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IMPROVED SENSITIVITY AND REPRODUCIBILITY OF THE PCR METHOD FOR DETECTION OF *Listeria spp*. AND *L. monocytogenes* IN MILK

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Listeria monocytogenes is a facultative intracellular Gram-positive bacterium, ubiquitous in nature and capable of causing listeriosis in humans and animals. Conventional microbiological techniques and modern molecular approaches are currently used for the isolation and detection of L. monocytogenes in food samples. The aim of this study was to improve the sensitivity and reproducibility of PCR for the detection of Listeria spp. in milk. For that purpose milk samples were artificially inoculated with serial dilutions of L. monocytogenes 4b ATCC 19115 and L. innocua ATCC 33090. The results obtained on artificially contaminated milk samples indicated that incubation time and target genes have an influence on the sensitivity of PCR detection. The best results were obtained after 24 h of pre-enrichment, with primers complementary to the hlyA gene, when it was possible to detect 1 CFU/mL of Listeria spp.

Key words: detection, Listeria spp., L. monocytogenes, milk, PCR

INTRODUCTION

Listeria monocytogenes is a Gram-positive opportunistic pathogen that has been isolated from many natural and human-created environments (Farber and Peterkin, 1991). Pathogenic and nonpathogenic strains of *Listeria* are ubiquitous in nature and can be isolated from the soil, vegetables and natural waters, as well as from healthy animals and man (Roberts and Wiedmann, 2003). *L. monocytogenes* is the most pathogenic species of this genus, although infections mainly occur in neonates, pregnant women, the elderly and immune-compromised individuals (McLaughlin, 1997). The primary mode of transmission of *L. monocytogenes* to humans is the consumption of contaminated minimally processed food (Schlech, 2000; Kathariou, 2002; Shen *et al*., 2006). Several large outbreaks of listeriosis have been associated with contaminated commercial foodstuffs, such as vegetables, milk, and meat products, on which these bacteria can multiply even at low temperatures (Schuchat *et al*., 1991). Usually, the presence of any *Listeria* species in food is an indicator of poor hygiene

(McLaughlin, 1997). Increased public awareness of the health-related and economic impacts of food contamination and food borne illnessess has resulted in greater efforts to develop sensitive methods for pathogen detection and identification. Conventional phenotypic assays are time-consuming, characterized by low sensitivity and may fail to detect strains of bacteria that are present in the samples at low concentrations or that have unusual phenotypic profiles (Tang *et al*., 1998). Advances in molecular technologies, particularly the PCR methodology, have allowed more reliable microbial identification and surveillance. PCR techniques are more sensitive, highly specific and allow more rapid processing times, and enhance the likelihood of detecting bacterial pathogens without the need for isolating pure cultures (Aznar and Alarcón 2003; Cocolin, *et al*., 2002).

In this paper, a direct identification of *Listeria* spp. in milk samples by a molecular method is described.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The standard strains of *Listeria monocytogenes* 4b ATCC 19115 and *Listeria innocua* ATCC 33090 were obtained from The American Type Culture Collection (ATCC; Manassas, Va., USA), and used for artificial contamination of pasteurized milk samples. Standard strains were grown on brain-heart agar (BHA) (Merck, GmbH Darmatadt, Germany) and buffered peptone water (Merck) at 37°C.

Samples. Artificially inoculated samples were prepared as follows: 40 mL of pasteurized milk were homogenized in 360 mL of half-concentrated Fraser broth, using a stomacher (MIX 2, AES Chemunex, France) for 1 min. The resulting mixture was distributed in 40 mL aliquots inoculated with 400 μL of 10-fold serial dilutions of standard strains in sterile saline (0.9% NaCl), covering the range from 1 to $1x10^7$ CFU mL^{-1} (determined by plate count on BHA). DNA was extracted from 10 mL of mixture, after 0 h, 2 h and 6 h, and from 1 mL after 24 h of incubation at 37°C.

DNA extraction. Two methods for DNA extraction from milk were evaluated, one using enzymatic treatment for bacterial lysis by using DNeasy Tissue Kit (Qiagen GmbH, Germany) according to the manufacturer's protocol for Gram-positive bacteria and the other using zirconia beads for lysis (Randazzo *et al.,* 2002).

PCR amplification conditions. PCR was performed in a final volume of 50 μL containing 1xPCR buffer (10xPCR buffer: 500 mM KCl, 100 mM Tris-HCl, 0.8% Nonidet P40), 2.5 mM $MgCl_2$, 200 μM of each dNTP, 2.5 μM of each primer, 1 U of *Taq* polymerase (Fermentas UAB, Lithuania) and 0.1-1 μg of DNA template. The samples were amplified in a DNA thermal cycler (Flexigene, Techne, UK) with primers LM1 (5'-CCT AAG ACG CCA ATC GAA-3') and LM2 (5'-AAG CGC TTG CAA CTG CTC-3'), complementary to the *hlyA* gene and LI1 (5'-CTC CAT AAA GGT GAC CCT-3') and U1 (5'-CAG CMG CCG CGG TAA TWC-3') complementary to 16S rDNA, 5 min at 94°C, 35 cycles of 30 s at 94°C, 45 s at 50°C, 45 s at 72°C and the final extension of 5 min at 72°C (Aznar and Alarcón, 2003). List of primers

used in this study is given in Table 1. All PCR products were analyzed by agarose gel electrophoresis on 1% and 2% (wt/vol) agarose gels in 1xTBE buffer (10xTBE: 89 mM Tris, 89 mM boric acid, 2 mM EDTA) (Fermentas), at a constant voltage 80 V for 1 h and visualized by CCD camera Bio Doc Analyze Darkhood (Biometra, Gottingen, Germany). All PCR products were run next to the DNA molecular standards "MassRuler^{TM} DNA Ladder" (Fermentas) and "GeneRuler^{TM} DNA Ladder Mix" (Fermentas).

RESULTS

Assay design. The assay was designed to identify and distinguish *Listeria* spp. and *L. monocytogenes* from milk samples. Two sets of PCR primers were used to amplify two target genes (*hlyA* and 16S rDNA), with expected 702 bp and 938 bp amplicons, respectively. Two methods for DNA extraction from milk were evaluated: one using enzymatic treatment for bacterial lysis and the other using zirconia beads for lysis. Results showed that two methods yielded similar amounts of bacterial DNA. The DNeasy Tissue Kit, being faster and easier to implement, was used for extracting DNA from artificially contaminated samples and from pre-enrichment cultures.

Validation with standard strains. The assay was initially validated with standard strains *L. monocytogenes* 4b ATCC 19115 and *L. innocua* ATCC 33090. The assay correctly identified both strains. Moreover, when DNA from non-*Listeria* strains, such as *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Proteus mirabilis, Proteus vulgaris, Yersinia enterocolitica, Bacillus cereus, Bacillus subtilis, Enterococcus faecalis, Staphylococcus aureus* and *Lactobacillus plantarum*, was used in the PCR, no PCR amplicon, of any size, was obtained (data not shown). Therefore, using primers directly on DNA extracted from microbial mixtures containing *Listeria* spp. facilitates specific detection of *Listeria* by PCR amplification.

Determination of the limit of detection. Different pre-enrichment incubation times (0 h, 2 h, 6 h, and 24 h) and target genes for PCR assay (*hlyA* and 16S rDNA) were tested on artificially contaminated pasteurized milk samples, in order to optimize the experimental conditions for detection of *Listeria* spp., suitable for routine laboratory analysis. The limit of detection was first determined using serial dilutions of the cell cultures diluted 10-fold from 1:10 to 1:10^7 (the number of cells in the undiluted sample was 10^8 CFU mL^{-1}). The primers LI1/U1 and LM1/LM2 used in this study were proven highly specific for *Listeria* spp. and *L. monocytogenes*, respectively. The results obtained in the direct PCR reaction (0h) showed that sensitivity for the detection of *L. monocytogenes*, with primers LM1/LM2, was approximately 10^4–10^6 CFU mL^{-1}, obtained in three independent experiments (Fig. 1). In addition, the results showed that it was possible to detect *L. innocua* in all serial dilutions by using primers LI1/U1 (Fig. 1).

Further, direct PCR detection (0 h) of *Listeria* spp., without pre-enrichment, was compared with detection after 2 h, 6 h and 24 h of pre-enrichment. Results obtained with primers LM1/LM2 directed to the *hlyA* gene showed that the

detection of *L. monocytogenes* increases over time. The best sensitivity result was achieved after 24 h incubation, detecting one CFU mL^{-1} (Fig 2).

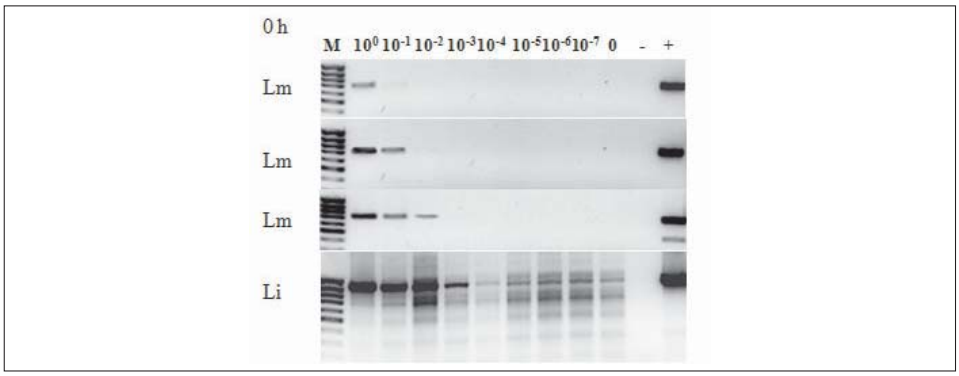

Figure 1. Agarose gel electrophoresis of the PCR products obtained by using primers LM1/LM2 and LI1/U1 on DNA isolated from artificially inoculated milk samples incubated for 0 h: M - MassRuler^{TM} DNA Ladder, 10^0-10^{-7} - serial dilutions of *L. monocytogenes* 4b ATCC 19115 (Lm) and *L. innocua* ATCC 33090 (Li), 0 - noninoculated sample, *"-"* - negative control, *"+"* - positive control

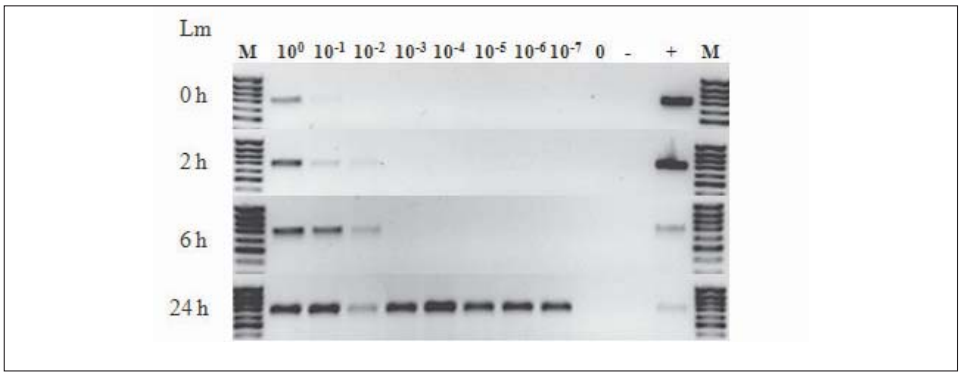

Figure 2. Agarose gel electrophoresis of the PCR products obtained by using primers LM1/LM2 on DNA isolated from artificially inoculated milk samples incubated for 0 h, 2 h, 6 h and 24 h: M - MassRuler^{TM} DNA Ladder, 10^0-10^{-7} - serial dilutions of *L. monocytogenes* 4b ATCC 19115 (Lm), 0 - noninoculated sample, *"-"* - negative control, *"+"* - positive control.

Interestingly, the results obtained after reamplification of the PCR products for 0 h of incubation, were similar to the result obtained in direct PCR reaction and did not have an influence on the sensitivity of the PCR detection (Fig 3).

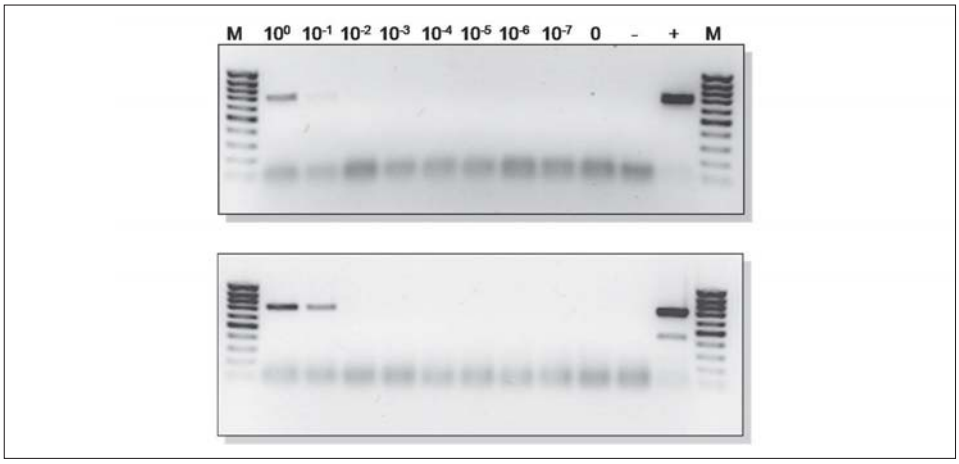

Figure 3. Agarose gel electrophoresis of the PCR products obtained after PCR amplification (A) and reamplification (B) reactions by using primers LM1/LM2 on DNA isolated from artificially inoculated milk samples incubated for 0 h: M - MassRuler^{TM} DNA Ladder, 10^0-10^{-7} - serial dilutions of *L. monocytogenes* 4b ATCC 19115, 0 - noninoculated sample, *"-"* - negative control, *"+"* - positive control

DISCUSSION

The detection and identification of *Listeria* spp. have attracted the attention of many researchers throughout the world (Aznar and Alarcón, 2003; Cocolin, *et al*., 2002; Curiale and Lewus, 1994; Kaur *et al*., 2007; Willis *et al*., 2006). This specific interest is related to the presence of *L. monocytogenes*, one of the most important food-borne pathogens. It is widely diffused in the environment and it can cause the contamination of food through the processing environment, from the raw material to the final product, and even persists after cleaning (Pan *et al*., 2006). The food industry needs a tool to trace sources of contamination and assess whether cleaning procedures are adequate. Recent developments in molecular diagnostics of *L. monocytogenes* include rapid and reliable methods for the detection of low concentrations of *L. monocytogenes* from a variety of food and environmental samples (Aznar and Alarcón, 2003; Cocolin *et al*., 2002; Kaur *et al*., 2007).

The aim of this study was to standardize a fast, reliable and highly sensitive method for the detection of *L. monocytogenes* and *Listeria* spp. in order to shorten the time for routine laboratory diagnostics of this pathogen in milk. The validation of the PCR method was performed on artificially inoculated milk samples with serial dilutions of *L. monocytogenes* 4b ATCC 19115 and *L. innocua* ATCC 33090. The obtained results indicated that incubation time and target genes have an influence on the sensitivity of the PCR detection method that was in concordance with the limits of detection reported by Aznar and Alarcón 2003 (limit of detection, 1 CFU/mL). The assay presented in this study shows very high

specificity for the detection and identification, with pure isolates, as well as with DNA isolated directly from milk. Negative controls incorporated at all stages of the assay showed no false positive identification. However, when 16S rDNA genes were used as target for PCR reaction, while seeming to be more sensitive, false positives may arise from residual nucleic acid contaminants of other bacteria in milk.

Since the assay has been designed for detecting food-borne pathogens, the technique was validated with artificially contaminated milk. Two methods for DNA extraction were tested in order to remove potential PCR inhibitors from milk during DNA extraction. Both protocols for DNA extraction were successful in removing potential PCR inhibitors from milk, as well as incorrectly identifying bacteria.

Finally, the time to final identification and determination of antimicrobial susceptibility is generally 1 to 3 days (Tang *et al*, 1998). In contrast, the PCR assay, including DNA extraction, PCR amplification and data analysis, can be completed in less than 8 h. This assay can also be adopted by small public health laboratories, food testing laboratories, and food industries, which cannot afford more expensive methods and equipment or trained technicians.

In conclusion, we have standardized high-throughput PCR assay for the detection of *Listeria* spp. and *L. monocytogenes* from milk samples. The assay has been successfully validated. The assay can provide "same-day" identification, which represents a significant reduction in time compared to conventional microbiological methods for identification of *Listeria* spp. from positive food samples. This assay represents a significant improvement over conventional techniques due to its rapid and non-tedious format.

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POBOLJŠANA SENZITIVNOST I REPRODUCIBILNOST PCR METODE ZA DETEKCIJU *Listeria spp*. I *L. monocytogenes* U MLEKU

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SADRŽAJ

Listeria monocytogenes, prouzrokovač listerioze kod ljudi i životinja, je fakultativan intraćelijski mikroorganizam široko rasprostranjen u prirodi. U cilju izolacije i detekcije *L. monocytogenes* iz hrane koriste se tradicionalne mikrobiološke i nove molekularno-genetičke metode. Cilj ovog rada je bio povećanje osetljivosti i ponovljivosti PCR metode u detekciji *L. monocytogenes* u mleku. U tu svrhu, uzorci pasterizovanog mleka su kontaminirani serijskim razblaženjima sojeva *L. monocytogenes* 4b ATCC 119115 i *Listeria innocua* ATCC 33090. Dobijeni rezultati na veštački kontaminiranim uzorcima pasterizovanog mleka, ukazuju da osetljivost PCR metode zavisi od perioda inkubacije i izbora prajmera. Najbolji rezultati su dobijeni nakon 24 h inkubacije, pomoću prajmera za *hlyA* gen, kada je bilo moguće detektovati 1 ćeliju *L. monocytogenes* tj. 1 CFU/ml.