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RAPID AND CONCURRENT DETECTION OF *LISTERIA* SPECIES BY MULTIPLEX PCR

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ABSTRACT

Listeria sps are ubiquitous in nature. Infection due to *L. monocytogenes* causes illness in animals and humans worldwide. Aim of the present study was to standardize a multiplex PCR for the identification and differentiation of important *Listeria* species, in particular, *Listeria monocytogenes* and to detect toxigenic potential of *L. monocytogenes*. Employing primers for truncated regions of seven genes namely, *inlC, llo, iap, prs, mpl, mogR, ispD* with an internal amplification control, a novel mPCR was developed. The mPCR was found to be robust and specific when tested against non-listerial organisms. The sensitivity of the assay for detection of *Listeria* sps in spiked food samples was 10^2-10^3 cfu/ml. The assay was evaluated with widely used API listeria kit for identification of 107 *Listeria* organisms isolated from 238 food/soil samples. The mPCR correctly and promptly identified majority of the isolates. The assay was able to overcome the false positive results of two mutton isolates and two fish isolates that were identified by API listeria kit. Therefore, this mPCR has the potential to be employed as routine food microbiological and epidemiological investigation tool for *Listeria* sps.
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KEYWORDS: ***Listeria monocytogenes*, Multiplex PCR, PALCAM agar, Specificity, Sensitivity.**

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INTRODUCTION

Listeria monocytogenes, a motile, non–spore-forming, facultatively anaerobic gram-positive bacillus is the etiological agent of listeriosis, a leading food borne and zoonotic infection in the world responsible for nearly 500 deaths out of 2,500 cases that occur annually in the United States alone [1]. Milk and milk products, non vegetarian food products like mutton, poultry, beef, fish and pork, and processed Ready to Eat foods are the major food products contaminated by *L. monocytogenes* [2, 3, 4]. The organism can tolerate heat, pH, osmolarity, able to grow at refrigeration temperatures and viable even at freezing temperatures, thus making it an ubiqutous and formidable pathogen to control [2, 4]. *L. monocytogenes* primarily infects pregnant women, infants, elderly people and the immunocompromised. Two types of Listeriosis have been reported; Invasive listeriosis and non-invasive listeriosis. Invasive Listeriosis results in adverse outcome such as septicemia, meningitis, encephalitis, abortion or stillbirth, materno-fetal infection, endocarditis, cutaneous infections and though rare, it may cause infections, such as endophthalmitis, septic arthritis, osteomyelitis and pleural infection Non-invasive listeriosis causes gastrointestinal illness, which may result in chills, diarrhea, headache, abdominal pain and cramps, nausea, vomiting, fatigue, and myalgia [1, 2] .Listeriosis has the high case fatality rate (20-30% of cases), the long incubation time and the predilection for immunocompromised [5], thus emerging as an atypical foodborne illness of major public health concern. The genus *Listeria* consists of 6 closely related species (*L. monocytogenes, L. ivannovi, L. innocua, L. welshimeri, L. seeligeri, L. grayi*) and is divided into two major lines of descent as *L. grayi* and other *Listeria* species [5]. All *Listeria* species are morphologically identical they are short (0.5 × 1 - 2 micrometers), Gram-positive, catalase-positive, nonacid-fast, nonsporulating, facultative anaerobic rods. They exhibit a characteristic tumbling motility at 20 °C to 25 °C. *L. ivanovii* is the only other pathogenic bacteria besides *L. monocytogenes* [6]. *L. innocua* is not a pathogen but, a fatal bacteraemia caused by *L. innocua* in a 62-year-old Patient was reported as case study in France [7].

Different PCR and molecular typing methods were developed for the identification of *Listeria* species and gene targets that have been applied in the literature included *iap, llo* [3, 8] internalin genes (*inl A, inl B, inl C, inl J*), *lmo2821* [9, 10, 11] , *lmo0737, lmo1118, prs, ORF2110, ORF2819* [12], *prf A* [13]. By targeting these genes it was possible to identify toxigenic potential of *L. monocytogenes*. At the same time a few attempts were made to identify *Listeria* at species level or differentiating *L. monocytogenes* from other important *Listeria* species employing PCRs targeting *scr A, prs, iap, Lin0464,* Oxidoreductase gene and *N*-acetylmuramidase, fibronectin binding protein (*fbp*) genes [2, 14, 15, 16] . Except for a small number of sporadic case reports [17, 18], research on *L. monocytogenes* infections in India is scarce. Many of the food borne outbreaks in the world and in India, particularly, go unreported because of the huge variety of organisms involved and the co-occurrence infectious bacteria with the other non-infectious family members. Often, the cost involved in routine analysis of implicated foods or patient samples in the developing countries would be huge, thus venting for a venture into developing low cost, save reliable detection systems. In the present work, an mPCR was standardized for simultaneous detection of major *Listeria* species viz. *L. monocytogenes, L. ivannovi, L. innocua, L. welshimeri and L. seeligeri.* The current mPCR could provide a fast and reliable tool to study the incidence of the members of the genus *Listeria*, monitoring their spread in food and environmental samples, making it routinely usable in food investigation laboratories.

MATERIALS AND METHODS

(i) Bacterial strains

The standard bacterial strains of *Listeria* and other bacterial species that were used for developing the mPCR assay and specificity tests are given in Table 1. *Listeria* strains were isolated from mutton, poultry, fish, pork, milk and ice cream soil samples as described later.

Table 1
Bacterial species and strains used in the study.

Sl.no	Bacterial strains	
1	*L. monocytogenes* ATCC 13593	Himedia
2	*L. monocytogenes* ATCC 15313	Himedia
3	*L. monocytogenes* ATCC 19115	Himedia
4	*L. monocytogenes* ATCC 19114	Himedia
5	*L. monocytogenes* ATCC 7644	Himedia
6	*L. monocytogenes* NCIM 839	MTCC
7	*L. monocytogenes* NCIM 1143	MTCC
8	*L. monocytogenes* ATCC 19111	Himedia
9	*L. monocytogenes* ATCC 19112	Himedia
10	*L. monocytogenes* ATCC BAA 751	Himedia
11	*L. innocua* ATCC 33090	Himedia
12	*L. ivannovi* ATCC BAA 139	Himedia
13	*L. ivannovi* ATCC 19119	Himedia
14	*L. welshimeri* ATCC 35897	Himedia
15	*L. seeligeri* ATCC 35967	Himedia
16	*L. grayi* ATCC 25401	Himedia
17	*L. grayi* ATCC 700545	Himedia
18	*Staphylococcus aureus* ATCC 600799	Himedia
19	*Staphylococcus aureus* NCIM 2120	MTCC
20	*Bacillus cereus* ATCC 14579	Himedia
21	*Bacillus thurengenesis* ATCC 16872	Himedia

(ii) Genomic DNA preparation

Bacterial cells pelleted from 2mL of overnight culture grown in BHI broth was used to prepare genomic DNA by DNA extraction kit (Mackery-nagel) or by thermal lysis method. The bacterial cells resuspended in 100 µl TE buffer (10 mM Tris and 1 mM EDTA) were incubated at 37 ^{o}C for 30 min along with Lysozyme (10 mg/ml) and then boiled for 15 minutes. The bacterial cell lysates were centrifuged at 12,000 rpm for 2 min and the supernatant stored in -20 ^{o}C for further use.

(iii) Designing of Primers and IAC

The primer pairs were designed to amplify conserved regions of the targeted genes *inl C, llo, iap, prs, mpl, mog R, isp D* using Gene runner software (http://www.generunner.com). The gene bank accession numbers of the gene sequences used to design the primers and Tm values are given in Table 2. Appropriate care was taken to ensure that the primer pairs had similar annealing temperature and the resulting amplicons had a minimum 70 bp difference among themselves (Table 2).

The primer pairs were custom synthesized from Eurofins Biotech, Bangalore, India. A synthetic competitive internal amplification control DNA fragment was synthesized using primer pair complementary to pUC 19 plasmid along with *mpl* gene overhangs at their 5' ends. PCR amplification was performed in an Eppendorf master thermal cycler (Hamburg, Germany) with the following amplification conditions; initial denaturation at 94 ^oC for 5 min followed by 30 cycles of denaturation at 94 ^oC for 30 sec, annealing at 54 ^oC for 1 min and extension at 72 ^oC for 1.30 min. A final extension of 72 ^oC for 8 min was given at the end of PCR reaction. The reaction in 20 µl volume containing 0.5 µM of IAC primers, 2.0 mM of $MgCl_2$, 1X PCR buffer (MBI fermentas), 60 µM of each dNTP (MBI fermentas), 0.5 units of *Taq* polymerase and 50 ng pUC 19 plasmid DNA. The PCR amplicon was purified using PCR purification kit (Mackery Nagel) and the concentration of IAC DNA chosen for use was determined spectrophotometrically at 260 nm and the copy number was calculated using the equation: Genomic copy number/µl = W x $6.023 \times 10^{23}/660$ g mol^{-1} x N, where W is weight of PCR fragment (in g μl^{-1}) and N is number of base pairs of PCR fragment.

Table 2
List of primers used in this study.

Primer designation	Primer sequence 5'-3'	Tm (^0C)	Product size in (bp)	Accession number
ispD	F - AGATGCTAACAACAACGAG R - TAAGAGCATTCCATCCCAG	54 56	1029	EF409983.1
mogR	F- GTTCTTCATACCCAATCCTT R- GTCATGGTTTCGTTTTGTTG	54 52	890	AY590468.1
mpl	F- ATCATAAAACGGACGCATC R- AATCTTTCGCTGCTTGTTGA	54 54	720	EU073159.1
prs	F- CGTGAACTAGCTGAAGAG R- CAGCTGTGTCGATAATGTC	54 56	641	EF110564.1
iap	F - ATACAAATGCAAGCACACC R - CTGTGAAGCGAACTTCCT	54 54	507	AY729921.1
llo	F- GTGGTTCCGCAAAAGATGA R- TTACCGTTCTCCACCATTC	56 54	441	EU073158.1
inlC	F - CAGGCTGCGAGTATTCTA R - CAGTATCTCTGAGTTCGTT	54 54	370	Y07639.1

(iv) Standardization of Multiplex PCR

Multiplex PCR was optimized in an Eppendorf master thermal cycler (Hamburg, Germany) with a reaction volume of 30 µl. The amplification mixture consisted of 50 ng of template DNA, seven pairs of primers complementary to individual genes (*inlC, llo, iap, prs, mpl, mog R, isp D*), IAC DNA, $MgCl_2$, 1X PCR buffer, 200 mM dNTP mix, and 1.5U of *Taq* polymerase (MBI fermentas). The concentration of $MgCl_2$ and primer pairs, were optimized to achieve uniform and efficient amplification of all the targeted genes. The copy number of IAC incorporated in every PCR reaction was adjusted as described elsewhere. PCR amplification was performed with the following amplification condition; initial denaturation at 94 ^oC for 5 min followed by 30 cycles of denaturation at 94 ^oC for 30 sec, annealing at temperatures from 52 ^oC to 58 ^oC for 1 min and extension at 72 ^oC for 60s, 90s, 120s. The annealing temperature and extension time yielding discreet amplicons was adopted for the mPCR. A final extension of 72 ^oC for 8 min was given at the end of every PCR reaction.

(v) Determination of sensitivity and specificity of mPCR

The specificity of mPCR was examined using standard strains of the bacterial species given in table1. Sensitivity of the mPCR was estimated by performing mPCR onto tenfold serial dilution of overnight *L. monocytogenes* ATCC 19115 cultures raised in BHI Broth. CFU counts were recorded by plating 100 µl of each dilution of the bacterial culture on PALCAM agar plates (Mannitol- 10 g/lt, Glucose- 0.5 g/lt, Esculin- 1.0 g/lt, Ferric Ammonium Citrate- 0.5 g/lt, Lithium Chloride- 15 g/lt, Phenol Red- 0.08 g/lt, Acriflavine HCL- 0.005 g/lt, Polymyxin B Sulfate- 0.01 g/lt Ceftazidime- 0.008 g/lt, Agar- 2.0 g/lt pH- 7.2 ± 0.2) [19] with supplements procured from Himedia. Grey-green colonies positive for mannitol fermentation and aesculin hydrolysis and incubating overnight at 30°C. The culture dilutions were allowed to stabilize for 30 min and 1 mL sample from each dilution was processed for DNA extraction by thermal lysis method as described earlier. One microliter of the DNA sample was used as template in the standardized mPCR to determine the minimum no of bacterial cells required for mPCR detection. Concentration of DNA used in the mPCR was standardized by nanodrop method (Thermo Scientific Pvt. Ltd, USA). To achieve rapid identification of the organism directly from culture plates, colony PCR was carried out using a single colony from the BHI agar plate.

(vi) Detection from artificially contaminated food samples

Meat samples (Mutton, Poultry, Fish) (25g) or milk (25 mL) obtained from retail market was stomached in 225 mL of UVM (Proteose peptone- 5 g/lt, Tryptone- 5 g /lt, `Lab-Lemco' powder-5 g /lt, Yeast extract- 5 g /lt, Sodium chloride- 20 g /lt, Disodium hydrogen phosphate- 12 g /lt, Potassium dihydrogen phosphate- 1.35 g /lt, Aesculin-1 g /lt. Final pH 7.4 ± 0.2) [20.] Listeria enrichment broth for 30 min and inoculated with 10 fold dilution series of *L. monocytogenes* ATCC 19115, *L. innocua*, *L. ivanovii*, *L. welshmeri*, *L. seeligeri* overnight BHI culture broth (1mL) and held at room temperature for 30 min. Genomic DNA isolated from 1 ml sample drawn from the homogenates was used as template for mPCR. Simultaneously 100 µl sample from each dilution was plated to assess the CFU's. Similar sampling and analysis were done after 8 h incubation also.

(vii) Investigation of Listeria sps. from food and environmental sources

Meat, chicken, mutton, fish, ice cream and milk samples obtained from local retail vendors were examined for the presence of *L. monocytogenes* by conventional biochemical methods and mPCR method (table 4). Twenty five gram of individual food or 25ml of milk samples were stomached in 225 mL University of Vermount (UVM) enrichment broth containing listerial supplements (Himedia) broth and incubated/enriched at 37 ^{o}C overnight. A loop full of the overnight enrichment culture was inoculated onto PALCAM agar and maintained at 37 ^{o}C for 16h. The presumptive grey colour colonies with black halo were examined for their ability to hydrolyse aesculin. The presumptive Listeria isolates were analyzed by biochemical profiling using API *Listeria* system [21] (BioMerieux. Pvt Ltd). Overnight culture of the *Listeria* isolates grown in BHI broth were adjusted to 0.5 McFarland standards for inoculating the test strips and incubated at 35 ^{o}C overnight. The samples that yielded with atleast one positive *Listeria* isolate were characterized as positive. Genomic DNA (1 uL) prepared from 1 mL enrichment broth by thermal lysis method was used in mPCR reaction.

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Table 4
Listeria Isolates correlated with conventional tests and mPCR.

	No of samples	No. of listeral species	*L. monocytogenes*		*L. innocua*		*L. ivannovi*		*L. welshimeri*		*L. seeligeri*	
			API 20	mPCR	API 20	mPCR	API 20	mPCR	API 20	mPCR	API 20	mPCR
Mutton	70	37	28	26	3	5	4	4	1	1	1	1
Chicken	29	16	8	8	5	5	2	2	1	1	-	-
Fish	68	25	15	17	2	2	4	2	2	2		
Pork	18	9	5	5	3	3	1	1	-	-	-	-
Ice cream	20	11	5	5	2	2	3	3	-	-	1	1
soil	18	11	7	7	2	2	1	1	1	1	-	-
Milk	15	-	-	-	-	-	-	-	-	-	-	-
Total	238	109	68	68	16	19	15	13	5	5	2	2

RESULTS

(i) Bioinformatics analysis/Primer design

Nucleotide sequences of the gene targets/markers associated with virulence in *L. monocytogenes* (*inlC, llo, iap, prs, mpl, mog R, isp D*) were used for BLAST analysis and the homologues of the respective genes from other Listeria species were acquired from public (NCBI) databases. The homologues were aligned using the multiple sequence alignment tool CLUSTAL W and primers were designed in such a way that selective amplification could be obtained.

(ii) Standardization of Multiplex PCR

The genomic DNA of standard ATCC cultures (table 1) was used to optimize the multiplex PCR as described in the materials and methods section. Concentrations of primer sets were optimized to have clear intense amplified products at an annealing temperature of 54 ^{o}C in the presence of 2 mM $Mgcl_2$. For each PCR reaction, 100 nm, 50 nm, 250 nm, 150 nm, 150 nm, 300 nm and 180 nm concentrations of primer pairs specific to the targeted genes *inlC, llo, iap, prs, mpl, mog R* and *isp D* respectively were added. Serial dilution of IAC incorporated in the mPCR assays revealed that 2000 copies of the same resulted in unhindered amplification of *mpl* gene for which the IAC primer pair was competitive, besides meticulously amplifying other mPCR genes (Fig 1). The ability of the mPCR to identify *L. monocytogenes* and differentiate it from other Listeria sps. was validated using listerial species and other bacterial strains listed in table 2. All the 7 genes including the IAC amplified from *L. monocytogenes* strains used in the study. From *L. innocua* 3 genes (*mog R, prs iap*), *L. welshimeri* 2 genes (*mog R, iap*) *L. ivannovi* 1 gene (*inl C*), *L. seeligeri* 1 gene (*mog R*) were amplified along with the IAC. All the other bacterial strains including *L. grayi* amplified only the IAC. The mPCR assay yielded predicted amplification pattern for the *Listeria* species that were used to validate the assay. Consistent results were obtained when the mPCR was repeated atleast 3 times using the genomic DNA from standard listerial strains.

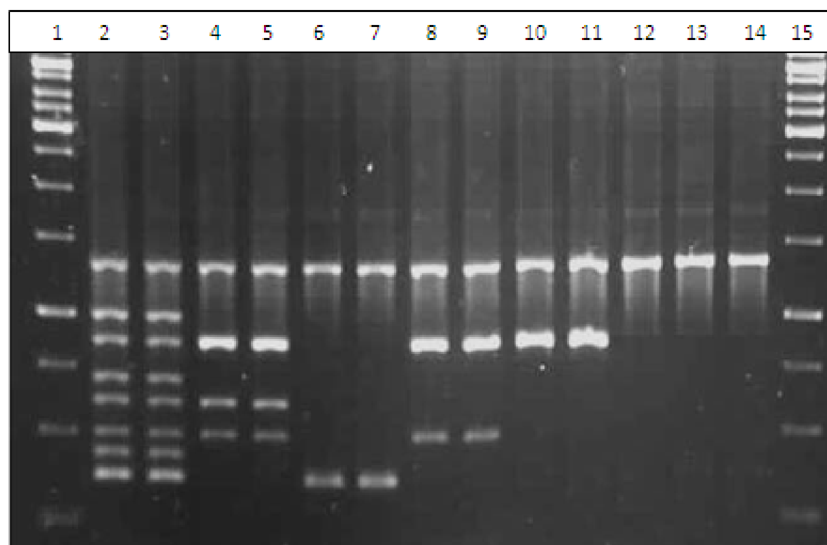

Figure 1
1% agarose gel showing mPCR pattern for the major Listeria species.

Lane 1 & 15- 1 Kb DNA Ladder, Lane 2- *Listeria monocytogenes* ATCC 19115, Lane 3 – *L.monocytogenes* NCIM 1143, Lane 4 – *L. innocua* ATCC 33090, Lane 5 – *L. innocua* MT16 (Isolate), Lane 6 – *L. ivannovi* ATCC BAA 139, Lane 7 – *L. ivannovi* ATCC 19119, Lane 8 – *L. welshimeri* ATCC 35897, Lane 9 – *L. welshimeri* (isolate) , Lane 10 – *L. seeligeri* ATCC 35967, Lane 11 – *L. seeligeri* Sardine3/3 (Isolate), Lane 12 – *L. grayi* ATCC 25401, Lane 13, 14 – Negative control,

Determination of specificity and sensitivity of mPCR

All the *Listeria* species used in the study yielded predicted amplification patterns. *L. grayi* and other non listerial species used in the study and the sample without any genomic DNA amplified only the IAC. The mPCR assay could detect *L. monocytogenes* from culture dilutions with $2X10^2$ CFU/mL. Homogenates of artificially contaminated food samples were examined to assess the sensitivity of mPCR to detect *Listeria* sps directly from food matrices. The detection sensitivity of the assay was found to be 10^2-10^3cfu/ml (table 3). After 6 hours of incubation at 30 ^oC the detection limit from the homogenates was found to be 10^1 CFU/ml. The intensity of individual amplified products was found to be the same when the mPCR was performed onto serial dilutions of the overnight culture or artificially contaminated food homogenates. Detection of *Listeria* from food and environmental sources: The multiplex PCR was then used to ascertain the presence of *L. monocytogenes* and other *Listeria* species in raw food samples obtained from retail outlets and environmental samples in Mysore region of Southern India. A total of 107 listeria isolates recovered from food samples is given in table 4 were examined by conventional biochemical and mPCR methods. Two listeria isolates recovered from mutton and two isolates from fish samples were identified as *L. innocua* and *L. monocytogenes* by mPCR but were identified as *L. monocytogenes* and *L. ivannovi* respectively by API listeria system. The monoplex PCRs [2, 14, 15, 22, 23] of these isolates revealed that they are *L. innocua* and *L. monocytogenes* as shown by mPCR. IAC was amplified in all the samples. mPCR was performed using the thermal lysis DNA of bacterial cells enriched from food samples.

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Table 3
Sensitivity of mPCR to detect Listeria spp. from spiked food samples.

Sl. No	Organism	Chicken	Mutton	Fish	Milk
1	*L. monocytogenes*	9×10^2	5×10^2	6×10^2	2×10^2
2	*L. ivanovii*	$8X\ 10^2$	$7X\ 10^2$	$7X\ 10^2$	$9X\ 10^2$
3	*L. innocua*	$8X\ 10^2$	$6X\ 10^2$	$6X\ 10^2$	$5X\ 10^2$
4	*L. seeligeri*	$9X\ 10^2$	$2X\ 10^3$	$8X\ 10^2$	$1X\ 10^3$
5	*L. welshmeri*	$1X\ 10^3$	$2X\ 10^3$	$2X\ 10^3$	$4X\ 10^3$

DISCUSSION

In the current study, we developed a novel multiplex PCR for the direct detection and differentiation of five major *Listeria* species (*L. monocytogenes, L. ivannovi, L. innocua, L. welshimeri, L. seeligeri*) from food matrices. A combination of differentially distributed virulence associated genes and group specific markers were utilized in the multiplex PCR for reliable and reproducible discrimination. The proficiency of the individual primers to yield specific amplification for the target bacterial species was confirmed initially by monoplex PCR's and then adapted into multiplex format. Current methods for identification of *Listeria* species rely on physiological and biochemical methods. *Listeria* species are motile by peritrichous flagella and when grown at <30°C, display a characteristic "tumbling" motility. They are catalase positive, oxidase negative and can ferment carbohydrates. On PALCAM agar, all species give grey-green colonies with a black precipitate following incubation for 24 - 48 hours at 35 °C. This is due to common biochemical characteristics among major listerial species such as esculin hydrolysis, α- Mannosidase production and fermentation of carbohydrates [21]. Therefore, identification of *Listeria* species by conventional detection procedures is cumbersome and lead to ambiguous results. According to API kit (Biomereux. Pvt. Ltd) differentiation between *L. innocua* and *L. monocytogenes* is based on the presence or the absence of arylamidase (DIM test), differentiation between other species is by acid production from D-arabitol, D-xylose, L-rhamnose, a-methyl-D-glucoside, D-ribose, glucose-1-phosphate, and D-tagatose. Though the kit is easy to handle, interpretation of the result becomes messy because of weak/no colour development and acid production from sugar fermentation. The mPCR developed in this study was compared with the API listeria kit commonly used for the identification of *Listeria* species. Listerial species recovered from different food matrices were characterized by both the methods. The mPCR identification results correlated with the API system and identified as *L. monocytogenes, L. innocua, L. ivannovi, and L. welshimeri,* but two mutton isolates were read as *L. innocua* and two fish isolates as *L. monocytogenes* by API kit. The newly standardized mPCR identified these mutton and fish isolates as *L. innocua* and *L. monocytogenes* respectively. This ambiguity would be because of almost 90% similarity between *L. monocytogenes* and *L. innocua* genetically and physiologically [24]

An extensive literature survey and *in silico* analysis of the genome sequences of five major *Listeria* species viz. *L. monocytogenes, L. ivannovi, L. innocua, L. welshimeri, L. seeligeri,* and *L. grayi* spotted out seven target genes that could be used in combination to differentiate the species. Seven genes, each one encoding listeriolysin (*llo*), immunodominant surface protein D (*isp D*), metalloprotease (*mpl*), phosphoribosyl pyrophosphate synthetase

(*prs*), internalin C (*inl C*), invasion assosiated protein (*iap*) and motility gene repressor (*mog R)* were proven (by using BLAST algorithm) to be specific markers for the identification and differentiation of *Listeria* species. Variations exist in the common gene within different listerial species [3]. Listeriolysin (*llo*) gene, a classical species specific marker utilized in the present mPCR to detect *L. monocytogenes*. The *llo* protein mediates the escape of organism from the primary phagocytic vacuoles of the host [25]. However, in rare cases the infecting strain was found to be having mutation in *llo* gene [26] thus making the escape of the organism difficult. Therefore, to achieve a more reliable confirmation of *L. monocytogenes,* virulence associated gene; namely, metalloprotease (*mpl)* [27] which is an enzyme marker and a more stable structural protein Immunodominant Surface Protein D (*isp D*) gene were also targeted in the current mPCR for complete and reliable identification of *L. monocytogenes* [28]. Internalin C (*inl C*) which is important for cell to cell spread of both *L. monocytogenes* and *L. ivannovi* [29] was also included in the mPCR. A conserved region of 890 bp in motility gene repressor (*mog R)* gene that regulates temperature-dependent flagellin expression in *Listeria* was incorporated to amplify *L. monocytogenes, L. innocua, L. welshimeri* and *L. seeligeri* [30]. Invasion assosiated protein gene (*iap*) has already been reported as *Listeria* genus specific gene present in all the six species [4, 31], but bioinformatics analysis revealed a region of 507 bp in this gene that is conserved only in *L. monocytogenes, L. innocua* and *L. welshimeri,* thus demonstrating its potential in discrimination of aforementioned species from others*.* Phosphoribosyl pyrophosphate synthetase (*prs*) gene (641 bp) facilitated in identification of *L. innocua.* The specificity of the designed mPCR was evaluated by testing the primer sets against DNA from non-listerial bacteria and was found to be robust and specific. Generally bacteriological methods of isolation and characterization are considered as gold standard, but are time consuming and less sensitive. This sensitive and specific mPCR developed in the current study on the other hand can rapidly identify and differentiate the *Listeria* organism to species level. Therefore, this assay can serve as routine investigation tool for identification of *Listeria* sps.

CONCLUSION

The syndicate of the seven genetic markers described here allowed simultaneous species identification and differential detection of major *Listeria* species. By using this single-tube multiplex PCR; one can quickly discern which *Listeria* species does an isolate belongs to. This multiplex PCR assay can be used for screening a large number of food samples for listerial contamination without a time consuming prerequisite of obtaining pure cultures, thus significantly reduced the expenditure on isolation and conventional biochemical characterization. This assay can be adopted by food- testing laboratories, food industries, hospitals, and diagnostic labs which cannot afford more expensive methods and equipment or trained technicians.

REFERENCES

1. Zhu M, Du M, Cordray J, and Ahn DU, Control of *Listeria monocytogenes* contamination in ready-to-eat meat products. Comprehensive rev in food scien and food safe, 4:34-42, (2005).
2. Gilot P and Content J, Specific identification of *Listeria welshimeri* and *Listeria monocytogenes* by PCR assays targeting a gene encoding fibronectin binding protein. J Clin Microbiol 40(2): 698-703, (2002).
3. Haiyan Z, Xiaofeng Z, Zhen S and Weihuan F, Multiplex PCR identification of *Listeria monocytogenes* isolates from

milk and milk-processing environments, J of the Science of Food and Agriculture, 86(3): 367-371, (2005).

4. Jozi FM, Karen E, Ana PGF, Marisa C and Jeverson F, Molecular analysis of the *iap* gene of *Listeria monocytogenes* isolated from cheese in rio grande do sul, Brazil. Brazi J of Microbiol, 39(1):169-172, (2008).
5. Franz A, *Listeria*: growth, phenotypic differentiation and molecular microbiology. Immunol and Med Microbiol, 35(3):183-189, (2003).
6. Melamed SY, Vaisbein E and Nassar F, Low virulence but potentially fatal outcome —*Listeria ivanovii.* European Journ of Internal Medicine, 17(4):286-287, (2006).
7. Perrin M, Bemer M and Delamare C, Fatal case of *Listeria innocua* bacteremia. J of clinic microbiol 41(11): 5308. (2003).
8. Jofre A, Martin B, Garriga M, Hugas M, Pla M, Rodriguez-Lazaro D and Aymerich T, Simultaneous detection of *Listeria monocytogenes* and *Salmonella* by multiplex PCR in cooked ham. Food Microbiol, 22(1):109–115, (2005).
9. Dongyou L, Jerald AA, Frank WA, Lawrence ML, Characterization of virulent and avirulent *Listeria monocytogenes* strains by PCR amplification of putative transcriptional regulator and internalin genes. Journ of Medi Microbiol, 52:1065–1070, (2003).
10. Dongyou L, Lawrence ML, Austin WF and Jerald AA, A multiplex PCR for species- and virulence- specific *Listeria monocytogenes*. J of Micro Meth, 71(2):133-140, (2007).
11. Caterina M, Aurora A, Cristina R, Nathalie P, Pierluigi N, Patrizia P, Antonino N and Mirella MP, Characterization of *Listeria monocytogenes* isolates from human listeriosis cases in Italy. J of Clinical Microbiol, 47(9):2925–2930, (2009).
12. Michel D, Carmen B, Philippe G, Christine J and Paul M, Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. Jour of Clinical Microbiol, 42(8):3819-3822, (2004).
13. Bansal NS, McDonell FHY, Smith A, Arnold G and Ibrahim GF, Multiplex PCR assay for the routine detection of *Listeria* in food. Intern J of Food Microbiol, 33(2-3):293-330, (1996).
14. Dongyou L, Jerald AA, Austin, FW and Lawrence ML, PCR detection of a putative *N*-acetylmuramidase gene from *Listeria ivanovii* facilitates its rapid identification. Veterinary Microbiol, 101(2):83-89, (2004).
15. Dongyou L, Lawrence ML and Hitchens AD, Molecular characterization of *Listeria monocytogenes* strains harboring *Listeria innocua* putative transcriptional regulator *LIN0464*. J of rapid meth and automation in microbial, 16(4):412–427, (2008).
16. Bubert A, Kohler S, Goebel W, Inge H, Marcus R, Angelika L, Byoungsu Y, Martin W Detection and Differentiation of *Listeria* spp. by a Single Reaction Based on Multiplex PCR Applied and environmental microbiology, 65(10):4688–4692, (1999).
17. Gupta V, Gautam V, Mehta N, Kumari I, Joshi RM. Listeriosis in second trimester of pregnancy: case report from India. Jpn J Infect Dis, 56(2):60-1, (2003).
18. Peer MA, Nasir RA, Kakru DK, Fomda BA, Wani MA and Hakeem QN, *Listeria monocytogenes* meningoencephalitis in an immunocompetent, previously healthy 20-month old female child. Indian J Med Microbiol, 28(2):169-71, (2010).
19. Van Netten P, Van Gaal B, D and Mossel DAA, Selection, differentiation and counting of haemolytic *Listeria* spp. on PALCAM medium. Letters in Applied Microbiology 12(1): 20–22, (1991).
20. Busch SV and Donnelly CW, Development of a repair-enrichment broth for resuscitation of heat injured *Listeria monocytogenes* and *Listeria innocua*. Applied and Environmental Microbiology 58(1): 14- 20 (1992).
21. Bille J, Catimel B, Bannerman E, Jacquet C, Yersin MN, Caniaux I, Monget D and Rocourt J, API *Listeria*, a

new and promising one-day system To identify *Listeria* isolates. Appl and environ microbiol, 58(6): 1857-1860, (1992).
22. Dongyou L, Jerald AA, Frank WA and Lawrence ML, Identification of *Listeria innocua* by PCR targeting a putative transcriptional regulator gene. FEMS Microbiol letters, 223(2):205-210, (2003).
23. Dongyou L, Lawrence ML, Jerald AA and Austin FW, Isolation and PCR amplification of a species – specific oxidoreductase- coding gene region in *Listeria grayi.* Can. J. Microbiol, 51(1):95–98, (2005).
24. Chen J, Jiang L, Chen X, Luo X, Chen Y, Yu Y, Tian G, Liu D and Fang W, *Listeria monocytogenes* serovar 4a is a possible evolutionary intermediate between *L. monocytogenes* serovars 1/2a and 4b and *L. innocua*. J Microbiol Biotechnol, 19:238-49, (2009).
25. Cossart P, Vicente MF, Mengaud J, Baquero F, Perez-Diaz JC and Berche P, Listeriolysin O is essential for virulence of *Listeria monocytogenes*: direct evidence obtained by gene complementation. Infect Immun, 57(11):3629-36, (1989).
26. Ian J, Glomski A, Decatur L and Daniel PA, *Listeria monocytogenes* mutants that fail to compartmentalize listerolysin O activity are cytotoxic, avirulent, and unable to evade host extracellular defenses. Infect and immune, 71(12):6754–6765, (2003).
27. Alan PB, Min C and Marquis H, The metalloprotease of *Listeria monocytogenes* is activated by Intramolecular autocatalysis. J of bacterial, 190(1):107–111, (2008)
28. Wei LY, Hanhong D and Min L Novel protein targets of the humoral immune response to *Listeria monocytogenes* infection in rabbits Animal Diseases. J of Med Microbiol, 56:888–895, (2007).
29. Shaynoor D, Pierre D, Maryse L, Pierre L, Goossens and Pascale C, Identification of four new members of the internalin multigene family of *Listeria monocytogenes* EGD. Infect and Immun, 65(5):1615–1625, (1997).
30. Angelika G, Laura S, Burrack HG, Archie B and Darren EH, *Listeria monocytogenes* regulates flagellar motility gene expression through *MogR,* a transcriptional repressor required for virulence. PNAS, 101(33):12318-12323, (2004).
31. Bubert A, Kohler S and Goebel W, The homologous and heterologous regions within the *iap* gene allow genus- and species-specific identification of *Listeria spp*. by polymerase chain reaction. Appl Environ Microbiol, 58(8):2625–2632, (1992).