Food Microbiology 80 (2019) 1–8

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Development and application of Peptide Nucleic Acid Fluorescence *in situ* Hybridization for the specific detection of *Listeria monocytogenes*

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ARTICLE INFO

Keywords:
Listeria monocytogenes
PNA-FISH
Blocker probe
Enrichment procedure

ABSTRACT

Listeria monocytogenes is one of the most important foodborne pathogens due to the high hospitalization and mortality rates associated to an outbreak. Several new molecular methods that accelerate the identification of *L. monocytogenes* have been developed, however conventional culture-based methods still remain the gold standard. In this work we developed a novel Peptide Nucleic Acid Fluorescence *in situ* Hybridization (PNA-FISH) method for the specific detection of *L. monocytogenes*. The method was based on an already existing PNA probe, LmPNA1253, coupled with a novel blocker probe in a 1:2 ratio. The method was optimized for the detection of *L. monocytogenes* in food samples through an evaluation of several rich and selective enrichment broths. The best outcome was achieved using One Broth Listeria in a two-step enrichment of 24 h plus 18 h. For validation in food samples, ground beef, ground pork, milk, lettuce and cooked shrimp were artificially contaminated with two ranges of inoculum: a low level (0.2–2 CFU/25 g or mL) and a high level (2–10 CFU/25 g or mL). The PNA-FISH method performed well in all types of food matrices, presenting an overall accuracy of ≈99% and a detection limit of 0.5 CFU/25 g or mL of food sample.

1. Introduction

Listeria spp. are Gram-positive, facultative anaerobic, non-spore-forming, rod-shaped bacteria with a low G + C content (Ponniah et al., 2010). The *Listeria* genus is composed of seventeen species and among them, *Listeria monocytogenes* is a primary human pathogen. Nonetheless, there have been rare reports of illnesses caused by *Listeria seeligeri*, *Listeria ivanovii* and *Listeria innocua* (Gasanov et al., 2005; Guillet et al., 2010; Perrin et al., 2003; Weller et al., 2015).

L. monocytogenes is recognized worldwide as an important foodborne pathogen due to high morbidity, hospitalization (over 90%) and mortality (25–30%) rates in vulnerable populations (pregnant, neonates, elderly and immunocompromised people) (Zunabovic et al., 2011). Symptoms of listeriosis range from flu-like illness to severe complications including meningitis, septicemia and spontaneous abortion (FAO/WHO, 2004). In 2015 the European Food Safety Authority (EFSA) and the European Center for Disease Prevention and Control (ECDC) reported 2206 confirmed human cases of listeriosis in the 28 European Member States (0.46 cases per 100,000 population), 97.4% of which needed hospitalization. More importantly, 270 of these cases were fatal. EFSA/ECDC also reported an increasing trend of listeriosis since 2008, but the number of cases has stabilized from 2014 onwards (EFSA/ECDC, 2015; 2016). Infection with *L. monocytogenes* in humans occurs in 99% of the cases as a result of consumption of contaminated ready-to-eat and raw food products such as vegetables, milk, soft cheese, meat, poultry, seafood and dairy products (Mead et al., 1999; Schlech and Acheson, 2000; Volokhov et al., 2002).

The detection, differentiation and identification of *Listeria* spp. usually depends on phenotypic, biochemical and immunological assays as well as genotypic methodologies (Zunabovic et al., 2011). Conventional culture-based methods for detecting *Listeria* spp. in foods, ISO 11290–1:1996, are simple to perform, but they are also time-consuming and take too long to deliver the results. Consequently, culture-independent approaches, such as Peptide Nucleic Acid Fluorescence *in situ* Hybridization (PNA-FISH) techniques, have become important tools for the specific, reliable and fast detection of human pathogens (Rohde

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https://doi.org/10.1016/j.fm.2018.12.009
Received 20 September 2017; Received in revised form 25 February 2018; Accepted 14 December 2018
Available online 15 December 2018
0740-0020/

et al., 2015).

Several 16S or 23S rRNA probes have been developed for the detection of *Listeria* spp. by FISH methods (Almeida et al., 2011; Brehm-Stecher et al., 2005; Fuchizawa et al., 2008, 2009; Moreno et al., 2011; Schmid et al., 2003; Wang et al., 1991; Zhang et al., 2012) but only a few of them are able to specifically detect *L. monocytogenes* (Almeida et al., 2011; Fuchizawa et al., 2009; Moreno et al., 2011; Wang et al., 1991; Zhang et al., 2012). These methods have been described as being highly specific and sensitive but there is no comparison between the probes. Additionally, due to the advent of genome sequencing technologies, public databases are now much more updated and accurate and thus theoretical estimation is more realistic.

In this study we started by performing a theoretical evaluation of the published probes for the specific detection of *L. monocytogenes*. The most promising probe was further evaluated in a representative set of bacterial strains and the addition of a blocker probe was assessed to increase method specificity. Finally, the enrichment step was optimized for the detection of *L. monocytogenes* in food matrices through PNA-FISH and its performance compared to ISO 11290.

2. Materials and methods

2.1. Bacterial strains and culture maintenance

A total of 67 bacterial strains from both the genus *Listeria* and other related genera were included in this study (Table 1). The *Streptococcus* strain was maintained on Columbia agar (Oxoid, United Kingdom) supplemented with 5% (vol/vol) defibrinated horse blood (Probiológica, Portugal) and incubated at 37 °C in a CO_2 incubator (HERAcell 150; Thermo Electron Corporation, United States of America) set to 10% (vol/vol) CO_2 and 5% (vol/vol) O_2. Single colonies were streaked onto fresh plates every 2 or 3 days. *Gemella morbillorum* was grown in Brain Heart Infusion (BHI) (Liofilchem, Italy) supplemented with 5% bovine calf serum; *Brochothrix thermosphacta* was maintained in Corynebacterium agar (1% casein peptone; 0.5% yeast extract; 0.5% Glucose; 0.5% NaCl and 1.5% agar) (Liofilchem); *Lactobacillus paracasei* was maintained in MRS agar (Liofilchem) and *Lactococcus lactis* was maintained in YGLPB medium (1% peptone; 0.3% yeast extract; 0.5% glucose; 0.5% lactose; 0.8% beef extract; 0.25% KH_2PO_4; 0.25% K_2HPO_4; 0.02% $MgSO_4{\cdot}7H_2O$ and 0.005% $MnSO_4{\cdot}4H_2O$) (Liofilchem). *Bacillus thurigiensis* and *Bacillus thermosphacta* strains were grown at 26 °C, while *L. paracasei* and *G. morbillorum* strains were grown under anaerobic conditions. All remaining bacterial species were maintained on BHI at 37 °C and streaked onto fresh plates every 24 h.

2.2. Theoretical evaluation of the probes

The theoretical specificity and sensitivity of the probes described for *L. monocytogenes* were evaluated using the TestProbe analysis software at SILVA database (Quast et al., 2013). The probes were aligned with a total of 1922213 sequences present in the SILVA 16SREF database (last accession in September 2017). They were also tested against the large subunit (23S/28S, LSU) database, to evaluate the existence of possible cross-hybridization. Specificity was calculated as $nLm/(TnLm) \times 100$, where nLm stands for the number of non-*L. monocytogenes* strains that did not react with the probe and TnLm for total of non-*L. monocytogenes* strains examined. Sensitivity was calculated as $Lm/(TLm) \times 100$, where Lm stands for the number of *L. monocytogenes* strains detected by the probe and TLm for the total number of *L. monocytogenes* strains existent in the databases (Almeida et al., 2010).

The selected sequence, 5′- GACCCTTTGTACTAT -3' (Almeida et al., 2011), was synthesized (PANAGENE, South Korea) and the oligonucleotide N terminus attached to Alexa Fluor 568 via a double AEEA linker (-8-amino-3,6-dioxa octanoic acid).

Table 1
Specificity and sensitivity test for PNA-FISH including simultaneously both LmPNA1253 and *Listeria* blocker probe at a 1:2 ratio at 60ºC. The PNA-FISH test was repeated three times for each strain with similar outcomes.

Microorganism	Serotype	PNA-FISH outcome
L. monocytogenes CECT 5873	1/2a	+
L. monocytogenes CECT 5725	4c	+
L. monocytogenes CECT 938	3c	+
L. monocytogenes CECT 911	1/2c	+
L. monocytogenes CECT 933	3a	+
L. monocytogenes CECT 934	4a	+
L. monocytogenes CECT 937	3b	+
L. monocytogenes CECT 936	1/2b	+
L. monocytogenes CECT 4031T	1a	+
L. monocytogenes 747[a]	1/2b	+
L. monocytogenes 812[a]	1/2b	+
L. monocytogenes 832[a]	1/2b	+
L. monocytogenes 924[a]	1/2b	+
L. monocytogenes 925[a]	1/2b	+
L. monocytogenes 930[a]	1/2b	+
L. monocytogenes 994[a]	4 ab	+
L. monocytogenes 1559[a]	1/2b	+
L. monocytogenes 1562A[a]	4b	+
L. monocytogenes 1014[a]	1/2a	+
L. monocytogenes 1360[a]	4b	+
L. monocytogenes 2241[a]	4b	+
L. monocytogenes 2020[a]	1/2c	+
L. monocytogenes 1809[a]	1/2a	+
L. monocytogenes 2723[a]	–	+
L. monocytogenes L1B1[b]	–	+
L. monocytogenes L1D1[b]	–	+
L. monocytogenes L1F3[b]	–	+
L. monocytogenes L1L1[b]	–	+
L. monocytogenes L1L12[b]	–	+
L. innocua CECT 910	6a	–
L. innocua CECT 5376	–	–
L. innocua CECT 4030	–	–
L. innocua 1325[a]	–	–
L. innocua 2110[a]	–	–
L. innocua 1141[a]	–	–
L. ivanovii CECT 913	5	–
L. ivanovii londoniensis CECT 5375	–	–
L. ivanovii ivanovii CECT 5368	5	–
L. ivanovii londoniensis CECT 5374	–	–
L. ivanovii ivanovii CECT 5369	–	–
L. ivanovii 1326[a]	–	–
L. seeligeri CECT 917	1/2b	–
L. seeligeri CECT 5340	–	–
L. seeligeri CECT 5339	6b	–
L. seeligeri 2136[a]	–	–
L. welshimeri CECT 919	6a	–
L. welshimeri CECT 5370	1/2b	–
L. welshimeri CECT 5380	–	–
L. welshimeri CECT 5371	6a	–
L. grayi CECT 942	–	–
L. grayi CECT 931	–	–
Brochothrix thermosphacta CECT 847	–	–
Bacillus cereus[c]	–	–
Bacillus thuringiensis CECT 197	–	–
Enterococcus faecalis CECT 183	–	–
Enterococcus faecium CECT 410	–	–
Lactococcus lactis CECT 188	–	–
Lactobacillus paracasei CECT 277	–	–
Gemella morbillorum CECT 991	–	–
Staphylococcus aureus CECT 259	–	–
Staphylococcus aureus[c]	–	–
Staphylococcus epidermidis CECT 4184	–	–
Staphylococcus epidermidis CECT 231	–	–
Streptococcus mutans[c]	–	–
Escherichia coli CECT 533	–	–
Escherichia coli CECT 515	–	–
Salmonella dublin SGSC 2470	–	–

[a] Isolated strain provided by Professor Paula Teixeira from the Catholic University, Porto - Portugal.
[b] Isolated strain provided by Professor Marta Cabo from the Institute of Marine Research, Vigo - Spain.
[c] Isolated strain from our group.

2.3. Blocker probe design

A blocker probe suppresses the binding of the detecting probe to an unwanted target sequence (Stender et al., 2001). In this work a blocker probe was designed to block non-specific binding to non-*L. monocytogenes Listeria* species. The LmPNA1253 probe was aligned with the 16S rRNA sequences from both *L. monocytogenes* and other *Listeria* species. Sequences were obtained from SILVA database and the alignments were performed using the Clustal Omega program available at the EBI website (http://www.ebi.ac.uk/). Both blocker and detection probes were evaluated regarding their melting temperatures and free energy (Giesen et al., 1998; Yilmaz and Noguera, 2004) to ensure a similar affinity to the corresponding target sequences. The blocker probe was also synthesized as described above but no linker or fluorochrome were added to the probe.

2.4. Application of the PNA-FISH procedure

The hybridization procedure was performed as previously reported in Almeida et al. (2010) with some modifications. Smears of each strain were prepared by standard procedures, immersed in 4% (wt/vol) paraformaldehyde (Sigma) followed by 50% (vol/vol) ethanol (Fisher Scientific) for 10 min each and allowed to air dry. The smears were then covered with 20 μL of hybridization solution containing 10% (wt/vol) dextran sulfate (6500–10,000 Molecular Weight - Sigma), 10 mM NaCl (Sigma), 5.5% (vol/vol) formamide (Sigma), 0.1% (wt/vol) sodium pyrophosphate (Sigma), 0.2% (wt/vol) polyvinylpyrrolidone (average 10,000 Molecular Weight - Sigma), 0.2% (wt/vol) Ficoll (Sigma), 5 mM disodium EDTA (Sigma), 0.1% (vol/vol) Triton X-100 (Sigma), 50 mM Tris-HCl (pH 7.5; Sigma), 200 nM PNA probe and 400 nM of blocker probe. Samples were covered with coverslips, placed in moist chambers and incubated for 60 min at 60 °C. Subsequently, the coverslips were removed and the slides were submerged in a pre-warmed (60 °C) washing solution containing 15 mM NaCl (Sigma), 0.1% (vol/vol) Triton X (Sigma) and 5 mM Tris Base (pH 10; Sigma). Washing was performed at 60 °C for 30 min and the slides were subsequently air-dried. During protocol optimization, the hybridization was performed at different hybridization and washing temperatures (55–65 °C), hybridization times (45–90 min), formamide concentrations (5.5%, 30% and 50% [vol/vol]) and blocker and detection probe concentrations (1:1 and 2:1 ratio). The above described procedure was the one that allowed a better discrimination between *L. monocytogenes* and non-*L. monocytogenes* strains and used throughout the rest of the work.

Prior to microscopy visualization, pure culture smears of *L. monocytogenes* CECT 933 and *L. innocua* CECT 910 were additionally stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma). Samples were covered with 20 μL (0.1 mg/mL) of DAPI and incubated for 10 min in the dark. Excess DAPI was gently removed and samples were allowed to air dry.

2.5. Optimization of an enrichment step for L. monocytogenes detection in food samples

After PNA-FISH optimization, several enrichment broths were tested in order to obtain a positive PNA-FISH output for artificially contaminated samples with concentrations as low as 1 CFU/25 g or mL of food. Several time points, from 8 to 48 h at 30 °C or 37 °C were also tested. Both universal and selective enrichment broths were evaluated. Universal broths included: BHI, Tryptic Soy Broth (TSB) (Liofilchem), Buffered Peptone Water (BPW) (Liofilchem) and Universal Preenrichment Broth (UPB) (Becton Dickinson). Selective enrichment broths for *Listeria* spp. and *L. monocytogenes* tested were: University of Vermont (UVM) (Liofilchem), Demi-Fraser Broth (DFB) (Liofilchem), Fraser Broth (FB) (Liofilchem), Buffered Listeria Enrichment Broth (BLEB) (Liofilchem) and One Broth Listeria (OBL) (Oxoid). Two-step enrichment protocols were also tested using selective broths, as follows: UVM-UVM, UVM-BLEB, UVM-FB, UVM-OBL, OBL-OBL, OBL-UVM, OBL-BLEB, OBL-FB. The first medium was always used to dilute the matrix using 225 mL of broth + 25 g or mL of food sample in a stomacher bag; while the second medium was used in a 9 mL-tube, inoculated with 1 mL of the previous enrichment.

At specific time points, 8, 12, 18, 24, 36 and 48 h of the enrichment step, 20 μL of the sample were placed directly in the microscope slide, dried in the incubator and then hybridization was performed as described above.

2.6. Detection of L. monocytogenes in artificially contaminated food matrices

To assess the performance of the pre-enrichment step in the detection of *L. monocytogenes* by PNA-FISH, five different food matrices from a local retailer (Pingo Doce, Portugal) were tested: ground beef, ground pork, milk, lettuce and cooked shrimp. These matrices were selected to evaluate the suitability of method in a diverse array of matrices including meats, seafood, vegetables and dairy products, known for a recurrent prevalence of *L. monocytogenes* and/or frequently associated with listeriosis outbreaks (Adzitey and Huda, 2010; Larivière-Gauthier et al., 2014; Navratilova et al., 2004; Paranjpye et al., 2008; Rebagliati et al., 2009; Ryser and Marth, 2007; Shantha and Gopal, 2014; Smith et al., 2011; Thévenot et al., 2006; Wan Norhana et al., 2010; Zhu et al., 2017).

Three bulk batches for each matrix were prepared, one non-inoculated (NI) used to check for the presence of *L. monocytogenes* (≈300 g of matrix for each NI batch), a low level (LL) inoculum batch with 0.2–2 CFU/25 g or mL of sample (≈1400 g of matrix for each LL batch) and a high level (HL) inoculum batch with 2–10 CFU/25 g or mL of sample (≈400 g of matrix for each HL batch). The inoculum was prepared using overnight grown colonies of *L. monocytogenes* diluted in phosphate-buffered saline (137 mM NaCl; 2.7 mM KCl; 10 mM $Na_2HPO_4{\cdot}2H_2O$ and 1.8 mM KH_2PO_4 [Sigma]) and subsequently spread and mixed into the matrix bulk batches to achieve the desired concentration. For the cooked shrimp, before matrix inoculation, the inoculum was placed at 50 °C for 10 min to mimic the stress that natural microflora passed during the cooking. Then, the prepared bulk batches were placed at 4 °C for 48–72 h to allow the stabilization of the inoculum and simulate refrigerated conditions.

For all matrixes, the test design included 5 control samples, 20 low level samples and 5 high level samples, each containing a portion of 25 g or mL retrieved from the correspondent bulk batch prepared as described above. Test portions were diluted in 225 mL of OBL and homogenized in a stomacher (VWR/PBI, Italy) for 15 s at high speed. Sample pre-enrichment was performed in the optimal conditions obtained in the present study. More precisely, a two-step procedure in OBL (incubation for 24 h followed by a 1/10 dilution [1 mL of pre-enriched sample + 9 mL OBL] and a second incubation step for 18 h), was used. To confirm the presence of *L. monocytogenes*, the secondary enriched media was plated on ALOA agar (Biomerieux) and Oxford agar (Liofilchem). When presumptive positive colonies appeared, a biochemical characterization was performed according to the ISO 11290. Biochemical characterization included xylose and rhamnose sugar fermentation (Liofilchem), hemolysis and CAMP test profiles.

To evaluate the performance of the PNA-FISH method the parameter of accuracy was estimated for all tested matrices (Baratloo et al., 2015):

$$AC\,(\%) = \frac{TP + TN}{TP + TN + FP + FN} \times 100$$

Where TP or True Positive stands for the number of cases where *L. monocytogenes* detection occurs simultaneously by PNA-FISH and ISO confirmation; TN or True Negative stands for the number of cases where *L. monocytogenes* was not detected simultaneously by PNA-FISH and ISO confirmation; FP or False Positive stands for the number of cases where

L. monocytogenes detection occurs by PNA-FISH and not by ISO confirmation; FN or False Negative stands for the number of cases where *L. monocytogenes* detection occurs by ISO confirmation and not by PNA-FISH.

2.7. Most Probable Number (MPN) estimation

For the estimation of the inoculation level in the low and high level matrix samples, a Most Probable Number (MPN) evaluation was performed following ISO 11290 protocol. For low level (LL) estimation, samples of 50 (×5 replicates), 25 (×20 replicates) and 10 (×5 replicates) g or mL each were taken from the corresponding bulk batch, prepared as described above, and mixed with 450, 225 and 90 mL of DFB, respectively. For high level (HL) estimation, samples of 25 (×5 replicates), 10 (×5 replicates) and 5 (×5 replicates) g or mL each were taken from the corresponding bulk batch and mixed with 225, 90 and 45 mL of DFB, respectively. The samples were homogenized in a stomacher (VWR/PBI, Italy) for 15 s at high speed processes according ISO 11290. More precisely, samples were incubated in DFB for 24 h at 30 °C. From this a 100 µL sample of the DFB enrichment was placed in 10 mL of FB and incubated for 48 h at 37 °C. To confirm the presence of *L. monocytogenes*, the FB medium was plated on ALOA agar (Biomerieux) and Oxford agar (Liofilchem). When presumptive positive colonies appeared, a biochemical characterization was performed according to the ISO 11290. Biochemical characterization included xylose and rhamnose sugar fermentation (Liofilchem), hemolysis and CAMP test profiles. The number of positive samples obtained for each level were used to estimate real contamination levels (LaBudde, 2008).

2.8. Microscopy visualization

This step was performed using an OLYMPUS BX51 (OLYMPUS Portugal SA, Portugal) epifluorescence microscope equipped with a filter sensitive to the Alexa Fluor 568 molecule attached to PNA probe (Excitation 530–550 nm; Barrier 570 nm; Emission LP 591 nm) and DAPI (Excitation 365–370 nm; Barrier 400 nm; Emission LP 421 nm). Other filters present in the microscope were used to confirm that cells did not present autofluorescence. For every experiment, a negative control was performed simultaneously, where all the steps described above were carried out, but where no probes were added during the hybridization procedure.

The outcome of a PNA-FISH sample was only assessed after the analysis of the entire area of the glass slide well where the 20 µL sample was present. A positive outcome was determined when at least 10 fluorescent cells were visualized/microscopic field, which implies a concentration of $\approx 2.0 \times 10^5$ cell/mL (this calculation considered a microscopic field area of 0.1364 mm^2 and well area of 50.27 mm^2). All images were acquired using the Olympus CellB software with equal exposure time.

3. Results and discussion

Extensive research has been carried out to develop molecular methods that could accelerate identification of *L. monocytogenes* on both food and clinical settings (Ponniah et al., 2010; Välimaa et al., 2015; Zunabovic et al., 2011). The optimal test for routine procedure should be simple to perform even by non-specialized technicians, sensitive enough to detect an inoculum level as low as 1 CFU/sample of food product, and fast (providing results within a few hours). Most researchers focused on PCR-based procedures, however, it is well known that PCR is susceptible to inhibitors, cross-contamination and can amplify DNA from non-viable cells (or even naked DNA), resulting in the appearance of both false negative and false positive results (Adzitey et al., 2013; Oikarinen et al., 2009; Rådström et al., 2008; Singer et al., 2006). Additionally, it requires specialized personnel and involves more complex procedures than the traditional culture methods. Some of these limitations have been solved in the meantime by improving the DNA extraction protocols, including internal controls and use RNA instead of DNA as template (Mangal et al., 2016; Marlony et al., 2008; Rådström et al., 2008).

Further research has been focusing in the development of alternative molecular technologies that are not susceptible to the previously stated factors. FISH is an alternative molecular method used to identify and quantify microbial populations (Costa et al., 2017). The combination of this method with peptide nucleic acid (PNA) probes has shown to have many advantages, including higher robustness, increased specificity and faster procedure, when compared to conventional DNA-FISH procedures. The use of PNA probes allowed a standardization of FISH procedures and this methodology has already been applied for the detection of several clinical relevant microorganisms on a broad range of samples (Cerqueira et al., 2008; Rohde et al., 2015).

3.1. Evaluation of the L. monocytogenes probes described in literature

There are already FISH procedures developed for *Listeria* spp. detection, but only a few probes are specific for *L. monocytogenes* (Almeida et al., 2011; Fuchizawa et al., 2009; Moreno et al., 2011; Wang et al., 1991; Zhang et al., 2012) (Table S1 of supplementary material). Most of the existing probes are not simultaneously specific and sensitive because of the high number of non-target strains or the limited coverage of the target strains. Only two probes, LmPNA1253 and Lmon-16S-2, present adequate theoretical values considering the following thresholds: Number of non-target strains detected < 10, Specificity > 99.9% and Sensitivity > 95%. These probes are nearly identical, as probe Lm-16S-2 shifted by only one nucleotide in relation to the 16S target sequence when comparing to the LmPNA1253. Therefore, these two probes detect both *L. monocytogenes*, *L. marthii* and also one *L. welshimeri* sequence (out of the 1922213 sequences available at the database). *L. marthii* is a relatively new species that has, so far, only been isolated in a specific area of the New York State in the USA (Orsi and Wiedmann, 2016).

Analyzing the target sequences for those probes, some closely related species (*Listeria* and *Bacillus* spp.) differ by only one nucleotide, which can hinder the discrimination between these species. For both probes, mismatches are placed near the probes 5′ or 3′ ends which can difficult even more the discrimination. However, since discrimination from closely-related *Listeria* species is usually a major challenge, as discussed in more detail in the next section (3.2.), LmPNA1253 was selected for further tests as, in this case, the mismatch with other *Listeria* species is placed at the probe third position (Table S2 of supplementary material).

3.2. Improving the L. monocytogenes PNA-FISH procedure specificity by including a blocker probe

Laboratory testing on representative strains have shown that the best hybridization conditions for LmPNA1253 were achieved using hybridization solution containing 5.5% (vol/vol) of formamide for 1 h, from 55 to 60 °C. However, LmPNA1253 still detected a few strains of *Listeria* non-*monocytogenes*, even after increasing the hybridization temperature (Table S3 of supplementary material). Increasing the temperature above 60 °C improved hybridization specificity but a decrease in fluorescence intensity of the target species was also noticed.

While PNA has been described as highly effective for the discrimination of single-base mismatch sequences (Fontenete et al., 2015; Lefmann et al., 2006), the fact is that effective discrimination might also be dependent on the mismatch position. Those at the center are usually associated with an easier discrimination, while those near the 5′ or 3′ ends, which is the case in here, are reported as less effective for discrimination purposes (Amann, 1995; Lefmann et al., 2006). In fact, our results have shown some cross-reactivity with *L. innocua*, *L. welshimeri* and *L. ivanovii* (Table S3 of supplementary material). In order to

block non-specific hybridization and hence increase robustness, an unlabeled blocker probe was used. PNA probes are particularly efficient blocker probes due to their particular thermodynamic properties (Fiandaca et al., 1999; Stender et al., 2001). The alignment with closely related strains, as stated before, have shown two positions potentially important for ensuring the method specificity and robustness (Table S2 of supplementary material). Blocking position 3 would bring important advantages on preventing cross-hybridization with other *Listeria* spp., while blocking position 15 would avoid cross-hybridization with *Bacillus* spp. A more detailed evaluation of these two possibilities have shown that the most relevant *Bacillus* species do not present this risk (Table S4 of supplementary material). On the other hand, the other *Listeria* spp. are widespread in nature and can be present in food and food processing plants (Ryser and Marth, 2007; Sauders et al., 2012). The most prevalent in this type of environments are usually *L. monocytogenes* and *L. innocua* (MacGowan et al., 1994) and several researchers have observed that *L. innocua* can outcompete *L. monocytogenes* if the two species are cultivated together in commonly used enrichment media (Carvalheira et al., 2010). Consequently, the non-*L. monocytogenes Listeria* species represent an increased risk of cross-hybridization in detection methods for *L. monocytogenes*. The addition of a blocker probe (in a 2:1 ratio), was able to effectively block the cross-hybridization for this set of strains. This effect was more efficient at 60 °C (Fig. 1 and Table S3 of supplementary material).

Subsequently, the final protocol was tested in 67 strains (including 50 *Listeria* strains). The results showed 100% agreement between PNA-FISH and strain identification (Table 1). Based on this, a specificity value of 100% (95% Confidence Internal [CI], 85.4–100) and a sensitivity value of 100% (95% CI, 88.6–100), were obtained for this detection protocol.

3.3. Optimization of an enrichment step for Listeria monocytogenes detection in food samples

Single cell pathogen detection directly in the food samples is still a goal rather than a reality for old and newly developed methods (López-Campos et al., 2012). As such, pathogen enrichment in an enrichment medium before analysis is required. In traditional bacteriological methods, this is generally achieved by applying a two-step enrichment process. Typically, the first comprises a non-or semi-selective medium to recover injured organisms, dilute the inhibitory compounds and rehydrate the bacterial cells. The second is generally a selective medium that suppresses the background flora and increases the target pathogen, enabling its isolation and detection (Välimaa et al., 2015).

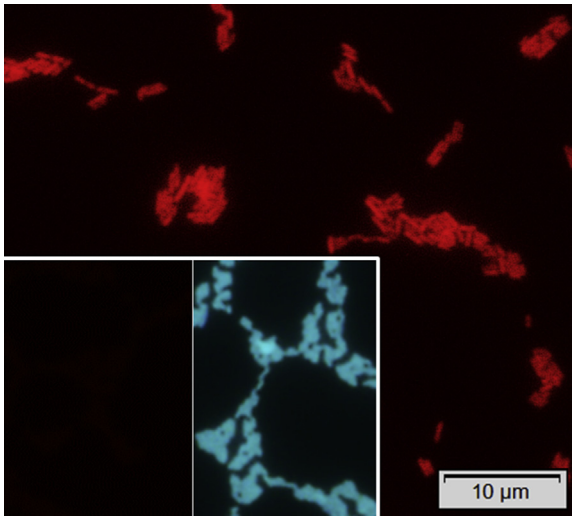

Fig. 1. Hybridization results for the LmPNA1253/blocking probes combination with a pure culture smear of *Listeria monocytogenes* CECT 933. In the white box a pure culture smear of *L. innocua* CECT 910 exhibiting absence of signal on the left and DAPI stained on the right. The experiments were performed simultaneously and images were obtained with equal exposure times.

PNA-FISH, like most other molecular and culture-based methods, requires an enrichment step to successfully detect as low as 1 CFU of the pathogen in a sample (López-Campos et al., 2012). Without the inclusion of a filtration step, a typical PNA-FISH procedure has a detection limit of 10^5 cells/mL (Almeida et al., 2009). In order to reach that concentration (ideally > 10^6 cells/mL), different enrichment broths were tested (Table S5 of supplementary material), starting with frequently-used rich enrichment broths, such BHI, TSB, BPW and UPB. Ground beef was used on these experiments due to the high load of background microflora found in this food matrix that potentially hinders *L. monocytogenes* growth (Gill and McGinnis, 1993). Initial experiments using non-selective enrichment broths were not able to detect *L. monocytogenes* even with an inoculum of up to 500 CFUs in 10 g of ground beef. This is in agreement with previous reports that indicated that after 24 h, the concentration of *L. monocytogenes* in meat matrices was approx. 10^4/mL, a value below the PNA-FISH detection limit (Duffy et al., 2001; Gehring et al., 2012).

Subsequently, a set of commonly used selective enrichment broths used for the detection of *L. monocytogenes*, namely, FB and DFB (ISO 11290–1:1996), BLEB (FDA) and UVM (USDA-FSIS) and a more recent commercial enrichment broth (OBL), were tested. From this set of enrichment broths, only UVM and OBL were able to recover *L. monocytogenes* at a concentration that met the detection requirements (Table S5 of supplementary material). However, a low fluorescence intensity of the bacteria was obtained, probably arising from the low metabolic state/decreased rRNA levels of the cells as the cultures enter the stationary phase. The lack of a positive outcome observed for BLEB, DFB and FB could originate from the competing microflora present in meat (similarly to the non-selective enrichment broths) or from increased lag phases as a result of bacteria adaptation to these media. In fact, similarly to non-selective enrichment broths, previous reports indicate that at 24 h *L. monocytogenes* levels only reaches around 10^3–10^4 CFU/mL (Gehring et al., 2012; Vlaemynck et al., 2000).

As none of the above-described strategies was successful, a two-step enrichment procedure of 18 h plus 8 h was tested, using in the first and second steps OBL and UVM. Two other selective broths, FB and BLEB, were also tested in the second step. All tested combinations were able to retrieve *L. monocytogenes* above the defined threshold of detection (Table S5 of supplementary material) using a 200 CFU/10 g *L. monocytogenes* inoculum. Combinations including FB were excluded due to the presence of autofluorescing microflora observed in the green channel. Combinations including BLEB were excluded due to the low fluorescence intensity and low numbers of *L. monocytogenes*. The OBL + OBL was preferred over UVM combinations due to the consistently higher concentrations of *L. monocytogenes* cells observed in the microscope after the PNA-FISH procedure.

Since the two-step enrichment with OBL provided the best results, further tests were performed with lower concentrations to confirm if the desired detection level of 1 CFU/25 g or mL of sample was achieved. Results have shown that, for low inoculation levels, a centrifugation step was needed to concentrate the cells. To avoid centrifugation, the time of both enrichment steps was extended from 18 to 24 and from 8 to 18 h. This modification allowed the detection of 1 CFU of *L. monocytogenes* in 25 g or mL of sample by PNA-FISH without the need for a centrifugation step in ground beef, milk and lettuce matrices (Fig. 2 and Table 2).

An interesting observation was that *L. monocytogenes* cells grown in OBL do not present the typical small rod-shaped bacilli (Ponniah et al., 2010). They are present in a chain-like elongated form (filamentous) (Fig. 2). We theorize that this morphology is due to the components present in the OBL broth. In fact, this behavior was already reported in *L. monocytogenes* strains in the presence of some antimicrobial agents (Giotis et al., 2007; Hazeleger et al., 2006).

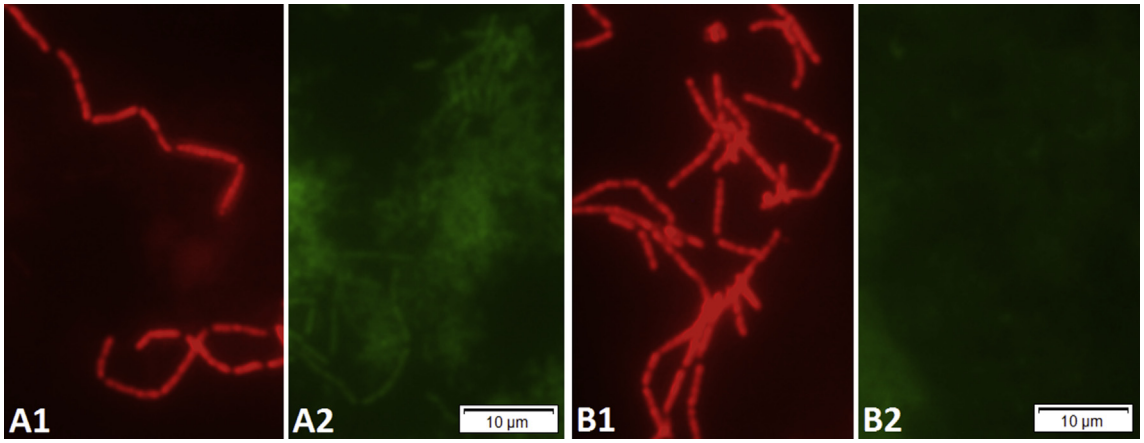

Fig. 2. Detection of *L. monocytogenes* in ground beef artificially inoculated with ≈1 CFU/25 g of sample, using LmPNA1253 probe attached to Alexa Fluor 568. A - Sample taken after 8 h in the second enrichment step with OBL and a 5min centrifugation step; B - Sample taken after 18 h in the second enrichment step with OBL. Cells of *L. monocytogenes* visible at the red channel (1), while the green channel (2) was used to check for the absence of autofluorescence. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Finally, a validation assay was performed to verify the applicability and specificity of the PNA-FISH protocol for the detection of *L. monocytogenes* in real scenarios of contamination. To that end, food samples were contaminated with two levels of inoculum, a LL (0.2–2 CFU/sample) and a HL (2–10 CFU/sample). Different *L. monocytogenes* strains were selected for each food matrix (Table 3). *L. monocytogenes* was detected in all tested matrices in levels as low as 0.5 CFU/25 g or mL of sample. For all 150 samples, an overall accuracy of 99% was obtained with the PNA-FISH method. This value results from the observation of only 2 false negative results, one in low level ground beef and the other in high level cooked shrimp assays.

4. Conclusions

This work describes the development of a new detection method for *L. monocytogenes* in food matrices. The method is based on a PNA-FISH procedure that combines the use of a previously described probe by Almeida et al. (2011), LmPNA1253, with a blocker probe resulting in an overall accuracy of 99%. In order to be able to detect 1 CFU of *L. monocytogenes* in 25 g or mL of sample, several selective and non-selective enrichment broths were evaluated. Overall, a two-step enrichment procedure in OBL, provided the most reliable results at the desired limit of detection. The total time-to-result of the method is 29 h, if a centrifugation step is included, or 45 h without a centrifugation step. A validation assay in five different food matrices showed that the method presents a high accuracy performance. The method high specificity, sensibility, accuracy and faster time-to-result makes it a good candidate for routine application in food safety laboratories.

Disclosure statement

Rui Rocha and José M. Sousa work for Biomode S.A, a company that develops and commercializes *in vitro* diagnostic kits for microorganism detection, using PNA-FISH technology. Also, Laura Cerqueira, Carina Almeida and Nuno F. Azevedo are founders and shareholders of Biomode SA.

Acknowledgments

This work was funded by the following projects:

POCI-01-0145-FEDER-006939 (Laboratory for Process Engineering, Environment, Biotechnology and Energy - UID/EQU/00511/2013), POCI-01-0145-FEDER-006684 (Center of Biological Engineering - UID/BIO/04469) funded by the " Fundo Europeu de Desenvolvimento Regional (FEDER)", and Coded-FISH (PTDC/DTP-PIC/4562/2014) POCI-01-0145-FEDER-016678, through COMPETE2020 - Programa Operacional Competitividade e Internacionalização (POCI) and by national funds, through FCT - Fundação para a Ciência e a Tecnologia.

NORTE-01-0145-FEDER-000005 - LEPABE-2-ECO-INNOVATION and NORTE-01-0145-FEDER-000004 - BioTecNorte operation, supported by North Portugal Regional Operational Programme (NORTE 2020), under the Portugal 2020 Partnership Agreement, through the European Regional Development Fund (ERDF).

PhD Fellowship SFRH/BDE/51910/2012 and Post-Doc Fellowship SFRH/BPD/74480/2010 supported by national funds through FCT - Fundação para a Ciência e a Tecnologia.

ENMed/0003/2014 - NanoDiaBac funded by national funds through FCT - Fundação para a Ciência e a Tecnologia.

The authors also thank to Professor Paula Teixeira from the Catholic University, Porto - Portugal and Professor Marta Cabo from the Institute of Marine Research, Vigo - Spain for providing some of the *Listeria* isolates used in the study.

Table 2
List of assays performed to successfully detect *L. monocytogenes* in food matrices to 1 CFU/25 g or mL of sample. Optimizations were conducted in ground beef and subsequently validated in milk and lettuce matrices. All OBL enrichments steps were performed at 30 °C. Three replicates of each assay were performed with two different *L. monocytogenes* strains - CECT 938 and CECT 5873, with an inoculation level of 2.9 ± 2.0 CFU/25 g or mL of sample.

Matrix	Ground Beef			Milk	Lettuce
Enrichment Procedure	OBL 18 h + OBL 8 h.	OBL 18 h + OBL 8 h; 5 min 10 000 g centrifugation.	OBL 24 h + OBL 18 h.	OBL 24 h + OBL 18 h.	OBL 24 h + OBL 18 h.
L. monocytogenes PNA-FISH outcome	–	+	+ +	+ +	+ +

- No presence of *L. monocytogenes*; + Presence of *L. monocytogenes* near the defined detection limit; + + Presence of *L. monocytogenes* above the defined detection limit.

Table 3
PNA-FISH, confirmation and accuracy levels obtained for the detection of *L. monocytogenes* on different food matrices inoculated with LL and HL ranges in 25 g or mL samples.

Matrix/Tested Strain	LL samples			HL samples			Accuracy %
	MPN estimation (CFU/25 g or mL)	PNA-FISH positives	Confirmed positives*	MPN estimation (CFU/25 g or mL)	PNA-FISH positives	Confirmed positives*	
Ground Beef *L. monocytogenes* L1F3	0.5 (0.2–0.8)	11/20	12/20	2.0 (1.0–4.1)	4/5	4/5	96
Ground Pork *L. monocytogenes* 2723	0.7 (0.4–1.2)	13/20	13/20	2.3 (1.1–4.7)	5/5	5/5	100
Milk *L. monocytogenes* 812	0.8 (0.4–1.3)	10/20	10/20	6.4 (3.8–14.5)	5/5	5/5	100
Lettuce *L. monocytogenes* 1360	1.6 (1.0–2.9)	18/20	18/20	5.6 (2.6–11.8)	5/5	5/5	100
Cooked Shrimp *L. monocytogenes* L1D1	1.8 (1.2–3.2)	15/20	15/20	2.3 (1.2–4.6)	4/5	5/5	96
					Overall accuracy		≈99

*According to ISO 11290.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2018.12.009.

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