

Rapid detection of food-borne pathogens by using molecular techniques

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Traditional methods of identification of food-borne pathogens, which cause disease in humans, are time-consuming and laborious, so there is a need for the development of innovative methods for the rapid identification of food-borne pathogens. Recent advances in molecular cloning and recombinant DNA techniques have revolutionized the detection of pathogens in foods. In this study the development of a PCR-based technique for the rapid identification of the food-borne pathogens *Salmonella* and *Escherichia coli* was undertaken. Suitable primers were designed based on specific gene *fimA* of *Salmonella* and gene *afa* of pathogenic *E. coli* for amplification. Agarose gel electrophoresis and subsequent staining with ethidium bromide were used for the identification of PCR products. The size of the amplified product was 120 bp as shown by comparison with marker DNA. These studies have established that *fimA* and *afa* primers were specific for detecting *Salmonella* and pathogenic *E. coli*, respectively, in the environmental samples. Thus a rapid, sensitive and reliable technique for the detection of *Salmonella* and pathogenic *E. coli* was developed.

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INTRODUCTION

Many high-risk pathogens that cause disease in humans are transmitted through various food items. Due to increased morbidity and mortality leading to time lost in the work place and reduced productivity, food-borne disease across the world costs billions of dollars annually (Todd, 1989). Because outbreaks of food-borne illnesses may be under-reported by as much as a factor of 30, the number of cases of gastroenteritis associated with food is estimated to be between 68 million and 275 million per year. Even at the lower end of this range, food-borne disease constitutes a major public health problem.

Assessment of the quality and safety of foods is important in human health. Common pathogenic bacteria that are the causes of food-borne diseases include strains of *Salmonella* and *Escherichia coli* (Sockett, 1991). The conventional microbiological methods for detection of these bacteria, however, usually include multiple subcultures and biotype- or serotype-identification steps and, thus are laborious and time-consuming (Swaminathan & Feng, 1994; Feng, 1993; Blackburn, 1993).

One of the inherent difficulties in the detection of food pathogens is that they are generally present in very low numbers (< 100 c.f.u. g^{-1}) in the midst of up to a million or more other bacteria. These microbes may be lost among a background of indigenous microflora, and substances in the foods themselves may hinder recovery. There is also the difficulty of demonstrating that the strains recovered from a

food sample are, indeed, pathogenic to human beings (Sockett, 1991). Rapid and easy detection of pathogenic organisms will facilitate precautionary measures to maintain healthy food (Feng, 1992).

The advent of gene probe techniques has allowed the development of powerful tests by which particular bacterial strains can be rapidly identified without the need for isolating pure cultures (Rasmussen *et al.*, 1994; Cohen *et al.*, 1993). The polymerase chain reaction (PCR) is a technique for *in vitro* amplification of specific segments of DNA by using a pair of primers (Nguyen *et al.*, 1994). A million-fold amplification of a particular region can often be realized, allowing, among a myriad of other uses, the sensitive detection of specific genes in samples. PCR can be used to amplify genes specific to taxonomic groups of bacteria and also to detect genes involved in the virulence of food-borne bacteria (Finlay & Falkow, 1988; Bej *et al.*, 1994). The recently developed techniques for amplifying specific DNA sequences *in vitro* allow the detection of very small amounts of target DNA in various specimens. Theoretically these procedures can detect even one molecule of target DNA. By amplifying a sequence that is unique to the pathogenic micro-organism of interest, the *in vitro* amplification methods can be used to indirectly detect extremely low concentrations of microbes.

METHODS

Bacteria and reagents. Bacterial strains of *Salmonella* MTCC98 and *E. coli* JM109 were purchased from MTCC, Culture Center, Chandigarh,

India. Taq DNA polymerase and other reagents required for PCR were purchased from Promega. All other chemicals were of analytical grade and from Sigma.

Isolation of DNA from bacteria. The DNA was isolated from different micro-organisms by using the phenol–chloroform method. A single colony of the particular strain was incubated in 10 ml Luria broth (LB) and grown overnight at 37 °C in a shaker. A 1.5 ml sample of the culture was taken in a microcentrifuge tube and centrifuged at 5000 r.p.m. and the pellet was collected. The pellet was resuspended in TE buffer (10 mM Tris/HCl, pH 8.0), 15 µl 20 % SDS and 3 µl proteinase K (concentration, 20 mg ml⁻¹). The mixture was incubated at 37 °C for 1 h. After incubation 100 µl of 5 M NaCl was added and mixed thoroughly. To this solution 80 µl of CTAB reagent [4.1 g of NaCl, 10 g of hexadecyltrimethylammonium bromide (CTAB) in 100 ml of distilled water] was added and incubated at 65 °C for 10 min. DNA was collected by centrifugation after phenol–chloroform extraction and was washed with 70% ethanol and dried in a speed Vac concentrator. The purity of the DNA was checked by agarose gel electrophoresis, and PCR was carried out by using the isolated DNA.

Isolation of DNA from a single colony. The bacterial isolates from different feed and food samples were grown on agar-LB plates overnight at 37 °C. A single colony was picked and placed in a microcentrifuge tube containing 50 µl of Triton-X 100 (2 %) and heated to 100 °C for 5 min. The sample was cooled to room temperature and centrifuged at 10 000 r.p.m. for 10 min and the supernatant was directly used for PCR.

Isolation of DNA from animal feed. DNA was isolated from different feed samples using the procedure described above. These feed samples are commercially available and were taken from feeds used for feeding the animals in our laboratory. A feed sample (0.5 g) was taken and frozen in a pestle and mortar at -20 °C and was ground to a fine powder and suspended in TE buffer (1.5 ml). This was centrifuged and the supernatant was collected. The DNA was isolated using the procedure described above after treatment with proteinase K and CTAB solution, and finally precipitated with 2-propanol. The isolated DNA was checked for its purity by gel electrophoresis and used for PCR.

Similarly DNA was isolated from different food samples and used for detecting the pathogenic bacteria by PCR.

Design of PCR primers from *Salmonella* and *E. coli*. Primers were designed for *S. typhimurium* based on the *fimA* gene sequence. The *fimA* gene in *S. typhimurium* encodes the major fimbrial subunit (Clegg & Gerlach, 1987; Nichols *et al.*, 1990). This gene has been cloned and sequenced from *S. typhimurium* (Swenson *et al.*, 1991), and a particular region was found to be specific for *Salmonella*. The nucleotide sequence of the *fimA* gene is available from GenBank and the sequence was retrieved for designing the primer (GenBank accession no. M18283). The sequence of the primers designed is: forward primer, 5' CCT TTC TCC ATC GTC CTG AA 3'; reverse primer, 5' TGG TGT TAT CTG CCT GAC CA 3'.

For *E. coli* the primer sequence was based on the gene sequence of *afa*. This gene is responsible for pathogenicity and is specific to *E. coli* (Jothikumar & Griffiths, 2002). The primer sequence for the amplification of the *afa* gene from *E. coli* is: forward primer, 5' GCT GGG CAG CAA ACT GAT AAC TCT C 3'; reverse primer, 5' CAT CAA GCT GTT TGT TCG TCC GCC G 3'.

PCR. PCR was carried out using a thermal cycler (MJ Research). The reaction mixture consisting of 50 mM Tris/HCl (pH 8.3), 200 µl each of dATP, dCTP, dGTP and dTTP, 0.075 µM of each of the primers, 0.65 Units of Taq DNA polymerase, 2.5 mM MgCl₂ and chromosomal DNA at the concentration of 4 ng/µl was taken in a sterile 0.5 ml microcentrifuge tube. The reaction volume was made up to 25 µl with sterile

nuclease-free water. The reaction mixture was centrifuged to bring all the additions to the bottom of the tube and layered with 25 µl of mineral oil. The reaction mixture tubes were placed in a thermal cycler and the reaction was performed for 20 cycles of PCR with each cycle consisting of 1 min at 94 °C (denaturation), 30 s at 56 °C (annealing) and 1 min at 72 °C (primer extension). An additional step of 5 min at 72 °C was also included for primer extension at the end of the reaction. After the reaction was complete the mineral oil was removed carefully and the PCR products were detected by agarose gel electrophoresis followed by visualization under a UV transilluminator.

RESULTS

The DNA was isolated from different micro-organisms and the purity and integrity of the isolated DNA was examined by agarose gel electrophoresis. A high-molecular-mass band with minimum shearing was observed. DNA isolated from different organisms such as *Salmonella*, *E. coli* and unknown bacteria isolated from feed and food samples also exhibited the same property. Using specific primers PCR was carried out on DNA isolated from different micro-organisms. The conditions of the PCR were carefully standardized and all the parameters were established. The optimum annealing temperature was found to be 56 °C and within 20 cycles a substantial band was amplified only from *Salmonella* strains and not from non-*Salmonella* strains. The size of the amplified product was 120 bp as shown by comparison with marker DNA (Fig. 1).

A method for the rapid identification of *Salmonella* was also standardized. In this procedure DNA was isolated from a single colony. PCR was conducted to detect the specific DNA. The agarose gel electrophoresis pattern of the PCR products showed that DNA from all the *Salmonella* colonies was amplified with the specific sets of primers, but under these PCR conditions DNA from non-*Salmonella* colonies

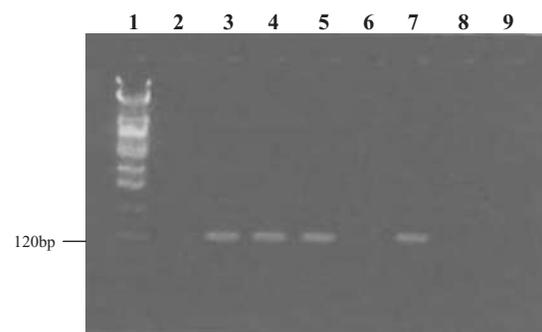


Fig. 1. Agarose gel electrophoresis of the PCR products. DNA from the isolated bacteria, colonies, feed and food were used for PCR as follows: lane 1, molecular mass marker DNA sample; lane 2, *E. coli* DNA sample; lane 3, *Salmonella* DNA sample; lane 4, *Salmonella* DNA sample isolated from food; lane 5, *Salmonella* DNA sample isolated from animal feed; lane 6, unknown DNA sample isolated from food; lane 7, *Salmonella* DNA sample isolated from colonies; lane 8, unknown DNA sample isolated from unknown colonies; lane 9, unknown DNA sample isolated from feed.

was not amplified, and also no non-specific products were amplified (Fig. 1). PCR amplification was found only in *Salmonella* strains and not in non-*Salmonella* strains regardless of the method of template preparation. Thus a simple and rapid technique was developed for the identification of *Salmonella*.

DISCUSSION

The method employed for the isolation of DNA in this study was fast and reproducible. By adopting this method it is possible to isolate DNA within 2 h. These studies have established that *fimA* primers were specific for detecting *Salmonella* in the environmental samples. The specificity of the PCR was also established by using DNA isolated from non-*Salmonella* micro-organisms. These studies have shown that non-*Salmonella* DNA was not able to give PCR product (Aabo *et al.*, 1993).

Food-borne pathogen identification is an important aspect of human health care. PCR methods have been developed for the identification of *Salmonella* pathogens (Hill, 1996; Jones *et al.*, 1993; Tsen *et al.*, 1994). PCR is an effective, rapid, reliable and sensitive technique for the detection of *fimA* gene of *Salmonella* strains (Cohen *et al.*, 1993). Here, the primers selected were completely internal to the *fimA* gene, which meant that all non-*Salmonella* strains responded negatively to the amplicon of the *fimA* gene, making this a promising diagnostic tool for sensitivity and specificity.

The *fimA* gene demonstrates a high degree of sequence conservation among *Salmonella* serovars. All fimbriate *Salmonella* serovars possess a closely related *fimA* gene, even though some strains produce antigenically unrelated fimbriae (Duguid *et al.*, 1966). The antigenic determinants for fimbriae are conserved in most *Salmonella* strains (Duguid & Campbell, 1967). The *fimA* gene seems to be unique, at least in the region of our primers, to *Salmonella* strains and can differentiate between *Salmonella* and non-*Salmonella* species. This is very useful in the diagnosis of *Salmonella* organisms at the genus level but not at the species level. The lack of non-specific bands during amplification plus the lack of false-positive results makes this method unique.

For identification of pathogenic strains of *E. coli* primers based on the *afa* gene were designed. Several *E. coli* strains were examined by using these primers in PCR reactions. However, the primers were specific for pathogenic *E. coli*. Our studies have shown that none of the isolated strains of *E. coli* were pathogenic and hence amplification did not occur. This observation also confirms the specificity of the primers used for detecting the pathogenic *E. coli* (Jothikumar & Griffiths, 2002).

Thus in this study we have standardized some of the techniques essential for detecting food-borne pathogens by using PCR.

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