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AMENDMENT 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**

AMENDMENT 1: Modification of the isolation media and the haemolysis test, and inclusion of precision data

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

Partie 1: Méthode de recherche

AMENDEMENT 1: Modification des milieux d'isolement, de la recherche de l'hémolyse et introduction de données de fidélité

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Foreword

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Amendment 1 to ISO 11290-1:1996 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

The isolation media have been modified, as has the haemolysis test. Precision data have been added.

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Part 1:
Detection method

AMENDMENT 1: Modification of the isolation media and the haemolysis test, and inclusion of precision data

Page 2, Subclause 4.3

Replace this subclause by the following:

4.3 Plating out and identification

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

— Agar *Listeria* according to Ottaviani and Agosti (ALOA[1)]) (see Reference [1] and B.3);

— any other solid selective medium at the choice of the laboratory complementary to Agar *Listeria* according to Ottaviani and Agosti, such as Oxford or PALCAM.

Incubation of the Agar *Listeria* according to Ottaviani and Agosti at 37 °C ± 1 °C and examination after 24 h ± 3 h, and if necessary after a further 24 h ± 3 h, to check for the presence of characteristic colonies which are presumed to be *L. monocytogenes*.

Incubation of the 2nd selective medium at the appropriate temperature and examination after the appropriate time.

Page 2, Subclauses 5.4.1 and 5.4.2

Replace these subclauses by the following:

5.4.1 First medium: Agar *Listeria* according to Ottaviani and Agosti (ALOA[1)]) [1]

See B.3.

5.4.2 Second medium

The choice of the second medium is left to the discretion of the testing laboratory. If a commercial medium is used, the manufacturer's instructions shall be precisely followed regarding its preparation for use.

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1) ALOA is an example of a suitable medium available commercially. This information is given for the convenience of users of this part of ISO 11290 and does not constitute an endorsement by ISO of this product. The use of other media with the same formulation is allowed.

Page 4, Subclause 9.4

Replace 9.4.1 by the following:

9.4.1 From the primary enrichment culture incubated for 24 h ± 3 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, Agar *Listeria* according to Ottaviani and Agosti (5.4.1), so that well-separated colonies are obtained.

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

Replace 9.4.3 by the following:

9.4.3 Invert the dishes obtained in 9.4.1 and 9.4.2 and place them in an incubator set at 37 °C for Agar *Listeria* according to Ottaviani and Agosti (5.4.1) and at the appropriate temperature for the second selective medium (5.4.2). If a commercial medium is used for the second selective medium, follow the manufacturer's instructions.

Delete Note 6.

Replace 9.4.4 by the following:

9.4.4 After incubation for 24 h ± 3 h (and for an additional 24 h ± 3 h if the growth is weak or if no colony is observed after 24 h incubation) for Agar *Listeria* according to Ottaviani and Agosti or for the appropriate time (second selective agar), examine the dishes (9.4.3) for the presence of colonies presumed to be *Listeria* spp.

Replace 9.4.4.1 by the following:

9.4.4.1 Agar *Listeria* according to Ottaviani and Agosti: Consider as *L. monocytogenes* the green-blue colonies surrounded by an opaque halo (typical colonies). If growth is slight, or if no colony is observed, or if no typical colony is present after 24 h ± 3 h of incubation, re-incubate the plates for a further 24 h ± 3 h.

NOTE 1 Some strains of *L. monocytogenes* show a very weak halo (even no halo) in cases of stress, in particular acid stress.

NOTE 2 Some *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 (see Reference [2]).

Replace 9.4.4.2 by the following:

9.4.4.2 Second selective medium: Examine after the appropriate time to check for the presence of colonies which, from their characteristics, are considered to be presumptive *Listeria* spp. or *monocytogenes*, depending on the type of medium used.

Page 5, Subclause 9.6.1 Haemolysis test

Insert the subclause numbers 9.6.1.1 at the beginning of the text of 9.6.1.

Replace Note 9 by the following subclause:

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

Disperse the colony in 150 µl of TSYEB (B.6); incubate at 37 °C for 2 h. Add 150 µl of a suspension of sheep red blood corpuscles (B.4 of this Amendment). Incubate at 37 °C for 15 min to 60 min, then refrigerate at 3 °C ± 2 °C for approximately 2 h. Examine for haemolytic activity. If the reaction is not definite, leave at 3 °C ± 2 °C for up to 24 h ± 3 h.

Page 7, new Clause 11

Add the following clause after Clause 10:

11 Precision of the method

11.1 General

It is not possible to express the precision of a qualitative method by using the parameters of repeatability and reproducibility which can be calculated only for quantitative methods. Thus new performance characteristics have been selected (see Reference [3]). These characteristics are: accuracy (sensitivity for positive samples, specificity for negative samples), accordance and concordance (see 11.2, 11.3 and 11.4).

The values of these characteristics have been determined by an interlaboratory test on the method organized within the framework of a European project (see Annex D). Performance characteristics were determined using three types of food contaminated at various levels and for reference materials. The values derived from the interlaboratory test may not be applicable to analyte concentration ranges and matrices other than those given in Annex D.

WARNING — The method which was tested was without this amendment, i.e. the isolation was performed on PALCAM and Oxford agars. The precision data give some general guidance to the user on the global performance of the method and these precision data are applicable in particular to this part of ISO 11290 together with this amendment when the second isolation agar is either Oxford or PALCAM.

11.2 Accuracy

11.2.1 Definition

Accuracy is the percentage of samples correctly identified.

For positive samples, the accuracy is called sensitivity and is the percentage of samples correctly identified as positives. For the purpose of this calculation, it must be assumed that all supposedly positive samples do in fact contain the organism.

For negative samples, the accuracy is called specificity and is the percentage of samples correctly identified as negatives.

11.2.2 Overall values

As a general indication of specificity (Sp), the following value may be used when testing food samples in general: Sp = 97,4 %.

As a general indication of sensitivity (Se) the following value may be used when testing food samples in general: Se = 85,2 %.

For reference materials (capsules containing 23 CFU, prepared by RIVM, Netherlands, for the trial), the following value has been obtained: Se = 89,5 %.

These values may be interpreted to mean that a sample which contains *L. monocytogenes* will be recognized as positive when analysed with the method described in this part of ISO 11290-1 in 85,2 % of cases.

11.3 Accordance

11.3.1 Definition

Accordance is the percentage chance of finding the same result (i.e. both negative or both positive) from two identical test portions analysed in the same laboratory, under repeatability conditions (i.e. one operator using the same apparatus and same reagents within the shortest feasible time interval).

The accordance is therefore the equivalent of repeatability for quantitative methods.

To calculate accordance from the results of an interlaboratory test, the probability that two samples give the same result is calculated for each participating laboratory in turn, and this probability is then averaged over all laboratories.

11.3.2 Overall values

As a general indication of accordance (Ac), the following value may be used when testing food samples in general: Ac = 88,7 %.

For reference materials (capsules containing 23 CFU, prepared by RIVM, Netherlands, for the trial), the following value may be used: Ac = 88,2 %.

These values may be interpreted to mean that if two identical test portions of a sample containing *L. monocytogenes* are analysed by the same operator in a short time and with exactly the same operating conditions, there is an 88,7 % chance of obtaining the same result (presence of *L. monocytogenes*) for the two test portions.

11.4 Concordance

11.4.1 Definition

Concordance is the percentage chance of finding the same result for two identical samples analysed in two different laboratories.

The concordance is therefore the equivalent of reproducibility for quantitative methods.

To calculate concordance from the results of an interlaboratory test, each observation in each participating laboratory is taken in turn, pairing it with the results obtained for that particular sample by all the other laboratories. The concordance is the percentage of all pairings giving the same results on all the possible pairings of data.

11.4.2 Overall values

As a general indication of concordance (Cc), the following value may be used when testing food samples in general: Cc = 84,4 %.

For reference materials (capsules containing 23 CFU, prepared by RIVM, Netherlands, for the trial), the following value can be used: Cc = 80,8 %.

These values may be interpreted to mean that if two identical test portions of a sample containing *L. monocytogenes* are analysed by two laboratories, there is an 84,4 % chance of obtaining the same result (presence of *L. monocytogenes*) for the two test portions.

Page 7

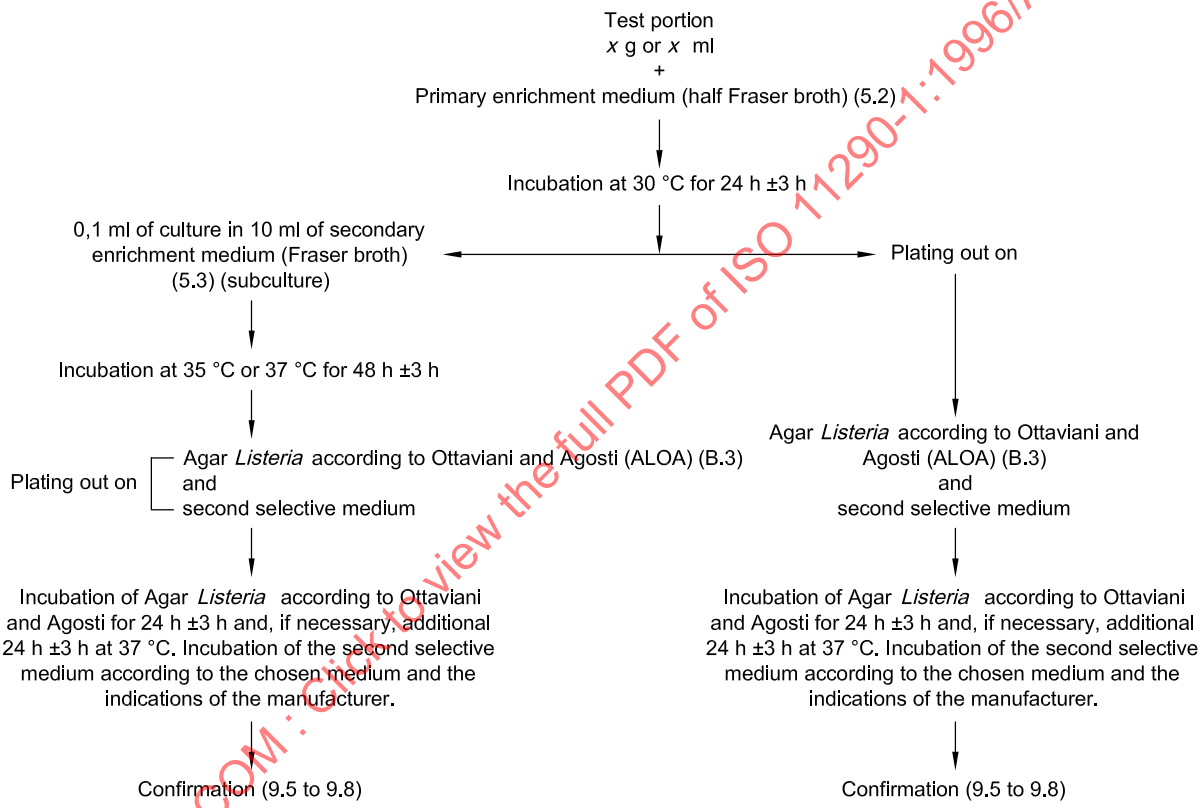
Renumber the existing Clause 11 as Clause 12.

Page 9, Annex A

Replace the exisiting Annex A by the following:

Annex A
(normative)

Diagram of procedure



Page 11, Annex B

Replace Clause B.3 by the following:

B.3 Agar *Listeria* according to Ottaviani and Agosti (ALOA [2])

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-β-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 set 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.

2) ALOA is an example of a suitable medium available commercially. This information is given for the convenience of users of this part of ISO 11290 and does not constitute an endorsement by ISO of this product. The use of other media with the same formulation is allowed.

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.

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

B.3.6 Supplement

Dissolve 2 g of L-α-phosphatidylinositol (Sigma P 6636® [3]) in 50 ml of cold water.

Stir for about 30 min until a homogeneous suspension is obtained.

Autoclave at 121 °C for 15 min and cool to 48 °C to 50 °C.

3) P 6636 is a trade name of a product supplied by Sigma. This information is given for the convenience of the users of this part of ISO 11290 and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

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)	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 approximately 50 °C, 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 opaque.

B.3.7.3 Preparation of agar plates

Place in each Petri dish 15 ml to 20 ml of the freshly prepared complete medium, then allow to solidify.

B.3.8 Performance testing for the quality assurance of the culture medium

For the definition of selectivity and productivity refer to ISO/TS 11133-2. Table B.1 gives details of performance testing relating to Agar *Listeria* according to Ottaviani and Agosti.

Table B.1 — Performance testing of Agar *Listeria* according to Ottaviani and Agosti

Function	Incubation	Control strains	Reference medium	Method of control	Criteria	Characteristic reactions
Productivity	37 °C for 48 h	*L. monocytogenes* 4b ATCC 13932 [a] and/or [b] *L. monocytogenes* 1/2a ATCC 19111 (or other equivalent strains in a recognized collection of microorganisms)	TSA	quantitative	PR ⩾ 0,5	blue green colonies with opaque halo
Specificity	37 °C for 48 h	*L. innocua* ATCC 33090 (or other equivalent strains in a recognized collection of microorganisms)		qualitative		blue green colonies without opaque halo
Selectivity	37 °C for 48 h	*E. coli* ATCC 25922 or 8739 [a] and/or [b] *E. faecalis* ATCC 29212 or 19433 (or other equivalent strains in a recognized collection of microorganisms)		qualitative	total inhibition	

[a] Strain to be used by the user laboratory (as minimum).

[b] Both strains to be used by media manufacturers.

Page 11, Clause B.4

Replace the existing clause by the following:

B.4 Sheep red blood corpuscle suspension

Maintain the sheep red blood corpuscles at 3 °C ± 2 °C before use.

Before use, examine for signs of haemolysis (reddening) in the superior layer of the serum.

If no haemolysis has occurred, introduce 2 ml of the inferior layer of blood corpuscles into 98 ml of PBS buffer (B.12).

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 5 days at 3 °C ± 2 °C. Discard it if haemolysis occurs.

Page 16, add a new Annex D.

Add a Bibliography after Annex D.

Annex D
(informative)

Results of the interlaboratory test

An international collaborative test was organized by the Central Science Laboratory of the UK Ministry of Agriculture, Fisheries and Food in 1998, in the framework of the European project SMT CT 96 2098 (see References [3], [4]). This test involved 19 laboratories in 14 countries in Europe and was carried out on fresh cheese, minced meat, dried egg powder and a reference material. The food samples were each tested at two different levels of contamination, plus a negative control. Samples were contaminated both by *L. monocytogenes* and *L. innocua* (see Table D.1), which means a low sensitivity. Due to lack of resources needed by laboratories to participate in this trial, it was not possible for all participants to use both Oxford and PALCAM isolation media: 11 participants used the PALCAM medium and 9 used the Oxford medium.

Table D.1 — Contamination of samples

Microorganisms (in 25 g)	Blank	Low level	High level
L. monocytogenes	—	5 to 100	50 to 100
L. innocua	50 to 100	5 to 100	50 to 100
Autochthonous flora		+	+

The values of the performance characteristics derived from this collaborative test are shown per type of sample in Tables D.2 to D.5. The data obtained with Oxford and PALCAM agars have been combined. Data obtained by some laboratories have been excluded from the calculations only on the basis of clearly identified technical reasons (deviations from the protocol).

Table D.2 — Results of data analysis obtained with dried egg powder samples

	Sample (level of contamination)		
	Dried egg powder (blank)	Dried egg powder (low level)	Dried egg powder (high level)
Year of interlaboratory test	1998	1998	1998
Number of laboratories having returned results	18	18	18
Number of samples per laboratory	5	5	5
Number of excluded laboratories	1	1	1
Number of laboratories retained after exclusion	17	17	17
Number of accepted samples	85	85	85
Accuracy (specificity), %	97,9	—	—
Accuracy (sensitivity), %	—	53,7	88,4
Accordance, %	96,6	58,7	86,5
Concordance, %	95,8	49,8	79,1