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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 gastro-enteritis 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% CO_2 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 -70ºC. 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 $MgCl_2$, 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 60ºC. PCR was carried out with the following reaction condition: an initial denaturation at 95ºC for 3 minutes followed by 30 cycles of denaturation (94º for 45 seconds), annealing (53/55/57/60ºC for 45 seconds) and extension (72ºC for 45 seconds). The final extension was carried out at 72ºC 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
Listeria monocytogenes	NCTC 11994
Listeria monocytogenes	MTCC 657
Listeria innocua	ATCC 33090
Listeria ivanovii	ATCC 19119
Listeria grayi	ATCC 19120
Listeria monocytogenes	Freshwater fish isolate
Listeria innocua	NCAFB-L-SD-1
Staphylococcus aureus	NCIM 5022
Bacillus cereus	NCIM 2106
Lactobacillus acidophilus	MTCC 447
Streptococcus pyogenes	NCIM 2608

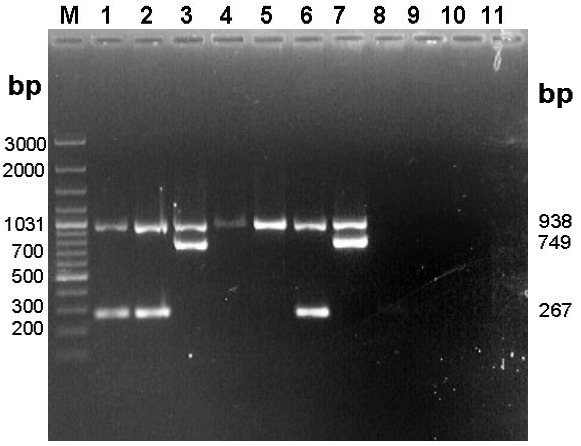

Fig. 1. Multiplex PCR for differentiation of Listeria monocytogenes and Listeria innocua: Lane M: 100 bp DNA ladder plus, Lane 1: Listeria monocytogenes NCTC 11994, Lane 2: Listeria monocytogenes MTCC 657, Lane 3: Listeria innocua ATCC 33090, Lane 4: Listeria ivanovil ATCC 19119, Lane 5: Listeria grayi 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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