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■ RESEARCH ARTICLE

A Novel Rapid Detection Method of *Escherichia coli* using PCR-based Nucleic Acid Amplification Using pH-sensitive Dyes

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ABSTRACT: Molecular diagnostic assays based on nucleic acid amplification are the primary choice for diagnosing *Escherichia coli*. However, it requires extended workflow time (more than 10 hours), sophisticated equipment, or both to detect and analyze the amplification product. Here, a novel method of amplification detection was developed that mediates the pH change resulting from amplification reactions performed with minimal buffering capacity. This novel method allows one to easily check the positive infection state of *Escherichia coli* by changing the color of the amplified DNA product, which mediates the low pH of the reaction buffer. Colony Polymerase Chain Reaction (PCR), a widely used method to determine the presence or absence of bacteria DNA, was performed to amplify the 16s rRNA sequence. In this paper, experiments were conducted several times to find the optimal conditions for the color change in the DNA-amplified reaction buffer. Firstly, 47 ˚C, 50 ˚C, and 53 ˚C annealing temperatures were tested, and all three conditions successfully amplified the target bands. Next, phenol red concentration was optimized with 0.35 mM condition among five different conditions tested: 0.14 mM, 0.35 mM, 0.71 mM, 1.07 mM. Also, various concentrations of HCl were added to the PCR reaction solution, and the addition of 0.6 µM HCl showed the optimal pH condition for color change detection. Using the optimal conditions, we successfully amplified the *Escherichia coli* DNA with the transparent color change of the PCR reactions. This colorimetric detection of amplification demonstrates a generally applicable approach for visual detection of nucleic acid amplification, which enables molecular diagnostic assays to be analyzed rapidly in 2 hours without the need for expensive lab equipment (agarose gel electrophoresis, gel documentation, and real-time PCR thermocycler). This novel method is also possible to be applied in diagnosing coronavirus.

KEYWORDS: Cellular and Molecular Biology. pH; Color change; *E. coli*; PCR; DNA amplification.

■ Introduction

Escherichia coli (*E. coli*) is a widely known gram-negative bacillus being a crucial member of the normal intestinal microflora of mammals, including humans.[1] *E. coli* causes various human diseases, such as bloody diarrhea, seizures, and dysentery.[2] These are enteric diseases caused by at least six different pathotypes. Further pathotypes can possibly cause extra-intestinal infections such as urinary tract infections and meningitis.[3] Therefore, rapid diagnosis of *E. coli* infection is crucial.

One method to identify *E. coli* is utilizing Polymerase chain reaction (PCR) with primers against the uidA gene encoding beta-D-glucuronidase from food samples.[4] Overall, this method requires a colony of bacteria on an agar plate, which takes 24 h. Another method to identify *E. coli* is Enzyme-Linked Immunosorbent Assay (ELISA).[5] This method detects the protein of *E. coli* and takes about 24 hours to pre-enrichment the culture samples. The two methods can be merged to produce even more robust results.[6] PCR is an excellent method of *E. coli* detection compared to standard culture and ELISA methods being extremely sensitive as well as accurate and promising real-time quantitative.[7]

We developed a new PCR-based nucleic acid amplification in this study using pH-sensitive dyes. This method only requires performing the PCR reaction with the samples. When the *E. coli* is presented in the sample, then the PCR reaction buffer decreases the pH by amplifying *E. coli* DNA. This new method can save time because DNA amplification can be detected by the color change of the PCR reaction buffer (red to orange). Through this study, we optimized the PCR conditions, such as annealing temperature, initial pH, and the concentration of phenol red (pH-sensitive dye) that detects the *E. coli* DNA by changing the color of the PCR reaction buffer.

■ Methods

Polymerase Chain Reaction (PCR)

Firstly, a mixed solution 11 times larger than 20 µl, which is the amount needed per one tube for the PCR, was prepared. Ten solutions of 20 µl in total to run five different amounts of phenol red indicator for bacteria-containing and not containing conditions were prepared. The following PCR reaction condition was prepared: Water (15.5 µl), 10x buffer (2 µl), dNTP (1 µl), F primer (0.5 µl), R primer (0.5µl), Enzyme (polymerase) (0.5µl), E *coli* bacteria (0.5 µl). For optimizing phenol red concentration, various volume of phenol red indicator (14.1 mM) was added to the PCR reaction buffer: 2µl, 1.5µl, 1µl, 0.5µl, and 0.2µl. Start the PCR program by denaturation at 95°C for 3 minutes, followed by a series of cycles of

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denaturation at 95˚C for 30 seconds, annealing at 50 ˚C for 30 seconds, and extension at 72˚C for 40 seconds. The number of cycles was set to 35 cycles.

Optimizing pH condition for PCR

A mix solution which was 11 times larger than the original solution, was made. Then using pipettes, this solution was divided into ten solutions. Then to each solution, different amounts of concentrated hydrochloric acid were added to alter the solution's acidity to easily detect color change after the PCR within the range of 0 µl to 1 µl. The following PCR reaction buffer was prepared: Phenol Red 0.5 µl, 10x buffer 2 µl, dNTP 1 µl, and water 17 µl. The solutions were mixed well, so there was no material lying on the bottom that was not getting thoroughly mixed.

Agarose Gel electrophoresis

First, the PCR samples with or without *E. coli* samples were prepared. In the gel tank, the agarose gel was ready to be poured into the TAE buffer in sinking the gel. Then to each well, the samples were inserted to visualize the band. In the end, we inserted a DNA size marker to check the band lengths. Then it was turned on for electricity, giving charge to the end so the DNA bands could move. It was left on for 20 minutes to move DNA bands from negative to positive charge flow. Smaller DNA travels faster; positioning on top and thicker DNA means it contains more DNA. Then agarose gel with DNA was placed under the UV light to check the results.

Results and Discussion

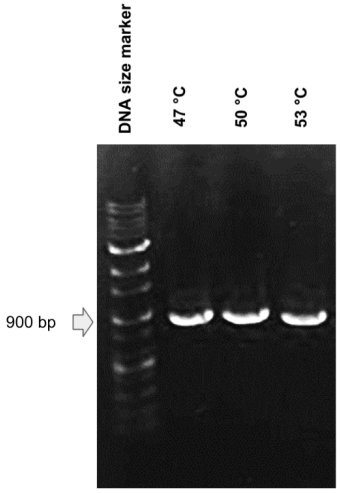

Figure 1: Optimization of annealing temperature for amplification of *E. coli* 16S rRNA gene. The agarose gel image of DNA amplified products using three different annealing temperature conditions: 47 ˚C, 50 ˚C, and 53 ˚C.

This experiment aims to find the optimal annealing temperature for PCR reaction. We tested three different annealing temperature conditions for PCR reaction: 47 ˚C, 50 ˚C, and 53 ˚C. Using agarose electrophoresis gel, we visualized the amplified DNA band in the blue light illuminator. All three temperatures amplified the *E. coli* DNA (Figure 1). The expected band size was about 900 bp, and the size of the DNA amplified product was confirmed with the DNA size marker (Figure 1). In conclusion, we used an annealing temperature of 50 ˚C for the PCR reaction.

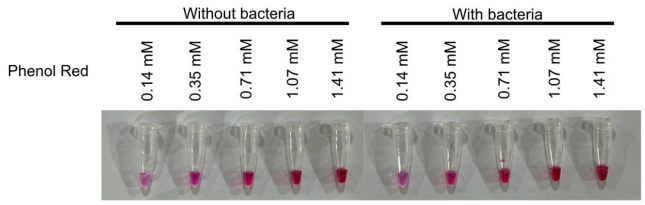

Figure 2: Optimization of phenol red concentration on PCR reaction buffer for inducing color change after DNA amplification. The image of the final PCR reaction buffer with different concentrations of phenol red after PCR reaction. Phenol red concentrations of 0.14 mM, 0.35 mM, 0.71 mM, 1.07 mM, and 1.41 mM were used in this experiment.

Phenol red is a widely used pH indicator because it is easy to use and can measure a wide range of pH values. It changes color depending on the pH of the solution, making it easy to determine the pH visually. It is also relatively inexpensive, stable, and non-toxic, making it a good choice for laboratory and industrial settings. Additionally, it can be used in liquid or solid form, making it versatile for different measurements. Since phenol red's versatility, stability, and a wide range of pH measurements, we selected phenol red as a pH indicator.

Next, we aimed to optimize phenol red concentration showing the color change after proceeding with the PCR reaction under bacteria-containing conditions. Therefore, we hypothesized that comparing of the negative and positive samples of bacteria would vividly portray apparent color changes in the PCR reaction buffer. Phenol red concentrations of 0.14 mM, 0.35 mM, 0.71 mM, 1.07 mM, and 1.41 mM were used in this experiment. We prepared five negative control samples without bacteria and five samples with bacteria. The color of the samples without the bacteria and with the bacteria showed no difference in all five different concentrations of phenol red (Figure 2). However, as the phenol red concentration increased, the color of the PCR reaction sample became darker red. The phenol red concentration is critical for transforming the color after the PCR reaction. Since the DNA amplification in PCR reaction decreases the pH of the samples slightly, a high concentration of phenol red may reduce the sensitivity of the color change. The concentration of 0.14 mM showed pale pink color, and 0.71 mM showed a dark red color (Figure 2). Therefore, we chose 0.35 mM phenol red concentration as the optimal concentration and fixed this condition in the following experiments. This result shows that we failed to induce the color change under a bacteria-containing sample.

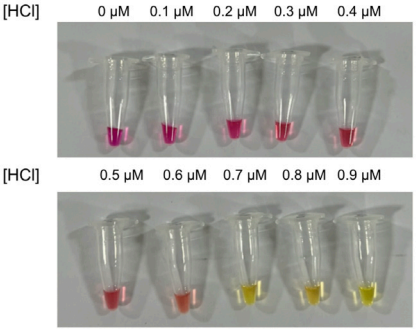

Figure 3: Ten HCl concentrations were added to each sample to find the range of color changes in the PCR reaction solution. The image portrays samples with the addition of HCl concentrations of 0 µM, 0.1 µM, 0.2 µM, 0.3 µM, 0.4 µM, 0.5 µM, 0.6 µM, 0.7 µM, 0.8 µM and 0.9 µM.

The intended purpose of this experiment was to deduce the range of pH conditions that allows clear visualization of the color changes. By adding different concentrations of HCl, a strong acid, the samples' initial pH conditions were decreased, indicating a more precise color range. Ten various HCl concentrations, including 0 μM, 0.1 μM, 0.2 μM, 0.3 μM, 0.4 μM, 0.5 μM, 0.6 μM, 0.7 μM, 0.8 μM and 0.9 μM were utilized to evoke the range of color change. As the added HCl concentration increases, the samples become more acidic, displaying brighter shades (Figure 3). For example, HCl concentrations of 0 μM to 0.2 μM showed dark red, 0.3 μM to 0.5 μM showed orange-red, 0.6 μM showed orange, and 0.7 μM to 0.9 μM showed yellow shades of the sample. In conclusion, we deduced that 0.5 μM - 0.6 μM and 0.6 μM - 0.7 μM concentrations display to most visible color changes, successfully concluding the optimal concentrations needed for the PCR reactions.

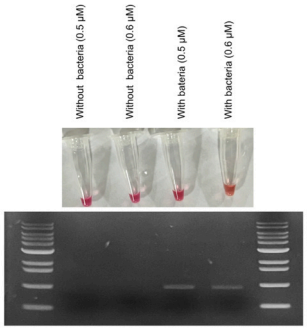

Figure 4: The PCR reaction sample with 0.6 μM HCl showed an apparent color change from red to orange. A total of four samples with or without bacteria supplemented with 0.5 μM and 0.6 μM HCl were prepared, and PCR was conducted. Then the PCR reaction tube image and the gel electrophoresis displaying the amplified bands of *E. coli* 16s rRNA are presented. The two bands on each side represent the DNA size marker indicating different lengths of the DNA bands.

This experiment aimed to display clear amplified bands of *E. coli* 16s rRNA from gel electrophoresis and induce color changes from pH change. Gel electrophoresis was performed by loading four different conditioned PCR reaction samples with either 0.5 μM or 0.6 μM HCl and with or without *E. coli*. DNA size markers were loaded on each side of the gel representing DNA band lengths comparison. After approximately 30 minutes, the gel was put under blue light to display the bands clearly. As expected, the two samples without bacteria did not show amplified DNA bands, meaning the *E. coli* 16s rRNA was not detected (Figure 4). The two samples containing bacteria, with 0.5 μM and 0.6 μM HCl, indicated 900 bp of the amplified DNA (Figure 4). In the 0.5 μM HCl condition, there was not an apparent color change shown, however in the 0.6 μM HCl condition, a color change from pink to orange was displayed, meaning 0.6 μM HCl is the optimized condition for detecting color change due to low pH produced by DNA amplification. However, since phenol red and HCl were added to the PCR reaction buffer solution, DNA amplification was not efficiently conducted, showing the lower intensity of DNA bands compared to Figure 1. In conclusion, the PCR reaction successfully induced pH color changes showing whether it contains *E. coli* bacteria.

Conclusion

In conclusion, our hypothesized predictions were in line with the concluded results. The annealing temperatures of 47 °C, 50 °C, and 53 °C were utilized successfully, amplifying the target bands and displaying an optimal condition for color change in the DNA amplified reaction buffer. The 0.35 mM phenol red concentration was the optimized concentration allowing the apparent color change after the PCR reaction. The optimized pH condition of the HCl concentration added was 0.6 μM HCl. Using such optimal conditions, *E. coli* DNA was successfully amplified, enabling a practical approach for visual detection and the further potential of this method.

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Author

Eugene Shim is a rising senior at Dulwich College Seoul. She is interested in studying biochemistry, especially nanotechnology and genetics. She hopes this research paper will provide her with wider perspectives as well as further sophisticated knowledge in her interested area in the near future for her career path.

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