

STANDARDISATION OF PAPER BASED PCR FOR DETECTION OF BACTERIA USING 16S rRNA GENE

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ABSTRACT

Reliable procedures are necessary to preserve, transport and test clinical, water or food samples, frequently requiring a challenging and costly cold chain to be in place. Effective disease surveillance or detection is thus severely hampered in resource-limited settings, such as those currently present in rural India, where procedures for appropriate laboratory based detection are suboptimal. Hence the recent scientific research is been focused to develop laboratory techniques/devices that can be applied in point of care settings. Point-of care (POC) techniques are getting increasingly popular, due to the advantages provided by them like ease of use, variety of applications, cost effectiveness and ease of disposal. This study deals with the standardization of method for microbial detection from sample collected on filter paper by direct amplification of a gene using PCR. Bacterial presence in a sample is confirmed by amplification of 16S rRNA gene as a proof of concept. 16S rRNA gene detection directly from sample spotted on Whatman filter paper no.3 without any pre-treatment to extract DNA is demonstrated, which is different from the currently practiced methods that include an additional step of DNA extraction from the paper. Robustness of the method was tested using Gram positive and Gram negative bacterial cultures and the sensitivity of detection on Whatman filter paper no. 3 was found to be 40-50 cells.

Keywords: 16S rRNA, PCR, Whatman paper, Point-of-care

INTRODUCTION

Point-of care (POC) techniques/devices are getting popularity worldwide because they can be effectively used in resource-limited settings. POC devices are used out of the laboratory settings and offer advantages like ease of use, variety of applications, cost effective and ease of disposal. The POC devices can be categorized as Instrumented and un-instrumented devices. Amongst un-instrumented devices, one of the commonly used methodology is filter paper. Samples stored on the filter papers are typically used for identification of microbes or parasites. Most common practice is extraction of DNA from these samples followed by PCR to amplify specific gene which aids in identification. Although there are some reports where direct PCR is carried out without prior DNA extraction step. Certainly the biggest advantage of direct PCR is the fact that the extraction and purification of DNA from filter paper can be omitted, resulting in an overall saving of the time and chemicals required for DNA extraction step. Additionally, it can potentially avoid loss of DNA during extraction due to manual error or poor sample conditions which can intern affect the PCR reaction.

The aim of this study was to develop and optimize a direct paper based PCR technique for the detection of bacteria from water sample without the DNA extraction process. To establish the proof of concept for this technique, method development and optimization was done using 16S rRNA gene as a model.

16S rRNA gene is the most common housekeeping genetic marker used to study bacterial phylogeny and taxonomy for a number of reasons. These include: (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1500 bp) is large enough for informatics purposes. This gene consists of two regions – conserved and variable. The conserved regions are targeted by universal primers for the detection of the presence of microorganism in a given sample whereas the variable regions are targeted for identification of genus or species using specific primers (Hassan, El Enany, & Rizk, 2014).

The current work is a proof of concept to establish direct amplification of 16S rRNA gene from a sample applied on a Whatman paper to detect presence of microbes without extraction of genomic DNA. Universal primers were used to

amplify the gene. The technique can be standardized for detection of particular organism using species specific /unique primers.

MATERIAL AND METHODS

Cultures Used

Genetically modified *E. coli* strains BL21 (DE3) and DH5 α were procured from Invitrogen (USA). Other bacterial cultures *Streptococcus pyogenes*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Citrobacter* and *Bacillus sp* were laboratory maintained strains.

Media and Reagents

PCR reaction components, *Taq* polymerase and buffer were from Invitrogen, dNTPs were from Invitrogen. Nitrocellulose membrane was procured from Invitrogen, USA and Whatman filter paper no. 3 was from GE Healthcare Life Sciences, Luria Bertani (LB) agar components were procured from Himedia.

Optimization of parameters for 16S rRNA gene PCR for detection of bacteria

For optimizing the conditions for 16S rRNA gene PCR, it was important to evaluate the various components used in PCR reaction. Genetically modified *E. coli* strains (DH5 α and BL21 DE3) were used as a source of template DNA. Following sections describe the details about optimization of each component

Primer Selection

Genomic DNA (gDNA) of *E. coli* strains DH5 α and BL21 (DE3) were isolated using QIAamp DNA Mini Kit from Qiagen following the protocol recommended by the manufacturer. Additionally, isolated colony of both the strains of *E. coli* mentioned above, was picked up from LB agar plates and was re-suspended in 25 μ L of water in 2 distinct tubes. These tubes were kept in boiling water bath to lyse the cells. The boiled cell suspension was spun down and supernatant containing the gDNA was used as template. The gDNA extracted using both the

above methods was used as template in the PCR. Two sets of universal primers with following sequences were synthesized (Sigma) for amplification of 16S rRNA gene.

Set 1: F1: 5'ACT CCT ACG GGA GGC AGC AGT 3', R1: 5'TCA CCG GCC GTG TGT ACA AG-3' (Matsuda et al., 2011)

Set 2: F2: 5'GTG TAG CCG TGA AAT GCG 3', R2: 5'ACG GGC GGT GTG TAC AA3' (Sauer, Gallo, Kesselova, Kolar, & Koukalova, 2005)

A typical PCR reaction was set up either using F1/R1 or F2/R2 primers, gDNA of DH5 α and BL21 (DE3) (from colony or extraction) along with optimal concentrations of dNTP, Taq polymerase and buffer. PCR cycle program was set as 94°C for 5 minutes for initial denaturation followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and final extension of 72°C for 7 minutes. Expected amplicon size for F1/R1 and F2/R2 primers was 1086 bp and 709bp respectively. Post amplification the amplified product was loaded on 1% agarose gel having ethidium bromide and the amplicon was visualized on UV transilluminator.

Cell Lysis Optimization for Release of gDNA during PCR Cycle

The major goal of the study was to establish a robust paper based PCR method without prior treatment of the sample.

Three isolated colonies of *E. coli* DH5 α were picked up from LB agar plates and were re-suspended in sterile water. 5 μ L of this suspension was directly spotted on the small strip of pre-sterilized Whatman filter paper no.3 in a laminar airflow unit (LAFU) without prior boiling. The paper was air dried in LAFU to avoid any contamination and was directly suspended in the master mix containing appropriate concentration of buffer, dNTP, F2/R2 primers and Taq polymerase. Along with this reaction, a standard colony PCR of DH5 α culture was set up using a colony suspended in water and the mixture was boiled and post cell lysis, suspension was used as template in PCR master mix. 5 μ L of lysed cell suspension post boiling was then spotted on a pre-sterilized Whatman filter paper no.3 as mentioned above and was allowed to air dry. This paper was also placed in a separate master mix and all the 3 reactions were processed for amplification. PCR cycling conditions and post amplification analysis was carried out.

Bacterial Culture Volume Optimization for Paper Based PCR

As the paper based PCR method for amplification of 16S rRNA gene was established using Whatman filter paper no. 3, it was important to optimize the lowest volume of culture that is sufficient to amplify 16S rRNA gene. Since the paper was directly dipped in 25 μ L of PCR master mix in a PCR tube, there was limitation of the size of the paper that can be used for the reaction which further limits the volume of culture that can be accommodated on that small piece of paper. Earlier experiments had shown that 5 μ L culture could be used to get desired amplicon; the volumes were reduced below 5 μ L. DH5 α colony suspension was prepared in sterile water. This suspension, without boiling was directly spotted on 3 independent presterilized small strips of Whatman filter paper no.3 as 1, 3 and 5 μ L. The strips were allowed to air dry in LAFU and were directly dipped in 25 μ L of PCR master mix and the amplification and detection was carried out. Standard colony PCR of the same culture was also carried out along with this reaction as a positive control.

Paper Based PCR Method Validation

Determination of Limit of detection (LOD) using *E. coli* DH5 α and BL21 (DE3) as model cultures

DH5 α suspension of 0.1 abs was prepared from overnight culture. It was diluted serially ten-fold up to 10⁻⁶ and 5 μ L of each suspension was spotted on pre-sterilized independent Whatman filter paper no.3 strips and the strips were air dried in LAFU. These strips were dipped in master mix having all reaction components and F2/R2 primers and PCR amplification was carried out, except the cycles were increased to 40 from 35 cycles and reaction volume was shifted to 30 μ L. Amplicon was loaded on agarose gel and was detected on UV transilluminator.

Viable count of all the dilutions was done by Miles and Misra's technique.

Optimal annealing temperature determination

To evaluate the optimal annealing temperature for primer set F2/R2, a gradient PCR program was set up in thermal cycler with annealing temperature range of 55-58°C where as other cycling conditions are maintained.

As a model organism, single colony of *E. coli* strain BL21 (DE3) was suspended in 15 μ L of sterile water and was further diluted 10 fold serially up to 10⁻⁶. All these dilutions were spotted on presterilized Whatman filter paper no.3 and processed as mentioned in LOD section. All these dilutions were exposed to 55 °C annealing temperature whereas the 6th dilution was also used at 56, 57 and 58°C annealing temperatures. The amplification was visualized.

Robustness

16S rRNA gene is a house keeping gene for all the bacteria, whether Gram positive or Gram negative. To ensure the specificity of the paper based PCR, various Gram positive and Gram negative bacterial colony suspensions were prepared and were spotted on pre-sterilized Whatman filter paper no.3 as mentioned earlier. The bacteria used for this study were *Streptococcus pyogenes*, *Klebsiella pneumoniae*, *Citrobacter* and *Bacillus sp*. Each of these cultures were streaked on sterile Luria agar plate to achieve single isolated colony. Single colony of each of these cultures was picked up and suspended in 15 μ L of sterile water and it was further serially diluted to get 40-50 cells/5 μ L for each of the five cultures. 5 μ L of each suspension was spotted on pre-sterilized independent Whatman filter paper no.3 strips and the strips were air dried in LAFU. These strips were dipped in master mix and PCR was carried out as mentioned earlier in case of *E. coli* DH5 α . The experiment was carried out with 2 different master mixes, one with F1/R1 primer set and another with F2/R2 primer set. An undiluted colony suspension of each culture served as a positive control along with paper based PCR with 40-50 cells.

RESULTS AND DISCUSSION

Optimization of parameters for 16S rRNA gene PCR for detection of bacteria

Primer Selection

A pair of universal primers was selected from the highly conserved region of the 16S rRNA sequence. The up-stream primer corresponded to C1 region nucleotides 358 to 378 and the downstream primer corresponded to C6 region nucleotides 1444 to 1425 (Labeled as F1/R1 for this study). The primers were used to amplify the region which gave the amplicon of size 1086 bp. Both genetically modified *E. coli* DH5 α and BL21 (DE3) strains could give desired amplicon irrespective of whether genomic DNA was extracted or a colony was used as a source of DNA (Fig 1). Earlier, these primers were also used by Matsuda et al. (Matsuda et al., 2011) for suspected blood infections and by Liu et al. (Liu et al., 2005) for the bacterial infection in CSF. The aim here was to establish the primer pair which is suitable for amplification of 16S rRNA gene which can be used in paper based PCR method that was being developed for detection of microbes. Also another set of universal primer was evaluated for their efficiency to amplify the bacterial DNA. 16S rRNA primers used by Sauer et al. (Sauer, Gallo, Kesselova, Kolar, & Koukalova, 2005) for detection of pathogenic bacteria causing prosthetic joint infections were synthesized. The authors have claimed that the primer pair is highly specific for broad range of bacterial pathogens and can be used with variety of clinical specimens. Since the ultimate goal of the current study was to establish a robust paper based PCR method to detect microbes from variety of food and clinical samples, it was essential to demonstrate the primer pair that can be used commonly with broad range of samples. In the current study this primer pair was labeled as F2/R2.

When F2/R2 was used in the PCR for amplification of 16S rRNA gene from genetically modified *E. coli* strains, amplicon of 709 bp was detectable on the agarose gel electrophoresis (Fig1). Since both the primer pairs were highly specific for 16S rRNA gene, one primer pair (F2/R2) was used in subsequent experimentation.

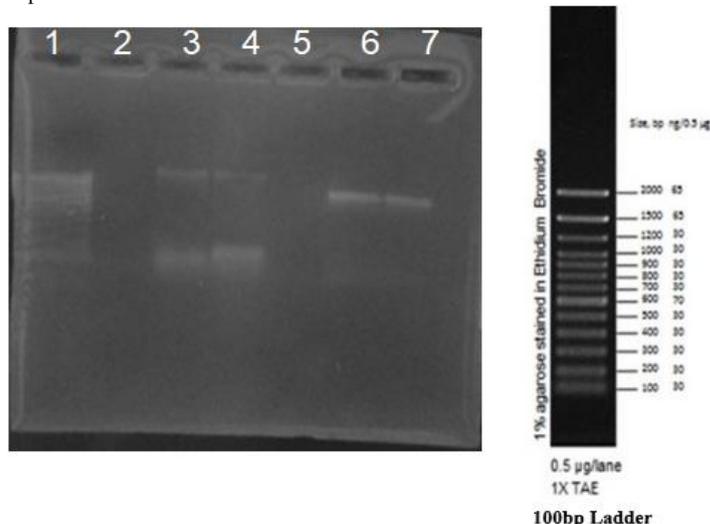


Figure 1 Amplification of 16S rRNA gene of *E. coli* strain DH5 α and BL21DE3 using universal primers
Lane 1: 100 bp ladder, Lane 2: Negative control for F1/R1, Lane 3: 16S rRNA gene of DH5 α amplified with F1/R1, Lane 4: 16S rRNA gene of BL21DE3 amplified with F1/R1, Lane 5: Negative control for F2/R2, Lane 6: 16S rRNA gene of DH5 α amplified with F2/R2, Lane 7: 16S rRNA gene of BL21DE3 amplified with F2/R2.

Optimization of Cell Lysis for PCR

In a standard colony PCR reaction the colony suspension is boiled to lyse the cells and release the genomic DNA which is subsequently amplified with gene specific primers. To optimize this cell treatment for paper based PCR reaction, the colony suspension was boiled first and then spotted on pre-sterilized Whatman filter paper no.3. To evaluate the possibility of eliminating this lysis step, a reaction was also set up where the colony suspension was not boiled and directly spotted onto pre-sterilized Whatman filter paper no.3. All the three conditions could give the amplification of 16S rRNA (Data not shown) which implies that the boiling treatment for cell lysis and release of genomic DNA can be eliminated. This may be because the first denaturation step in PCR cycling condition helps in cell lysis and release of genomic DNA which is subsequently amplified. Hence the boiling of cell culture was not carried out in further experiments and the cultures were directly spotted on pre-sterilized Whatman filter paper no.3 for paper based PCR.

Culture volume Optimisation

Paper-based PCR for amplification of 16S rRNA was optimized with Whatman filter paper no.3 and without prior cell treatment. Since the final PCR reaction is carried out in small volumes (30 µL), it was essential to reduce the size of the Whatman filter paper no.3 which is being dipped in the PCR tube and stays in the tube throughout the amplification. It was hence decided to load different volumes of cultures on the paper to evaluate the lowest possible volume of culture that can give the positive reaction. The amplification of 16S rRNA gene could be achieved with as low volume as 1 µL of culture on paper, using the PCR conditions optimized so far. Standard colony PCR reaction without use of Whatman filter paper no.3 served as a positive control.

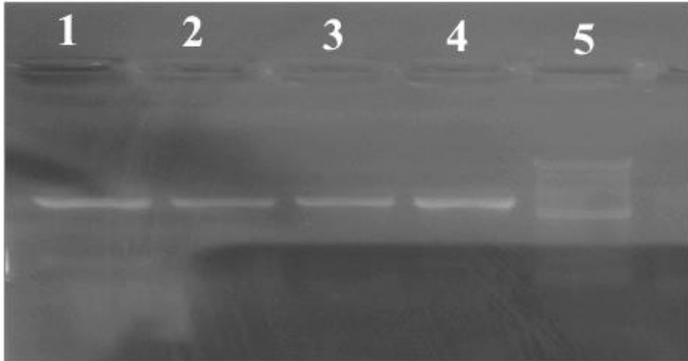


Figure 2 *E. coli* DH5α culture volume optimization for amplification of 16S rRNA gene

Lane 1: DH5α – colony PCR, Lane 2: Amplicon when 5 µl culture spotted, Lane 3: Amplicon when 3 µl culture spotted, Lane 4: Amplicon when 1 µl culture spotted on pre-sterilized Whatman paper No 3, Lane 5: 100 bp ladder

Determination of limit of detection (LOD) for 16S rRNA gene in *E. coli* strains DH5α and BL21 (DE3)

A single colony of *E. coli* strains DH5α contains $\sim 9 \times 10^8$ cells mL⁻¹ which was serially diluted up to 10⁻⁶ to give ~ 900 cells mL⁻¹. Since for amplification reaction, 5 µL of this suspension was spotted on pre-sterilized Whatman filter paper no.3 and 16S rRNA amplification could be achieved, that indicates the LOD to be 5 cells for *E. coli* strains DH5α. Since the intensity of amplicon band is very low in case of 10⁻⁶, ideally a previous dilution of 10⁻⁵ can be safely detected which is approximately 50 cells. Similarly for BL21 (DE3), a single colony suspension contains $\sim 4.5 \times 10^9$ cells mL⁻¹ which was serially diluted ten-fold up to 10⁻⁶ to give ~ 4500 cells mL⁻¹. Since 5 µL of this suspension on paper could amplify the gene, the LOD for BL21 (DE3) was also same as that of DH5α (Data not shown but dilution scheme was followed for detection of other organisms as mentioned in robustness section).

Annealing temperature optimization for 16S rRNA amplification from *E. coli* DH5α culture

Earlier data has shown weak amplification when the *E. coli* DH5α culture was serially diluted to 10⁻⁶ and spotted on paper. It was hence necessary to optimize the primer annealing temperature to get clearly detectable amplicon on agarose gel. The recommended annealing temperature of F2/R2 universal primers was 55°C. To optimize this annealing temperature for better amplification, a gradient PCR was set up with the range 56-58°C with only 10⁻⁶ dilution spotted on the paper which served as a template DNA. Good intensity amplicon was visible in PCR with annealing temperature of 57°C (Data not shown). So here after, 57°C was used as the optimal annealing temperature.

16S rRNA gene amplification in Gram positive and Gram negative bacteria using paper based PC (Robustness)

To ensure the robustness of the developed paper based PCR method, Gram positive and Gram negative single colony bacterial suspensions were prepared and were diluted up to 10⁻⁵ dilutions which corresponds to ~ 50 cells. These were spotted on the pre-sterilized paper and were amplified using both sets of primers (F1/R1 and F2/R2).

For every culture, all dilutions and undiluted colony suspensions were independently amplified using F2/R2 primer pair. It is clearly seen in (Fig 3a) that 16S rRNA gene can be amplified even post dilution of both Gram positive and negative bacterial culture up to 50 cells. Although, in some cases of diluted cultures, the intensity was relatively low; this could be due to the cell number being just around or below the detection limit. The robustness of the method was also tested using different set of primers (F1/R1) with undiluted cultures of gram positive and negative bacteria spotted on paper. All the cultures could give the amplicon of ~ 1 Kb which is the expected size with F1/R1 primers (Fig 3b).

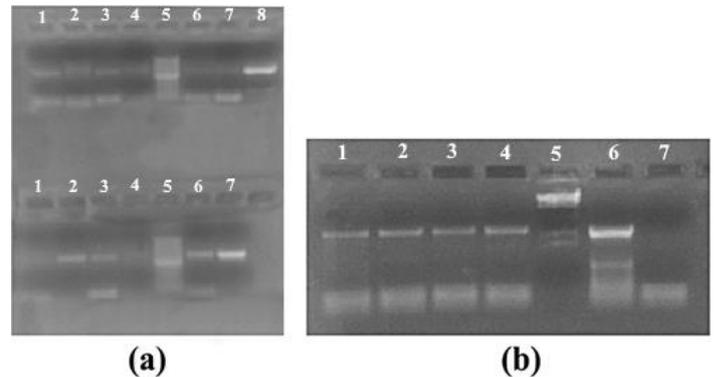


Figure 3 16S rRNA gene amplification in Gram positive and Gram negative bacteria using paper based PCR

(a) Amplification with F2/R2 primer pair **Top half:** For all the cultures positive control is single colony suspension and test is 40-50 cells spotted on Whatman filter paper no.3. (Left to Right) Lane 1 & 2: *K. pneumoniae* positive control and Test, Lane 3 & 4: *Citrobacter* positive control and Test, Lane 5: 100 bp ladder, Lane 6: negative control with Whatman, Lane 7: negative control water, Lane 8: positive control (DH5α genomic DNA PCR); **Bottom half:** (Left to Right) Lane 1 & 2: *S. pyogenes* Test and positive control, Lane 3 & 4: *Bacillus* positive control and test, Lane 5: 100 bp ladder, Lane 6 & 7: *E. coli* DH5α Test and positive control; (b) Amplification with F1/R1 primer pair (Left to Right) Lane 1: *S.pyogenes*, Lane 2: *Bacillus*, Lane 3: *Citrobacter*, Lane 4: *K. pneumoniae* Lane 5: 100 bp ladder, Lane 6: DH5α genomic DNA PCR (positive control) , Lane 7: negative control

CONCLUSION

16S rRNA gene detection directly using Whatman filter paper no.3 without any pre-treatment to extract DNA from paper is demonstrated above as a proof of concept. The method is robust, sensitive and can detect up to 40-50 cells on the paper. The primers used for the amplification were universal primers and can be used for detection of presence or absence of bacteria in any sample. This technique can detect bacterial contamination in samples which are meant to be sterile like injectables, oral syrups, tissue culture media etc. The technique can be standardised for detection of particular species by using species specific variable regions in the gene.

This method can be used for analysis of samples brought from point of care in remote locations. The processing can be done in the central laboratories in tier 2 or tier 3 cities which have basic laboratory facilities such as basic PCR machine which is not very costly. The procedure does not need highly skilled workers for conducting the test. Additionally, specialized filter paper which is normally used for collection of samples like Whatman 903 is also replaced in this method by a normal Whatman filter paper no. 3 which is easily available at a lower cost. The detection of microbes can be done in a short span as compared to traditional microbiological methods. Therefore this direct paper based PCR method is versatile and can be used for applications in resource limited settings to applications in fully equipped central laboratories in developed countries.

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