

Research Note

Detection and Differentiation of *Listeria monocytogenes* and *Listeria innocua* by Multiplex PCR

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An important and emerging food borne pathogen, *Listeria monocytogenes*, is a Gram positive facultative intracellular organism, causing many serious illnesses including abortion, meningitis, septicemia and gastroenteritis in human beings and animals (Vázquez-Boland *et al.*, 2001). In ruminants, the organism has been reported to cause 'circling disease', due to the attack on central nervous system (CNS) (Vishwanathan & Ayyar, 1950). *Listeria* spp. including *L. monocytogenes* are frequently being isolated from different seafood with report of occasional outbreaks (Farber *et al.*, 2000; Jeyasekaran *et al.*, 2003). A 'zero tolerance' policy for *L. monocytogenes* has been adopted by most of the regulatory bodies including United States Food and Drug Administration (USFDA) (Hitchins, 1998).

In contrast, another species of *Listeria* i.e. *L. innocua* is a totally non-pathogenic organism (Rocourt, 1999). Hence, it is very important to differentiate the potential pathogen *L. monocytogenes* from the apathogenic species *L. innocua*. Both the species share common biochemical characteristics and the conventional method for differentiation of these two species is based on the expression of hemolytic activity by *L. monocytogenes* on sheep blood agar (Hitchins, 1998). But the detection of hemolysis is very

often ambiguous (Skalka *et al.*, 1982; 1983). Although ELISA has been successfully used for detection of *Listeria* spp., this test can not differentiate *L. monocytogenes* from *L. innocua* (Lovett *et al.*, 1987; Mattingly *et al.*, 1988). Polymerase chain reaction (PCR) has been found to be a very sensitive and specific tool for detection of *L. monocytogenes* as well as *L. innocua* (Border *et al.*, 1990; Herman *et al.*, 1995; Liu *et al.*, 2003; Rawool, 2005). Keeping these in view, the present study has been designed to differentiate *L. monocytogenes* from *L. innocua* in a single tube Multiplex PCR reaction.

The bacterial strains used in the study have been listed in Table 1. All the strains used with the exception of *Lactobacillus acidophilus* were grown on Tryptic soya broth (Difco) and confirmation of the *Listeria* strains was done by standard biochemical tests and hemolysis (Hitchins, 1998). *L. acidophilus* was grown in MRS broth in presence of 5% CO₂ tension.

Lysate from the bacteria was prepared by boiling method as described by Fitter *et al.* (1992) with slight modifications. Overnight grown bacterial culture was centrifuged at 7000 X g for 10 minutes at 4°C and the resultant pellet was washed once in normal saline. The pellet was suspended in

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150 µl autoclaved Millipore distilled water, kept in boiling water bath for 10 minutes and frozen immediately at -70°C. Before use as PCR template, the lysed bacterial suspension was thawed and centrifuged at 4000 X g to pellet the debris. Five µl of the supernatant was used as template in the PCR reaction.

Three sets of primers have been used in this study viz. *Listeria* genus specific primer UI/LII (Border *et al.*, 1990), *L. monocytogenes* species specific primer LL5/LL6 (Herman *et al.*, 1995) and *L. innocua* species specific primer lin0464F/lin0464R (Liu *et al.*, 2003). For standardization of concentration of primers, 3 sets of primers were used in two different concentrations (10 pmol or 20 pmol) in various combinations and it was observed that combination of 10 pmol of each LL5/LL6 and lin0464F/lin0464R with 20 pmol of UI/LII yielded the best result. In PCR, 5 µl of template was amplified in a reaction volume of 25 µl containing 10mM Tris-HCl (pH 9), 50 mM KCl, 1.5 mM MgCl₂, 200 µM of each dNTP (Finnzyme, Finland), 1 U *Taq* DNA polymerase (Bangalore Genei, India), 10 pmol of each primers LL5, LL6, lin0464F & lin0464R and 20 pmol of each primers UI & LII. PCR

was performed in different annealing temperatures i.e. 53, 55, 57 and 60°C. PCR was carried out with the following reaction condition: an initial denaturation at 95°C for 3 minutes followed by 30 cycles of denaturation (94° for 45 seconds), annealing (53/55/57/60°C for 45 seconds) and extension (72°C for 45 seconds). The final extension was carried out at 72°C for 5 minutes. Analysis of PCR products was performed on 1.5% agarose gel containing 0.3 µg/ml of ethidium bromide. Electrophoresis was carried out in 1X TAE buffer (40mM Tris-acetate and 1mM EDTA, pH 8.0) at 7 volts/cm for 1 hour 30 minutes. The gel was photographed using UV gel documentation system (Alpha Innotech Corporation, USA).

Table 1: Bacterial strains used in the study

Organism	Strain
<i>Listeria monocytogenes</i>	NCTC 11994
<i>Listeria monocytogenes</i>	MTCC 657
<i>Listeria innocua</i>	ATCC 33090
<i>Listeria ivanovii</i>	ATCC 19119
<i>Listeria grayi</i>	ATCC 19120
<i>Listeria monocytogenes</i>	Freshwater fish isolate
<i>Listeria innocua</i>	NCAFB-L-SD-1
<i>Staphylococcus aureus</i>	NCIM 5022
<i>Bacillus cereus</i>	NCIM 2106
<i>Lactobacillus acidophilus</i>	MTCC 447
<i>Streptococcus pyogenes</i>	NCIM 2608

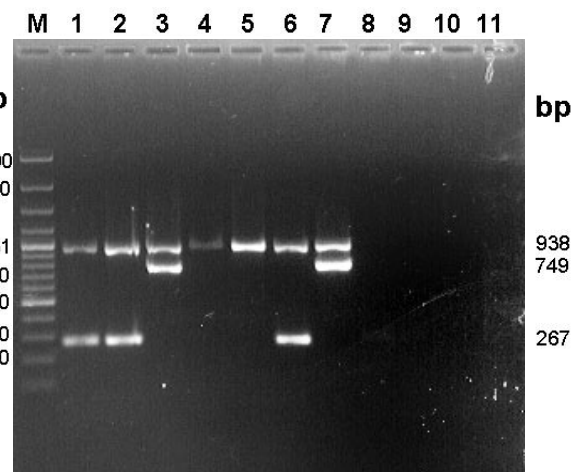


Fig. 1. Multiplex PCR for differentiation of *Listeria monocytogenes* and *Listeria innocua*: Lane M: 100 bp DNA ladder plus, Lane 1: *Listeria monocytogenes* NCTC 11994, Lane 2: *Listeria monocytogenes* MTCC 657, Lane 3: *Listeria innocua* ATCC 33090, Lane 4: *Listeria ivanovii* ATCC 19119, Lane 5: *Listeria grayi* ATCC 19120, Lane 6: *Listeria monocytogenes* freshwater fish isolate, Lane 7: *Listeria innocua* NCAFB-L-SD-1, Lane 8: *Staphylococcus aureus* NCIM 5022, Lane 9: *Bacillus cereus* NCIM 2106, Lane 10: *Lactobacillus acidophilus* MTCC 447 and Lane 11: *Streptococcus pyogenes* NCIM 2608.

In this study, a multiplex PCR was developed for the detection and differentiation of *L. monocytogenes* and *L. innocua*. Among the different annealing temperatures

tested, 55°C was found to give the best results. Hence, this annealing temperature was used for all further experiments. The multiplex PCR gave a *Listeria* genus specific product of 938 bp with all the *Listeria* spp. tested. The multiplex PCR did not give any PCR product with *Staphylococcus aureus*, *Bacillus cereus*, *L. acidophilus* and *Streptococcus pyogenes*. Along with the genus specific product, a second product of 267 bp was obtained for *L. monocytogenes* while a product of 749 bp was obtained for *L. innocua* (Fig. 1).

Due to similar biochemical characteristics, the differentiation of *L. monocytogenes* and *L. innocua* is very difficult (Hitchins, 1998). Differentiation of these two species is very much essential from the public health point of view. In this study, a multiplex PCR was developed that can detect and differentiate *L. monocytogenes* and *L. innocua* using a single PCR reaction. The PCR assay was also able to differentiate genus *Listeria* from other bacteria as evidenced by the presence of 938 bp band in case of *L. monocytogenes*, *L. ivanovii*, *L. innocua* and *L. grayi*, but not in bacteria other than genus *Listeria* (Fig. 1).

The Multiplex PCR assay can be used in identification of *Listeria* spp. as well as in differentiation of *L. monocytogenes* from *L. innocua* within a very short period of time.

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References

- Border, P. M., Howard, J. J., Plastow, G. S. and Siggins, K. W. (1990) Detection of *Listeria* spp. and *Listeria monocytogenes* using Polymerase Chain Reaction. *Lett. Appl. Microbiol.* **11**, pp 158-162
- Farber, J. M., Daley, E. M., MacKie, M. T. and Limerick, B. (2000) A Small Outbreak of Listeriosis Potentially Linked to the Consumption of Imitation Crab Meat. *Lett. Appl. Microbiol.* **31**, pp 100-104
- Fitter, S., Heuzenroeder, M. and Thomas, C. J. (1992) A Combined PCR and Selective Enrichment Method for Rapid Detection of *Listeria monocytogenes*. *J. Appl. Bacteriol.* **73**, pp 53-59
- Herman, L. M. F., De Block, J. H. G. E. and Moermans, R. J. B. (1995) Direct Detection of *Listeria monocytogenes* in 25 Milliliters of Raw Milk by a Two Step PCR with Nested Primers. *Appl. Environ. Microbiol.* **61**, pp 817-819
- Hitchins, A. D. (1998). Detection and enumeration of *Listeria monocytogenes* in foods. In: *Bacteriological Analytical Manual*, Centre for Food Safety and Applied Nutrition, United States Food and Drug Administration. Accessed online through <http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm071400.htm> on 3-3-2006
- Jeyasekaran, G., Karunasagar, I. and Karunasagar, I. (2003) Incidence of *Listeria* spp. in Seafood Handling Environments. *Ind. J. Fish.* **50**, pp 211-214
- Liu, D., Ainsworth, A. S., Austin, F. W. and Lawrence, M. L. (2003) Identification of *Listeria innocua* by PCR Targeting Putative Transcriptional Regulator Gene. *FEMS Microbiol. Lett.* **223**, pp 205-210
- Lovett, J., Francis, D. W. and Hunt, J. M. (1987) *Listeria monocytogenes* in Raw Milk: Detection, Incidence and Pathogenicity. *J. Food Prot.* **50**, pp 188-192
- Mattingly, J. A., Butman, B. T., Plank, M. C., Durham, R. J. and Robison, B. J. (1988) Rapid Monoclonal Antibody-based Enzyme Linked Immunosorbent Assay for

- Detection of *Listeria* in Food Products. *J. Assoc. Off. Anal. Chem.* **71**, pp 679-681
- Rawool, R. B. (2005) *Study on rapid detection and prevalence of pathogenic Listeria monocytogenes in bovine mastitis cases*. Ph.D. Thesis, Indian Veterinary Research Institute, Izatnagar, India
- Rocourt, J. (1999) The genus *Listeria* and *Listeria monocytogenes*: Phylogenetic position, taxonomy and identification. In: *Listeria, Listeriosis and Food Safety*. (E. T. Ryser and E. H. Marth, Eds) Marcel Dekker, Inc. Newyork, Basel pp 1-20
- Skalka, B., Smola, J. and Elischerocva, K. (1982) Routine Test for in-vitro Differentiation of Pathogenic and Apathogenic *Listeria monocytogenes* Strains. *J. Clin. Microbiol.* **15**, pp 503-507
- Skalka, B., Smola, J. and Elischerocva, K. (1983) Hemolytic Phenomena under the Cultivation of *Listeria innocua*. *Zentral. Bakteriolog. Mikrobiolog. Hyg. Abt. 1 Orig. Reihe B*, **29**, pp 642-649
- Vázquez-Boland, J. A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., Gonzalez-Zorn, B., Wehland, J. and Kreft, J. (2001) *Listeria* Pathogenesis and Molecular Virulence Determinants. *Clin. Microbiol. Rev.* **14**, pp 584-680
- Vishwanathan, G. R. and Ayyar, V. V. (1950) Circling Disease of Sheep in Madras State- Etiology Established. *Ind. Vet. J.* **26**, pp 395-402