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Determination of *Listeria monocytogenes* numbers at less than 10 cfu/g

K. Hunt, M. Vacelet, K. Jordan†

Food Safety Department, Teagasc Food Research Centre, Moorepark, Fermoy, County Cork, Ireland

Abstract

Listeria monocytogenes **is a foodborne pathogen that causes a relatively rare foodborne disease called listeriosis, with a high mortality rate of 20%–30% and an undefined dose response. Current European Union regulations permit up to 100 colony-forming units (cfu)/g in food at the end of its shelf life, where the food has been shown not to support the growth of this pathogenic bacterium. Therefore, enumeration of *L. monocytogenes* at low numbers in food is important. The objective of this study was to reduce the detection limit of *L. monocytogenes* in food by a factor of 10. The International Organisation for Standardisation (ISO) 11290-2 method for enumeration of *L. monocytogenes* in food recommends spreading 0.1 mL of a 1:10 dilution of the food on the surface of an agar plate (detection limit 100 cfu/g), or 1.0 mL spread in equal parts on the surface of three agar plates (detection limit: 10 cfu/g). The pour-plate method (using 1 or 10 mL of an appropriate dilution) was compared to the spread-plate method using the ISO-approved chromogenic medium Agar Listeria according to Ottaviani and Agosti (ALOA). Using the pour-plate method, the colony morphology and halo formation were similar to the spread-plate method from pure cultures and inoculated foods. Using the pour-plate method in a 140 mm Petri dish, 10 mL of a 1:10 dilution of food allowed determination of numbers as low as 1 cfu/g. Applying this method, *L. monocytogenes* in naturally contaminated food samples were enumerated at numbers as low as 1–9 cfu/g.**

Keywords

ALOA agar • Listeria monocytogenes • low bacterial numbers • microbial detection limits in food • pour plate

Introduction

Listeria monocytogenes is a Gram-positive bacterium, in the division Firmicutes, which is a non-spore-forming, catalase-positive, oxidase-negative and motile foodborne pathogenic rod widely dispersed in the environment, being found in soil, water and plant material. It can survive desiccation (Takahashi *et al*., 2011) and grows at refrigeration temperature (Schmid *et al*., 2009), at pH 4.7 and at 10% salt, as well as being able to grow optimally at 35°C and 37°C. Thus, it can persist in the harsh conditions of the food-processing environment, from which it can contaminate food (Lin *et al*., 2006). Due to the lack of cooking, or similar bacterial inactivation step, *L. monocytogenes* can persist in ready-to-eat food. *L. monocytogenes* represents a problem in the food industry because this ubiquitous bacterium is a causative agent of listeriosis, a disease that can be particularly severe, and even fatal, in high-risk groups, such as the elderly, neonates and the immunocompromised, with a mortality rate of 25% worldwide (de Noordhout *et al*., 2014).

The genus *Listeria* includes many different species, including *L. monocytogenes*, *L. ivanovii, L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. grayi,* as well as some recently described species, such as *Listeria floridensis sp. nov*., *Listeria aquatica sp. nov*. and *Listeria cornellensis sp. nov* (den Bakker *et al*., 2014). *L. monocytogenes* is the only member of the species that has been associated with human illness, although there are some reports of illness associated with *L. ivanovii* (Snapir *et al*., 2006). Strains of *L. monocytogenes* are subdivided into 13 serotypes, all of which can cause disease, but with >90% of disease-causing clinical isolates belonging to only four serotypes: 1/2a, 1/2b, 1/2c and 4b (Orsi *et al*., 2011).

Food products are usually contaminated at low levels (Auvolat and Besse, 2016). In many cases, food is a favourable environment to support the growth of *L. monocytogenes* and, although contaminated at low levels, numbers can increase to levels high enough to cause infection. When bacterial numbers can increase in foods or if the food is used for feeding high-risk groups, European Union (EU) regulations require the absence of *L. monocytogenes* in 5 × 25 g of food. If food does not support the growth of *L. monocytogenes,* then the regulations allow up to 100 colony-forming units (cfu)/g during the shelf life. In

†Corresponding author: Kieran Jordan
E-mail: kieran.jordan@teagasc.ie

such foods, it is important to monitor *L. monocytogenes* at low numbers, less than this limit of 100 cfu/g (European Commission [EC], 2005).

Agar Listeria according to Ottaviani and Agosti (ALOA) is a chromogenic medium used in the detection and enumeration of *L. monocytogenes*. This medium shows *L. monocytogenes* forming regular round colonies, with a blue-green colour and an opaque halo, which allows differentiation from most non-pathogenic species of *Listeria*. This halo is due to the activity of a phospholipase (phospholipase C) involved in the infection process of pathogenic *Listeria* (Ottaviani *et al*., 1997). The International Organisation for Standardisation (ISO) standard method 11290-2 specifies that a sample (diluted 1:10, if solid or opaque) should be spread on the surface of the agar by the spread-plate technique using 100 µL spread on one plate (detection limit: 100 cfu/g) or 1 ml spread evenly on three plates (detection limit: 10 cfu/g) (ISO, 1998). Even with a detection limit of 10 cfu/g, there is only a one-log difference between the detection limit and the regulatory limit, a small window of opportunity to monitor growth.

The aim of this study was to determine whether the pour-plate method could detect *L. monocytogenes* on ALOA agar with 10 times greater sensitivity, giving a two-log window of opportunity for monitoring growth.

Materials and methods

Strains used in this study

Five strains of *L. monocytogenes*, which were isolated from different foods or food environments, were used. Strains 6179 and 999 (serotype 1/2a) were isolated from cheese and the processing environment, respectively; strain 1055 (serotype 1/2b) was isolated from the floor in a food-processing environment; strain 1157 (serotype 1/2c) was isolated from a drain in a meat-processing environment; and strain 1181 (serotype 4b/4e) was isolated from a farm environment. The strains were conserved in cryovials (VWR, West Chester, PA, USA), in the freezer at –20°C.

For each experiment, cultures of these strains were grown at 37°C for 18 h in brain heart infusion (BHI; Merck, Darmstadt, Germany) broth. Thus, 10 µL of the culture was suspended in 10 mL of BHI broth, which was incubated at 37°C for 24 h. This culture was serially diluted in maximum recovery diluent (MRD; Oxoid, Chester, UK), as appropriate.

Spread-plate method

For each appropriate dilution, 0.1 mL or 1 mL was spread on the surface of a pre-poured ALOA (Oxoid) Petri dish, 90 mm or 140 mm, respectively, using a sterile plastic spreader. The plates were incubated at 37°C for 24–48 h.

Pour-plate method

For each appropriate dilution, 1 mL was added to a 90 mm Petri dish and approximately 30 mL of ALOA agar medium was added. Alternatively, 10 mL was added to a 140 mm Petri dish and approximately 90 mL of ALOA was added. In each case, the ALOA agar medium was made up with 10% less water. The plates were mixed well by swirling, allowed to solidify and incubated at 37°C for 24–48 h.

Comparison with alternative chromogenic agar Brilliance Listeria Agar (BLA)

Using the spread-plate and pour-plate methods, on a sample with a target inoculum of 150 cfu/mL of the test strain *L. monocytogenes* 6179, ALOA was compared with BLA (Oxoid) in 90 mm agar plates.

Artificial inoculation of food samples

Pate, milk, feta cheese and salmon were purchased from a local supermarket. Prior to inoculation, all foods were tested for the presence of *L. monocytogenes* by the ISO 11290-1 method for detection (ISO, 1997). The test strain *L. monocytogenes* 6179 was inoculated by spreading the appropriate dilution in MRD (Oxoid) onto each food item (or mixed in the case of milk) to give the required level of inoculation. Following inoculation, 10 g of sample was mixed with 90 mL of MRD and 0.1 or 1 mL was spread on pre-poured ALOA agar plates (either 90 mm or 140 mm), while 1 and 10 mL was tested using the pour-plate method, using 90 mm or 140 mm Petri dishes, respectively. The plates were incubated at 37°C for 24–48 h.

Naturally contaminated food samples

Naturally contaminated smoked salmon was obtained from a local producer. Thus, 25 g of salmon was added to 225 mL of half Fraser broth and tested by spreading or pouring the appropriate volume on or in ALOA, respectively, as described. The plates were incubated at 37°C for 24–48 h.

Statistical analysis

Statistical analysis of the data was carried out using Graph Pad InStat (Version 6.0; Graph Pad Software, Inc., San Diego, CA, USA), for calculation of mean values, standard deviations and regression analysis, and Predictive Analytics SoftWare (PASW) V18 (IBM Corp., Armonk, NY, USA), for determining the *P*- values using the Mann–Whitney test.

Results

Strain difference

The results showed that colony morphology, colour and halo formation were similar when using either the spread-plate or the pour-plate method. No significant difference in numbers

($P > 0.05$) was seen between the pour-plate method and the spread-plate method for all the five *L. monocytogenes* isolates tested, with a target inoculum of 20 cfu/mL (Figure 1).

Comparison of pour-plate and spread-plate methodologies

As there was no strain difference, a control strain 6179 was selected for further study using 90 mm and 140 mm Petri dishes and the pour-plate method. No significant difference ($P > 0.05$) in colony counts was observed between the pour-plate and spread-plate methods, with a target inoculum of low and high inoculation levels in 140 and 90 mm agar plates, respectively (Figure 2).

Comparison of ALOA and BLA

Using both spread-plate and pour-plate methods with ALOA and BLA at 150 cfu/mL, no significant ($P > 0.05$) difference was seen between the different agars (data not shown).

Artificially inoculated food

All the foods used were initially negative for the presence of *L. monocytogenes*. For all four food types tested, there was no significant difference ($P > 0.05$) in the numbers detected when the samples were analysed by pour-plate (1 mL) and spread-plate (0.1 mL) techniques (Figure 3).

Naturally contaminated food samples

Table 1 shows the results obtained by direct detection and enrichment from two batches of naturally contaminated smoked salmon. The numbers were detected at <10 cfu/g on one batch, while the second batch contained >100 cfu/g.

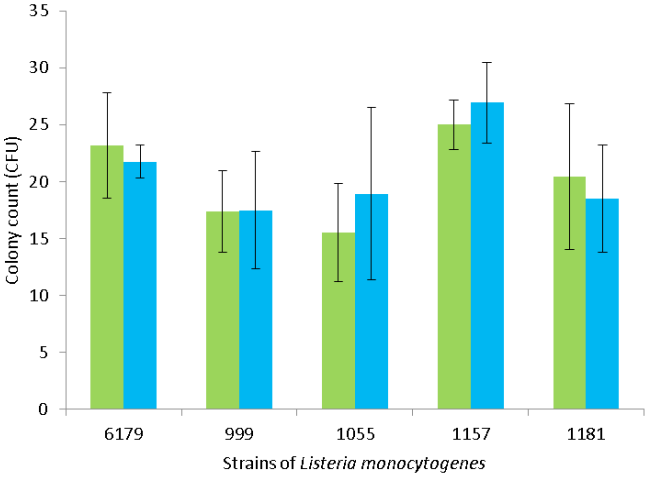

Figure 1. Comparison of colony counts with five strains of *L. monocytogenes* determined by two methods, namely, the pour-plate (green) and spread-plate (blue) methods. The average and standard deviation values of five replicates are shown for each strain. CFU = colony-forming units.

Discussion

Commercially available agars such as COMPASS Listeria Agar (Biokar, 2016), ALOAÒ, chromID™ Lmono Agar (both BioMérieux, 2016) and RAPID'L.mono Agar (Bio-Rad, 2016) have all been validated by the companies for use with the pour-plate method, using ISO 16140 (ISO, 2016). However, an independent verification of the pour-plate method using chromogenic agar is needed.

The ISO 11290-2 recommends the use of the spread-plate method; therefore, this study looked at the pour-plate method to increase the sensitivity of detection of *L. monocytogenes* in food by 10-fold. Increased sensitivity is beneficial when a food item does not support the growth of *L. monocytogenes* as defined by EU regulations, allowing bacterial numbers to

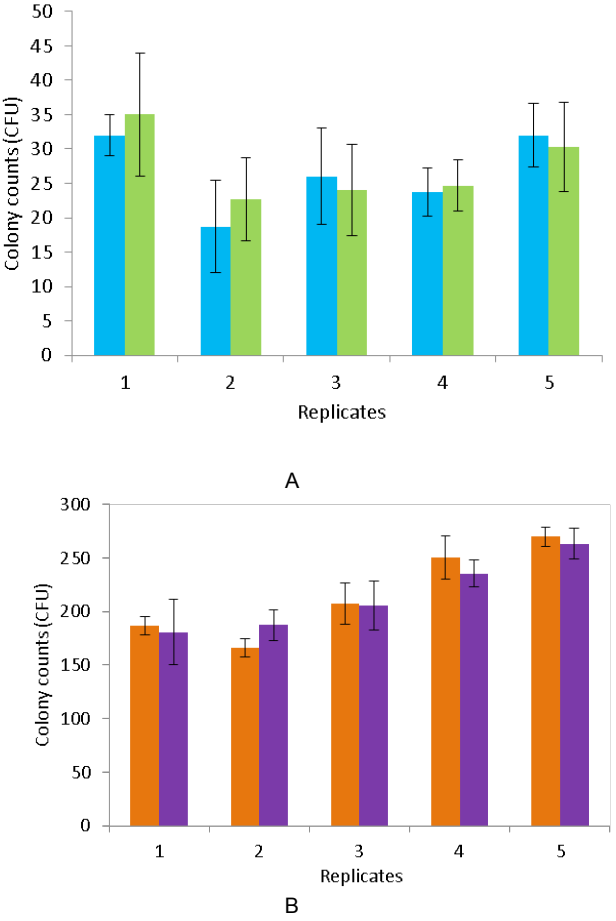

Figure 2. Comparison of colony counts of *L. monocytogenes* strain 6179 determined by (A) pour-plate (blue) and spread-plate (green) methods on ALOA using 140 mm and (B) pour-plate (orange) and spread-plate (purple) methods on ALOA using 90 mm Petri dishes. The average and standard deviation values of four replicates are shown for each the five experiments. ALOA = Agar Listeria according to Ottaviani and Agosti; CFU = colony-forming units.

be present up to 100 cfu/g (EC, 2073/2005). However, the ability to detect at 1–10 cfu/g results in an opportunity to study the growth over two log cycles, giving regulators and food business owners more control over food safety in their products.

When making up the agar for the pour-plate method, 10% less water was added to account for the dilution of the agar with the sample. Although there was 8% difference in the dilution rate between the 90 mm and 140 mm Petri dishes, this was not considered a significant difference (European Pharmacopoeia, 2006).

Using the pour-plate method, colonies were embedded in the agar; however, this had no influence on the blue-green colour of the colonies or on the development of the opaque halo. Similar results were obtained while using the pour-plate method for detection of *E. coli* O157 by Jordan and Maher (2006), using the *E. coli* O157 ID medium. From a practical point of view, recovering colonies that are embedded in agar is more challenging than recovering surface colonies from a spread plate; however, recovering embedded colonies to transfer to new agar is feasible, particularly when balanced against a 10-fold increase in sensitivity.

When determining the numbers of *L. monocytogenes* in milk samples, it can sometimes be difficult to observe the halo associated with *L. monocytogenes*, resulting in possible false negatives. Although it is difficult to see the halo, it was not impossible when diluted 1:10 as food, especially after 48 h.

Similar to *L. monocytogenes*, *L. ivanovii*, another *Listeria* sp., has a blue-green colony and an opaque halo on chromogenic agars, like ALOA and BLA. In this study, *L. ivanovii* isolates were not tested for their colony morphology using the pour-plate method. Further studies to determine the colony

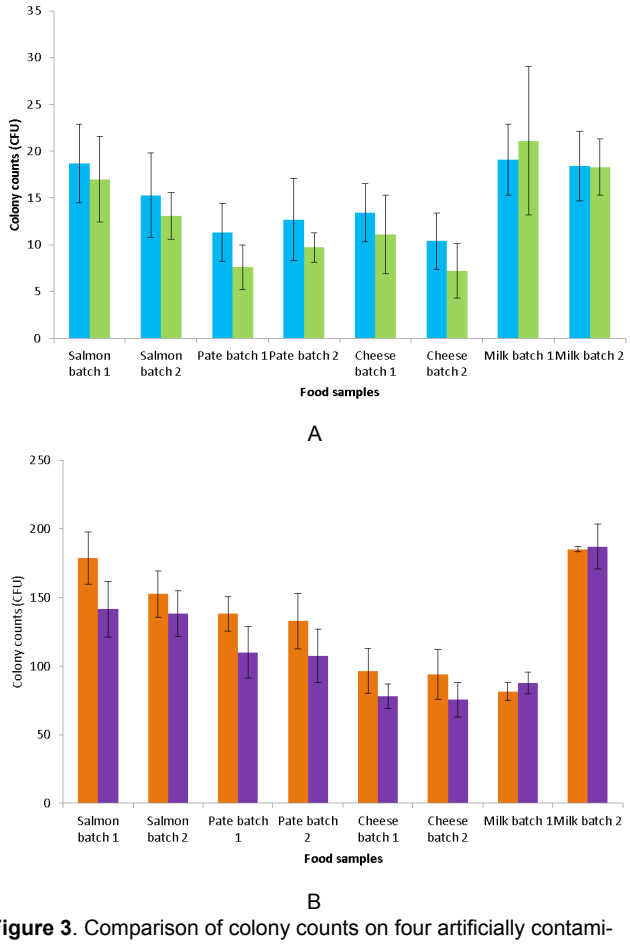

Figure 3. Comparison of colony counts on four artificially contaminated food products determined (A) by pouring (blue) and spreading (green) methods at low and (B) by pouring (orange) and spreading (purple) methods at high inoculum levels. CFU = colony-forming units.

Table 1. Analysis results of six samples of naturally contaminated smoked salmon from two different batches from one local supplier

Samples	Enumeration (in triplicate), CFU/g[1]	Plate size, mm	Method	Enrichment
		Batch 1		
1	12, 10, 9	140	Pour 10 mL	+
2	<1, 1, 3	140	Pour 10 mL	+
3	4, 2, 1	140	Pour 10 mL	+
		Batch 2		
1	>100	140	Pour 10 mL	+
2	>100	140	Pour 10 mL	+
3	>100	140	Pour 10 mL	+

[1]CFU = colony-forming units.

morphology of *L. ivanovii* with the pour-plate method are required. A potential disadvantage of the pour-plate method is that it is possible that some bacteria could grow in the semi-anaerobic environment of the pour-plate but not in the aerobic environment of the spread plate. Such bacteria are unlikely to result in false positives but could cause overcrowding of the plate and result in false negatives. To prevent this occurrence, the macerated sample should be kept in the refrigerator and, if the problem arises, it could be pour plated at a different dilution.

EU challenge studies with *L. monocytogenes* recommend an inoculation level of 100 cfu/g (Beaufort *et al*., 2014). Studying the growth potential of *L. monocytogenes* in food using this method allows the sensitivity of 1 cfu/g a better opportunity to determine growth. Detection at a lower limit of 10 cfu/g (the current detection limit) gives a one-log window of opportunity for growth before the regulatory limit is reached, whereas detection at a limit of 1 cfu/g gives a much greater two-log window of opportunity for studying growth.

From a cost perspective, three 90 mm Petri dishes are required to achieve a sensitivity of 10 cfu/g of food using the ISO 11290 method (0.33 mL of homogenate spread on the surface of each plate). Using a single 140 mm Petri dish with 10 mL of homogenate, the amount of agar used is approximately the same as three 90 mm plates. Therefore, there are no increased costs associated with using larger agar plates.

The results of this study demonstrate the option of using the pour-plate method for enumeration of *L. monocytogenes* at a 10-fold greater sensitivity and should be considered in future revisions of standard methods.

Conclusions

A 10-fold greater sensitivity for enumeration of *L. monocytogenes* from food can be achieved using 10 mL of a 1:10 dilution of food homogenate in a 140 mm agar plate by the pour-plate method using chromogenic agar. Colony colour and halo formation were not affected, and there are no cost implications in terms of media usage. Greater sensitivity is an advantage in studying lower, more realistic numbers of *L. monocytogenes* in naturally contaminated food.

Acknowledgements

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