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Note

A multiplex PCR for detection of *Listeria monocytogenes* and its lineages

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ABSTRACT

A novel multiplex PCR assay was developed to identify genus *Listeria*, and discriminate *Listeria monocytogenes* and its major lineages (LI, LII, LIII). This assay is a rapid and inexpensive subtyping method for screening and characterization of *L. monocytogenes*.

Listeria monocytogenes, an opportunistic food-borne pathogen responsible for listeriosis, is associated with high mortality rates. Invasive listeriosis is usually severe and clinically manifested as spontaneous abortion, sepsis, and meningoencephalitis, and also as a febrile gastroenteritis syndrome (Mackiw et al., 2016; Barbuddhe and Chakraborty, 2009). *L. monocytogenes* can cause case-fatality as high as 30% in specific high-risk population groups such as immuno-compromised individuals, the elderly, fetuses and newborns (Lomonaco et al., 2015). Estimation of the global burden of listeriosis in 2010 revealed listeriosis to be responsible for 23,150 illnesses, 5463 deaths and 172,823 disability-adjusted life-years (DALYs) globally (De Noordhout et al., 2014).

In contrast to many other foodborne pathogens, *L. monocytogenes* has the ability to grow at refrigeration temperatures and high salt concentration, persist and multiply in the food environment making it difficult to control (Havelaar et al., 2010; De Noordhout et al., 2014). Therefore, precise identification and classification of the pathogen is fundamental in understanding its epidemiology (Jadhav et al., 2015). The delineation of lineages and clonal groups is a prerequisite to examine the within-species genetic variations particularly with respect to pathogenic potential (Doijad et al., 2015). Of the 12 identified serotypes of *L. monocytogenes*, serotypes 1/2a, 1/2b and 4b were mainly (>97%) associated with human diseases (Swaminathan and Gerner-Smidt, 2007). *L. monocytogenes* reveals four serotype-associated lineages, lineages I to IV (Roberts et al., 2006; Ward et al., 2008). Lineage I consists of strains with serotype 4b, 1/2b, and 3b (Doumith et al., 2004a), while, lineage II strains are of serotype 1/2a, 1/2c, 3a, and 3c (Roberts et al., 2006; Ward et al., 2004). Lineage III includes strains of serotype 4a and 4c, as well as certain strains of serotype 4b (Roberts et al., 2006; Ward et al., 2004). Recently, a 4a serotype strain from a subgroup of lineage III has been assigned to an independent lineage, lineage IV (Orsi et al., 2011; Ward et al., 2008). Lineage I isolates include major epidemic clones of *L. monocytogenes* associated with human listeriosis cases (Sauders et al., 2006), while, lineage II isolates are isolated mostly from foods and the environment (Kathariou, 2002), and lineage III isolates are mostly found in animal hosts (Kathariou, 2002).

An accurate, inexpensive, rapid and a high-throughput method for identification of *L. monocytogenes* and its lineage is needed to study the population genetics, ecology, and epidemiology of this food-borne pathogen. Such assays can help in understanding the biological and regulatory significance of the evolutionary lineages that have been identified within this species. Also, species identification combined with molecular subtyping would simplify characterization, tracking, and control of *L. monocytogenes*, especially those lineages, serotypes or epidemic clones responsible for the most of the food-borne outbreaks. In recent past, microarray based differences identified in the genomic content among strains of different serotypes have led to the development of a PCR-based method for discrimination of four clinically significant serovars (1/2a, 1/2b, 1/2c, and 4b) into four distinct serogroups (Doumith et al., 2004b). In addition to this, to determine the lineage of individual *L. monocytogenes* isolates an ASO PCR was developed by Ward et al. (2004). The accuracy of this test was evaluated by comparing the ASO-PCR results with lineage identifications based on *p*VGC sequence data (Ward et al., 2004). However, the developed assay failed to

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Table 1
Nucleotide sequences of primer sets used in the study.

Gene	Primer sequences	Product size (bp)	Protein encoded	Source
prs	F - 5′-AGCTGAAGAGATTGCGAAAGA-3′ R - 5′-TTCACCAAGAAGAGCTGCAA-3′	844	lmo4a_0215 phosphoribosyl pyrophosphate synthetase	This study
isp	F - 5′-TGCAGCGAATGCTCTTAGTG-3′ R - 5′-AGCCAAGCACGGCTACTTTA-3′	713	lmo1441: similar to putative peptidoglycan acetylation protein	This study
L1	F - 5′-GGCGCATTCAAATCCAAGAG-3′ R - 5′-GTGGTTGCTTGGTACAATGAG-3′	384	LMOF2365_RS13380 (old_locus_tag LMOf2365_2638) cell wall surface anchor family protein	This study
L2	F - 5′-CAGAAAATGGCTGGGGATTA-3′ R - 5′-GCGGAACATTGGTCTGAACT-3′	476	lmo0525 hypothetical protein	This study
L3	F - 5′-GTAAGCGAGCTTTAGGAGAGTT-3′ R - 5′-CGTATATGCCTAAACCTACACCA-3′	261	LMO4A_RS05595 (old locus tag lmo4a_1083) hypothetical protein	This study

detect genus *Listeria*, *L. monocytogenes* and the lineages of *L. monocytogenes* in a single tube reaction. Later, a multiplex PCR assay which can detect bacteria of the genus *Listeria*, *L. monocytogenes* and epidemic clones I, II, and III of *L. monocytogenes* was also developed (Chen and Knabel, 2007), The major drawback of this assay was its limitation to detect *L. monocytogenes* serotypes 4b and 1/2a which either belong to lineage 1 and II, respectively. In addition to this, most of the earlier studies were based on the partial information of the strains or limited numbers of the genomes available in the public domain. However, in recent years with the advancement in the technologies, the cost of whole genome sequencing t has reduced considerably and this has led to the inclusion of more number of whole genome sequences of either genus *Listeria* and *L. monocytogenes* in public domain. In this study, we have screened whole genomes of 113 strains of *L. monocytogenes* with a comparative genomic approach to understand the overall phylogenetic relation of *L. monocytogenes* strains (Doijad et al., 2015) and also to delineate specific lineage wise differences, with an objective to develop a novel multiplex PCR which can detect genus *Listeria*, *L. monocytogenes*, and its major lineages in a single tube for rapid ease and simplicity.

The gene targets for genus *Listeria*, *L. monocytogenes* and the three lineages of *L. monocytogenes* were identified by using comparative genomics GECO software (Kuenne et al., 2007). The identified genes targets were also crossed checked by use of Gegenees tool considering the seed value of 200 bp (Agren et al., 2012). The genes that were uniqueto *Listeria* species or lineage were used to design the genus, species and lineages I, II, III specific primers by using online Primer Quest software tool of Integrated DNA Technologies (http://www.idtdna.com/Primerquest/Home/Index). Their characteristics were analysed by PCR Primer Stats of Sequence Manipulation Suite software (SMS) (http://www.bioinformatics.org/sms2/pcr_primer_stats.html), and synthesized from Sigma Aldrich (St. Louis Mo., USA). The sequences of the primers used in the study along with the putative functions of the selected genetic markers and the lengths of PCR products are listed in Table 1.

The overnight grown test cultures in brain-heart infusion broth were used for DNA isolation by using QIAamp DNA kit as per manufacturers instructions. The multiplex PCR assay was standardized and performed in a 25 μl reaction volume; containing PCR buffer (100 mM Tris-HCl buffer, pH 8.3 containing 500 mM KCl, 15 mM MgCl2 and 0.01% gelatin), 1 mM dNTP mix, 25 mM $MgCl_2$ and 50 μM of five primer sets (Table 1), 3 units of Taq DNA polymerase (3B Black Bio, Spain), 50 ng of DNA template and sterilized nuclease free water to make up the reaction volume. The cycling conditions for PCR included an initial denaturation of DNA at 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s, 56 °C for 1 min and 72 °C for 2 min. At the end of all cycles, a final extension at 72 °C for 10 min was performed in a Mastercycler Thermal cycler (Eppendorf, GmbH, Germany). The resultant amplified PCR products were resolved in 1% agarose gel containing ethidium bromide (10 μg ml^{-1}) by electrophoresis using Tris-Acetate-EDTA as running buffer and visualised by gel documentation system employing UVP Gel Seq software (Fig. 1).

The specificity of the PCR was evaluated with 46 previously characterized *L. monocytogenes* strains recovered from human and animal clinical cases and from foods, 10 reference strains of *L. monocytogenes* serotypes and 4 non-*L. monocytogenes* strains. Also, the strains were confirmed and cross checked using serogrouping PCR (Doumith et al., 2004b). The reproducibility of the method was evaluated by performing PCR on all the strains at least three times.

Figs. 1 and 2 show some typical examples of multiplex PCR results obtained following conditions mentioned earlier with *L. monocytogenes* strains and its different serotypes. Table 2 summarizes the results for all the test isolates including reference strains of *L. monocytogenes* and other *Listeria* species. All primer pairs specifically amplified the desired

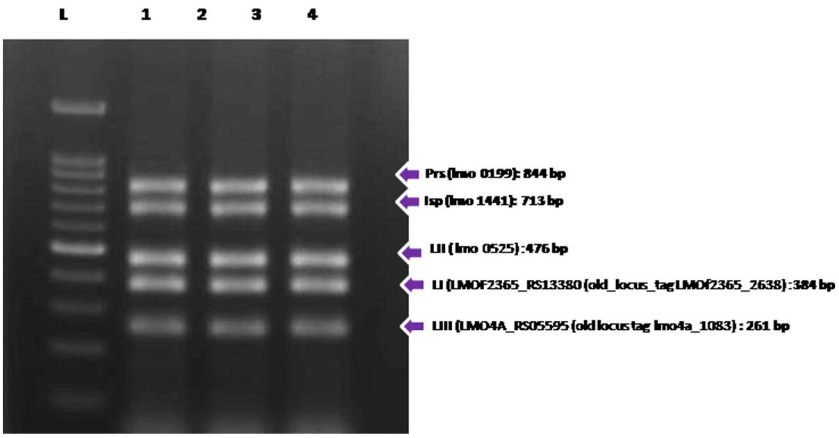

Fig. 1. Multiplex PCR assay revealing detection of genes specific for genus (*prs*), species (*isp*) and lineages (LI, LII, LIII) of *Listeria monocytogenes*. Lane L: 100 bp DNA marker; Lane 1: Cocktail of *Listeria monocytogenes* reference strains of 3b (LI), 1/2a (L II), 4a (LIII)); showing amplification of *prs* (844 bp), *isp* (713 bp), LI (384), LII (476 bp), LIII (261); Lane 2: Similar cocktail of *Listeria monocytogenes* reference strains 1/2b (LI), 3c (LII), 4c (LIII); Lane 3: Similar cocktail of *Listeria monocytogenes* reference strains 4b (LI), 3a (LII), 4a (LIII); Lane 4: negative control.