
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Contents

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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 liason 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 11290-1 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 9, *Microbiology*.

ISO 11290 consists of the following parts, under the general title *Microbiology of food and animal feeding stuffs — Horizontal method for the detection and enumeration of* Listeria monocytogenes:

— *Part 1: Detection method*

— *Part 2: Enumeration method*

Annexes A and B form an integral part of this part of ISO 11290. Annex C is for information only.

Introduction

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, every attempt should be made to apply this horizontal method as far as possible and that deviations from this will only be made if absolutely necessary for justified technical reasons.

When this part of ISO 11290 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 part of ISO 11290 so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

Microbiology of food and animal feeding stuffs — Horizontal method for the detection and enumeration of *Listeria monocytogenes* —

Part 1:
Detection method

WARNING — In order to safeguard the health of laboratory personnel, it is strongly recommended that tests for detecting *Listeria monocytogenes* are 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. In particular, it is strongly recommended that pregnant personnel do not manipulate cultures of *L. monocytogenes*.

1 Scope

This part of ISO 11290 specifies a horizontal method for the detection of *Listeria monocytogenes*.

Subject to the limitations discussed in the introduction, this part of ISO 11290 is applicable to products intended for human consumption or animal feeding.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this part of ISO 11290. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this part of ISO 11290 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 7218:1996, *Microbiology of food and animal feeding stuffs — General rules for microbiological examinations.*

3 Definitions

For the purposes of this part of ISO 11290, the following definitions apply.

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 part of ISO 11290.

3.2 detection of *Listeria monocytogenes*: Determination of the presence or absence of these microorganisms, in a given mass or volume of product, when tests are carried out in accordance with this part of ISO 11290.

4 Principle

Within the limits of this part of ISO 11290, the detection of *L. monocytogenes* necessitates four successive stages (see annex A for a flowchart).

NOTE 1 *Listeria* spp. may be present in small numbers and are often accompanied by considerably larger numbers of other genera, therefore selective enrichment is necessary. It is also necessary to detect injured *Listeria* spp. and the primary selective enrichment medium, with reduced inhibitor concentration, fulfils at least part of this function.

4.1 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 one volume of lithium chloride and half a volume of both acriflavine and nalidixic acid (half Fraser broth), which is also used as a dilution fluid for the test portion (9.1).

Incubation of the test portion at 30 °C for 24 h.

4.2 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 4.1.

Incubation of the Fraser broth at 35 °C or 37 °C for 48 h.

4.3 Plating out and identification

From the cultures obtained in 4.1 and in 4.2, plating out on the two selective solid media:

a) Oxford agar;

b) PALCAM agar.

Incubation at 30 °C, 35 °C or 37 °C and examination after 24 h and, if necessary, after 48 h to check for the presence of characteristic colonies which are presumed to be *L. monocytogenes*.

4.4 Confirmation

Subculturing of the colonies of presumptive *L. monocytogenes*, plated out as described in 4.3, and confirmation by means of appropriate morphological, physiological and biochemical tests.

5 Culture media and reagents

5.1 General

For current laboratory practice, see ISO 7218.

NOTE 2 Because of the large number of culture media and reagents, it has been considered preferable, for clarity of the text, to give their composition and preparation in annex B.

5.2 Selective primary enrichment medium: Fraser broth with reduced concentration of selective agents (half Fraser broth)

See clause B.1.

5.3 Selective secondary enrichment medium with full concentration of selective agents (Fraser broth)

See clause B.2.

5.4 Selective solid plating-out media

5.4.1 First medium: Oxford agar

See clause B.3.

5.4.2 Second medium: PALCAM agar

See clause B.4.

5.5 Solid culture medium: Tryptone soya yeast extract agar (TSYEA)

See clause B.5.

5.6 Liquid culture medium: Tryptone soya yeast extract broth (TSYEB)

See clause B.6.

5.7 Sheep blood agar

See clause B.7.

5.8 Carbohydrate utilization broth (rhamnose and xylose)

See clause B.8.

5.9 Motility agar (optional)

See clause B.9.

5.10 CAMP (Christie, Atkins, Munch-Petersen) medium and test strains

See clause B.10.

5.11 Hydrogen peroxide solution

See clause B.11.

5.12 Phosphate-buffered saline (PBS)

See clause B.12.

6 Apparatus and glassware

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

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

See ISO 7218.

6.2 Drying cabinet or **incubator,** capable of being maintained at between 25 °C ± 1 °C and 50 °C ± 1 °C.

6.3 Incubators, for maintaining the inoculated media, plates and tubes within the following temperature ranges:

a) 25 °C ± 1 °C;

b) 30 °C ± 1 °C; and

c) 35 °C ± 1 °C or 37 °C ± 1 °C.

6.4 Water bath, capable of being maintained at 47 °C ± 2 °C.

6.5 Loops, of platinum/iridium or nickel/chromium, approximately 3 mm in diameter, and **wires** of the same material, or **hockey-stick-shaped glass rods** or **single-use loops**.

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 Test tubes or **flasks,** of appropriate capacity, for sterilization and storage of culture media and incubation of liquid media.

6.8 Measuring cylinders, of capacity 50 ml to 1 000 ml, for preparation of dilutions and complete media.

6.9 Total-delivery graduated pipettes, of nominal capacities 10 ml and 1 ml, graduated respectively in 0,5 ml and 0,1 ml divisions.

6.10 Petri dishes, of diameter 90 mm to 100 mm.

6.11 Jars, suitable for microaerobic incubation (optional).

6.12 Gas mixture (optional), of specified composition for microaerobic incubation:

5 % to 12 % CO_2, 5 % to 15 % O_2, and 75 % N_2.

6.13 Equipment for the Henry illumination test (optional).

See annex C.

6.14 Microscope, preferably with phase-contrast, and with slides and coverslips.

7 Sampling

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

Sampling is not part of the method specified in this part of ISO 11290. If there is no specific International Standard dealing with sampling of the product concerned, it is recommended that the parties concerned come to an agreement on this subject.

8 Preparation of 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 the parties concerned come to an agreement on this subject.

9 Procedure

9.1 Test portion and initial suspension

See ISO 6887 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 5.2.

In general, to prepare the initial suspension, add a test portion of x g or x ml to $9x$ ml or $9x$ g of the selective primary enrichment medium (5.2), to obtain a ratio of test portion to selective primary enrichment medium of 1/10 (mass to volume or volume to volume).

9.2 Primary enrichment

Incubate [6.3 b)] the initial suspension, prepared in accordance with 9.1, at 30 °C for 24 h ± 2 h.

NOTE 3 A black coloration may develop during the incubation.

9.3 Secondary enrichment

9.3.1 After incubation of the initial suspension (primary enrichment) for 24 h ± 2 h (9.2), transfer 0,1 ml of the culture obtained in 9.2 (regardless of its colour) to a tube (6.7) containing 10 ml of secondary enrichment medium (Fraser broth) (5.3).

9.3.2 Incubate the inoculated medium (9.3.1) for 48 h ± 2 h at 35 °C or 37 °C.

NOTE 4 The temperature of the inoculated medium should be agreed upon between the parties concerned and recorded in the test report.

9.4 Plating out and identification

9.4.1 From the primary enrichment culture incubated for 24 h ± 2 h at 30 °C (9.2), take, by means of a loop or glass rod (6.5), a portion of the culture and inoculate the surface of the first selective plating medium (Oxford agar) (5.4.1) so that well-separated colonies are obtained.

Proceed in the same way with the second selective plating-out medium (PALCAM agar) (5.4.2).

NOTE 5 This plating out is carried out regardless of the colour of the medium.

9.4.2 From the secondary enrichment medium incubated for 48 h ± 2 h at 35 °C or 37 °C (9.3.2), repeat the procedure described in 9.4.1 with the two selective plating-out media.

9.4.3 Invert the dishes obtained in 9.4.1 and 9.4.2 and place them in an incubator (6.3) set at 30 °C, 35 °C or 37 °C. PALCAM agar plates are incubated either microaerobically in a jar (6.11) containing the gas mixture (6.12) or aerobically.

NOTE 6 Incubation of the Oxford agar at 30 °C is suitable for foodstuffs only lightly contaminated by a supplementary flora. For products heavily contaminated by a supplementary flora, it is preferable to incubate the Oxford agar at 35 °C or 37 °C because *Listeria* spp. tend to develop at the same time as the supplementary flora. In all cases, the incubation temperature of the agar should be agreed upon between the parties concerned and recorded in the test report.

9.4.4 After incubation for 24 h and for an additional 18 h to 24 h (if growth is slight or if no colonies are observed after 24 h of incubation), examine the dishes (9.4.3) for the presence of colonies presumed to be *Listeria* spp.

9.4.4.1 Oxford agar: Typical colonies of *Listeria* spp. grown on Oxford agar for 24 h are small (1 mm) greyish colonies surrounded by black halos. After 48 h colonies become darker, with a possible greenish sheen, and are about 2 mm in diameter, with black halos and sunken centres.

9.4.4.2 PALCAM agar: For plates incubated microaerobically, after incubation expose the PALCAM agar plates to air for 1 h to allow the medium to regain its pink to purple colour. After 24 h *Listeria* spp. grow as small or very small greyish green or olive green colonies, 1,5 mm to 2 mm in diameter, sometimes with black centres, but always with black halos. After 48 h *Listeria* spp. appear in the form of green colonies about 1,5 mm to 2 mm in diameter, with a central depression and surrounded by a black halo.

9.5 Confirmation of *Listeria* spp.

9.5.1 Selection of colonies for confirmation

9.5.1.1 For confirmation, take from each plate of each selective medium (see 9.4.4.1 and 9.4.4.2), five colonies presumed to be *Listeria* spp.

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

9.5.1.2 Streak the selected colonies onto the surface of pre-dried plates of tryptone soya yeast extract agar (TSYEA) (5.5) in a manner which will allow well-separated colonies to develop.

Place the plates in the incubator [6.3 c)] set at 35 °C or 37 °C for 18 h to 24 h or until growth is satisfactory.

NOTE 7 The temperature of the inoculated medium should be agreed upon between the parties concerned and recorded in the test report.

Typical colonies are 1 mm to 2 mm in diameter, convex, colourless and opaque with an entire edge. If the colonies are not well separated, pick a typical *Listeria* spp. colony onto another TSYEA plate. Carry out the following tests from colonies of a pure culture on the TSYEA.

NOTE 8 The Henry illumination test (see annex C) may be conducted, if necessary. For this test, it is important that the agar medium is thin (15 ml/plate).

9.5.2 Catalase reaction

Take an isolated colony obtained in 9.5.1.2 and suspend it in a drop of hydrogen peroxide solution (5.11) on a slide. The immediate formation of gas bubbles indicates a positive reaction.

9.5.3 Gram staining

Perform the Gram stain on a colony separated in 9.5.1.2. *Listeria* spp. are revealed as Gram-positive slim, short rods.

9.5.4 Motility test (if necessary)[1)]

Take an isolated colony obtained in 9.5.1.2 and suspend it in a tube containing TSYEB (5.6).

Incubate in the incubator [6.3 a)] set at 25 °C for 8 h to 24 h until a cloudy medium is observed.

Deposit a drop of the above culture using a loop (6.5) onto a clean glass microscope slide. Place a coverslip on top and examine it with the microscope (6.14). *Listeria* spp. appear as slim, short rods with tumbling motility.

Cultures grown above 25 °C may fail to exhibit this motion. Always compare them to a known 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), stab the motility agar (5.9) with a culture taken from a typical colony on TSYEA (9.5.1.2). Incubate it for 48 h in the incubator [6.3 a)] set 25 °C.

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 5 days and observe the stab again.

9.6 Confirmation of *L. monocytogenes*

9.6.1 Haemolysis test

If the morphological and physiological characteristics and catalase reaction are indicative of *Listeria* spp., inoculate the sheep blood agar plates (5.7) to determine the haemolytic reaction.

Dry the agar surface well before use. Take a colony separated in 9.5.1.2 and plate and stab one space for each culture, using a wire (6.5). Simultaneously stab positive (*L. monocytogenes*) and negative control cultures (*L. innocua*).

After incubation at 35 °C or 37 °C for 24 h ± 2 h, examine the test strains and controls. *L. monocytogenes* show narrow, clear, light zones (β-haemolysis[2)]; see figure 1); *L. innocua* show no clear zone around the stab. *L. seeligeri* show a weak zone of haemolysis. *L. ivanovii* usually show wide, clearly delineated zones of β-haemolysis. Examine the plates in a bright light to compare test cultures with controls.

NOTE 9 The haemolytic reaction may also be carried out using red blood corpuscles. Disperse the colony in 150 µl of TSYEB (5.6); incubate at 35 °C or 37 °C for 2 h. Add 150 µl of sheep red blood corpuscles in a 2 ‰ solution of PBS (5.12). Incubate at 35 °C or 37 °C for between 15 min and 60 min, then refrigerate at 3 °C ± 2 °C for about 2 h. Then examine for the presence or absence of haemolysis. If the reaction is not definite, leave the culture at 3 °C ± 2 °C for up to 24 h.

9.6.2 Carbohydrate utilization

Inoculate using a loop (6.5) each of the carbohydrate utilization broths (5.8) with a culture from TSYEB (9.5.4). Incubate at 35 °C or 37 °C for up to 5 days. Positive reactions (acid formation) are indicated by a yellow colour and occur mostly within 24 h to 48 h.

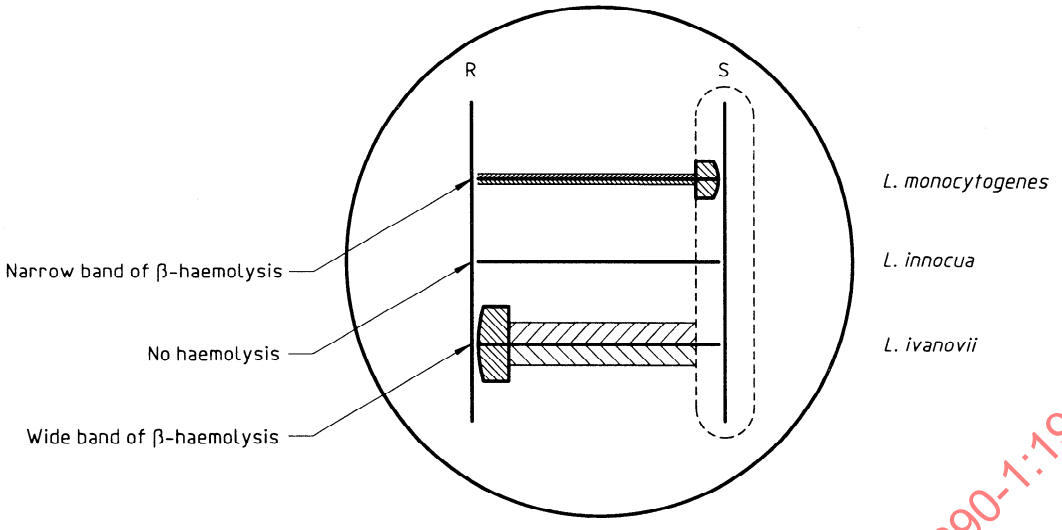
9.6.3 CAMP test

Streak each of the *Staphylococcus aureus* and *Rhodococcus equi* (B.10.4) cultures in single lines across the sheep blood agar plate (5.7 or B.10.3) 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 the test stain separated in 9.5.1.2 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.

1) This examination is not necessary in all cases if the analysis is carried out by a microbiologist who regularly works on the detection of *L. monocytogenes*.

2) This is more readily seen by removing any colony grown on the surface of the agar around the inoculum mark.



NOTES

1 Inoculate thin blood agar plates (5.7 or B.10.3) as shown in the diagram. Vertical lines 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.

2 The dotted area indicates the zone of influence of the *S. aureus* culture.

Figure 1 — Inoculation and interpretation of CAMP test plates

Simultaneously, streak control cultures of *L. monocytogenes, L. innocua* and *L. ivanovii*. If blood agar (5.7) is used, incubate the plates at 35 °C or 37 °C for 18 h to 24 h. If double-layer plates (B.10.3) are used, incubate at 35 °C or 37 °C for 12 h to 18 h.

An enhanced zone of β-haemolysis at the intersection of the test strain with each of the cultures of *S. aureus* and *R. equi* is considered to be a positive reaction.

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*.

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

All *Listeria* spp. are small, Gram-positive rods that demonstrate motility. They are catalase positive. *L. monocytogenes* are distinguished from other species by the characteristics listed in table 1.

9.8 Definitive confirmation

Strains which are considered to be *L. monocytogenes* (9.7) may be sent to a recognized *Listeria* reference laboratory for serological or, possibly, lysogenic typing. The despatch shall be accompanied by all possible information concerning the strain(s).

Table 1 — Reactions for the identification of *Listeria* spp.

Species	Haemolysis	Production of acid		CAMP test	
		Rhamnose	Xylose	*S. aureus*	*R. equi*
L. monocytogenes	+	+	–	+	–
L. innocua	–	V	–	–	–
L. ivanovii	+	–	+	–	+
L. seeligeri	(+)	–	+	(+)	–
L. welshimeri	–	V	+	–	–
L. grayi subsp. *grayi*	–	–	–	–	–
L. grayi subsp. *murrayi*	–	V	–	–	–

V: variable reaction
(+): weak reaction
+: > 90 % of positive reactions
–: no reaction

NOTE — 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 part of ISO 11290.

9.9 Control cultures

In order to check the ability of the enrichment and identification media to support the selective growth of *L. monocytogenes*, a dilution of the reference culture of recently isolated strains of *L. monocytogenes* and negative control strains (e.g. rods, *Streptococcus*) should be introduced in a control flask of the selective primary enrichment medium (see 9.2). Add 10 to 100 *L. monocytogenes* cells or negative control strains per flask.

Proceed with the control flasks as for the test cultures to demonstrate that the positive control culture is recovered.

10 Expression of results

In accordance with the interpretation of the results, report the presence or absence of *Listeria monocytogenes* in the test portion, specifying the mass in grams, or the volume in millilitres, of the sample tested.

NOTE 10 If other *Listeria* species are isolated, these may be noted in the test report, if agreed between the parties concerned.

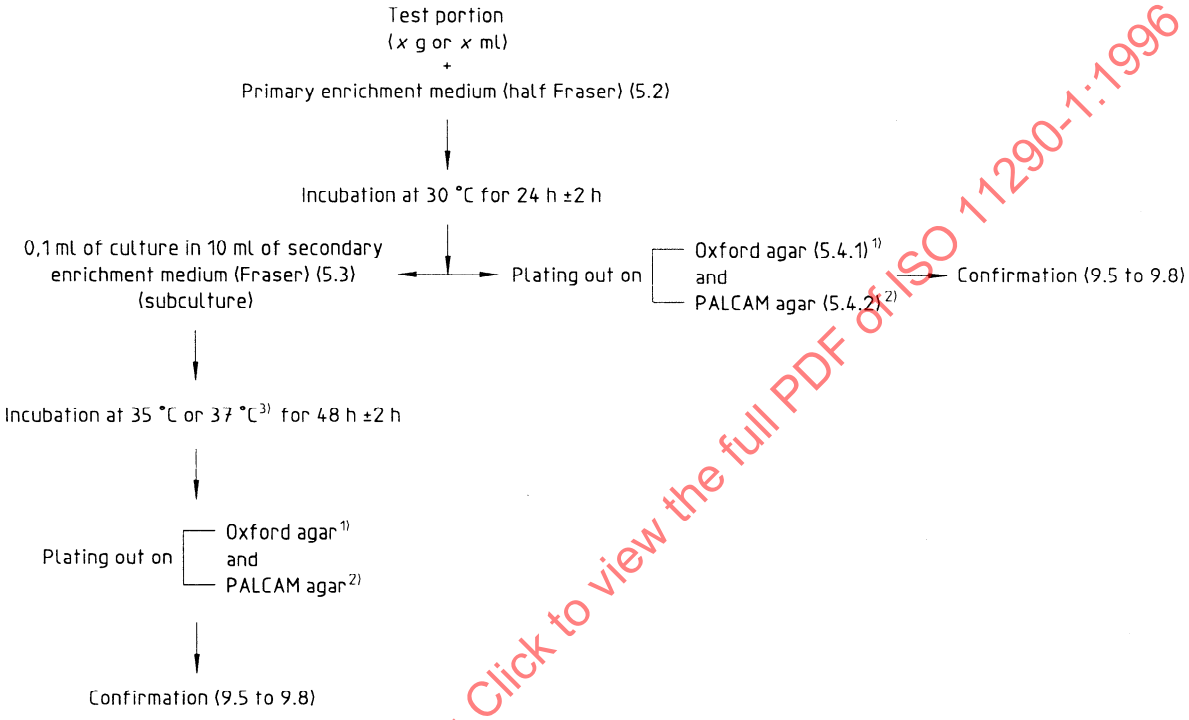
11 Test report

The test report shall specify the method used, the temperatures of incubation and the results obtained. It shall also mention any operating details not specified in this part of ISO 11290, or regarded as optional, together with details of any incidents likely to have influenced the results.

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

Annex A
(normative)

Diagram of procedure



1) Oxford agar plates are incubated for 24 h to 48 h at 30 °C, 35 °C or 37 °C, aerobically.
2) PALCAM agar plates are incubated for 24 h to 48 h at 30 °C, 35 °C or 37 °C, microaerobically if necessary.
3) The temperature shall be agreed between the parties concerned.

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

Meat peptone (peptic digest of animal tissue)	5,0 g
Tryptone (peptic digest of casein)	5,0 g
Beef 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[3)] at 25 °C.

Dispense the base in flasks (6.7) of suitable capacity to obtain portions appropriate for the test (see 9.1).

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

NOTE 11 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.

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.

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.

3) See ISO 7218.

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.

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 (B.1.3)	0,1 ml
Acriflavine hydrochloride (B.1.4)	0,5 ml
Ammonium iron(III) citrate (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

Meat peptone (peptic digest of animal tissue)	5,0 g
Tryptone (peptic 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 set 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 First selective plating-out medium: Oxford agar

B.3.1 Agar base

B.3.1.1 Composition

Columbia agar[1)]	39 g
Aesculin	1 g
Ammonium iron(III) citrate	0,5 g
Lithium chloride	15 g
Water	1 000 ml

1) Proteose peptone	23,0 g
Starch	1,0 g
Sodium chloride	5,0 g
Agar (depending on the gel strength of the agar)	9 g to 18 g

B.3.1.2 Preparation

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

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

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

B.3.2 Supplement for 1 000 ml medium

B.3.2.1 Composition

Cycloheximide	400 mg
Colistin sulfate	20 mg
Acriflavine hydrochloride	5,0 mg
Cefotetan	2,0 mg
Fosfomycin	10 mg
Ethanol	5,0 ml
Water	5,0 ml

B.3.2.2 Preparation

Dissolve the components or the dehydrated complete medium in the ethanol/water mixture.

Sterilize by filtration.

B.3.3 Preparation of complete medium

Cool the base (B.3.1) to about 47 °C and aseptically add the supplement (B.3.2).

Dispense the medium into sterile Petri dishes in quantities of about 15 ml and allow to solidify.

Store the medium away from light.

B.4 Second selective plating-out medium: PALCAM agar[4)]

B.4.1 Agar base

B.4.1.1 Composition

Peptones	23,0 g
Starch	1,0 g
Sodium chloride	5,0 g
Yeast extract	3,0 g
Agar	9 g to 18 g[1)]
D-glucose	0,5 g
D-mannitol	10,0 g
Aesculin	0,8 g
Ammonium iron(III) citrate	0,5 g
Phenol red	0,08 g
Lithium chloride	15,0 g
Water	960 ml

1) Depending on the gel strength of the agar.

B.4.1.2 Preparation

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

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

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

4) Details are given in: VAN NETTEN P., PERALES I., VAN DE MOOSDIJK A., CURTIS G.D.W. and MOSSEL D.A.A. Liquid and solid selective differential media for the detection and enumeration of *L. monocytogenes* and other *Listeria* spp. *Int. J. Food Microbiol.*, **8**, 1989, pp. 299-316.

B.4.2 Polymyxin B sulfate solution

B.4.2.1 Composition

Polymyxin B sulfate (100 000 IU)	0,1 g
Water	100 ml

B.4.2.2 Preparation

Dissolve the polymyxin B sulfate in the water.

Sterilize by filtration.

B.4.3 Acriflavine hydrochloride solution

B.4.3.1 Composition

Acriflavine hydrochloride	0,05 g
Water	100 ml

B.4.3.2 Preparation

Dissolve the acriflavine hydrochloride in the water.

Sterilize by filtration.

B.4.4 Sodium ceftazidime pentahydrate solution

B.4.4.1 Composition

Sodium ceftazidime pentahydrate	0,116 g
Water	100 ml

B.4.4.2 Preparation

Dissolve the sodium ceftazidime in the water.

Sterilize by filtration.

B.4.5 Complete medium

B.4.5.1 Composition

Base (B.4.1)	960 ml
Polymyxin B sulfate solution (B.4.2)	10 ml
Acriflavine hydrochloride solution (B.4.3)	10 ml
Sodium ceftazidime pentahydrate solution (B.4.4)	20 ml

B.4.5.2 Preparation

To the molten base (B.4.1) at 47 °C, add solutions B.4.2 to B.4.4, mixing gently between each addition.

B.4.6 Preparation of the agar plates

Place in each of an appropriate number of Petri dishes (6.10) about 15 ml of the freshly prepared complete medium (B.4.5). Allow to solidify.

Store the medium away from light.

B.5 Solid culture medium: Tryptone soya yeast extract agar (TSYEA)

B.5.1 Composition

Tryptone soya broth[1)]	30 g
Yeast extract	6 g
Agar	9 g to 18 g[2)]
Water	1 000 ml

1)	Tryptone	17,0 g
	Soya peptone	3,0 g
	Sodium chloride	5,0 g
	Dipotassium phosphate	2,5 g
	Glucose	2,5 g
2)	Depending on the gel strength of the agar.	

B.5.2 Preparation

Dissolve the components or the 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.

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

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

Allow to set in a sloping position.

For the preparation of agar plates, dispense the medium into sterile Petri dishes in portions appropriate for the test. Allow to solidify.

B.6 Liquid culture medium: Tryptone soya yeast extract broth (TSYEB)

B.6.1 Composition

Tryptone soya broth[1)]	30 g
Yeast extract	6 g
Water	1 000 ml

1) See B.5.1.

B.6.2 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 7,3 ± 0,2 at 25 °C.

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

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

B.7 Sheep blood agar

B.7.1 Base

B.7.1.1 Composition

Meat peptone	15 g
Liver digest	2,5 g
Yeast extract	5 g
Sodium chloride	5 g
Agar	9 g to 18 g[1)]
Water	1 000 ml

1) Depending on the gel strength of the agar.

B.7.1.2 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 set at 121 °C.

B.7.2 Defibrinated sheep blood

B.7.3 Complete base

B.7.3.1 Composition

Base (B.7.1)	100 ml
Defibrinated sheep blood (B.7.2)	5 ml to 7 ml

B.7.3.2 Preparation

Add the blood to the base previously cooled to about 47 °C. Mix well.

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

B.8 Carbohydrate utilization broth (rhamnose and xylose)

B.8.1 Base

B.8.1.1 Composition

Proteose peptone	10 g
Meat extract	1 g
Sodium chloride	5 g
Bromocresol purple	0,02 g
Water	1 000 ml

B.8.1.2 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 set at 121 °C.

B.8.2 Carbohydrate solutions

B.8.2.1 Composition

Carbohydrate[1)]	5 g
Water	100 ml

1) L-Rhamnose or D-xylose.

B.8.2.2 Preparation

Dissolve separately each carbohydrate in 100 ml of water.

Sterilize by filtration.

B.8.3 Complete media

For each carbohydrate, add aseptically x ml of solution B.8.2 to $9x$ ml of the base (B.8.1).

B.9 Motility agar

B.9.1 Composition

Casein peptone	20,0 g
Meat peptone	6,1 g
Agar	3,5 g
Water	1 000 ml

B.9.2 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 set at 121 °C.

B.10 CAMP (Christie, Atkins, Munch-Petersen) medium and test strains

Sheep blood agar plates (B.7) may be used for this test, but it is preferable to use double-layered agar plates with a very thin blood layer (B.10.3).

B.10.1 Base

See B.7.1.

B.10.2 Sheep blood medium

See B.7.3.1.

B.10.3 Complete medium

Dispense the base (B.10.1) into sterile Petri dishes in quantities of about 10 ml and allow to solidify. Pour a very thin layer of the blood medium (B.10.2) 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.10.4 CAMP reaction strains

A β-haemolytic strain of *S. aureus* (e.g. NCTC 1803 or ATCC 25923) and a strain of *R. equi* (e.g. NCTC 1621 or ATCC 6939) are required to undertake the CAMP test. Not all strains of *S. aureus* are suitable for the CAMP test.

Maintain stock cultures of *S. aureus*, *R. equi*, *L. monocytogenes*, *L. innocua* and *L. ivanovii* by inoculating the TSYEA slopes (B.5.2), incubating them at 35 °C or 37 °C for 24 h to 28 h, or until growth has occurred, and storing in the refrigerator at 3 °C ± 2 °C. Subculture at least once per month.

B.11 Hydrogen peroxide solution

Use a 3 % (m/m), i.e. 10 volume solution.

B.12 Phosphate-buffered saline (PBS)

B.12.1 Composition

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

B.12.2 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.