DEVELOPMENT OF MULTIPLEX PCR BASED ASSAY FOR THE CONCURRENT DETECTION OF PATHOGENIC MICROORGANISMS

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ABSTRACT

Salmonella enterica is an important enteric pathogen which causes gastroenteritis and enteric fever in humans and is widely spread in nature. Outbreaks of Salmonella infections are frequently reported in both developed and developing countries, as this pathogen spreads very rapidly by means of water and the food chain.

A multiplex PCR (mPCR) assay was developed for the detection of multiple Salmonella serotypes in different kind of food products. To increase specificity of this molecular method, three sets of oligonucleotide primer were used to detect the most prevalent salmonella species. Different primer pairs were used for the optimization of the multiplex PCR. The primer pair, ST FW and ST RV, was specific to *Salmonella typhi* and targeted a randomly selected sequence of 600bp. The primer pair SPFW and SPRV specific to *Salmonella paratyphi* for a sequence of 800bp in length. Likewise, the primers pairs SEFW and SERV were designed to amplify a sequence of about 1000bp from *S. enterica*.

The Salmonella strains tested in this study were *Salmonella typhi, Salmonella enterica* and *Salmonella Paratyphi*. The STFW/STRV primer pair amplified 600bp DNA fragments when the DNA templates from *Salmonella typhi, Salmonella enteritidis, Salmonella enterica* and *Salmonella paratyphi* were tested. The DNA template of *Salmonella paratyphi* specifically amplified 800-bp fragments. Likewise, the DNA template for *Salmonella enterica* amplified 1000-bp fragment. This technique can be used routinely for the rapid detection of Salmonella spp. in food materials at low level of contamination with an enrichment time that depend in complexity of food product.

Keywords: Multiplex PCR, Salmonella, Optimization of PCR.

INTRODUCTION

Microbial spoilage of food is an area of global concern, causing serious foodborne intoxications and resulting in high economic losses in the food production sector [1]. The microbiological safety of food has become an increased importance for the consumers, as well as for the food producing industry [2, 3]. Research in the food safety and quality field has been continuously focused on the search for sensitive, accurate, rapid, and cost-effective methods to determine and detect the potential microbial risks. Conventional cultivation methods and phenotypical tests are still commonly applied in the food microbiological field but are time consuming and labour intensive [4, 5]. Over the past years, the tools for molecular diagnosis have greatly improved.

Typhoid fever is caused by infection of humans with the microorganism Salmonella enteric subspecies enterica serotype Typhi (*S. typhi* for short). It is a systemic disease characterized by a prolonged fever, malaise and weight loss. Salmonellosis is responsible for large numbers of infections in both humans and animals [6]. Typhoid fever and paratyphoid fever are still serious public health problems in many geographic areas and are endemic in most countries [7, 8]. *Salmonella enterica* serotype typhi is a member of the family Enterobacteriaciae. Conventional methods of isolation of Salmonella strains take 4-7 days to complete and are therefore laborious and require substantial manpower [9, 10, 11].

During the progressive development of the PCR over the last two decades, this method became one of the central techniques for nucleic acid analyses and is the most widely used method for pathogen detection [12]. Rapid pathogen testing is very important to the food industry and this helps the increased public health concern [13, 14].

Applying rapid multiplex PCR technique, we have developed very fast PCR by using conventional thermocyclers and rapid electrophoresis that can be applied in any diagnostic laboratory for detection and identification of *S. typhi*. In the present study we tried to design and validate oligonucletide primers for the identification of Salmonella species and to characterize multiple serovars of Salmonella spp. in food samples using highly sensitive and specific Multiplex PCR.

MATERIALS AND METHODS

Genomic DNA Isolation

Total genomic DNA from the bacteria was isolated by N- Cetyl- N, N, N-trimethyl- ammonium bromide (CTAB) method. The bacterial isolates were generously donated by the bacterial repository of Credora Life sciences, Bangalore, India.

Total genomic DNA from the bacteria was isolated by N- Cetyl- N, N, N-trimethyl- ammonium bromide (CTAB) method described elsewhere (Wilson, K. 2001). In brief, the culture was centrifuged at 10000 rpm at 4°C and lysed with 675μl extraction buffer (100 mM Tris HCl, 100 mM EDTA, 1.4M NaCl, 1% CTAB and Proteinase K - 0.03μg/μl). The suspension was incubated at about 37°C for 30 minutes. To the mixture 75μl of 20% SDS was added and incubated at 65°C for 2 hours. The suspension was then centrifuged and the supernatant was extracted with equal volumes of Chloroform and Isoamyl alcohol (24:1). The aqueous phase obtained after centrifugation was then extracted with 0.6 volumes of isopropyl alcohol. The mixture was allowed to stand undisturbed at RT for 1hour. The suspension was then centrifuged again and the DNA was pelleted with 500μl of 70% ethanol. The DNA collected was then quantified using UV spectrophotomer (Shimadzu 1800 series).

PCR Amplification

Specific oligonucletide primers were designed to each of the Salmonella serovars (*Typhi, Paratyphi* and *enterica*) using Primer Plus software and the designed oligonucleotides were synthesized in Sigma Corporation USA.

The multiplex PCR amplification was performed using a Biotron 96-Well Thermal Cycler (Applied Biosystems). The reaction was carried out according to the protocol prescribed by the kit. A negative control containing sterile distilled water instead of the template DNA solution was included in each PCR assay.

Different primer pairs were used for the optimization of the multiplex PCR. The primer pair, ST FW and ST RV, was specific to *Salmonella typhi* and targeted a randomly selected sequence of 600bp. The primer pair, SPFW and SPRV specific to *Salmonella paratyphi* for a sequence of 800bp in length. Likewise, the primers pairs SEFW and SERV were designed to amplify a sequence of about 1000bp from *S. enterica*. The primer pairs were first optimized for the different serovars of Salmonella, *typhi. paratyphi and enterica* in separate individual PCR reactions. Subsequently, the optimization of primer pairs was performed for Salmonella spp. Finally, the three primer pairs were assessed in the final multiplex PCR using three repetitions to ensure reproducibility of the assay.

The PCR mixture consisted of 10x reaction buffer with $MgCl_2$ (1.5 mM), 2μL of dNTP mix (2.5 mM), 2μL each of forward and reverse primers (10picomoles/μl each primer), 0.3μL of Taq DNA polymerase (5U/μL), and 50ng/μL of template DNA in a total volume of 20μL. The PCR was performed with the following cycling profile: initial denaturation at 94°C for 2 min, followed by 35 cycles of 1 min denaturation at 94°C, annealing at 53°C for 30s, and extension at 72°C for 1 min. The time for the final extension step was increased to 6 min. The PCR products amplified were then qualitatively analyzed on 1% agarose gel. The PCR product was recovered using the QIA quick gel extraction kit, and the amplified product was then purified and used for cloning purpose.

Primers

Primer sets	Length	Primer sequence	Product (bp)
ST FW	21	AGCTACAGCTATTCGGGAAC	600
ST RV	21	CACAGCTCCAGTTTGAGCAAC	
SP FW	21	ACTGGCTTGAGCATGTCTCTC	800
SP RV	21	CCAGGCAGAGTTTCTGCAGTT	
SE FW	21	AACACCTCGTCGATGGACTAC	1000
SE RV	21	CATGAGAGGGTCCGTAACAGA	

Optimization of the multiplex PCR

Parameters to be optimized included $MgCl_2$ concentration (1.5 to 3.5mM), primer concentration (0.2μM to 1.2μM) and annealing temperature (50 to 60°C). Each component was optimized while others were kept constant. Then concurrent optimized parameters were used in subsequent experiments. A panel of Salmonella and non-Salmonella strains were tested for specificity using the optimized multiplex PCR conditions. The procedure was done in duplicates. The Salmonella strains tested in this study were *Salmonella typhi*, *Salmonella enterica* and *Salmonella Paratyphi*; while non-Salmonella strains included *Escherichia coli.*

RESULTS

DNA isolation and quantification

The total genomic DNA was isolated by modified CTAB method from the infected blood samples. A total of seven blood sample was collected from the Govt. Hospital, Bangalore. After RBC lysis, the obtained pellet was used for the total genomic DNA isolation. The isolated DNA was subjected to 1% Agarose gel electrophoresis (Fig.1).
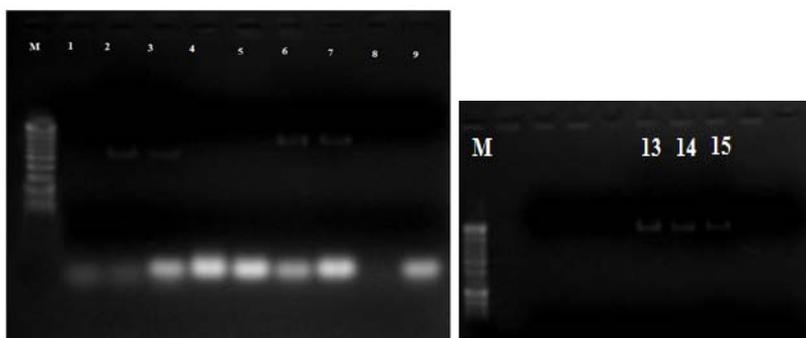

Fig. 2: PCR amplification of Salmonella spp (Top: M-100bp ladder. Lanes 2 and 3 are *S. paratyphi*. Lanes 6 and 7 *S. Typhi).* Right: Lane 13,14,15 *S. enteric.*
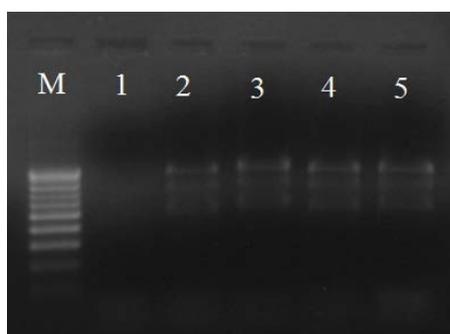

Fig. 4: Multiplex PCR amplification of Salmonella species. The primer sets got amplified at the approximate band length. Lane M: Standard (1000bp ladder), Lane 1: Negative control, Lane 2: *Salmonella typhi*, Lane 3: *Salmonella enteritidis*, Lane 4: *Salmonella enteric*, Lane 5: *Salmonella paratyphi.* The bands obtained are of 1000bp, 800bp and 600bp.

PCR primer designing and PCR amplification

The $MgCl_2$ concentration and the annealing temperature (T^m) for the detection of Salmonella spp. were optimized. The amplicon analysis with agarose gel electrophoresis are shown in Figure 2. The primer concentration was adjusted by increasing the concentration to a point where a faint amplicon band was obtained or by decreasing the concentration a point where a strong band was obtained. The adjustments were made to obtain the best combination for optimum primer concentrations of all target DNAs in the multiplex PCR. We found the optimum primer pair concentrations to be: 0.2μM for STFW/STRV, 1.2μM for SPFW/SPRV and 1.2μM for SEFW/SERV.

The thermal cycling conditions were tested at different concentrations of DNA. The annealing temperature that yielded the greatest amount of amplicons was 56°C or 53°C for both *Salmonella typhi* and *Salmonella paratyphi*. However, the annealing temperature best for all three Salmonella groups *(Salmonella typhi, Salmonella paratyphi and Salmonella enterica* was 53°C (Fig.4). Therefore a 53°C annealing temperature was judged to be suitable for the multiplex PCR for these species.

DISCUSSION

Overall, the optimized multiplex PCR reaction contained the following: 10 μL 5X PCR buffer, 0.2 mM dNTP mix, 0.2μM STFW/STRV and 1.2μM SPFW/SPRV and SEFW/SERV primers, 2.5 mM $MgCl_2$, 1.5U Taq DNA polymerase and 4 μL DNA template. The optimized thermocycling conditions were found to be initial denaturation at 94°C for 2 min; followed by 35 cycles: denaturation at 94°C for 1 min, primer annealing at 53°C for 1 min and extension at 72°C for 1 min; an additional cycle at 72°C for 7 min; and maintenance at 4°C until analysis.

The STFW/STRV primer pair amplified 600bp DNA fragments when the DNA templates from *Salmonella typhi*, *Salmonella enteritidis*, *Salmonella enterica* and *Salmonella paratyphi* were tested. The DNA template of *Salmonella paratyphi* specifically amplified 800-bp fragments. Likewise, the DNA template for *Salmonella enterica* amplified 1000-bp fragment. Figure 4 shows that the three primer pairs are very specific to the target microorganisms and their respective serotypes.

CONCLUSION

Salmonella is one of the major pathogens of food-borne disease outbreaks; This genus includes more than 2400 different known serovars. In view of limited rules in maintaining very high standards of hygiene, the chance of bacterial contamination of food at different levels of processing is always very high. Traditional Salmonella detection methods are based on cultures using selective media and characterization by biochemical methods. These methods are generally time delaying. Therefore a rapid method is necessary for identification of Salmonella serotypes from food. In this way, the normal traditional methods must be supplemented by a range of molecular genotyping methods.

In recent studies done, molecular-based methods (Ribotyping &RAPD) have been published. But these methods are not too sensitive in detecting the contamination at the specific level. In the present study we not only established a rapid, most sensitive and specific multiplex PCR method using highly conserved primers to detect different serovars of Salmonella spp. but also optimized the PCR conditions for specific amplification.

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