SHORT COMMUNICATION

An Improve Protocol for PCR Using LM1 and LM2 Primers for *Listeria monocytogenes* Detection in Food Matrices

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A b s t r a c t

Several studies have observed that use of a conventional PCR protocol with primers LM1 and LM2 for the identification of the *hly*A gene of *Listeria monocytogenes* generates non-specific PCR amplifications and false positives. For this reason, in this study we provide a modified PCR protocol that improves the specificity of the results obtained with LM1 and LM2 primers.

K e y w o r d s: *Listeria monocytogenes*, *hly*A, PCR

The *hly*A gene encoding for listeriolysin O, a secreted pore-forming toxin, is a conventional genetic marker for the identification of *Listeria monocytogenes* in foods. The LM1 and LM2 primers, designed by Border *et al.* (1990) are among the most commonly used primer sets for the detection of this pathogen as cited in at least 51 publications (Lawrence and Gilmour, 1994; Karpiskova *et al.*, 2000; Van Coillie *et al.*, 2004; Kawasaki *et al.*, 2005; Marian *et al.*, 2012; Jamali *et al.*, 2013; Kuan *et al.*, 2013). A conventional PCR protocol using these primers was originally designed by Aznar and Alarcón (2002) and has been commonly used in some investigations (Aznar and Alarcón, 2003; Aznar and Solis, 2006; Aznar and Elizaquível, 2008; Brankica *et al.*, 2010; Peres *et al.*, 2010; Elizaquível *et al.*, 2011). However, the use of this PCR protocol results in non-specific bands after detection of *L. monocytogenes* and other *Listeria* species from pure cultures (Aznar and Alarcón, 2002). Moreover, during in-house validation, we also observed non-specific bands using DNA extracted from *Salmonella enterica* and *Escherichia coli*. For this reason, the goal of the present study was to improve the specificity of the LM1 and LM2 primers used for the amplification of the *hly*A gene from *L. monocytogenes*.

To validate the specificity of the Aznar and Alarcon (2002) PCR protocol, we used a collection of laboratory strains: *L. monocytogenes* ATCC 19115, *L. monocytogenes* CDC, *L. monocytogenes* Scott A, *Listeria innocua*

ATCC 33090, *L. innocua*, *Listeria seeligeri*, *E. coli* ATCC H10407, *S. enterica*, and *Salmonella* Thompson ATCC 8391. The bacterial strains were cultivated in Trypticase Soy Broth (Dibico, México) and incubated at 35°C for 19 h. One milliliter of these cultures was used for DNA extraction using the DNeasy® Blood & Tissue Kit (QIAGEN, USA) with a minor modification. Briefly, before addition of AL buffer, 200 μl of ASL buffer was added, and the mix was incubated at 95°C for 15 min. In addition to pure cultures, DNA was extracted from enriched samples (Universal Preenrichment Broth, Difco, USA) of jalapeno peppers and tomatoes inoculated previously with a mixture of *S. enterica*, *Shigella* spp., *E. coli*, and *L. monocytogenes* cultures. One milliliter of enrichment broth was used to extract DNA using the QIAamp® DNA Stool kit (QIAGEN, USA). In all cases, the extracted DNA was quantified, and the purity was determined using a nanodrop (Thermo Scientific 2000c, USA).

DNA was mixed independently with two types of master mix: mic with TaKaRa Taq™ DNA Polymerase (Takara Shuzo Co., Japan) or with Bio-Rad DNA Polymerase (Bio-Rad, USA). PCR amplifications were performed at two laboratories from the Universidad Autónoma de Querétaro, México: Food Safety Laboratory, Chemistry Faculty and Animal Nutrition Laboratory, Natural Sciences Faculty. At the Food Safety Laboratory, the PCR reactions were conducted using

a Techne TC-512 Thermal Cycler (Techne, UK), whereas a T100 Thermal Cycler (Bio-Rad, USA) was used at the Animal Nutrition Laboratory. PCR was performed using the conditions established by Aznar and Alarcon (2002). To optimize the PCR protocol, gradients of DNA concentrations (40 to 0.0625 ng/µl) and annealing temperatures (55–64.7°C) were evaluated; additionally, the annealing and extension times and the number of cycles were reduced to 25 s and 25 cycles, respectively. The electrophoresis gel was prepared with 2% agarose and 0.0028% ethidium bromide (10 mg/ml) in 1X TBE buffer (89 mM Tris base, 89 mM Boric acid, 2 mM EDTA). The lanes were loaded with 7 µl of the PCR products and 3 µl of 6X gel-loading buffer (30% glycerol, 0.25% bromophenol blue in 10 ml dH$_2$O), and gel images were taken using a MiniBis Pro photo documenter (Bio-Imaging Systems, Israel).

The electrophoresis gel obtained after running a PCR using the conditions from Aznar and Alarcon (2002) showed non-specific amplification products from pure cultures of all *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *E. coli*, and *Salmonella* strains and from DNA obtained from inoculated vegetables, when the expected product was a single band of 702 bp corresponding to *L. monocytogenes* (Fig. 1A). In DNA from *L. innocua*, *L. seeligeri* and *Salmonella*, a similar expected fragment (720 bp) was observed. The presence of atypical bands can lead to false-positive results. The observation of artifacts in *L. monocytogenes* and other *Listeria* species was also reported by the authors that established this PCR protocol.

The original PCR protocol was modified, and the optimized conditions were an initial denaturation of 5 min at 94°C, 25 cycles of 30 s at 94°C, 25 s at 60.5°C
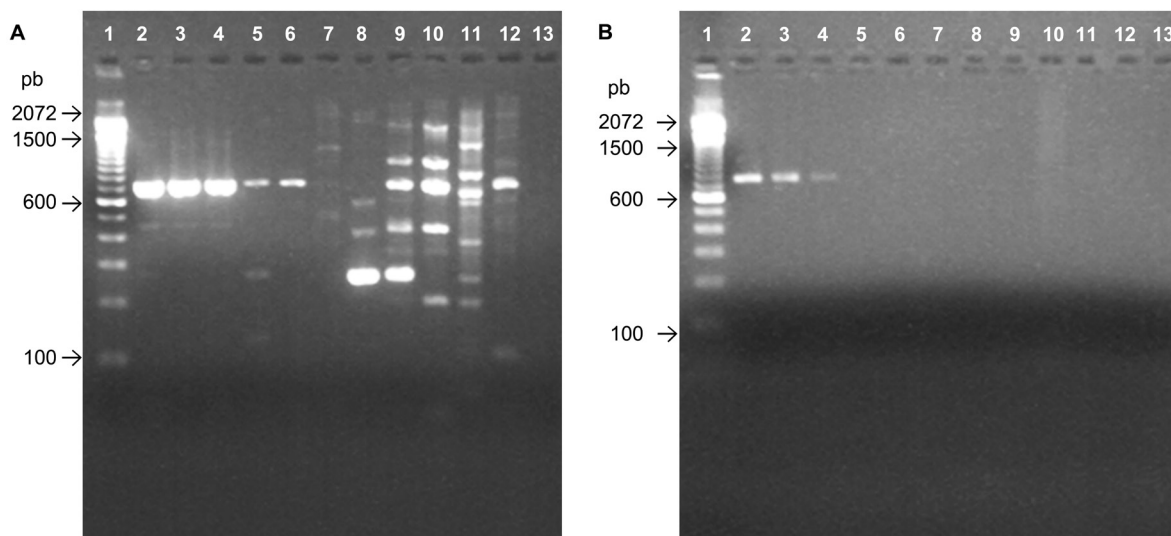

Fig. 1. PCR identification of *L. monocytogenes* strains using LM1 and LM2 primers.
A: PCR protocol established by Aznar and Alarcón (2002); B: Modified PCR protocol. Lane 1, DNA ladder; lane 2, *L. monocytogenes* Scott A; lane 3, *L. monocytogenes* CDC; lane 4, *L. monocytogenes* ATCC 19115; lane 5, *L. seeligeri*; lane 6, *L. innocua* ATCC 33090; lane 7, *L. innocua*; lane 8, *E. coli* ATCC H10410; lane 9, *S.* Thompson ATCC 8391; lane 10, *Salmonella* spp.; lane 11, inoculated jalapeno pepper; lane 12, inoculated tomato; lane 13, negative control.
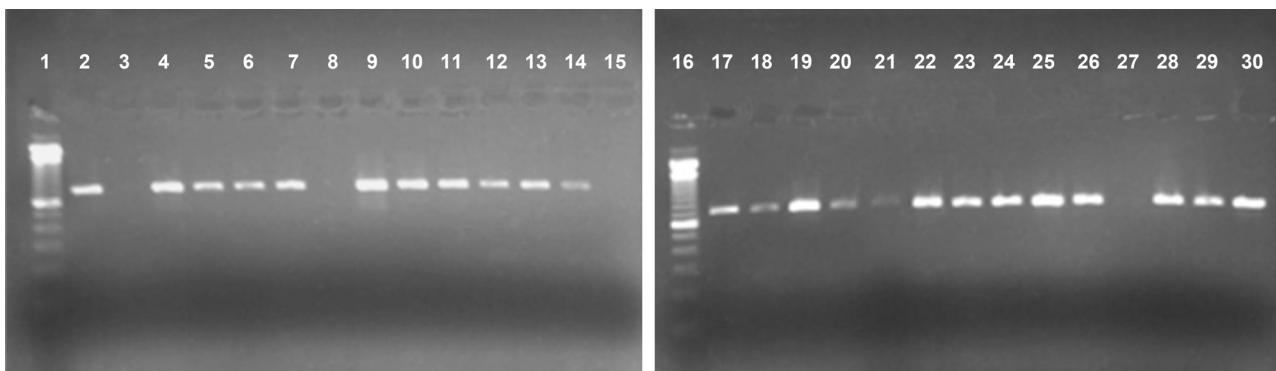

Fig. 2. Identification of *L. monocytogenes* strains isolated from a frozen vegetable processing plant using an optimized PCR protocol.
Lane 1 and 16, DNA ladder; lane 2 and 17, *L. monocytogenes* Scott A; lanes 3 to 15 and 18 to 30; environmental samples.

and 25 s at 72°C, and a final extension of 5 min at 72°C. The optimal concentration of DNA was set from 0.125 to 0.8 ng/µl. Using the optimized protocol, the expected fragment of 702 bp corresponding to the *hly*A gene only appeared in all *L. monocytogenes* strains, and no atypical bands were present in any sample (Fig. 1B). No amplifications were observed in inoculated vegetables and pure cultures of microorganisms different from *L. monocytogenes*. The absence of the expected fragment in DNA extracted from inoculated vegetables with the pathogens, including *L. monocytogenes* strains, may be due to the presence of PCR inhibitors in the food matrix.

Xiacyum *et al.* (2001) indicated that artifacts and chimeras increment significantly as the number of cycles is increased, reported that DNA template concentration affected PCR amplification kinetics, and demonstrated that PCR artifacts disappeared as the template concentration of each strain decreased. In this study, similar results were obtained; atypical bands disappeared as the number of cycles decreased and DNA concentration was reduced.

Finally, to validate the proposed optimized PCR protocol, 26 isolates presumptively identified as *L. monocytogenes*, were confirmed. The isolated strains were cultivated in Trypticase Soy Broth (Dibico, México) and incubated at 35°C for 19 h. The DNA was extracted as described above for pure cultures. In 22 samples (85%), the expected fragment and the absence of atypical bands were observed (Fig. 2).

In conclusion, the standardized PCR protocol can be used to confirm *L. monocytogenes* strains without producing false positives.

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