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Modified Colony PCR using Genome Level Common Primers for Differential Detection of Five Bacteria

R. Anuradha, C. Naveen Prasanth, A. Vidhya Parkavi, S.M. Kavitha and S. Babu

School of Biosciences and Technology, VIT University, Vellore - 632 014, India.

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Large number of PCR and DNA Hybridization techniques have been validated for bacterial diagnosis but still the detection technology lacks effective translation for practical use. Hence, we have developed a simplified colony PCR technique for amplification of target regions from genome of *Salmonella enterica*, *Escherichia coli, Aeromonas hydrophila, Staphylococcus aureus* and *Streptococcus pyogenes* for identifying different pathogenic bacteria. A common primer set was designed based on comparative sequence analysis of genomic regions of these bacteria. Three different colony PCR methods were evaluated and compared with isolated genomic DNA as template. Directly using the cell suspension or heat lysed culture as DNA sample resulted in non specific amplifications. When the cells were pelleted (to remove media), boiled after suspending in sterile water and the lysate used in PCR, expected size amplicons were obtained for all the five bacteria without any non specific amplifications. The intensity of amplified fragment - which could be roughly correlated with the number of copies amplified - was several folds higher than it was observed in the PCR with 200 ng of isolated genomic DNA. Our colony PCR technique is simple than the previously reported methods which require long time boiling or treatments with enzymes like proteinase K.

Key words: Bacteria, Colony PCR, Detection, Food-borne.

DNA based detection of bacteria has advanced rapidly in recent years with the advent of new techniques like real-time and multiplex PCR methods. However, the adoptability of these expensive and laborious techniques in diagnostic laboratories of developing countries still remains mater for debate. A systematic review of the cost-effectiveness of rapid diagnostic tests for the detection of bacterial pathogens in food and faeces[1] suggested that adoption of rapid tests in combination with routine culture is unlikely to be cost-effective, however, as the cost of rapid technologies decreases, total replacement with rapid technologies may be feasible. PCR has proved potentially very successful in bacterial detection with more accuracy than biochemical or immunological methods.

To circumvent tedious DNA isolation procedures for PCR, direct colony PCR techniques have been developed and reported[2-5]. It is important to make a note of the less wide practicing of colony PCR method as evident from the deficiency in literature on further advancement of this approach. The setback encountered is largely due to the unaccountability of the number of cells and in turn the DNA molecules used as template. It is well-known that the ratio of DNA to primer is the key to successful amplification of target genomic region of diagnostic importance.

* To whom all correspondence should be addressed.
Mob.: +91-9790166084; Fax: +91-416-2243092;
E-mail: babu.s@vit.ac.in

Colony PCR technique is still valid as it not only by-passes the DNA extraction procedures but also encompasses the potential to be translated to direct amplification and identification of specific bacteria in complex biological samples. In such a case, the challenge is also to avoid the PCR inhibitory compounds in the complex environmental, food or clinical samples for which several approaches have been developed including enrichment, heating, proteinase K treatment (for detailed review, Fredriksson-Ahomaa and Korkeala[6]). When the release and number of DNA molecules in the pure culture colony PCR is a problem, this would be compounded when biological samples are directly used.

We have been routinely using colony PCR approach in our attempts to develop universal primer probe for multiple food-borne bacterial detection. Inconsistency in the colony PCR results experienced by us forced to optimize and develop a simplified and reproducible colony PCR method. It is also usually unlikely to obtain expected copies of amplified DNA based on theoretical calculation of 2^n number of amplicons at the end of n number of cycles. This could be attributed to template DNA-primer ratio, PCR inhibitory compounds and the presence of lysed cell debries in the PCR reaction mixture in case of bacterial culture directly used as template. Therefore, optimization of colony PCR in comparison with amplification of same target in purified genomic DNA would be a validation of colony PCR over purified DNA template PCR. With this objective, we have compared different types of colony PCR template preparations of selected food borne bacteria *viz., Salmonella enterica*, *Aeromonas hydrophila*, *Escherichia coli, Streptococcus pyogenes* and *Staphylococcus aureus*.

MATERIALS AND METHODS

Genome sequences used

Whole genome sequence of *Escherichia coli* K12 substr.MG1655, *Streptococcus pyogenes* MGAS5005, *Aeromonas hydrophila* subsp. *hydrophila* ATCC7966, *Salmonella enterica* subsp.*enterica* serovar Newport str.SL254, *Staphylococcus aureus* subsp.*aureus* MRSA252 were retrived from NCBI microbial genomes database (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_taxtree.html). The sequences of first rRNA operon (covering the 16S, 23S and 5S and their spacer regions) in the genome of all these bacteria was chosen.

Bacterial cultures

Type cultures synonymous to the strains chosen from database were obtained from Institute of Microbial Technology, Chandigarh, India. The lyophilized forms were revived as per the instructions and sub cultured and maintained.

Design of common PCR primer set

The sequences of rRNA operon region were aligned using ClustalW online tool version 2.1 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Highly conserved regions among the five bacteria were selected for design of primers. The primers were designed manually in such a way they anneal to conserved region and amplify variable region in the genome. Oligonucleotide synthesis was carried out at Chromos Biotech Private Limited, Bangalore, India. The primers are 5'GTTGGGTTAAGTCCCGCAAC3' and 5'GGTACTGGTTCACTATCGGTC 3'(forward and reverse, respectively).

Preparation of template DNA for PCR

Method 1

Single colony from overnight culture of bacterial strains was suspended separately in 30 µL of sterile distilled water using toothpick. About 2 µL of the suspension was directly used as template in the PCR.

Method 2

Single colony from overnight culture of bacterial strains was suspended separately in 30 µL of sterile distilled water using toothpick. The suspension was heated at 95°C for 10 min followed by brief centrifugation in microfuge for 2 min. About 2 µL of the supernatant was used in PCR.

Method 3

Bacterial cultures were inoculated in liquid LB broth and incubated overnight at 37°C with 100 rpm. From the culture, 200 µL was transferred into 1.5 mL microcentrifuge tubes and centrifuged at 12,000 rpm for 5 min. The supernatant was discarded and pellet dissolved in 30 µL of sterile distilled water. The bacterial cell suspension was boiled at 100°C for 5 min and 2 µL of the boiled suspension was used as template in PCR.

Method 4

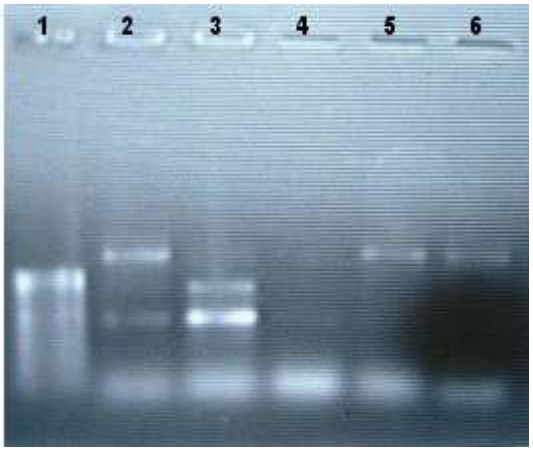
Overnight culture of bacterial strains was centrifuged at 8000 rpm for 5 min at 4°C. The pellet was resuspended in 467 µL of TE buffer and 30 µL of 10% SDS. After incubation for 1 h at 37°C, the suspension was extracted with equal volume of phenol:chloroform and centrifuged at 12,000 rpm for 10 min. Double the volume of ice cold ethanol was added to aqueous phase and the DNA was pelleted by centrifuging at 14,000 rpm for 10 min at 4°C. The pellet was washed with 70% ethanol, air dried and dissolved in 30 µL of TE buffer. About 2 µL of this genomic DNA preparation was used in PCR.

PCR reaction mixture and conditions

Reaction volume of 20 µL contained 2 µL of DNA preparation, 0.5 µM each primer, 0.5 U of *Taq* polymerase (Genei, Bangalore, India), 25 mM each dNTP, 10 mM Tris-HCl (pH 8.3), 5 mM KCl and 1.5 mM $MgCl_2$. For PCR with genomic DNA, volume corresponding to 200 ng of DNA was used. The temperature cycling was as follows: after an initial denaturation at 95°C for 5 min, the subsequent 32 cycles consisted of a denaturation at 95°C for 1 min, annealing of the primer at 53°C for 1 min, an extension at 72°C for 1.5 min with a final extension step of 72°C for 7 min. The PCR products were separated by gel electrophoresis in 1% agarose (HiMedia, India) gels containing ethidium bromide with 1XTAE buffer, and visualized under a UV illuminator. The annealing temperature was chosen after gradient PCR reactions with the genomic DNA samples.

RESULTS AND DISCUSSION

Detection of bacteria by conventional culture methods generally takes 3-4 days and these methods are labor intensive and less sensitive. PCR is a powerful method in bacterial detection because of the rapidity and accuracy[7-9]. Real-time PCR methods have been reported to detect food-borne pathogens with more sensitivity[9]. However, use of expensive real-time PCR methods by small scale food industries needs to be debated. To simplify the PCR detection without genomic DNA extraction, different types of colony PCR have been reported[2,5,10-12]. Barletta et al. [13] developed a five-colony pool analysis using multiplex real-time PCR for detection of diarrheagenic *E. coli*. However all the studies report the specific detection of either single bacteria or strains of the same species. In the present study, we have optimized a common primer set based on comparative genome analysis



Single colony from overnight culture of bacterial strains was suspended separately in 30 µL of sterile distilled water using toothpick. About 2 µL of the suspension was directly used as template in the PCR.
Lanes 1: 100-1000 bp DNA ladder; 2: *S. enterica*; 3: *E. coli*; 4: *S. pyogenes*; 5: *S. aureus*; 6: *A. hydrophila*

Fig. 1. Colony PCR amplification by method 1

Single colony from overnight culture of bacterial strains was suspended separately in 30 µL of sterile distilled water using toothpick. The suspension was heated at 95°C for 10 min followed by brief centrifugation in microfuge for 2 min. About 2 µL of the supernatant was used in PCR.
Lanes 1: 100-1000 bp DNA ladder; 2: *S. enterica*; 3: *E. coli*; 4: *S. pyogenes*; 5: *S. aureus*; 6: *A. hydrophila*

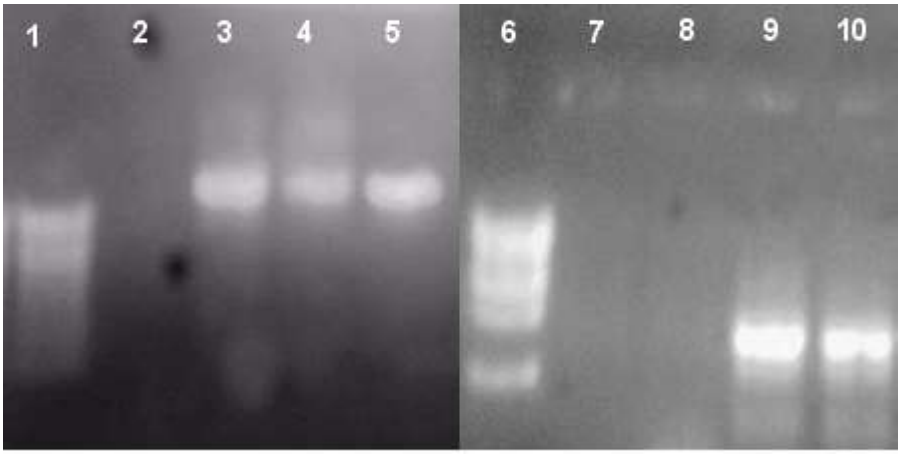
Fig. 2. Colony PCR amplification by method 2

and optimized colony PCR method for all the five bacteria under study and compared with the results of PCR with isolated genomic DNA.

In a parallel study, our primers which are designed on the conserved regions of the rRNA operon region proved to amplify similar size fragments in PCR from all the five bacteria under testing. Fragmentation of these PCR products by restriction digestion resulted in polymorphism unique to each genus (unpublished data). The same primers were used to optimize the colony PCR method which can yield either comparable or better amplification. Since DNA isolation from samples is the most time consuming factor in PCR[11], developing an efficient colony PCR method will circumvent the issue and can form the base for direct identification of pathogens in the food. Nevertheless, the colony PCR conditions have to be optimized to amplify target bacterial DNA directly from food sample in the presence of any possible inhibitory compounds.

The results of different colony PCR methods performed in the study are presented in Fig. 1-3. The results of PCR with isolated genomic DNA are presented in Fig. 4. When the single colony suspension of bacteria were directly used in PCR, expected size amplification of 1.5 kb was obtained in *S. enterica*, *S. aureus* and *A. hydrophila*. (Fig. 1). Low molecular weight non specific amplifications were also observed in *S. enterica* and *E. coli* and there was neither specific nor non specific amplification in *S. pyogenes*. The second method (lysed bacterial suspension by heating at 95°C) resulted in amplification of the expected size fragment only in *E. coli* and all the other bacteria showed non specific amplication (Fig. 2.). The hot boiling PCR (method 3) resulted in not only a specific single expected size amplicon but also the intensity of amplified DNA bands were several folds more than the other methods (Fig. 3). To confirm the size, *S. enterica, E. coli* and *S. pyogenes* were checked in agarose gel with 100-1000 bp DNA ladder. *S. aureus* and *A. hydrophila* amplification were resolved in gel with 1 kb – 10 kb DNA ladder. Amplification using isolated genomic DNA showed single amplicons which are more than the expected size in all bacteria except for *E. coli* (Fig. 4). Moreover, the intensity of the amplified products in the gel was comparatively low than observed in hot boiling PCR method.

Difficulties in obtaining high-quality DNA is a critical task in bacterial genotyping and detection. This is attributed to the degradation of DNA during isolation by a high level of endonuclease activity in bacteria[14-15]. Plourde-Owobi et al.[5] used colony PCR followed by pulse

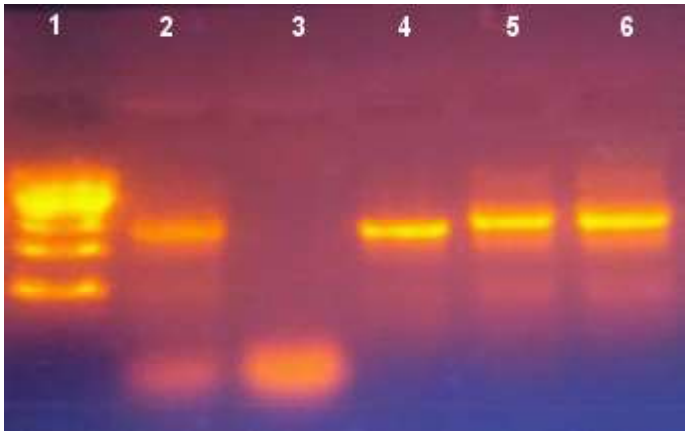


Bacterial cultures were inoculated in liquid LB broth and incubated overnight at 37°C with 100 rpm. From the culture, 200 µL was transferred into 1.5 mL microcentrifuge tubes and centrifuged at 12,000 rpm for 5 min. The supernatant was discarded and pellet dissolved in 30 µL of sterile distilled water. The bacterial cell suspension was boiled at 100°C for 5 min and 2 µL of the boiled suspension was used as template in PCR.
Lanes 1: 100-1000 bp DNA ladder; 3: *S. enterica*; 4: *E. coli*; 5: *S. pyogenes*; 6: 1 kb-10 kb DNA ladder; 9: *S. aureus*; 10: *A. hydrophila*

Fig. 3. Colony PCR amplification by method 3

field electrophoresis for characterization of *Clostridium tetani* strains. They have reported suspending the colonies in TE buffer and boiling for 30 min at 60°C and then for 10 min at 95°C. Jin et al.[11] and Pillai et al. [16] were able to amplify *Salmonella* sp specific gene directly from the inoculated chicken meat sample by PCR. They have reported have reported suspending the meat sample in phosphate buffered saline, treating with proteinase K, heating for 60 min at 60°C and finally boiling for 15 min. It has also been proposed that a higher sensitivity of detection can be obtained by optimizing the aforesaid conditions for PCR.

In this study, we have optimized the different simple procedures of direct colony / cell lysate PCR without the need to heat for long time and without any treatments like proteinase K. Our hot boiling PCR procedure (method 3) was able to produce amplification better than when pure genomic DNA was used as template. This suggests



Bacterial cultures were inoculated in liquid LB broth and incubated overnight at 37°C with 100 rpm. From the culture, 200 µL was transferred into 1.5 mL microcentrifuge tubes and centrifuged at 12,000 rpm for 5 min. The supernatant was discarded and pellet dissolved in 30 µL of sterile distilled water. The bacterial cell suspension was boiled at 100°C for 5 min and 2 µL of the boiled suspension was used as template in PCR.
Lanes 1: 1 kb -10 kb DNA ladder; 2: *S. enterica*; 3: *E. coli*; 4: *S. pyogenes*; 5: *S. aureus*; 6: *A. hydrophila*

Fig. 4. PCR with isolated genomic DNA

that the procedure optimized in the study is applicable for the more sensitive detection of these five bacteria in food samples even if the colony forming units are less. In direct PCR on food samples, it has been suggested that the major problem is the inhibitory compounds[17-18]. But the PCR based methods can clearly detect pathogenic bacteria in food, clinical and environmental samples with higher sensitivity than with culture methods[6]. As the intensity of amplified fragment in gel reflects the number of copies at the end of amplification, high number of amplified copies in our study strongly suggests that this method might be applicable in food-borne bacterial detection even without clarification of inhibitory substances.

Ethical Considerations

Ethical issues including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc. have been completely observed by the authors.

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