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Multiplex PCR Assay for Specific, Sensitive and Rapid detection of three food borne pathogens *Salmonella sp, Shigella sp* and *Listeria monocytogenes*

KEYWORDS: Multiplex PCR, *Salmonella, Shigella, Listeria monocytogenes*

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ABSTRACT *Food-borne illnesses caused by pathogenic bacteria still occur at high frequencies in industrialized nations and developing countries. Conventional microbiological analysis using biochemical tests are time consuming (turn around time up to 7 days), less sensitive and non reliable in most of the cases. This time lag inherent in traditional methods can be replaced by rapid, sensitive detection techniques like multiplex PCR. To achieve an effective detection of Salmonella sp, Shigella sp and Listeria monocytogenes in food materials a multiplex PCR method was standardized in this study. Primers were designed targeting pathogen specific DNA sequence in the genes ipaH, hlyA and sefA of Shigella sp, Listeria monocytogenes and Salmonella sp respectively. Specificity of the primers designed was confirmed by uniplex and multiplex PCR with bacteria (30 different species), fungi and yeast. The multiplex PCR aasay was able to detect all three organisms simultaneously in food materials at a detection sensitivity of 30 ng DNA obtained from each of the three bacteria. Further, the results from examination of food samples from vendor using traditional cultivation methods and multiplex PCR assay were comparatively analyzed. With this assay, simultaneously, all the above three food borne pathogens can be reliably detected with in less than 24 h.*

Introduction

The objective of global health is achieved by food safety and the major health risks are because of food borne diseases. Food borne pathogens are necessary to be examined in farms, food industries and markets. There is a need to identify pathogen quickly so that preventive and controlling measures can be taken up quickly. The conventional method for identification involves pre-enrichment, enrichment and finally selective media which are time consuming, tedious, low throughput and invariably mono specific (detecting only one type of pathogen at a time). Depending on type of pathogen it may take at least 5-10 days for identification and selective enumeration of a pathogen. (Echeverria et al. 1991; ISO 1991; USFDA 1998)

Rapid and dependable methods are needed for detection and identification of these pathogens. Advances in molecular techniques and tools, particularly PCR have allowed more reliable microbial identification and surveillance. Identifying a pathogen through DNA sequence analysis gives accurate results for biochemically inert species, uncultivable organisms and those having long cultivation periods. (Edwards and Gibbs 1994; Simon 1999; White et al. 1992)

Multiplex PCR (m-PCR) is a variant of PCR enabling the simultaneous amplification of many targets of interest in a single reaction using more than one pair of primers thereby conserving reaction components, saving time and minimizing expenses. Multiplex PCR detection of food borne pathogens was earlier published by many authors for different organisms (Zhou et al. 2013; Chen et al. 2012; Jiang et al. 2011)

There is various food borne pathogens that have been identified for food borne illness (Vijayalakshmi et al; 2010). Among them listeriosis caused by *Listeria monocytogenes*, shigellosis (bacillary dysentery) caused by *Shigella* (4 species) and salmonellosis caused by *Salmonella* sp are common pathogens responsible for human health havoc during outbreaks. Scant literature is available for identification of these organisms in a single reaction (Garrido et al. 2012; Hendriksen et al. 2011; Susumu kawasaki et al. 2011; Hamid reza tavakoli et al. 2010; Kwai lin thong et al. 2005). Efforts are made in the present study to develop method for multiplex PCR detection of these three organisms in a single reaction.

Materials and Methods

Reference cultures and cultural conditions

Thirty three known bacterial species, two yeast species and one Aspergillus species listed in Table 1 were obtained from culture bank of NCIM Pune and IMTECH, Chandigarh, India. They were cultivated and maintained on nutrient broth unless and otherwise specified. Purity of all cultures was confirmed by their growth on selective media and biochemical characters. Strains of distantly related genera of yeasts and fungi were also tested for contamination and cultivated on media specified for them.

Conventional methods

Identification of selected three bacterial species from food material was done according to the standard traditional methods (Bacteriological analytical manual, 1992; Compendium of methods for the microbiological examination of foods, 1992; AOAC, 1995)

Bacterial Genomic DNA isolation

Column based bacterial genomic DNA isolation kit (Cat No 2022) from Bioserve Biotechnologies, India Pvt Ltd was used for extraction of genomic DNA from gram positive and gram negative strains grown overnight in Luria-Bertani

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media at 37^0 C according to instructions of manufacturers. High quality genomic DNA was obtained from overnight broth cultures and typical yields were up to 20 mg with an A_{260}/A_{280} of 1.7-1.9 representing optimal DNA purity. Organic solvents are not present in extracted genomic DNA hence used directly in PCR.

Bacterial genomic DNA isolation from different food matrices

Twenty five grams each of different food matrices purchased from local stores were initially subjected to non-selective enrichment in buffered peptone water medium (35^0 C for 24 hr). Medium was centrifuged to get cell pellet containing bacteria of interest along with other background microflora. Cell pellets are then washed several times and used for obtaining high quality genomic DNA.

Oligonucleotide primer Design

The target genes selected for the study and the expected amplification products from multiplex PCR are listed in Table 2. All primers (ipaH F, ipaH R, LM-F, LM-R, SE-F and SE-R) were designed using Gene Tool Lite version (Advanced Bioinformatics solutions) software and were synthesized at Bioserve Biotechnologies Pvt Ltd (India) using Mermade IV (Bioautomation, Texas, USA) and purified by HPLC (Waters, Milford, MA01757, USA).

Uniplex and Multiplex PCR Amplification

Uniplex and multiplex amplifications were made in a 50 ml reaction mixture. A 3 µl sample DNA (appr 100 ng/µl of each bacterial species) were added to a mixture containing 10mM Tris HCl (pH 8.3), 500mM KCl (pH 8.3), 2.0 µl $MgCl_2$ (25mM), 2.5 µl dNTPs, 1.0 µl forward and reverse primers of each species (each of 10 pM/µl) and 2.0 µl of Taq polymerase (IU/µl) (Bioserve Biotechnologies , India). All PCR amplification reactions were performed in a Gene Amp PCR system 9700 (Applied Biosystem, California, USA). An initial incubation at 94^0 C for 5 min was used to denature the template and activate Taq polymerase. Then 35 cycles of PCR was performed involving denaturation for 94 ^{0}C for 30s, primer annealing at 55^{0}C for 30s and DNA extension for 60s at 68^{0}C. Final extension of the incompletely synthesized DNA was carried on at 68^{0}C for 7 min. Template DNA was replaced with sterile distilled water in negative control reaction mixtures.

Detection of PCR products

PCR products (6-10µl) were resolved by gel electrophoresis in 2% agarose (Lonza, Rockland, ME USA, Seakem LE) containing 1µg/ml ethidium bromide run in 1XTAE buffer at 80V for 30-40 min. The DNA bonds were observed by irradiating the pre-stained gel under UV transilluminator (c-80, Epi-illumination UV dark room) and photographed.

Results and Discussion

Virulence associated genes of the bacteria namely *ipaH, hlyA* and *sefA* were selected for the simultaneous detection of *Shigella* sp, *Listeria monocytogenes* and *Salmonella* sp respectively (Andreas Bubert et al. 1999; Kwai lin thong et al. 2005; Murugkar et al. 2003; Cossart et al. 1989; Sharon et al. 1993; Wang et al. 1997). The expected sizes of the amplicons are listed in Table 2. To facilitate PCR product detection and size discrimination by gel electrophoresis the primers were designed such that the sizes of the amplification product would be different. The theoretical analysis of primer with respect to self complimentarity, inter-primer annealing, specificity to the targets and design were accomplished by gene tool lite software.

Specificity of amplification

In order to verify and evaluate the specificity of the primers designed, each primer pair was tested by uniplex PCR on DNA template prepared from different groups of microorganisms (strains of same species, closely related genera and distantly related genera- yeast and molds). A total of 36 microorganisms comprising of 33 bacteria, 2 yeasts and one fungus were tested. The analysis indicated that all primers were specific for their corresponding target organisms (Table 1).

Table 1 Bacterial strains used for the evaluation of specificity of PCR primers

S No	Name of the Organism	Source	ipaH	hlyA	sefA
1	Listeria monocytogenes	MTCC 657	-	+	-
2	Listeria monocytogenes	MTCC 839	-	+	-
3	Listeria monocytogenes	MTCC 1143	-	+	-
4	Listeria ivanvoii	MTCC 7056	-	-	-
5	Shigella flexineri	MTCC 1457	+	-	-
6	Shigella sonnei	MTCC 2957	+	-	-
7	Salmonella enteritidis	MTCC 3219	-	-	+
8	Salmonella enterica typhimurium	MTCC 98	-	-	+
9	Salmonella enterica subsp arizonae	MTCC 660	-	-	+
10	Salmonella enteric ser paratyphi	MTCC 735	-	-	+
11	Salmonella typhimurium	MTCC 3224	-	-	+
12	Salmonella typhimurium	NCIM 2501	-	-	+
13	Salmonella abony	NCIM 2257	-	-	+
14	Escherichia coli	MTCC 1089	-	-	-
15	Enterobacter aerogenes	MTCC 2822	-	-	-
16	Klebsiella aerogenes	NCIM 2282	-	-	-
17	Yersinia enterocolitica	MTCC 840	-	-	-
18	Pseudomonas aeruginosa	NCIM 5029	-	-	-
19	Vibrio cholerae	MTCC 3906	-	-	-
20	Vibrio parahaemolyticus	MTCC 451	-	-	-
21	Enterobacter Sakazaki	MTCC 659	-	-	-
22	Enterobacter intermedia	NCIM 2490	-	-	-
23	CCc Campylobacter coli	MTCC 1126	-	-	-
24	Enterobacter spp	MTCC 7087	-	-	-
25	Citrobacter fruendii	MTCC 1658	-	-	-
26	Citrobacter brakii	MTCC 2690	-	-	-
27	Aeromonas hydrophila	MTCC 1739	-	-	-
28	Bacillus cereus	NCIM 2458	-	-	-
29	Bacillus licheniformis	MTCC 429	-	-	-
30	Bacillus subtilis	MTCC 121	-	-	-
31	Streptococcus faecalis	NCIM 2404	-	-	-
32	Staphylococcus aureus	MTCC 737	-	-	-
33	Clostridium perfringens	NCIM 2677	-	-	-
34	Saccharomyces cerevisiae	NCIM 3494	-	-	-
35	Saccharomyces cerevisiae	NCIM 3220	-	-	-
36	Aspergillus niger	NCIM 588	-	-	-

PCR amplification with each set of primers yielded single, detectable DNA fragment of the expected molecular weight only in presence of each of their respective template DNAs, whereas other bacteria did not show any product. This indicated that

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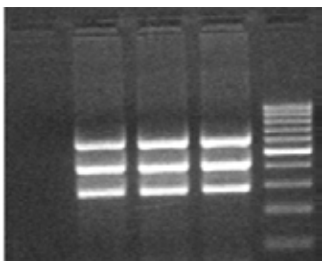
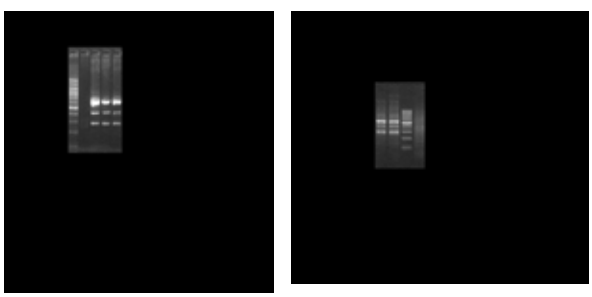
each of the selected oligonucleotide primer was specific for its respective target microbial pathogen and that DNA extraction method yielded sufficient DNA template to permit m-PCR to detect the three pathogens. Further, a multiplex test for detection of these three pathogens in food matrices, that is in presence of background microflora gave single DNA fragment of expected molecular weights (Fig 1)



Sensitivity of m-PCR

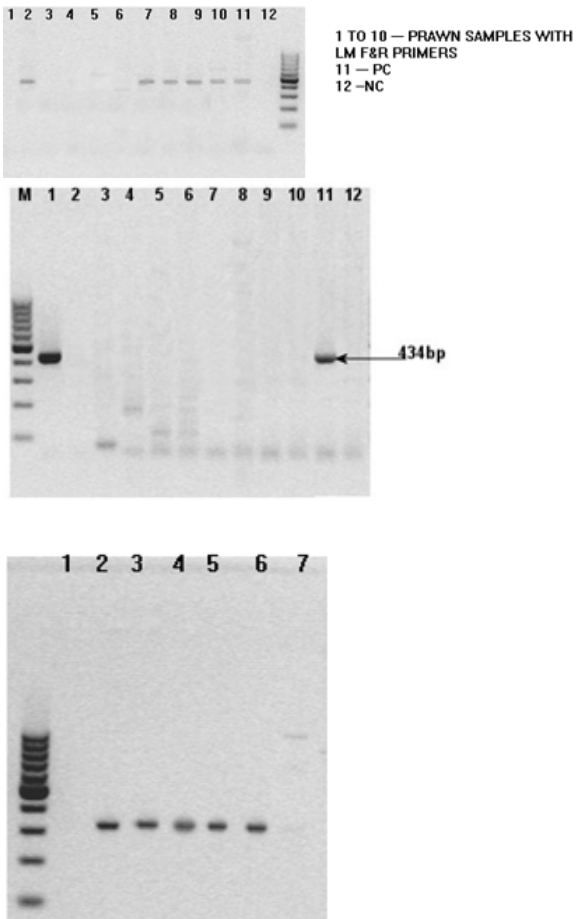
The sensitivity of m-PCR in food pathogen detection was evaluated in terms of reaction sensitivity (ISO 1991). The minimum amount of nucleic acid of each species required for successful PCR amplification implies reaction sensitivity. In the present context 100 nanograms of genomic DNA has worked well for detectable amplification product (Fig 2).



Further it was observed that the reaction was successful when a total of 100 nanograms (30ng from each of the three species and remaining from non target background microflora of food matrix) was used resulting in clear bands of amplification products (Fig 3). Further the amplification products were sequenced and submitted to NCBI gene bank (AC No GU945261, GU 945269).

Detection of pathogens in foods from local market

To evaluate practical use of m-PCR assay in street foods an attempt was made to compare the usage of conventional methods and PCR method for detecting these three pathogens. The results presented in Table 3 indicate that m-PCR is more effective compared to regular methods. It was observed that conventional method was unable to detect *Listeria* in Spinach 2, *Listeria* and *Salmonella* in Prawn 1 and 2 and *Salmonella* in Raw meat 1 (Fig 4,5,6). This may be because of low number of cell population.



Conclusion

Early detection of pathogen outbreak can ease the ways of tackling the disease onset. Traditional culture based methods are time consuming which do not serve the purpose of time frame detection of pathogens. In view of increased demand for rapid detection of food borne pathogens, multiplex PCR assay with newly designed primers can be a reliable alternative for traditional time consuming methods.

Table 2 Target gene and primers used

Organism	Primer used	Sequence Text(5′-3′)	Gene Targeted	Amplicon Size
Shigella sp	ipaH F ipaH R	GTTCCTTGACCGCCTTTCCG ATACCGTC GCCGGTCAGCCACCCTCTGAGAGTAC	ipaH gene	619 bp
Listeria monocytogenes	LM-F LM-R	GCTGCTTTTGATGCTGCCGTAA CTTGCTTTTATTGTTTTCGCTCCA	hlyA	434bp
Salmonella sp	SE-F SE-R	GCAGAGGTTCAGGCAGCGGTTA GCCAACCTGCAAGCCCGTCA	sefA	300bp

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Table 3 Detection of pathogens in foods from local market

		m-PCR			Conventional		
S.No	Food Item	Salmonella	Shigella	Listeria	Salmonella	Shigella	Listeria
1	Spinach 1	+	--	--	+	--	--
2	Spinach 2	--	--	+	--	--	--
3	Prawn 1	+	--	--	--	--	--
4	Prawn 2	--	--	+	--	--	--
5	Fish meal 1	--	+	--	--	+	--
6	Fish meal 2	+	--	--	+	--	--
7	Puffed rice 1	--	--	--	--	--	--
8	Puffed rice 2	--	--	--	--	--	--
9	Raw meat 1	+	--	--	--	--	--
10	Raw meat 2	--	+	--	--	+	--

Fig. 1 Multiplex PCR product obtained using DNA extracted from mixture of three bacterial sps *L.monocytogenes, Shigella sp* and *Salmonella sp* in presence of background microflora. 100ng DNA was used as template. Lanes: 1, Negative PCR Control; 2, *Shigella flexineri* (MTCC1457), *L.mocytogenes* (MTCC 657) and *Salmonella enteritidis* (MTCC 3219); 3, *Shigella flexineri* (MTCC1457), *L.mocytogenes* (MTCC 839) and *Salmonella typhimurium* (MTCC 98); 4, *Shigella sonnei* (MTCC 2957), *L.mocytogenes* (MTCC 1143) and *Salmonella enterica subsp arizonae* (MTCC 660) 5, 100bp Marker

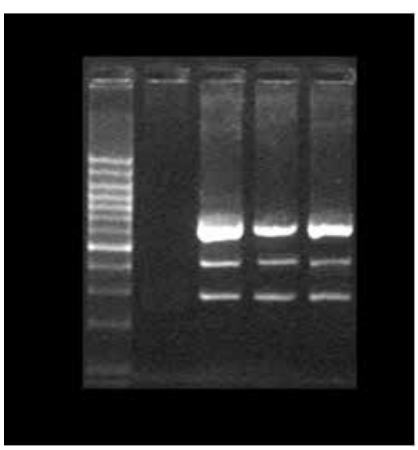

Fig. 2MultiplexPCR product obtained using 100ng DNA from each of the three bacterial sps *L.monocytogenes, Shigella* sp and *Salmonella* sp. Lanes: 1, 100bp Marker; 2, negative PCR control; 3, *Shigella flexineri* (MTCC1457), *L.mocytogenes* (MTCC 657) and *Salmonella enteritidis* (MTCC 3219); 4, *Shigella flexineri* (MTCC1457), *L.mocytogenes* (MTCC 839) and *Salmonella typhimurium* (MTCC 98); 5, *Shigella sonnei* (MTCC 2957), *L.mocytogenes* (MTCC 1143) and *Salmonella enterica subsp arizonae* (MTCC 660)

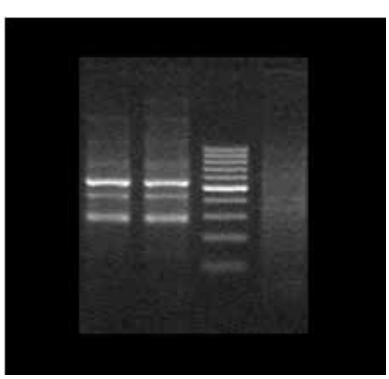

Fig. 3 Sensitivity of m-PCR assay performed using 30 ng of DNA from each of the three bacterial sps *L.monocytogenes, Shigella* sp and *Salmonella* sp in presence of background microflora when a total of 100ng DNA was used as template. Lanes: 1, *Shigella flexineri* (MTCC1457), *L.mocytogenes* (MTCC 657) and *Salmonella enteritidis* (MTCC 3219); 2, *Shigella sonnei* (MTCC 2957), *L.mocytogenes* (MTCC 1143) and *Salmonella enterica subsp arizonae* (MTCC 660); 3, 100bp Marker; 4, negative PCR control

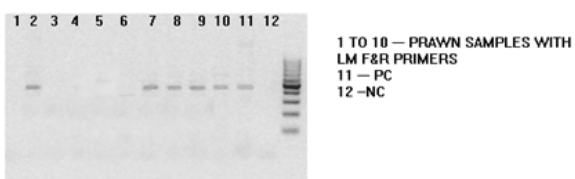

Fig. 4 Listeria monocytogenes specific PCR product obtained in prawn samples from retail store in presence of background micro flora using m-PCR (100 ng DNA template was used). Lanes: 1-11, Prawn samples with all primers; 12, negative control; 0, 100bp Marker; 1, 3-6, Negative for presence of all the three bacteria

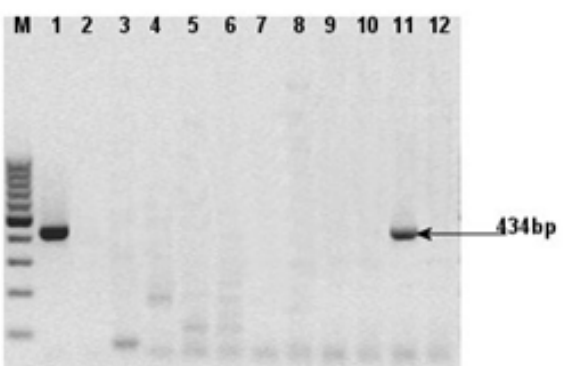

Fig. 5 Listeria monocytogenes specific PCR product obtained in spinach samples from retail store, in presence of background micro flora using m-PCR (100 ng DNA templates were used). Lanes: M, 100bp Marker; 2-10, Spinach samples with all primers; negative for presence of all the three bacteria; 1, 11, Spinach samples with all primers; positive for *L.monocytogenes*; 12, negative control

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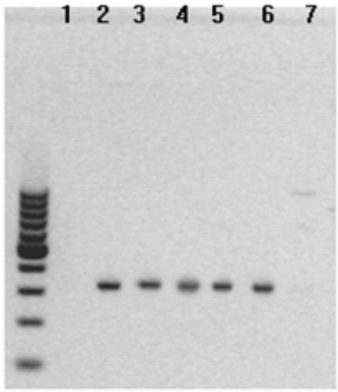

Fig. 6 *Salmonella* sp specific PCR product obtained in prawn samples from retail store, in presence of background micro flora using m-PCR (100 ng DNA templates were used). Lanes: 0, 100bp Marker; 1, Negative PCR control; 2-6, Prawn samples with all primers; positive for *Salmonella* sp; 7, Prawn sample with all primers; negative for all the three organisms

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