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PCR detection of *salmonella typhimurium* using Hin GENE

Sherly Williams E[1], Beena V S[2] and Vishnu Nair M S*[1]

1. Environmental Sciences, Aquaculture and Fish Biotechnology Lab, Department of Zoology, Fatima Mata National College, Kollam.
2. Saraswathy Vidyalayam, Thiruvananthapuram.

E-mail : msvishnunair@gmail.com Contact No : +91 - 9746170749, 9495036719.

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Abstract

Salmonella cause disease in a wide range of species of vertebrates. The knowledge of the serotypes helps to define the sources and vehicles of infection in outbreaks of food poisoning. The isolated genomic DNA from *Salmonella typhimurium* was amplified with the designed oligonucleotide primers for the amplification of Hin gene. The genomic DNA was serially diluted and the least expressed concentration of each genomic DNA for amplification was selected and performed the multiplex PCR, to detect the presence of Hin gene thereby detecting *Salmonella typhimurium*. The genomic DNA from bacterial cells was recovered using the salting out method. Oligonucleotide primers were designed for PCR amplification of *Salmonella* spp. Primer annealing temperatures were calculated by oligo version computer programme. PCR amplification was performed with a model Gene Amp PCR system 9700(Perkin Elmer) by using 1X PCR buffer ,2.5mM $MgCl_2$, deoxynucleoside triphosphate at a concentration of 200µM.primer at a concentration of 0.5 M,100ng of target DNA and 2.5U of DNA Polymerase . PCR amplified DNA was analyzed by gel electrophoresis. At an annealing temperature of 55^0C a specific amplification was obtained with a size of 230bp for Hin gene, 200bp for *Salmonella abony*,450 bp for *Pseudomonas aeruginosa* and 500bp for *E.coli*. The size of the PCR product was confirmed by comparing with a marker of 100bp which also stands true for the product size obtained by doing NCBI BLAST. The blast results suggested the amplifies sequence is showing 99% homology to the *S. typhimurium*.

Key words : disease, food , Hin Gene, PCR, poisoning, salmonella.

INTRODUCTION

Salmonella cause disease in a wide range of species of vertebrates.Out of the four clinical types of Salmonella infection distingushed,serotype of subspecies I are identified to be pathogenic to man.The knowledge of the serotypes helps to define the sources and vehicles of infection in outbreaks of food poisoning.Though the factors responsible for the virulence of Salmonella are ill defined[1] have stated that the lipopolysaccharides of cell wall prevents the killing of bacterial cell by phagocytic cells.

Generally *Salmonella* live in the intestines of mammals and move by rotating flagella on their cell surfaces. The many copies of the protein flagellin that make the flagella as the prominent targets of mammalian immune systems. In a mechanism that envades the immune response, *Salmonella* cells switch between two distinct flagellin proteins roughly once every 1000 generations, using a process called phase variation. Judy *et al*[2] have reported that Hin gene and H-li genes are involved in the control of phase variation of Salmonella species.

The switch is accomplished by periodic inversion of a segment of DNA containing the promoter for a flagellin gene. The inversion is site specific recombination reaction mediated by the recombinase called hin at specific sequences of 14 base pairs at either end of the DNA segment [3, 4]. When the DNA segment is in one orientation the gene for fljb flagellin and the gene encoding a repressor are expressed. The repressor shuts down expression of the gene for flic flagellin. When the dna segment is inverted the flja and fljb are no longer transcribed and the fljc gene is induced as the repressor becomes depleted. The Hin recombinase encoded by the Hin gene in the dna segment that undergoes inversion is expressed when the DNA segment is in either orientation, so it is always possible to switch from one state to other[4].

Although many different conventional culture media and enrichment regimes have been proposed for isolating *Salmonella* species from food or environmental samples these organisms are still difficult to culture, detect or enumerate from complex microbial communities of natural ecosystems. The ability of *Salmonella* species to enter a viable but nonculturable state after lengthy exposure to soil or ground water under ambient conditions of temperature and low nutrient concentration may contribute to this difficulty.

Polymerase Chain Reaction(PCR) has proved to be a valuable method and has the potential to solve the problems in the detection of pathogens in food[5].PCR is a rapid in vitro procedure for the enzymatic amplification of specific DNA sequences which increases the number of copies of the target sequence.This allows increased sensitivity of detection of a DNA sequence present in trace amounts in mixed populations[6,7,8].Numerous papers have been published on the PCR detection of food borne pathogens[5,9,10,11,12]. For *Salmonella* gene specific probes have been used for the monitoring of *Salmonella* species in food samples [13, 14].

Since its introduction, multiplex PCR has been successfully applied in many areas of nucleic acid diagnostics, including gene deletion analysis[15], mutation and polymorphism analysis [16], quantitative analysis [17], and RNA detection [2]. In the field of infectious diseases, the technique has been shown to be a valuable method for identification of viruses, bacteria, fungi, and/or parasites. Preferential amplification of one target sequence over another (bias in template-to-product ratios) is a known phenomenon in multiplex PCRs that are designed to amplify more than one target simultaneously [18].The identity of the pathogen is

one of the potential of DNA sequence analysis.The PCR products of the pathogens are confirmed by the sequencing.

MATERIALS AND METHODS

The genomic DNA from bacterial cells were recovered using the salting out method.Using this method,DNA from bacterial cells in 3ml overnight cultures were released by Tris and sodium dodecyl sulphate.Proteinase K was used to remove proteins and the DNA was precipitated using sodium chloride.The DNA was further purified by using chloroform-isoamylalcohol and extractions followed by precipitation with absolute alcohol.The pelleted DNA was washed in 70% alcohol and dried.Using this procedure we were able to recover 250 450µg of purified genomic DNA from each bacterial sample.

PCR amplification and detection

Oligonucleotide primers were designed for PCR amplification of *Salmonella* spp. DNAs on the basis of the previously reported nucleotide sequences of the Hin gene(Gene ID is 1254295 and GenBank accession number is AE008826 of *Salmonella typhimurium*[2] Hin gene codes for Hin recombinase which undergoes inversion and thereby accomplishing a switch over.The primers were synthesised by Bioserve Biotechnologies India.Primer annealing temperatures were calculated by oligo version computer programme.The HinF has 21 nucleotides(5'-CTAGTGCAAATTGTGACCGCA-3')and is located between 169 189bp on the Hin gene of *S.typhimurium*.HinR has 21 nucleotide (5'-CCCCATCGCGCTACTGGTATC-3') and is located between 384 and 404bp on the Hin gene.

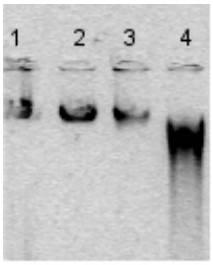
PCR amplification was performed with a model GeneAmp PCR system 9700(Perkin Elmer)by using 1X PCR buffer ,2.5mM MgCl2,deoxynucleoside triphosphate at a concentration of 200µM.primer at a concentration of 0.5 M,100ng of target DNA of target DNA and 2.5U of DNA Polymerase .35 cycles of amplification of target DNA were performed with initial denaturatuion at 94^{0}C for 5 minutes and post amplification extension at 68^{0}C for 10minutes.The parameters for each amplification cycle were as follows : template DNA denaturation at 94^{0}C for 30 seconds;primer annealing at 55^{0}C for 45 seconds;and primer extension at 68^{0}C for 60seconds.PCR amplified DNA was analysed by gel electrophoresis.

Multiplex PCR was performed with 2.5U of Taq DNA polymerase in 25µl reactions containing serialy diluted template DNA with a starting material of 100ng/ µl and different concentrations of primers.PCR was repeated with all the serially diluted genomic DNA samples.As per the results obtained from gel picture,the dilution at which the least good expressed band was selected for multiplex PCR along with other four selected serially diluted samples of genomic DNA from *Salmonella abony,Escherichia coli* and *Pseudomonas aeruginosa*. All the multiplex PCR components are mixed and PCR was set for 35 cycles at 94^{0}C for 30 seconds, 55^{0}C for 45seconds and 68^{0}C for 60seconds. For confirmation of the diagnosis of the Hin gene amplification, the gene was sequenced in Beckman Coulter CEQ 8000 Genetic Analysis System.

RESULTS

It was observed that the samples were of good quality and are between 1.7 and 1.8 ratios.

At an annealing temperature of 55^{0}C a specific amplification was obtained with a size of 230bp for Hin gene, 200bp for *Salmonella abony*,450 bp for *P.aeruginosa* and 500bp for *E.coli*.The size of the PCR product was confirmed by comparing with a marker of 100bpwhich also stands true for the product size obtained by doing NCBI BLAST.The samples were tested under mentioned standardised pcr conditions and were gel checked.(Fig:2)



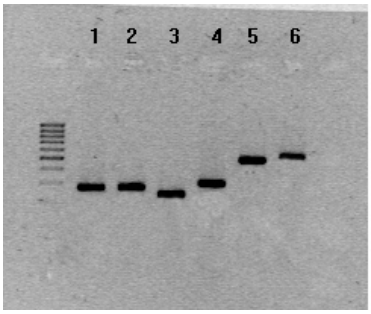
1 *Escherichia coli*

2 - Salmonella typhimurium

3 - Salmonella abony

4 - Pseudomonas aeruginosa

Fig:1. The gel picture of genomic DNA of the four selected sample



1, 2 - Salmonella abony (inhouse primer)

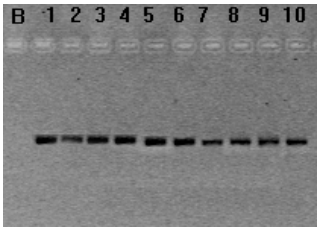
3 - Hin gene

5 - Pseudomonas aeruginosa(inhouse primer)

6 - E.coli (inhouse primer)

Fig:2

PCR amplification of serially diluted genomic DNA of Salmonella typhimurium amplified with Hin gene is shown in Fig: 3



B Negative control

1 - 100ng/ l

8 - least expressed good band

Fig: 3

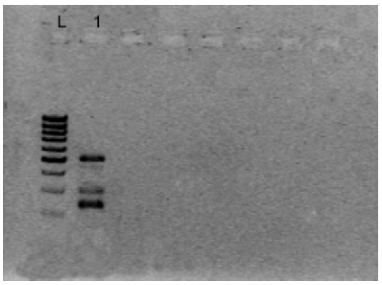


Fig : 4 Shows the multiplex PCR where the amplified Hin gene can be marked out along with the other three selected pcr products.

The sequencing of the amplified product was performed and the results were analyzed.The sequence is blasted against NCBI data bank. It was observed while blasting that there was 99% similarity with that of *Salmonella typhimurium*.

DISCUSSION

The aim of the present study was to develop a rapid and simple technique for the specific detection of pathogenic bacteria in the diagnosis of food borne pathogens. The pathogen selected for the present work is *Salmonella typhimurium.*The primer which is used to detect the pathogen is designed and synthesised. The results showed that the primers are very specific to the bacteria and had been identified that it belongs to *S.typhimurium*. The size of the PCR products was 230bp.

The results obtained from this study showed that the primer designed for *S.typhimurium* is very specific and is able to detect the pathogenic DNA in the mixture of other sample DNAs.The sequence of the PCR product were confirmed by sequencing it in the automated sequencer and compared with those in the databases.Thus it proved once again that PCR is a sensitive and rapid technique which is at the forefront of molecular diagnostic technology today.As reported by authors like [5,9,10,11] the present observations also reach a conclusion that PCR technique is at the forefront of molecular diagnostic technology today.

Also, the present study showed that multiplex PCR have increased diagnostic capacity to detect the pathogens in least quantities. As reported in the previous studies [3, 13, 16, 7] the present study also shows that multiplex PCR is applicable for molecular diagnosis. This could be applied for the development of diagnostic methods which will be highly significant for the detection of diseases in human rapidly and sensitively.

CONCLUSION

In the present study it was so easy to find out the presence of *s. typhimurium* using Hin gene even at its smaller concentration. Thus it could be pointed that Hin gene can be used as an effective means to detect the presence of this microbial infection. Hin gene based microbial presence detection will definitely form a stepping stone in the field of medical and food microbiology with the support of molecular biology tools. Diagnosis of diseases is a field which should be given much importance because disease itself can cause increased damage to human as well as other living organisms of commercial value which include agriculture also. So if proper techniques like multiplex pcr are developed for the diagnosis in time the disease outbreaks can be prevented by adequate treatment. Understanding and developing more on the technique leads to proper diagnosis which help us to protect from epidemics.

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