Conventional isolation methods: detection of *Listeria* spp. and *L. monocytogenes* according to ISO 11290-1:2017

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Introduction

- *Listeria* spp. are ubiquitous Gram+ bacteria, frequently isolated from a variety of raw/ processed food matrices and food processing plants environment.

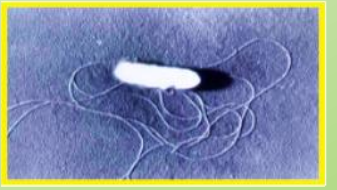
- More than 20 species are described, subdivided in two groups:

  - ***Listeria sensu strictu*** (*L. monocytogenes, L. innocua, L. welshimeri, L. seeligeri, L. ivanovii and L. marthii*);
  - ***Listeria sensu lato*** (*L. grayi, L. rocourtiae, L. fleischmannii, L. newyorkensis* and others).

Listeria

Species:

monocytogenes
ivanovii
seeligeri
innocua
welshimeri
grayi
murrayi



INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION

Quereda *et al.*, *Int. J. Syst. Evol. Microbiol.* 2020;70:5868–5879
DOI 10.1099/ijsem.0.004494

MICROBIOLOGY SOCIETY

Listeria valentina sp. nov., isolated from a water trough and the faeces of healthy sheep

Juan J. Quereda[1,*,†], Alexandre Leclercq[2,3†], Alexandra Moura[2,3,4†], Guillaume Vales[2,3], Ángel Gómez-Martín[1], Ángel García-Muñoz[1], Pierre Thouvenot[2,3], Nathalie Tessaud-Rita[2,3], Hélène Bracq-Dieye[2,3] and Marc Lecuit[2,3,4,5,*]

Introduction

Among all *Listeria* species, only *L. monocytogenes* and *L. ivanovii* are pathogenic:

- ***L. monocytogenes*** is known to be an important foodborne pathogen responsible of severe invasive disease (**Listeriosis**), mainly in immunocompromised individuals, pregnant women and neonates,

- ***L. ivanovii*** is pathogenic for animals (ruminants).

https://www.cdc.gov/listeria/faq.html

Introduction

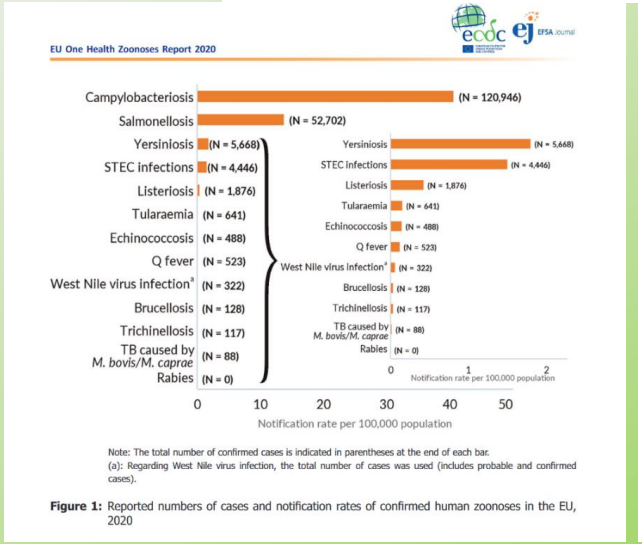
- One of the largest listeriosis outbreak detected (1060 cases) was reported in South Africa (2017-2018), due to ready to eat (RTE) processed meat product.

- Listeriosis is the **fifth** most commonly reported zoonosis in humans in the EU.

FOODBORNE PATHOGENS AND DISEASE
Volume 16, Number 7, 2019
Mary Ann Liebert, Inc.
DOI: 10.1089/fpd.2018.2586

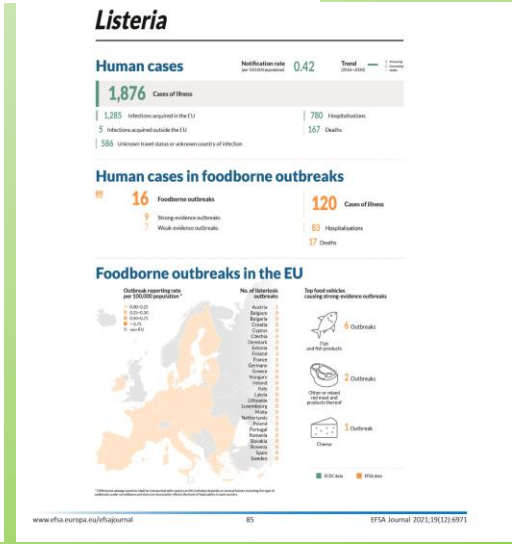
Outbreak of *Listeria monocytogenes* in South Africa, 2017–2018: Laboratory Activities and Experiences Associated with Whole-Genome Sequencing Analysis of Isolates

Anthony M. Smith,[1,2] Nomsa P. Tau,[1] Shannon L. Smouse,[1] Mushal Allam,[3] Arshad Ismail,[3] Ntsieni R. Ramalwa,[1] Bolele Disenyeng,[1] Mimmy Ngomane,[1] and Juno Thomas[1,2]



Note: The total number of confirmed cases is indicated in parentheses at the end of each bar.
(a): Regarding West Nile virus infection, the total number of cases was used (includes probable and confirmed cases).

Figure 1: Reported numbers of cases and notification rates of confirmed human zoonoses in the EU, 2020



Introduction

22.12.2005 EN Official Journal of the European Union L 338/1

I

(Acts whose publication is obligatory)

COMMISSION REGULATION (EC) No 2073/2005

of 15 November 2005

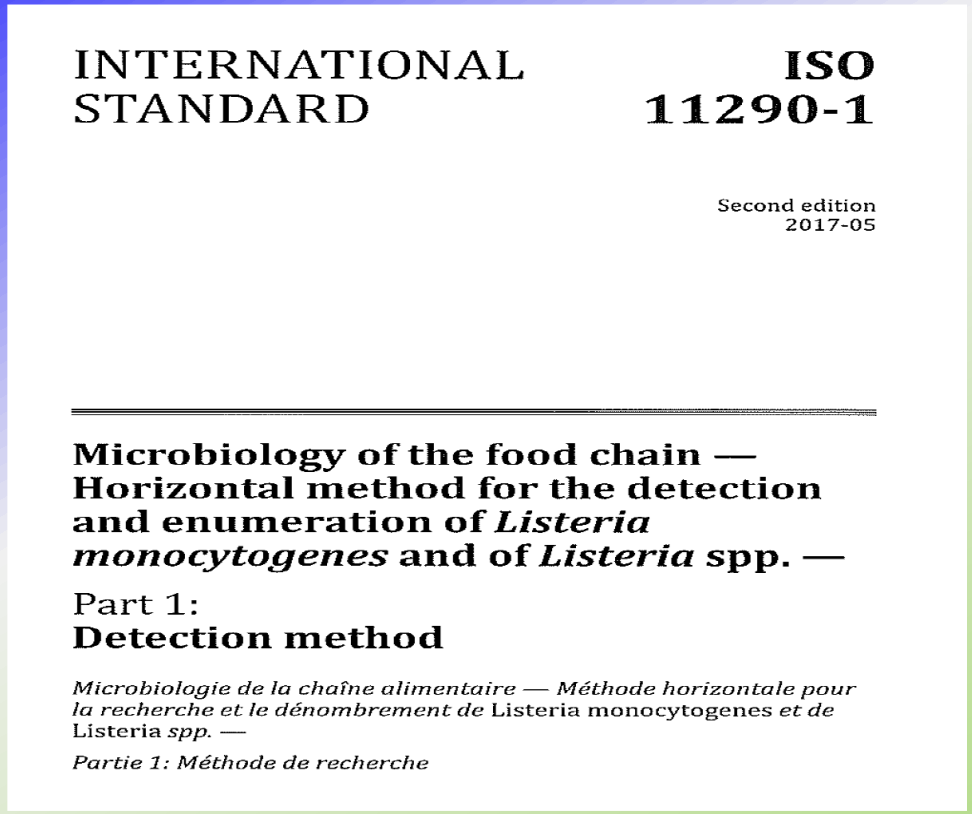
on microbiological criteria for foodstuffs

(Text with EEA relevance)

Chapter 1. Food safety criteria

Food category	Micro-organisms/their toxins, metabolites	Sampling-plan (1)		Limits (2)		Analytical reference method (3)	Stage where the criterion applies
		n	c	m	M		
1.1. Ready-to-eat foods intended for infants and ready-to-eat foods for special medical purposes (4)	*Listeria monocytogenes*	10	0	Absence in 25 g		EN/ISO 11290-1	Products placed on the market during their shelf-life
1.2. Ready-to-eat foods able to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes	*Listeria monocytogenes*	5	0	100 cfu/g (5)		EN/ISO 11290-2 (6)	Products placed on the market during their shelf-life
		5	0	Absence in 25 g (7)		EN/ISO 11290-1	Before the food has left the immediate control of the food business operator, who has produced it
1.3. Ready-to-eat foods unable to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes (4) (8)	*Listeria monocytogenes*	5	0	100 cfu/g		EN/ISO 11290-2 (6)	Products placed on the market during their shelf-life

ISO: International Organization for Standardization



INTERNATIONAL STANDARD

ISO 11290-1

Second edition
2017-05

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

Part 1:
Detection method

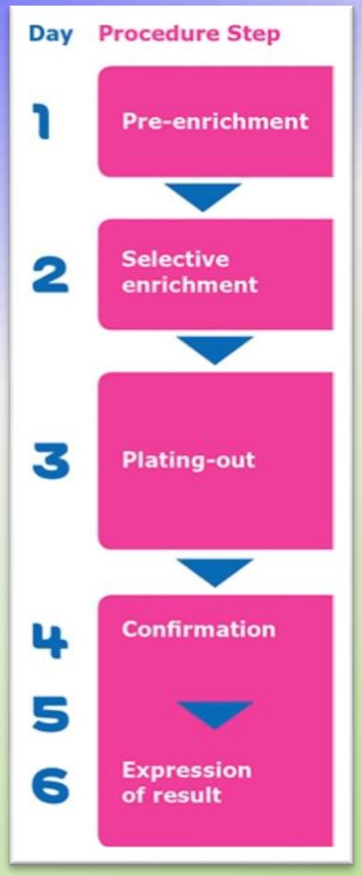
Microbiologie de la chaîne alimentaire — Méthode horizontale pour la recherche et le dénombrement de Listeria monocytogenes *et de* Listeria *spp.* —

Partie 1: Méthode de recherche

This document is applicable to:

- ✓ products intended for human consumption and for the feeding of animals;
- ✓ environmental samples in the area of food production and food handling.

ISO 11290-1:2017



Test portion (25 g or 25 ml)

+

Primary enrichment medium (half-Fraser broth; B1) (225 g or 225 ml)

↓

Incubate at 30 °C ± 1 °C for 24 h to 26 h

↓ +

0,1 ml of culture in 10 ml of secondary enrichment medium (Fraser broth ; B2)

↓

Incubation at 37 °C ± 1 °C for 24 h ± 2 h

↓

Plating out on
Agar *Listeria* according to Ottaviani and Agosti (B3) and second selective medium (B4)

↓

- Incubation of Agar *Listeria* according to Ottaviani and Agosti for 24 h ± 2 h and an additional 24 h ± 2 h at 37 °C ± 1 °C
- Incubation of second selective medium according to the chosen medium as specified by the manufacturer

↓

Confirmation of *L. monocytogenes* or *Listeria* spp. (9.5):

- inoculation of non-selective agar medium and incubation at 37 °C ± 1 °C (9.5.1)

- Confirmation of *L. monocytogenes* (see 9.5.2)
- Confirmation of *Listeria* spp.: (see 9.5.3)

ISO 11290-1:2017

INTERNATIONAL STANDARD ISO 11290-1:2017(E)

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.

Good Laboratory Practice

ISO 11290-1:2017: PREPARATION OF TEST SAMPLE

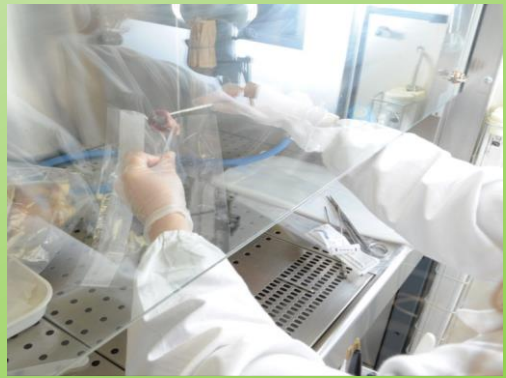
7 Sampling

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.



The test portion is defined by 25 g (for solid matrices) or 25 ml of sample (for liquid matrices).

ISO 11290-1:2017: PRIMARY ENRICHMENT

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).

- ✓ To prepare the initial suspension, add 225 ml of the selective primary enrichment medium (half-Fraser broth) to 25 g or 25 ml of sample.
- ✓ Incubate at 30°C for 25±1h.

ISO 11290-1:2017: PRIMARY ENRICHMENT

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 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.

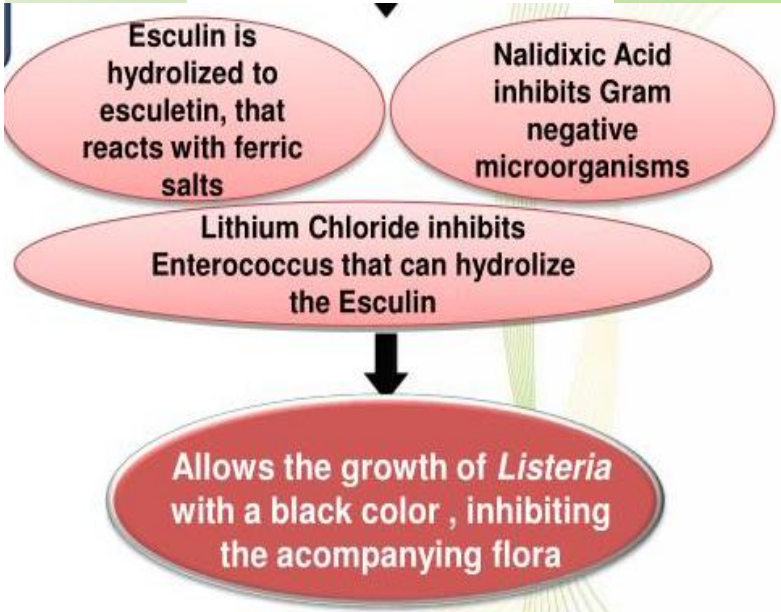
Adjust the pH, if necessary, so that after sterilization it is 7,2 ± 0,2 at 25 °C.

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.

Listeria Half Fraser Broth



ISO 11290-1:2017: SECONDARY ENRICHMENT

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.

- ✓ After incubation, transfer 0,1 ml from the primary enrichment broth into a tube containing 10 ml of secondary enrichment medium (Fraser broth).
- ✓ Incubate at 37°C for 24±2h.

In case of *Listeria* spp. detection, additional 24h can allow to recovery of more species.

ISO 11290-1:2017: SECONDARY ENRICHMENT

B.1.5 Ammonium iron(III) citrate solution

B.1.5.1 Composition

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

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

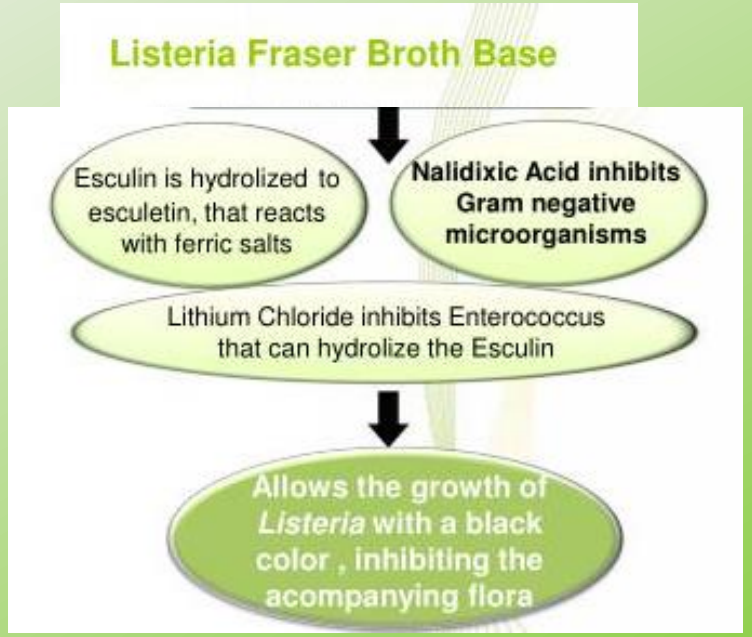
B.2.1.2 Preparation

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.



ISO 11290-1:2017: PLATING OUT

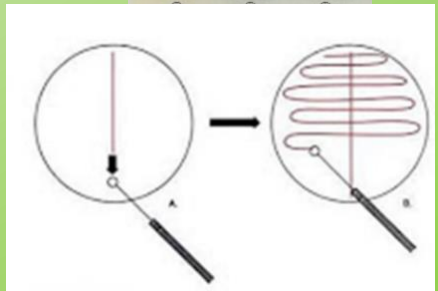
4.4 Plating out and identification

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.

- ✓ After incubation, from the primary enrichment (half-Fraser broth) and from the secondary enrichment (Fraser broth), inoculate by a loop the surface of the first selective plating medium (ALOA).
- ✓ Invert ALOA dishes obtained and incubate them at 37°C for a total of 48±2h. If colonies of presumptive *L. monocytogenes* or *Listeria* spp. are evident at 24±2h the incubation may be stopped at this stage.
- ✓ For the second selective medium follow the manufacture's instruction (Oxford agar, Palcam agar)



ISO 11290-1:2017: PLATING OUT

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).

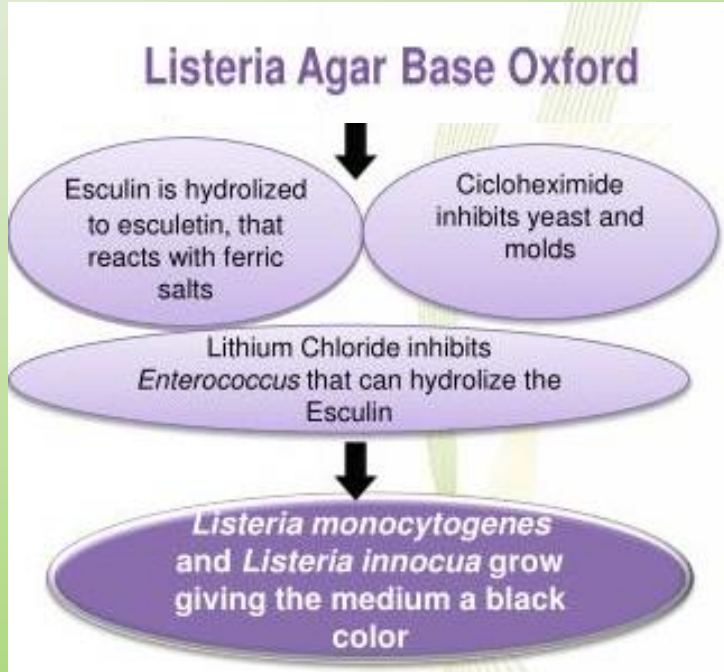
B.3.1.2 Preparation

Dissolve the dehydrated components or dehydrated complete base in the water by boili

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.

Enzymatic Activity	*L. monocytogenes*	*Listeria spp.*	Non-Listeria
PI-PLC detection	+	-	-
β-D-Glucosidase	+	+	-



ISO 11290-1:2017: CONFIRMATION

After incubation, examine the ALOA plates for the presence of presumptive colonies of *L. monocytogenes* or *Listeria* spp.

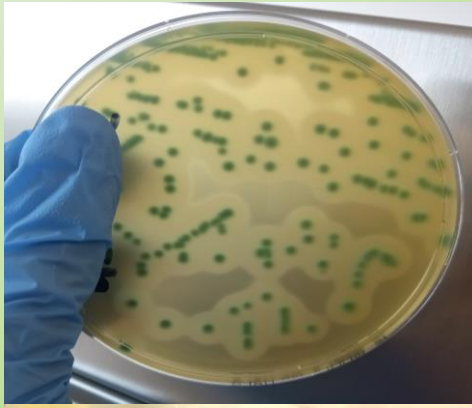
9.4.2 Agar *Listeria* according to Ottaviani and Agosti

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.



After incubation, examine the second selective medium for the presence of presumptive colonies of *L. monocytogenes* or *Listeria* spp., based on their characteristics for the type of the medium used.

ISO 11290-1:2017: CONFIRMATION

Presumptive colonies of *L. monocytogenes* on ALOA…

ISO 11290-1:2017: CONFIRMATION

- ✓ Streak the selected presumptive colonies onto the surface of a non-selective agar, as blood agar, and incubate the plates at 37°C for 18h to 24h or until the growth is satisfactory.

- ✓ After incubation, non-selective plates will be used to perform confirmation tests:

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)	+

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.

ISO 11290-1:2017: CONFIRMATION

For *Listeria* spp.

Microscopic aspect:

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.

Catalase:

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.

$2H_2O_2 \longrightarrow 2H_2O + O_2$

Hydrogen peroxyde → Water + Oxygen

Listeria spp. is catalase positive.

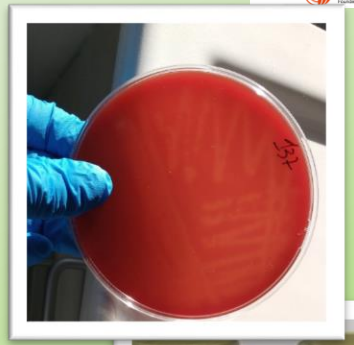
ISO 11290-1:2017: CONFIRMATION

For *L. monocytogenes*

Hemolysis:

The use of blood agar for pure culture enables interpretation of hemolysis, when is positive.

L. monocytogenes show narrow, clear, light zone of hemolysis (β- hemolysis).



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.

Carbohydrate utilization:

Using a loop, inoculate each of the carbohydrate broth (L-Rhamnose and D-Xylose) with the cultures obtained from the non-selective agar and incubate at 37°C for 24 h.

Positive reactions (acid formation) are indicated by a yellow color.

L. monocytogenes is L-Rhamnose positive and D-Xylose negative.

ISO 11290-1:2017: RESULTS

LISTERIA SPP. VS LISTERIA MONOCYTOGENES

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

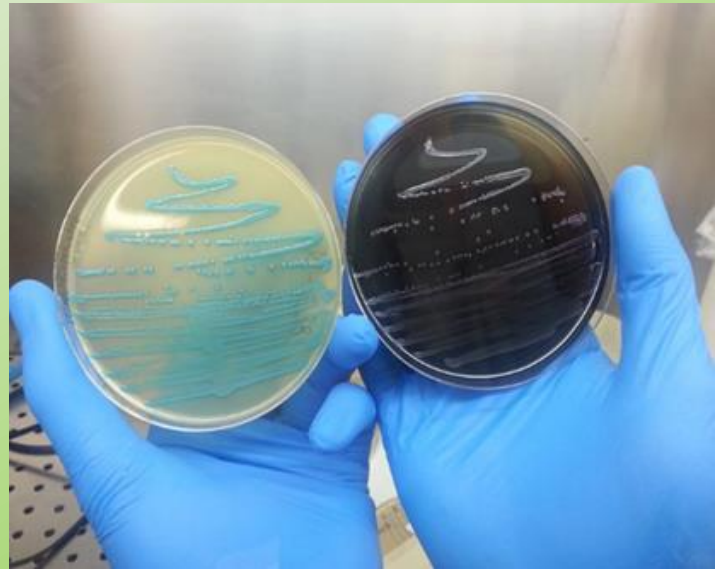
(+): weak reaction

+ : more than 90 % of positive reactions

-: no reaction

ISO 11290-1:2017: EXPRESSION OF RESULTS

In accordance with the interpretation of the results, report if *L. monocytogenes* and/or if *Listeria* spp. is detected or not detected in the test portion, by specifying the mass in grams or the volume in milliliters of the sample tested.



CONCLUSION

Additional characterization of isolated strains:

Isolates which are considered to be *L. monocytogenes* may be send for further **characterization** to a recognized national or regional *Listeria* Reference Laboratory.

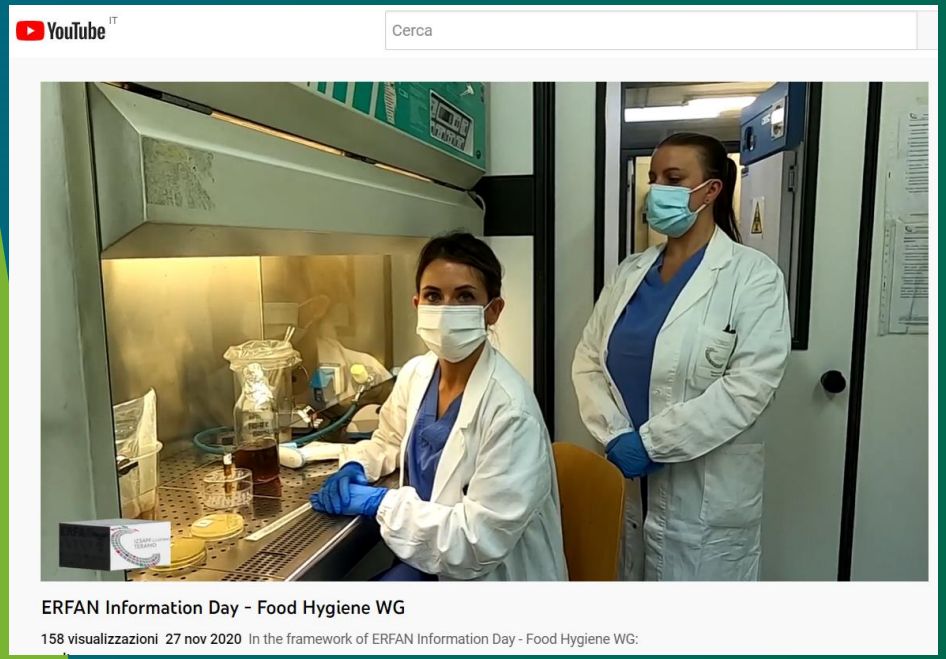
NATIONAL REFERENCE LABORATORY FOR *LISTERIA MONOCYTOGENES*

WGS (whole genome sequencing) is a DNA sequencing approach used to obtain the complete DNA sequences (genome).

The isolates sequence data can be compared via Internet to those in database (es. https://bigsdb.pasteur.fr/listeria/listeria.html) in order to:

- ✓ study differences between the same species;
- ✓ study *in silico* the presence of virulence genes, antibiotic resistance genes, detergent resistance genes, etc;
- ✓ study the isolates correlation during outbreaks cases.

THANK YOU!



g.centorotola@izs.it

https://www.youtube.com/watch?v=0JxlNGkGTJM&t=86s