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A PCR protocol using *inl* gene as a target for specific detection of *Listeria monocytogenes*

P.F. Almeida [a,*], R.C.C. Almeida [b]

[a] *Instituto de Ciências da Saúde, UFBA, Av. Reitor Mignel Calmon s/n, Canela CEP 40110-160, Salvador, Bahia, Brazil*
[b] *Escola de Nutrição, UFBA, Salvador, BA, Brazil*

Abstract

Polymerase chain reaction is a powerful technique for detection of pathogens in foods. It is a rapid procedure with both sensitivity and specificity for quick detection and identification of specific pathogenic bacteria from different sources. *Listeria monocytogenes* detection methods based on PCR amplification of the *iap*, *prf*A and *hly* gene sequences have been reported. The present study undertakes the development of an alternative PCR method using the *inl* gene sequences as a target to detect pathogenic *L. monocytogenes*. The presence of a unique and specific DNA amplification fragment of 760 bp for the intragenic repeats B of the *inl*A gene in all strains of *L. monocytogenes* as compared to none in other *Listeria* and unrelated Gram positive and Gram negative species confirms that this procedure is an alternative PCR protocol for detection of *L. monocytogenes*.

Keywords: PCR; *Listeria monocytogenes*; Internalin

1. Introduction

Listeria monocytogenes is a foodborne pathogen responsible for serious infections in immunocompromised individuals, newborns, pregnant women and older people. *Listeria* infections can result in stillbirth, meningitis, meningoencephalitis, septicemia, and death (Wiedman, Stolle & Batt, 1995). In recent years, a number of outbreaks of foodborne illness involving a wide range of foods have been linked to *L. monocytogenes* (Farber, 1989). The ubiquitous distribution of this pathogen in nature, its ability to grow at refrigeration temperatures and its tolerance to certain preservative agents make its elimination from food very difficult (Bansal, 1996). Given the severity of *Listeria* infections, the US FDA has adopted a "zero tolerance" policy (USDA, 1992). Inclusion of *L. monocytogenes* in the list of organisms subject to HACCP has recently driven the search for detection methods suitable for on-line monitoring (Klein & Juneja, 1997). Conventional methods for detection and identification of *L. monocytogenes* are laborious, time-consuming and are not very sensitive (Klein & Juneja, 1997). Additionally, as we do not know the infective dose for humans, the best methods have to detect its minimal presence in foods. Moreover, the difference in the level of virulence of *L. monocytogenes* strains, as suggested by epidemiological studies (Mclauchlin & Pini, 1989) and confirmed in an immunocompromised mouse model of infection (Tauboret, Rycke, Andurier & Poutrel, 1991) imply that future methods of diagnosis should be specific for pathogenic *L. monocytogenes*. Although new methods have been introduced (specially gene-based methods), and of course, the improvement in traditional microbiological methods, there is still a real need for a *L. monocytogenes* detection method adapted to industrial processing (Bubert, Schubert, Kõhler, Frank & Goebel, 1994). In recent years, a number of molecular biology-based methods for more rapid detection of *Listeria* cells have been developed including the immunoassays (Bubert et al., 1994), nucleic acid hybridization (Emond, Fliss & Pandian, 1994), nucleic acid amplification (Bessesen, Rotbart, Blaser & Elisson, 1990; Bsat & Batt, 1993; Klein & Juneja, 1997; Starbuck, Hill & Stewart, 1992; Wiedman et al., 1995) and *lux* phage assay (Loessner, Ress, Stewart & Scherer, 1996; Loessner, Rudolf & Scherer, 1997).

Since its introduction by Kary Mullis in 1983, the polymerase chain reaction (PCR) technology has proved

* Corresponding author. Tel.: +55-71-235-2416; fax: +55-71-2458917.
E-mail address: pfa@ufba.br (P.F. Almeida).

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to be an invaluable method for detection of pathogens in foods. It is a rapid procedure with both sensitivity and specificity for quick detection and identification of specific pathogenic bacteria from different sources. A number of studies have identified specific virulent DNA sequences in *L. monocytogenes*, which do not cross-react with other *Listeria* species or other bacterial genus (Fitter, Heuzenroeder & Thomas, 1992). Although the conventional PCR techniques could detect both viable and non-viable cells, the inclusion of an enrichment step or separation by immunomagnetic particles can solve this disadvantage (Cudjoe, Patel, Olsen, Skjerve & Olsvik, 1997). The PCR assays still do not discriminate strains from different origins and it is possible for the technique to give false-positives due to amplification of DNA from dead *L. monocytogenes* cells, which limits its application to food processes. This problem can be overcome by using reverse transcription amplification (RT-PCR) to detect messenger RNA based on its very short half-life. Several virulence-associated genes have been sequenced and used as PCR target for the detection of these bacteria. *L. monocytogenes* detection methods based on PCR amplification of the *iap*, *prf*A and *hly* gene sequences have been reported (Klein & Juneja, 1997; Herman, De Block & Moermanst, 1995; Makino, Okada & Maruyama, 1995). Recently a new method based on RT-PCR amplification of the *iap* mRNA for specific detection of viable *L. monocytogenes* showed a sensitivity of ca. 10 to 15 CFU/ml from pure culture, and the assay could be completed in 54 h (Klein & Juneja, 1997). It was validated to detect the pathogen in ready-to-eat, refrigerated meat products. However, the *iap* gene could not be the best target due to its presence in many species of *Listeria* and specially pathogenic strains because no humoral response against p60 could be found in listeriosis patients (Grenningloh, Darji, Wehland, Chakraborty & Weiss, 1997). Although most of the PCR and RT-PCR methods have been proved to be rapid, sensitive and specific for the detection of viable cells they still are unable to discriminate pathogenic from non-pathogenic strains of *L. monocytogenes* in a mixed microbiota. We believe that the *inl* genes should be further explored for this purpose because their products confer invasiveness to many mammalian cells (Gaillard et al., 1991), the first necessary step to produce disease.

The present study has been undertaken to explore the internalin gene sequences by a hot-start PCR for the detection of pathogenic *L. monocytogenes* cells.

2. Materials and methods

Bacterial strains. The bacteria used in this study are listed in Table 1. Other strains used were *L. monocytogenes* ATCC 15313^{T}(**T** = type strain) (serovar 1/2 a), *L. monocytogenes* Scott A (serovar 4b), and one of each *E. coli* ATCC 11229, *Staphylococcus aureus* ATCC 6538 and *Salmonella choleraesuis* ATCC 10708.

Culture conditions. Stock culture was obtained in heart infusion agar containing 0.5% of yeast extract (HIAYE) and stored at 4°C throughout the course of

Table 1
Listeria strains used in this study[a]

Organism	Origin	Source[b]
Listeria spp.		
L. monocytogenes, serovar 1/2 *a*	Food-borne illness	HPB 1671
L. monocytogenes, serovar 1/2 *b*	Food-borne illness	HPB 1377
L. monocytogenes, serovar 1/2 *b*	Food-borne illness	HPB 1327
L. monocytogenes, serovar 4 *b*	Food-borne illness	HPB 1469
L. monocytogenes, serovar 4 *b*	Food-borne illness	HPB 1488
L. monocytogenes, serovar 1/2 *c*	Environmental	HPB 12
L. monocytogenes	Environmental	UF
L. monocytogenes	Environmental	UF
L. monocytogenes	Environmental	Personal collection
L. monocytogenes	Environmental	Personal collection
L. seeligeri	Unknown	HPB 62
L. innocua	Unknown	HPB 124
L. grayi	Unknown	HPB 29
L. welshimeri	Unknown	HPB 32
L. ivanovii	Unknown	HPB 27
L. ivanovii	Wild type	WSLC 3009[c]

[a] Broth cultures containing ca 3×10^{8} CFU of various *Listeria* and non-*Listeria* bacteria per ml were submitted to the DNA extraction procedures described in the text followed by analysis with inlA PCR assay.

[b] HPB Health Protection Branch (Ottawa, Canada).

[c] WSLC Weihenstephan *Listeria* Collection, Freising, German; Seafood Microbiology Laboratory of the University of Florida (Gainesville, Fl, USA).

this study. Cultures were activated in heart infusion broth plus 0.5% yeast extract (HIBYE) overnight at 30°C. Shaking in the same media overnight at 30°C routinely grew each strain. All media and ingredients were obtained from Difco laboratories (Detroit, Michigan).

Sample preparation for specificity and sensitivity tests of the PCR assay. The specificity of the PCR assay was performed using strains of bacteria already mentioned. Bacterial cells were grown overnight at 30°C onto HIBYE, and 500 μl (3×10^8 UFC/ml) were harvested for template DNA extraction. For sensitivity of PCR detection an overnight culture of *L. monocytogenes* Scott A was centrifuged at 12 000 g for 10 min, washed once in phosphate buffered saline (PBS) and serial diluted. Viable cell numbers were determined by plating dilutions of cell suspensions onto HIAYE in triplicate and incubating the plates at 30°C for 24 h.

Template DNA preparation. Five hundreds microliters of the *Listeria* spp. and non-*Listeria* cultures were centrifuged 10 min at 12 000 g to collect the bacterial cells, and the supernatant was discarded. The pellets were dissolved in 100 μl of 1X PCR buffer containing 2 mg/ml of lysozyme (Sigma, USA), 10 mM tris-HCl pH 8.3, 50 mM KCl and 0.0017%(p/v) gelatin and incubated at room temperature for 15 min. Crude cells lysates were treated with 1 μl of proteinase K (Boehringer Mannheim, German) solution (20 mg/ml) and incubated for 1 h at 55°C. The lysates were boiled for 10 min to inactivate proteinase K. A 5% Chelex (Sigma) solution was added, then centrifuged at 13 000 g for 30 s. The supernatants were saved for the PCR reactions.

PCR primers. Oligonucleotide primers for the PCR assay were selected based on the published nucleotide sequence of the *inl*A gene (Nierderhause et al., 1992). We used the pair of primers 01 (5′-AGCCACTTAAGGCAAT-3′) and 02 (5′-AGTTGATGTTGTGTTAGA-3′) to amplify a 760 bp DNA fragment that corresponds to the region of repeats B of *inl*A gene which extends from 1472 to 2232 (Poyart et al., 1996). The primers were synthesized in the Interdisciplinary Center for Biotechnology Research at the University of Florida (Gainesville, FL).

Using a DNA thermal cycler (Perkin–Elmer model 9600) performed PCR Amplification – The amplification reaction of all bacterial DNA tested. Typical reaction mixture contained 47.5 μl of PCR supermix (2.5 μl of each primer at 10 μM, 5.0 μl of 10X PCR buffer, 2.0 μl of 25 mM $MgCl_2$, 2.5 μl of 2 mM of dNTPs mix, in 33 μl of ddH_20). Two μl of each supernatant were added to the PCR mix. For each PCR tube we added an Ampliwax™ gem (Perkin–Elmer). Then the tubes were capped carefully and the samples were heated in the thermal cycler to 80°C for 2 min. Samples were removed from the thermal cycler and set aside for 5 min. Finally we added 0.25 U of taq DNA polymerase to each sample. Forty amplification cycles were performed, each consisting of a denaturation of 45 s at 94°C, an annealing of 45 s at 50°C and an extension of 60 s at 72°C. Final extension was performed at 72°C for 5 min. The reaction products were cooled to 4°C until the assay of the amplifiers. PCR products were separated by electrophoresis at 80V on 1.3% TBE (w/v) agarose gel, followed by ethidium bromide staining. Molecular marker of 50–2000 kb ladders (AmpliSize, BioRad) was used as a size standard. When tenfold dilutions of the genomic DNA were tested, the predicted 760 bp segment was successfully amplified.

3. Results and discussion

The presence of a segment of *inl*A gene was studied by amplifying intragenic fragments of repeats B in 12 strains of *L. monocytogenes* as compared to 6 strains of other *Listeria* species (*L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri* and *L. grayi*) and 3 strains of unrelated Gram positive and Gram negative species (*Salmonella choleraesuis*, *Escherichia coli* and *Staphylococcus aureus*). For each pair of primers specific for intragenic fragments of repeats B of *inl*A, a single DNA fragment of the expected size (760 bp) was observed (Fig. 1) for all *L. monocytogenes* strains, including isolates from foodborne illnesses and the environment (Table 2). In contrast, no amplification product was detected with any strain belonging to other *Listeria*

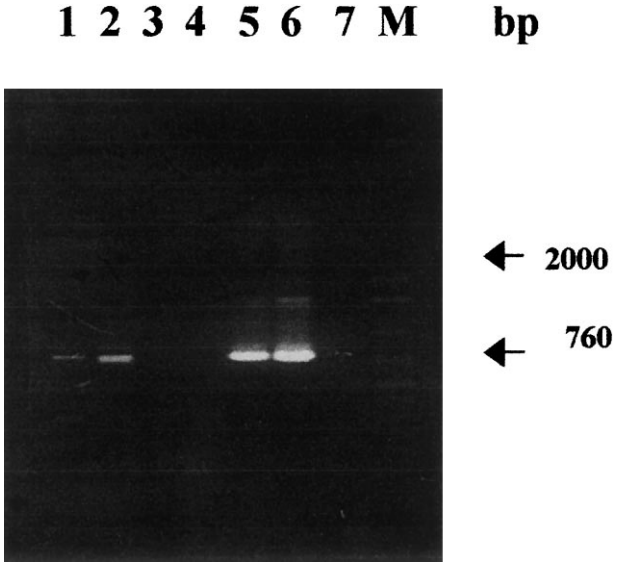

Fig. 1. Specificity and discriminatory results of the PCR assay in detecting the *inl*A gene from cells of representative *Listeria* species. Lanes 1 and 2, *L. monocytogenes* from environment; lane 3, *L. seeligeri*; lane 4, *L. ivanovii*; lanes 5 and 6 *L. monocytogenes* from two recent foodborne illnesses; lane 7, no cells; lane M Amplisize (BioRad) ladder.

Table 2
Specificity of the *inl*A PCR protocol in the assay of *Listeria* species and non-*Listeria* bacteria[a]

Organism	No. of positive strains/Total[b]
Listeria spp.	
L. monocytogenes	12 [c]/12
L. ivanovii	0/2
L. seeligeri	0/1
L. welshimeri	0/1
L. gravyi	0/1
L. innocua	0/1
Non-*Listeria*	
Escherichia coli	0/1
Salmonella choleraesuis	0/1
Staphylococcus aureus	0/1

[a] Broth cultures containing ca. 3×10^6 CFU of various *Listeria* and non-*Listeria* bacteria per ml were subcultured to the DNA extraction procedures described in the text followed by analysis with inlA PCR assay.

[b] Number of strains giving positive results in the PCR assay per total number tested.

[c] Two strains of *L. monocytogenes* from the environment produce weaker amplification signals.

species or to unrelated bacterial species by ethidium bromide staining (Table 2).

These results indicate that *inl*A is constantly present in *L. monocytogenes*, regardless of the origin and serovar of the isolates. Of great significance for future research are the findings that at least two strains of *L. monocytogenes* isolated from the environment which produced weaker signals (Fig. 1). This could elucidate a methodology to differentiate environmental from clinical strains.

These results confirm that *inl*A sequence targeted is specific for *L. monocytogenes* and the PCR assay is sufficiently discriminatory to enable detection of *L. monocytogenes* in pure culture. Experiments for sensitivity showed that the PCR assay could detect as few as 10 CFU of *L. monocytogenes* in a broth culture (data not shown). Studies are underway to turn this technique applicable for detection of *L. monocytogenes* in foods. Design of new primers or exploration of the PCR conditions are needed to discriminate between clinical and environmental strains of *L. monocytogenes* because many isolates of this species from the environment could show amplification signals without being a pathogenic strain. Although the PCR technique using *iap* gene as target is very sensitive it could not be appropriated to distinguish pathogenic from non pathogenic strains of *L. monocytogenes* (Poyart, Trieu-Cuott & Berche, 1996). Also, in studies conducted in human patients with known or suspected former contact to *Listeria*, none of the tested sera antibodies against p60 (the product of *iap* gene) could be found (Grenningloh et al., 1997). According to this group, led by Dr. Siegfried Weiss, the humoral response in listeriosis patients appeared to be more heterogeneous and included *hly*, *irp*, *inl*, and *act* as major targets. *Inl* genes seem to be controlled at the transcriptional level resulting in low expression, which could limit their application to *inl* mRNA by RT-PCR detection. It is still necessary to overcome this disadvantage. Therefore, an alternative technique for the detection of *L. monocytogenes* with the ability to differentiate between pathogenic and non-pathogenic strains is needed.

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