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Research Article

A PCR PROTOCOL USING LISTERIOLYSIN O (hlyA) GENE AS A TARGET FOR SPECIFIC DETECTION OF *LISTERIA MONOCYTOGENES*

***A. Sheeladevi and N. Ramanathan**
Deapartment of Microbiology, Annamalai University,
Annamalai Nagar, Chidambaram – 608 002
**Author for Correspondence*

ABSTRACT
Listeria monocytogenes is one of the important etiologic microorganisms of food poisoning. *Listeria monocytogenes* is a Gram-positive bacterium responsible for the severe foodborne illness listeriosis. This disease is primarily transmitted through various foods, fish, dairy products, cured or processed meat, egg, poultry, seafood, salad, fruits and vegetables. The preventive activity such as HACCP against *L. monocytogenes* contamination in food processing has been regarded as an effective measure for the prevention of listeriosis outbreaks. It requires rapid detection and quantification of contaminated pathogen for microbiological testing in verifying food safety. The development of rapid detection and rapid counting methods for the *L. monocytogenes* will play an important role for this goal. Regarding this, PCR technology has successfully shortened analysis time and has been widely applied for the detection of foodborne pathogens. In this study, to determine the detection of *L. monocytogenes* isolated from milk sample using the hlyA gene encoding listeriolysin O was chosen as a target for PCR amplification because this gene is a virulence determinant and has been tested for the specific detection of *L. monocytogenes*. Listeriolysin O (hlyA) gene detection gave positive result in which a band with 172bp size appeared on gel confirmed the detection and identification of *Listeria monocytogenes*.

Key Words: *Listeria Monocytogenes, Listeriolysin O (hlyA) Gene, PCR Technology, Milk*

INTRODUCTION
Listeria monocytogenes has become an issue of global concern because of its increased presence in milk and other food products (WHO 2002). *Listeria* species are Gram-positive, ubiquitous bacteria widely distributed in the environment. The genus *Listeria* contains six species namely *L. monocytogenes, L. ivanovii, L. innocua, L. welshimeri, L. seeligeri, L. grayi.*of which *Listeria monocytogenes* is the only species of public health significance (Swaminathan 2001 and Kumar 2011). With manufactured ready-to-eat foods being consumed in increasing quantities, it is no surprise that *L. monocytogenes* has become recognized as an important opportunistic human foodborne pathogen (Liu 2006). *L. monocytogenes* can cause serious diseases, such as septicemia, meningitis, meningoencephalitis in immunocompromised individuals, newborns and the elderly, and abortion and stillbirth in pregnant women (Vazquez-Boland *et al*., 2001).
Many different conventional testing methods have been developed for the detection and enumeration of *L. monocytogenes* from food; these have relied almost exclusively on the use of specific culture media followed by a series of tests for confirmation. Conventional plate counting methods are laborious, time-consuming and sometimes underestimate the numbers (Besnard *et al.,* 2000, Scotter *et al.*, 2001). To overcome these limitations, molecular biological, biochemical and immunological techniques have been applied for the rapid and specific detection of *L. monocytogenes* (Almeida and Almeida 2000, Solve *et al*., 2000).
Among these, PCR has been increasingly used for the rapid, sensitive and specific detection of foodborne pathogens (Olsen *et al.,* 1995). The aim of this study was to determine the prevalence of *L. monocytogenes* contamination in raw milk samples using a PCR protocol targetting Listeriolysin O (hlyA) gene for specific detection of *Listeria monocytogenes*.

MATERIALS AND METHODS

Bacteria

The standard strain of *L. monocytogenes* (MTCC 657) used in the study was obtained from IMTECH, Chandigarh, India.

Samples

The *L. monocytogenes* isolate included in the study was obtained from milk samples, collected from Cuddalore district, Tamilnadu. Morphologically typical colonies of recovered *L. monocytogenes* isolate was verified by the following tests Gram's staining, catalase reaction, oxidase reaction, tumbling motility at 20–25°C, Methyl red-Voges Proskauer (MR-VP) reactions, CAMP test, haemolysis on sheep blood agar (SBA), nitrate reduction, and fermentation of sugars (rhamnose, xylose, and α-methyl-D-mannopyranoside) along with reference strain MTCC 657.

Template DNA Preparation

Template DNA was prepared according to Sambrook *et al.,* (2001). In a 1.5ml microfuge vial 1.5ml of overnight grown bacterial cells was taken. The culture tube was spun at 10,000 rpm for 5 min. After discarding the supernatant, the pellet was dissolved in 400µl of extraction solution [0.1M Tris – HCl (pH-8), 0.1M EDTA (pH-8), 10% SDS (W/V) and Proteinase K (20mg/ml)] and incubated at 65° C for 10 minutes. The tube was allowed to cool to room temperature. The tube was spun at 10,000 rpm for 5 min. The supernatant was transferred into a new tube. To the supernatant double volume of ice cold ethanol and 5µl of 3M sodium acetate was added. After 5 min incubation on ice, the tube was spun at maximum speed for 10 min. The supernatant was discarded and the pellet was washed with 0.2ml of 70% ethanol. After discarding the wash solution the pellet was air dried at room temperature for 5 min. Pellet was dissolved in 0.1ml of sterile nuclease free water and used as template DNA for PCR amplification.

Selection of Primers

The primer sequences LL7 Forward, TTGCCAGGAATGACTAATCAAG, LL8 Reverse, and ATTCACTGTAAGCCATTTCGTC were used for detecting *Listeria monocytogenes* targeting Listeriolysin O (hlyA) – gene was synthesized according to Giammarini *et al.* (2003). Primer sequences used for the amplification were synthesized from Bioserve India Pvt. Ltd. Hyderabad, India. PCR was performed in Thermalcycler (Lark Research Model L125 +, India).

PCR Amplification

PCR Reaction Mix

Amplification reactions were performed in 50 µl volume in a 0.2 mL optical-grade PCR tubes (Tarsons, India). 50 µl reaction mixes contains 25µL Master Mix (2X) consist of 1.5U of Taq DNA Polymerase, 1.5 mM MgCl2, 100µM dNTPs (Genei, India). 2µl of 20 pM of each primer, 2µl of DNA sample was used for amplification and the final volume make up to 50 µl with sterile nuclease free water.

The PCR Thermal cycling conditions were: Initial Denaturation at 95°C for 15 min, Denaturation of primer at 95°C for 15sec., Primer annealing at 61°C for 20sec. and Extension at 72°C for 30sec for a total of 40 cycles, followed by a 4 min. final extension period. The expected size of the amplified product of Listeriolysin O (hlyA) – gene was 172bp. The product was analyzed in 2.0% Agarose gel.

Gel Electrophoresis

Ten to Fifteen microlitre aliquots from each PCR amplified product were analysed by 2% agarose gel electrophoresis in 1 X Tris-borate-EDTA buffer. Gels were stained with Ethidium bromide (0.5µg/ml). The DNA fragments were visualised by UV transillumination and photographed. The molecular sizes of the PCR products were compared with a 100bp DNA ladder.

RESULTS AND DISCUSSION

The detection of pathogenic bacteria is a fundamental objective of food microbiology ensuring food quality. The current microbiological culture procedures are laborious and time-consuming. Consequently, there is an increased need for a rapid and reliable detection method to guarantee food safety. Regarding this, PCR technology has successfully shortened analysis time and has been widely applied for the detection of foodborne pathogens (Bruce 1994). Several of these PCR based methods

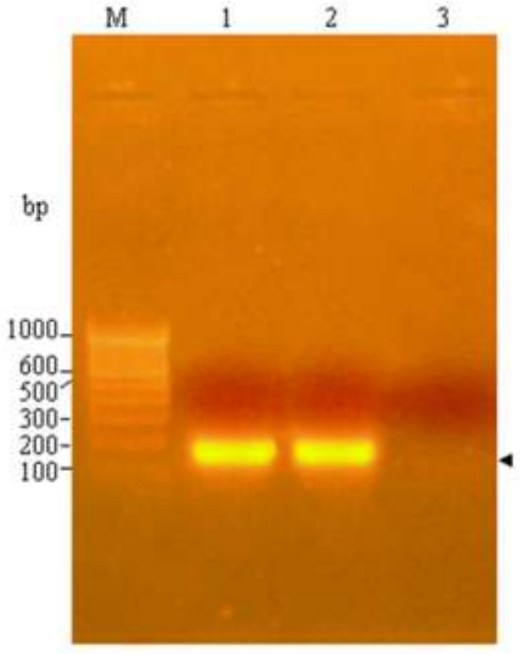
were developed for the detection of *L. monocytogenes* and involve a preenrichment step (Bansal, 1996, Manzano *et al.*, 1997), the use of monoclonal antibody-coated beads for bacteria concentration (Fluit *et al.*, 1993, Nogva *et al*., 2000) or the chemical extraction of food components (Herman *et al*., 1995).

Two nucleic acid-based methods for rapid and sensitive detection of the foodborne pathogen *Listeria monocytogenes* in milk were developed. These methods rely on paramagnetic nanoparticle-based isolation of bacterial DNA directly from milk and subsequent PCR with selective primers for the listeriolysin O (hlyA) gene. The hlyA specific product was reproducibly detected and showed a sensitivity of 10 cfu ml^{-1}. The magnetic-based system had a sensitivity 10-fold higher than that of commercially available column devices. The detection limit of both methods is sufficient for direct detection of *L. monocytogenes* DNA in milk avoiding the enrichment culturing step, reducing the time necessary to obtain results from samples to 7 h rather than the 5-day minimum required for the standard procedure. The methods developed are suitable for automation. (Amagliani *et al.*, 2004)

In this study, the primers LL7 and LL8 were selected to amplify a 172 bp region of the hlyA gene. On the basis of the published nucleotide sequence reported by Giammarini *et al.* (2003), primer location within the coding region of the listeriolysin O gene and the primer sequences are LL7 Forward, TTGCCAGGAATGACTAATCAAG, LL8 Reverse, and ATTCACTGTAAGCCATTTCGTC.

The PCR product size was determined with the 100 bp DNA ladder in the agarose gel and was verified with the same size of resultant product in the positive control lane. There was no amplification in the negative control lane. The PCR amplification using LL7 and LL8 primers generated a product of expected size from *L. monocytogenes* strains. These results clearly indicate that the primers LL7 and LL8 have a high affinity for the correct target sequence and are specific for *L. monocytogenes* and results are presented in Figure 1.

Figure 1: Showing 172 bp amplified Listeriolysin O (hlyA)-gene for the detection of Listeria *monocytogenes* by using Polymerase Chain Reaction (PCR)



Lane 1: Positive Control
Lane 2: Amplified Sample Product
Lane 3: Negative Control
Lane M: 100 bp DNA Ladder

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