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Applicability of the EN ISO 11290-1 standard method for *Listeria monocytogenes* detection in presence of new *Listeria* species

Léna Barre [a], Apostolos S. Angelidis [b], Djouher Boussaid [a], Emilie Decourseulles Brasseur [a], Eléonore Manso [a], Nathalie Gnanou Besse [a,*]

[a] Université Paris Est, ANSES, Maisons-Alfort Laboratory for Food Safety, F-94701 Maisons-Alfort, France
[b] Laboratory of Milk Hygiene and Technology, Department of Food Hygiene and Technology, School of Veterinary Medicine, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece

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ABSTRACT

During the past six years, new species of the genus *Listeria* have been isolated from foods and other environmental niches worldwide. The Standard method EN ISO 11290-1 that is currently under revision will include in its scope all *Listeria* species in addition to *L. monocytogenes*. The objective of this project was to evaluate the ability of the Standard EN ISO 11290-1 method to detect and identify the newly discovered *Listeria* spp., and to assess potential over-growth effects of the new species in mixed cultures with *L. monocytogenes* during each step of the enrichment process. This objective was addressed by the generation of necessary data on the behavior of the new species during the pre-enrichment and the enrichment steps of the reference method as well as data on their phenotypic characteristics on rich and selective media used for isolation and identification.
Most of the new *Listeria* species developed well on selective agar media for *Listeria*, however the recovery of some species was difficult due to poor growth in Half Fraser and Fraser broth. Good results (consistently positive) were obtained for confirmation at the genus level via the catalase test, the Gram test and the blueish appearance test on non-selective medium, but not with the VP test, as most of the new species yielded a negative result. In the light of results obtained in co-culture experiments and inhibition tests, and considering the growth rates in Half Fraser and Fraser broths, the new species do not seem to interfere with the detection of *L. monocytogenes*.

1. Introduction

L. monocytogenes is a Gram-positive bacterium responsible for listeriosis, a severe foodborne illness which may result in meningitis, septicemia, spontaneous abortion, perinatal infections and gastroenteritis. Despite the low incidence of infection in humans, listeriosis is associated with a high lethality, particularly in elderly and immuno-compromised individuals (Anon, 2000). Moreover, since 2000, an increase in the number of listeriosis cases has been observed in several European countries, but the reasons for this phenomenon still remain unclear (Anon, 2007; Anon, 2015; Rizzi, 2014). In addition, the detection of *L. monocytogenes* in food has important economic consequences, because it can lead to the withdrawal of incriminated products and subsequent decrease of sales. All six *Listeria* spp. described before 1985 (*L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. welshimeri*, *L. seeligeri* and *L. grayi*; hereafter referred to as "classic" *Listeria* spp.) can be isolated from food, and the distribution of species recovered may vary both according to food type and the detection methodology used, although *L. innocua* and *L. monocytogenes* are the most frequent food isolates.

Recently, 11 "new" species of the genus *Listeria* were identified. These new species were isolated from foods and other environmental niches around the world, including agricultural and natural environments: *L. marthii*, *L. rocourtiae*, *L. weihenstephanensis*, *L. cornellensis*, *L. riparia*, *L. grandensis*, *L. fleischmannii*, *L. aquatica*, *L. floridensis*, *L. newyorkensis* and *L. booriae* (Bertsch et al., 2013; den Bakker et al., 2014; Graves et al., 2010; Lang Halter et al., 2013; Leclercq et al., 2010; Sauders et al., 2012; Weller et al., 2015). Hence, the genus *Listeria* currently contains 18 species (Leclercq et al., 2016; Wiedmann, 2016). Based on published data on the absence of major virulence genes in their genome, the 11 new species of *Listeria* are considered to be non-pathogenic for humans (Wiedmann, 2016). Molecular analysis can currently distinguish between two genetically remote groups of *Listeria*: one group includes all but one (*L. grayi*) of the six "classic" *Listeria* spp. plus *L. marthii* (referred to as *Listeria* "sensu stricto"), and the other group includes all the new species (except *L. marthii*) plus *L. grayi* (referred to as *Listeria* "sensu lato") (Leclercq, 2014; Wiedmann, 2016). With the generalization of molecular methods, in particular sequencing, the discovery of new species is likely to continue in the future. For instance, recently, Leclercq et al. (2016) proposed to include a new species in the genus *Listeria*: *L. thailandensis*, isolated from fried chicken products in Thailand. Moreover, Orsi and Wiedmann (2016) proposed a reclassification of *Listeria* "sensu lato" in 3 new genera.

* Corresponding author.
E-mail address: nathalie.gnanou-besse@anses.fr (N. Gnanou Besse).

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Some of the new species of *Listeria* are of particular interest in food hygiene. Thus, *L. newyorkensis* and *L. booriae* have been isolated from seafood and dairy processing plants, respectively (Weller et al., 2015); *L. fleischmannii* was isolated from hard cheese (Bertsch et al., 2013), *L. rocourtiae* from pre-cut lettuce (Leclercq et al., 2010) and *L. marthii* from factories producing meat products in the United States (Leclercq A., personal communication). In addition, from a genetic viewpoint, *L. marthii* is very closely related to *L. monocytogenes* (den Bakker et al., 2010; Graves et al., 2010). The ability to readily detect and characterize the new *Listeria* species has practical food safety implications, because the presence of *Listeria* spp. in foods or food-processing environments is often used as an indicator of a potential contamination by *L. monocytogenes*. Commission Regulation (EC) No. 2073/2005 (Anon, 2005) lays down the safety criteria for *L. monocytogenes* in foods, both qualitative (absence in 25 g) and quantitative (less than 100 CFU/g). This Regulation cites as reference methods the standard methods EN ISO 11290-1 (Anon, 1996, 2004a) & 11290-2 (Anon, 1998, 2004b) for the detection and enumeration of *L. monocytogenes* in foods, respectively, which are currently being revised. Since the Standard methods under revision will include in their scope all other *Listeria* species in addition to *L. monocytogenes* (as indicators of potential contamination with *L. monocytogenes*), it is necessary to check the methods' ability to recover and identify the newly recognized *Listeria* species. In particular, certain characteristics of these newly discovered species remain unknown, such as their growth on commonly used *Listeria* selective isolation agars and in the selective enrichment broths, their reaction to the biochemical confirmation tests used in the Standard methods, and their possible impact on the detection of other *Listeria* spp., in particular on *L. monocytogenes*. Indeed, in the ISO 11290-1 Standard, the isolation of *L. monocytogenes* from foods is carried out using a double enrichment procedure. In cases where multiple *Listeria* species are present within the original sample (food), *L. monocytogenes* can be overgrown by other species of *Listeria* during the enrichment steps. From a practical perspective, this can result in a false-negative result and even complicate the ability of public health investigators to match food and clinical isolates. We have previously investigated this phenomenon by analysing the growth kinetics of single species and pairs of different species over the ISO 11290-1 enrichment process. Inter-strain competition mechanisms may be explained by the production of bacteriocins or bacteriophage, nutritional competition, or may be the result of different growth rates (Gnanou Besse et al., 2005, 2010). Culture overgrowth resulted primarily from interactions which occurred in late-exponential phase, where it was observed that the growth of all strains stopped when the dominant strain reached stationary phase. To date, there are no studies that have examined the evolution of the newly discovered *Listeria* spp. during the enrichment process, and their potential impact on *L. monocytogenes* growth.

The present work had two major objectives: a) to evaluate the inclusiveness of the Standard EN ISO 11290-1 method, i.e. its ability to detect a wide range of new *Listeria* spp., and b) to assess the potential impact of the new *Listeria* spp. and of strain over-growth on the detection of *L. monocytogenes*. This issue was investigated via the determination of growth rates, the assessment of production of inhibitory activities, and the detection performances in co-culture experiments.

2. Materials and methods

2.1. Strains

Strains belonging to the recently identified species of the genus *Listeria* (Table 1) were obtained from the German DSMZ collection (www.dsmz.de). A representative panel of *L. monocytogenes* strains previously isolated and characterized in our laboratory, was also included in this project in order to study the impact of the new *Listeria* species on the detection of *L. monocytogenes* (Table 2). *L. monocytogenes* strains were selected to reflect diversity of food origin and serotype or molecular serogroup. In addition, internal reference strains of *L. monocytogenes* (CHPL10), *L. innocua* (CHPL11), *L. ivanovii* (CHPL02) and *L. welshimeri* (CHPL04) were used as controls.

Table 1
Strains of the new *Listeria* species used in this work.

Listeria species	Strain
L. aquatica	DSM 26686
L. booriae	DSM 28860
L. cornellensis	DSM 26689
L. fleishmanii subsp. *coloradonensis*	DSM 25391
L. fleishmanii subsp. *fleishmanii*	DSM 24998, DSM 25003
L. floridensis	DSM 26687
L. grandensis	DSM 26688
L. marthii	DSM 23813
L. newyorkensis	DSM 28861
L. riparia	DSM 26685
L. rocourtiae	DSM 22097
L. weihenstephanensis	DSM 24698, DSM 24699

Stock cultures were maintained frozen at −80 °C using Cryobank tubes (BioMérieux, Combourg, France). Cultures were revived by plating onto Tryptone Soya Agar with Yeast Extract (TSAYE) and then propagated twice in Brain Heart Infusion (BHI, BioMérieux) broth at 30 °C before use. All dilutions were made in Tryptone Salt (TS) broth (BioMérieux).

2.2. Inclusiveness of the EN ISO 11290-1 standard method

The inclusiveness of the Standard method EN ISO 11290-1 (existing version, and draft of the ongoing revision), i.e. its ability to detect a wide range of new species, was tested with each of the new *Listeria* species. The method specifies a sequential double enrichment of the food under analysis in Half-Fraser and Fraser selective broths. The initial incubation in Half-Fraser is carried out for 24 h at 30 °C, followed by a second enrichment in Fraser broth for 48 h at 37 °C. After incubation and isolation on selective agars, typical colonies are submitted to confirmation tests described in the Standard. Agar *Listeria* according to Ottaviani and Agosti (ALOA), a chromogenic selective agar, which distinguishes pathogenic *Listeria* spp. (*L. monocytogenes* and *L. ivanovii*) from other species of *Listeria*, was adopted in 2004 by the International Organization for Standardization as the standard medium for the detection of *L. monocytogenes*. Following incubation at the fore-mentioned times and temperatures, the enrichment broths must be streaked both on ALOA and on another selective agar of own choice. In addition, the *Listeria* ad-hoc Technical Advisory Group (TAG) 17 of the CEN/TC 275/WG 6 European committee (Microbiology of the food chain), in charge of the ongoing revision and validation of the Standard, recommended to reduce the duration of the 2nd enrichment step by 24 h. The confirmation tests for the genus *Listeria* are the Gram staining and the catalase test (as mandatory tests), and the motility and Henry illumination tests (as optional tests). In a draft of the ongoing revision of the Standard, it

Table 2
Isolation origin and molecular serogroup or serotype of *Listeria monocytogenes* strains used in this work.

Strain	Origin	Molecular serogroup or serotype
09CEB387LM	Coppa (cured meat)	4b
07CEB139LM	Smoked sausage	IIb
09CEB633LM	Egg fried rice	IIc
10CEB580LM	Tomatos	IIa
08CEB87LM	Salmon	IIa
10CEB58LM	Merguez (spiced lamb sausage)	IIa
09CEB337LM	Cantal (regional French cheese)	IIa
10CEB56LM	Sausage	IIc
Lm ap 3	Turkey meat	1/2c
Lm ap 5	Smoked salmon	4b
Lm ap 4	Turkey meat	1/2a
Lm ap 7	Uncooked sausage	1/2c