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Development of HRPzyme-Integrated PCR Platform for Colorimetric Detection of Foodborne Pathogens

Bhagwan S. Batule, Seong U. Kim, Hyoyoung Mun,
Won-Bo Shim and Min-Gon Kim

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Abstract

In recent years, foodborne illnesses have become the most significant public health issue in both developed and developing countries. The World Health Organization (WHO) reported that in 2010, around 1.8 million people died due to foodborne illness. Therefore, the development of a cost-effective, sensitive, and selective detection method for identifying and monitoring foodborne pathogens is necessary for improved public health. Here, we describe a simple and ultrasensitive colorimetric method for the detection of foodborne pathogens based on HRPzyme-integrated PCR using PC-based ImageJ software. We present insights into different aspects of this method such as the importance of 16S rRNA detection, the modification of traditional PCR primers with a unique functional sequence for generating a color signal, and the application of ImageJ in colorimetric image data acquisition. The performance of the proposed strategy in detecting various foodborne pathogens is comparable to that of the commercial UV-Vis spectrophotometer Tecan Infinite 200 Pro. This detection platform exhibits linearity over wide range, high sensitivity, and high selectivity. The diagnostic capability of this colorimetric system to detect foodborne pathogens was demonstrated with spiked fruit and vegetable samples. This low-cost and effective colorimetric method can be conveniently employed for the analysis of DNA sequences arising from pathogenic bacteria.

Keywords: foodborne pathogens, 16S rRNA, PCR, primer, HRPzyme, colorimetric detection

1. Introduction

1.1. The importance of pathogenic foodborne pathogens

Food safety is critically important to consumer and public health and to the economic sustainability of the agro-food sector. Due to food poisoning incidents, consumers desire food safety assurances before they purchase food items. Foodborne illnesses, mainly caused by pathogens derived from contaminated water or contaminated and uncooked foods, cause many cases of death due to severe infection [1, 2]. The most widely recognized foodborne diseases are caused by pathogens such as *Escherichia coli* O157:H7, *Bacillus cereus*, *Listeria monocytogenes*, and *Vibrio parahaemolyticus*. Hence, it is important to detect the presence of pathogenic bacteria in food and water before they enter the body and cause serious outbreaks [2–4]. The standard microbiological methods for the detection of food pathogens are bacterial culture and biochemical staining, but the application of these is limited due to the time-consuming nature of analysis (up to 7 days) and their nonspecific results [5]. Therefore, there is a need for sensitive, selective, and point-of-care platforms that allow for both genotypic and phenotypic studies of foodborne pathogens. While the detection of foodborne pathogens has been significantly improved due to recent advances in molecular diagnostics, many of these methods require skilled persons and costly instruments. Therefore, point-of-need diagnostic methods are urgently needed to control the spread of food pathogen infections.

1.2. General overview of the importance and limitations of recent detection methods for foodborne pathogens

In recent years, a variety of diagnostic approaches, for example immunological methods, such as enzyme-linked immunosorbent assays (ELISAs) with detection limit of 10^3 cfu mL⁻¹ [6–8] and molecular biological methods such as polymerase chain reaction (PCR) with detection limit of 10^2 cfu mL⁻¹ [9–11] have been employed for the identification of foodborne pathogens. These methods vary in their sensitivity, specificity, cost, and efficacy. Owing to the recent availability of genomic information, molecular-based approaches have garnered considerable attention in terms of the development of molecular diagnostic techniques to detect and characterize pathogens [5, 12]. In the last decade, PCR, which can amplify a small amount of DNA through an amplification process, has been employed as a gold standard method for the molecular diagnosis of nucleic acids. Agarose gel electrophoresis [13] and real-time measurements [14] with DNA-binding dyes, such as the SYBR green and EvaGreen dyes, have been employed to detect amplified PCR products. Still, these strategies are expensive, time-consuming, and not user-friendly. Considering these limitations, there is a need to develop advanced methods that can overcome the aforementioned limitations. Thus, several research groups have reported new detection platforms based on molecular beacons [12] and nanoparticle-tagged probes [15, 16]. However, these advanced methods additionally require fluorescence conjugation or probe thiolation procedures [17–20], which are also costly and time-consuming. Therefore, a simple, fast, cost-effective, and user-friendly detection platform remains in high demand.

1.3. The introduction of HRPzyme-integrated PCR and its importance

Recently, novel detection platforms have been developed to detect genomic DNA as a target analyte based on colorimetric reactions generated by biocatalysts, such as horseradish peroxidase-mimicking DNAzymes (HRPzymes). HRPzymes consist of a folded structure of a G-quadruplex nucleotide sequence, and they exhibit peroxidase-like activity by folding with a hemin molecule [21, 22]. Importantly, the hemin-HRPzyme folded structure catalyzes the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) or 2,2-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) in the presence of H_2O_2 and produces a colored oxidized product [23, 24]. Based on this remarkable property, HRPzyme-based strategies have significantly improved the detection of proteins [25], small molecules [26], and heavy metal ions [27].

Recently, several researchers have reported the use of colorimetric HRPzyme-integrated PCR for the simple and rapid detection of bacteria [28–31]. This PCR platform can be employed for the simultaneous synthesis of a peroxidase-like DNAzyme using a primer including a complementary DNAzyme sequence. To overcome the need for primer labeling and expensive instrumentation, Cheglakov et al. [31] developed the HRPzyme-integrated PCR for the visual detection of bacteria. Similarly, Cheng et al. [28] reported the HRPzyme-based colorimetric PCR for the simple and cost-effective detection of *V. parahaemolyticus*. Bhadra et al. [29] reported a G-quadruplex-generating PCR for the naked-eye colorimetric analysis of SNPs associated with *Mycobacterium tuberculosis* drug resistance alleles. Finally, Seok et al. [30] devised a colorimetric signal generated by an amplified HRPzyme following the PCR amplification of the 16S rDNA of *Salmonella enterica* Typhimurium.

In the aforementioned studies, researchers utilized a combination of the HRPzyme sequence and a primer to amplify a target gene via PCR amplification. The HRPzyme sequences were generated through PCR amplification of the primer, which is integrated with an anti-HRPzyme sequence. After PCR amplification, in the presence of hemin, the unamplified HRPzyme sequence containing primers forms a catalytic hemin-G-quadruplex structure, which mimics peroxidase activity and produces a colorimetric signal via the oxidation of a peroxidase substrate such as TMB or ABTS. The HRPzyme sequence generated during PCR amplification thus produces an optical signal that can be identified by eyes or with a spectrophotometer. These developed colorimetric protocols have been adopted for the rapid and easy detection of various pathogens by integrating a unique functional sequence with the traditional primer set for the generation of the colorimetric signal. These studies thus demonstrated the broad applicability of a fast, simple, ultrasensitive, and selective detection method for DNA as a target analyte.

1.4. HRPzyme-integrated PCR-based detection of foodborne pathogens using PC-based ImageJ software

We have developed a PCR-based detection platform, termed G-quadruplex-blocking PCR, because the amplification of a specific target blocks the folding of the HRPzyme sequence, inhibiting the peroxidase activity of the HRPzyme. In contrast, in the presence of hemin and the absence of target-specific amplification, the G-quadruplex/hemin complex generates an oxidized substrate by oxidizing ABTS in the presence of hydrogen peroxide (H_2O_2) [32, 33].

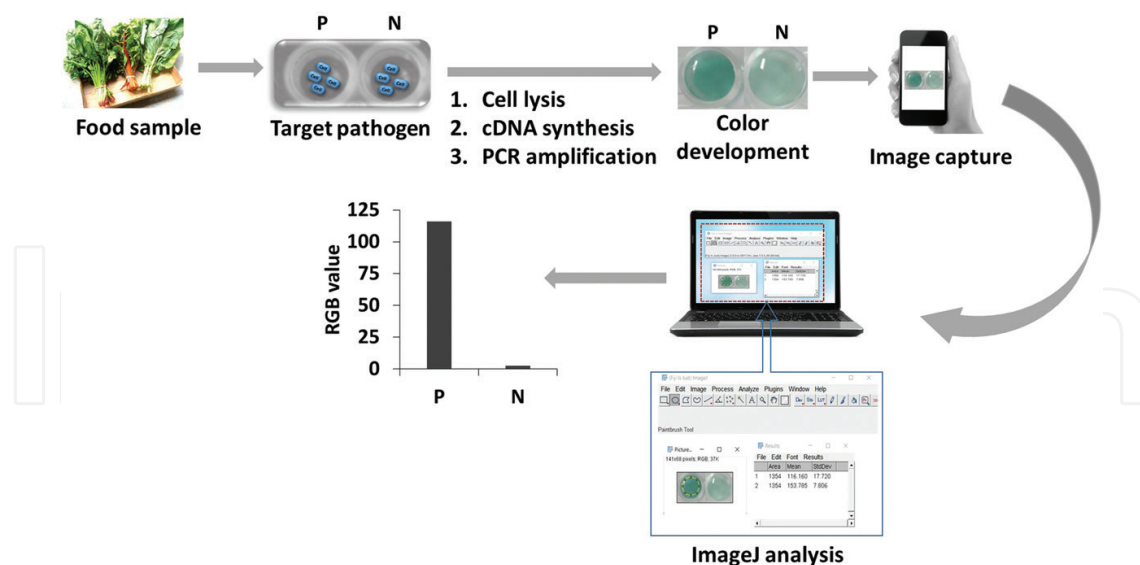


Figure 1. Schematic representation of HRPzyme-integrated PCR-based colorimetric detection of a foodborne pathogen. P: With target pathogen; N: Without target pathogen.

G-quadruplex/hemin complexes can also oxidize different peroxidase substrates such as fluorogenic (e.g., Amplex UltraRed (AUR) and QuantaBlu), electrochemical (e.g., 3-Indoxyl Phosphate and p-aminophenol (PAP)), and luminescent (e.g., luminol), allowing for the combination of the proposed method with fluorometric, electrochemical, and chemiluminescent detection platforms. The developed HRPzyme-integrated colorimetric detection platform allows for color development and provides a simple data analysis tool capable of the detection of specific common foodborne pathogens. Specifically, we used available ribosomal RNA (rRNA) sequence information from microorganisms to design the HRPzyme-integrated PCR assay. The proposed colorimetric detection platform allows the visual detection of food pathogens (even a single cfu per milliliter). In this chapter, we employed sequences of the 16S rRNA, a component of the 30S small subunit of the bacterial ribosome, as a target because several copies of 16S rRNA are present in each bacterium (1×10^3 to 1×10^5 copies) [34]. Further, we demonstrate the integration of this colorimetric HRPzyme-integrated PCR platform with a digital camera and desktop NIH ImageJ software, a simple data analysis tool that is able to measure the gray intensity (32-bit grayscale) of the colored images and further transmit the metadata to a centralized off-site laboratory (**Figure 1**).

2. Material and methods

2.1. Experimental material

DNA polymerase (*Thermus thermophilus* (Tth)) used for PCR reaction was obtained from Epicenter Technologies (Madison, WI, USA), and a DNA Ladder was obtained from Takara Bio (Seoul, South Korea) [35]. The oligonucleotides were obtained from Genotech (Daejeon, South Korea) and dissolved in sterile water and stored at -20°C [35]. Hemin and ABTS were purchased from Sigma-Aldrich (St. Louis, MO, USA). 10X TBE (Tris-borate-ethylene-diamine

tetracetic acid) buffer (for gel electrophoresis) was purchased from LPS Solution (Daejeon, South Korea). Phosphate buffered saline-1X (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 , 1.4 mM KH_2PO_4 , pH 7.2) was purchased from Bioseasang (Seongnam, Korea). The citrate buffer (0.1 M, pH -7) was prepared by mixing 59 mL of citric acid monohydrate (0.1 M) and 41 mL of trisodium citrate dihydrate (0.1 M). Agarose was purchased from Roche (Seoul, Korea). All reagents and solvents utilized in this study were of analytical grade and utilized without further purification. Target 16S rRNA sequences and primer sets designed for various foodborne pathogens are listed in **Table 1**.

2.2. Microorganism and culture conditions

Foodborne pathogens, including *E. coli* O157:H7 (ATCC 25922), *B. cereus* (KCTC 1092), *L. monocytogenes* (ATCC 19112), *V. parahaemolyticus* (ATCC 27969), *S. enterica* Typhimurium (ATCC 13311), and *C. sakazakii* (KTCT 2949), were grown in tryptic soy broth (Difco Laboratories, Franklin Lakes, NJ, USA) at 37°C. Concentrations of pathogenic bacteria were determined by cell counting on solid culture plates. Samples were collected with sterile plastic inoculating loops from solid culture plates, and culture solutions were prepared by serial dilution into 1× phosphate-buffered saline (PBS) to obtain 10^0 – 10^6 colony forming units per microliter (cfu mL^{-1}) and were stored at 4°C.

2.3. PCR amplification of 16S rRNA sequences specific to foodborne pathogens without genomic DNA isolation

Various concentrations of bacterial cultures were directly employed as template DNA for amplification with gene-specific primer sets (**Table 1**). Based on our previous report [35], PCR was performed in a total volume of 50 μL containing 5 μL bacterial culture at various concentrations (0 – 10^6 cfu mL^{-1}), 10× PCR buffer (2.5 μL), 25 mM MgCl_2 (6 μL), 2.5 mM dNTPs (deoxynucleotides) mix (8 μL), 10× PCR enhancer (5 μL), 25 mM MnSO_4 (1 μL), 20 μM forward primer (1 μL), 20 μM reverse primer (1 μL), and Tth DNA polymerase (0.5 μL). The PCR amplifications were performed under the following conditions: initial denaturation at 95°C for 5 min and 60°C for 20 min, then 35 cycles of 10 s at 94°C, 25 s at 60°C, and 10 s for 72°C adding last one cycle of 1 min for 72°C [35]. The PCR amplification was confirmed using 1.5% agarose gel electrophoresis. Then, to get colorimetric signal, PCR product (10 μL) was mixed with 300 μM hemin (5.5 μL), 5.5 mM ABTS (100 μL), 35% H_2O_2 (0.7 μL), and citrate buffer (1000 μL) with pH 4. After 10 min at room temperature (RT), the absorbance was measured at 410 nm with UV-Vis spectrophotometer (Infinite M2000pro, Männedorf, Switzerland) [35]. All images were taken using a digital camera (Samsung, Seoul, South Korea).

2.4. Spiked sample analysis

Fruit and vegetable samples such as apple, chicory, water dropwort, and white radish samples were purchased from the supermarket, washed with sterile water, and immersed in a plastic bag containing 20% ethanol and 1% lactic acid solution for 10 min. Then, sterilized food samples were cut to specific sizes and inoculated with different concentrations of bacteria, including *E. coli* O157:H7, *B. cereus*, *L. monocytogenes*, and *V. parahaemolyticus*, and kept in Petri dishes at 4°C overnight. Next, 0.1 mL of sterilized PBS was added to the inoculation site,

Pathogens	Target (GenBank number)	Primers (5'→3') (H-F: Forward primer; H-R: Reverse primer)	Product size (bp)
<i>Escherichia coli</i>	16S rRNA (AWXM02000001.1)	H-F: AAAAAAAAAA GGGTAGGGCGGGTTGGGT <u>AAAAAA</u> GCCTGATGCAGCCATGC H-R: AAAAAAAAAA GGGTAGGGCGGGTTGGGT <u>AAAAAA</u> CAATGAGTAAAGGTATTAACCTTTACTC	166
<i>Bacillus cereus</i>	16S rRNA (D16266.1)	H-F: AAAAAAAAAA GGGTAGGGCGGGTTGGGT <u>AAAAAA</u> CTAGTTGAATAAGCTGGCACCT H-R: AAAAAAAAAA GGGTAGGGCGGGTTGGGT <u>AAAAAA</u> GATAACGCTTGCCACCTACG	164
<i>Listeria monocytogenes</i>	16S rRNA (JF967621.1)	H-F: AAAAAAAAAA GGGTAGGGCGGGTTGGGT <u>AAAAAA</u> CAAGGATAAGAGTAACTGCTTGT H-R: AAAAAAAAAA GGGTAGGGCGGGTTGGGT <u>AAAAAA</u> CTTGCCACCTACGTATTACCG	162
<i>Vibrio parahaemolyticus</i>	16S rRNA (AE006468.1)	H-F: AAAAAAAAAA GGGTAGGGCGGGTTGGGT <u>AAAAAA</u> ACCTTCGGGGAACGATAACG H-R: AAAAAAAAAA GGGTAGGGCGGGTTGGGT <u>AAAAAA</u> GCCATCGTTTCCAATGGTTATC	162
<i>Salmonella enterica Typhimurium</i>	16S rRNA (NR_114632.1)	H-F: AAAAAAAAAA GGGTAGGGCGGGTTGGGT <u>AAAAAA</u> AGTTGTGGTTAATAACCGCAGCA H-R: AAAAAAAAAA GGGTAGGGCGGGTTGGGT <u>AAAAAA</u> ATTAACGCTTGACCCTCCG	166
<i>Cronobacter sakazakii</i>	16S rRNA (AE006468.1)	H-F: AAAAAAAAAA GGGTAGGGCGGGTTGGGT <u>AAAAAA</u> ATCCTGCAGAGATGCGGGA H-R: AAAAAAAAAA GGGTAGGGCGGGTTGGGT <u>AAAAAA</u> ACGGGACTTAACCCAACATTTC	160
Italic-protector sequence; Bold-HRPzyme sequence; Underline-spacer sequence			

Table 1. Oligonucleotide primers used in this work.

and the solution was recovered by pipetting and directly employed in PCR assay following the same experimental conditions mentioned in Section 2.3.

2.5. Data acquisition through ImageJ software

ImageJ is a simple, practical, and freely available downloadable program that can be used on any computer with Java 5 or on a virtual machine [36]. Recently, many researchers have employed the ImageJ software to quantify data [37, 38]. The green color intensities produced during the HRPzyme-TMB-H₂O₂ assay were captured using a digital camera (Samsung, Seoul, South Korea) equipped with a standard 18–55-mm objective lens. Color intensities were measured, quantified, and averaged from three experiments by using the digital camera and ImageJ (Wayne Rasband, National Institutes of Health, Rockville, MD, USA; <http://rsb.info.nih.gov/ij>). The Δ gray intensity value was obtained by subtracting the average gray intensity of the negative samples from the average gray intensity of the positive samples.

3. Results and discussion

3.1. Principle of HRPzyme-integrated PCR

In this chapter, we established a method for the colorimetric detection of a PCR product generated by HRPzyme-integrated primers, as shown in **Figure 1**. We modified the forward and reverse primers that contain four regions: a protector sequence, HRPzyme sequence, spacer sequence, and sequence complementary to the 16S rRNA sequence from one of several pathogens. The 16S rRNA sequence was employed as a target for the specific detection of food pathogens. During PCR amplification, cells were first lysed by heating PCR samples containing pathogenic bacteria. Then, Tth DNA polymerase was used to successfully transcribe 16S rRNA into complementary DNA (cDNA), which was further employed as a cDNA template for PCR amplification. The thermostable Tth DNA polymerase is obtained from *T. thermophiles* and exhibits optimal activity between 70 and 74°C; therefore, it is suitable for high-temperature PCR. Tth DNA polymerase has intrinsic 5' → 3' exonuclease activity but lacks 3' → 5' (proof-reading) nuclease activity. Tth polymerase is also very effective at reverse transcription in the presence of Mn metal ions, facilitating cDNA synthesis and PCR amplification in a one-step process. Further, as PCR proceeds, the HRPzyme sequence present in the primer is blocked by the formation of double-stranded DNA. Following PCR, the double-stranded HRPzyme sequence prevents the folding of the HRPzyme sequence in the presence of hemin. In contrast, primers that are unincorporated during PCR amplification fold into the G-quadruplex structure in the presence of hemin, oxidizing ABTS and generating a colored product in the presence of H₂O₂. Thus, images of the green PCR products generated from different food pathogens can be captured, and their color intensities were measured using the ImageJ software.

3.2. Feasibility study of the designed strategy

In order to demonstrate the feasibility of this concept, we employed 16S rRNA sequences from various foodborne pathogens as target analytes. According to our previous report [30],

this strategy requires two steps for the successful detection of target pathogens: the amplification of target sequences and the subsequent colorimetric signal development. For this reason, we designed 16S rRNA sequence-specific forward and reverse primers containing HRPzyme, protector, and spacer sequences. Under optimized conditions, we confirmed the amplification of 16S rRNA products from *E. coli*. In the agarose gel electrophoresis image, lanes 1 and 2 correspond to samples with and without *E. coli*, respectively (**Figure 2a**). Further, the PCR product could be detected by the generation of a colorimetric signal (**Figure 2b**) as well as the corresponding absorption data (**Figure 2c**). PCR of the negative control (containing no bacteria) did not result in a specific band for the target gene and showed a dark blue color reflecting the presence of unamplified primers.

3.3. Sensitivity and selectivity of proposed study

Based on the optimized conditions, we utilized the proposed detection strategy for the analysis of different foodborne pathogens with HRPzyme-integrated primer sets specific to the 16S rRNA sequences of various bacteria, as shown in **Table 1**. A detailed explanation of the ImageJ-based semiquantitative analysis is illustrated in **Figure 3**. First, a digital image of the green solution is captured using a digital camera. Then, captured images are converted into 32-bit grayscale utilizing the PC-based ImageJ software, and images are inverted (**Figure 3a**). Next, spot areas are individually selected, and their gray intensities are measured (**Figure 3b**). As shown in **Figure 3b**, the performance of the proposed system in the detection of *E. coli* was compared with that of a commercial UV-Vis spectrophotometer, the Tecan Infinite 200 Pro [39]. The results obtained from both systems showed the same linear range from 10^0 to 10^6 cfu mL⁻¹ and negligible change in the R^2 value, indicating the applicability of the ImageJ-derived quantitative data for detecting foodborne pathogens.

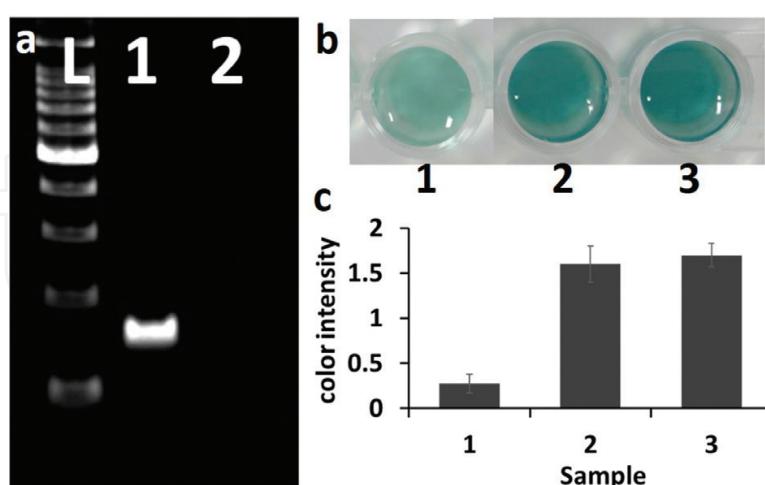


Figure 2. Feasibility study of the colorimetric method involving HRPzyme-blocked PCR for the specific detection of the foodborne pathogen *E. coli* as a model target. (a) Results of agarose gel electrophoresis of diluted PCR products generated using the HRPzyme-integrated primer set. L: DNA marker (100 bp); lane 1: *E. coli* As model target; lane 2: No target bacteria (negative control). (b) Photographs of HRPzyme-integrated PCR colorimetric product. Sample 1: *E. coli*; Sample 2: Negative control; Sample 3: HRPzyme-integrated primer set only. (c) Quantitative results of (b). Error bars represent the standard deviations from three representative experiments ($n = 3$).

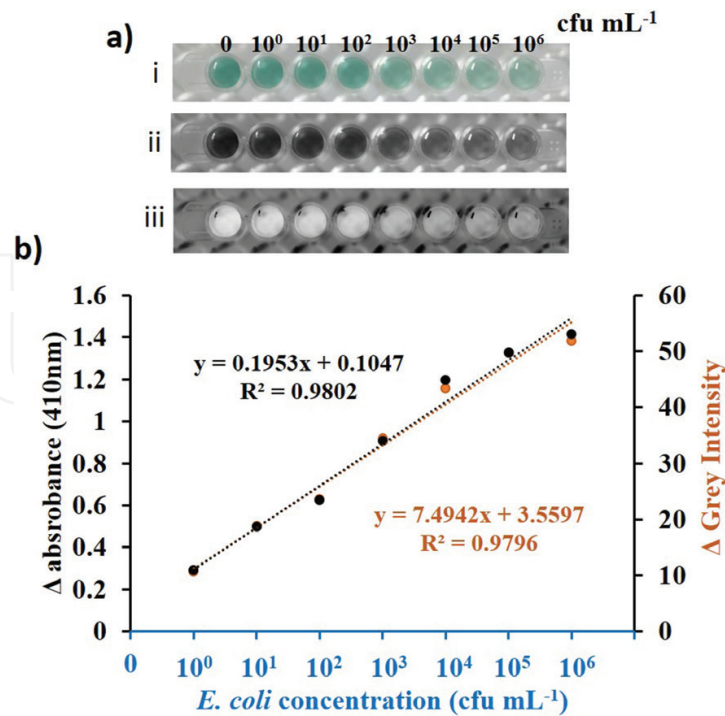


Figure 3. (a) Procedure for ImageJ analysis. (i) a digital image of the colored solution is generated using a digital camera. (ii) the image is converted to 32-bit grayscale utilizing ImageJ. (iii) the image is then inverted. (b) the performance of the proposed system in the detection of *E. coli* was compared with that of a commercial UV-vis spectrophotometer, the tecan infinite 200 pro. Δ absorbance at 410 nm = A_0 (without target) – A (with target); Δ gray intensity = blank gray intensity (without target pathogen) – Sample gray intensity (with target pathogen).

Next, we employed this proposed strategy to the detection of different food pathogens. First, we diluted bacteria to different concentrations in the range 0 to 1.0×10^6 cfu mL⁻¹ in 1× PBS buffer. We then directly employed these diluted samples in PCR amplification without first extracting genomic DNA. After PCR amplification, PCR products of different pathogens were used for colorimetric signal generation. As shown in **Figure 4a**, the electrophoresis gel data reveal the amplification of target gene bands with different concentrations of bacteria. In addition, the results depicted in **Figure 4b** (grayscale images) show that the color intensity signal decreased with increasing concentrations (number of cfu mL⁻¹) of bacteria. The color intensity data show a linear relationship with the concentration of the target pathogens (**Figure 4c**). The standardization curve of Δ gray intensity versus the bacterial concentration (cfu mL⁻¹) exhibited good linearity in the range from 1.0 to 1.0×10^6 cfu mL⁻¹ (**Figure 4c**). We observed clear differences between negative control samples and those containing various concentrations of bacteria. This strategy showed a 10-fold better performance than the gel electrophoresis-based assay. The proposed method could therefore be used in the diagnosis of pathogenic bacteria without needing to first isolate bacterial genomic DNA.

3.4. Detection specificity

In practical application, the specificity of the detection platform is critical. In order to assess the specificity of the proposed detection strategy, we performed a specificity test using

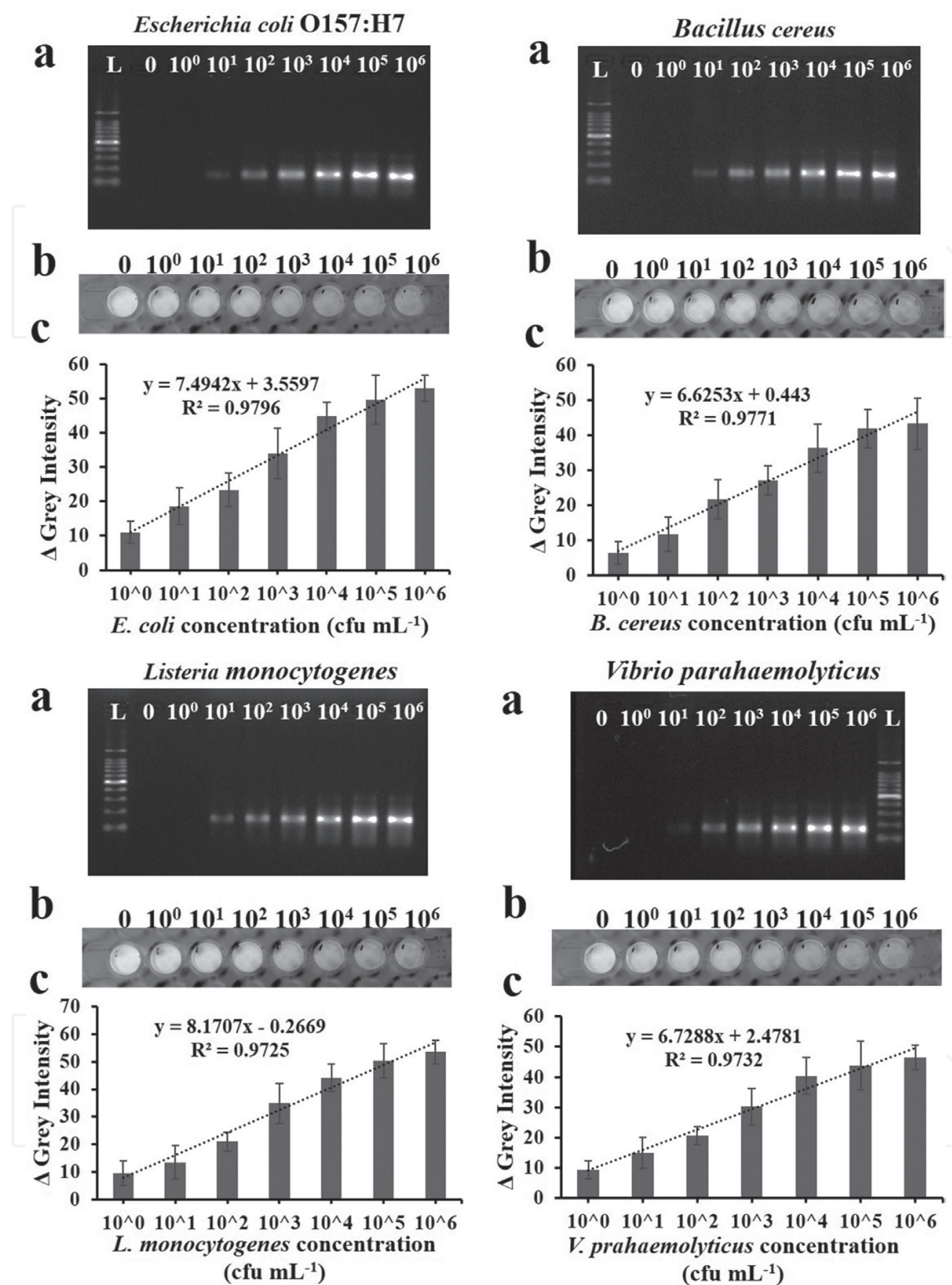


Figure 4. Sensitivity study of HRPzyme-integrated PCR performance in PBS containing different food pathogens (*E. coli* O157:H7, *B. cereus*, *L. monocytogenes*, and *V. parahaemolyticus*). (a) Agarose gel (1.5%) electrophoresis-based analysis. (b) Grayscale images showing different concentrations of bacteria, derived from ImageJ analysis. (c) Δ gray intensities of samples with different concentrations of bacteria, derived from ImageJ analysis. Δ gray intensity = blank gray intensity – Sample gray intensity.

different food pathogens. We successfully demonstrated the selectivity of the method for the detection of several bacteria, including *E. coli* O157:H7, *B. cereus*, *L. monocytogenes*, and *V. parahaemolyticus*, which contain highly homologous 16S rRNA sequences [40, 41]. Selectivity tests including these four pathogens were carried out using the same reaction procedures with the concentration of all pathogens set at 10^4 cfu mL⁻¹. The results are shown in **Figure 5**. We observed specific bands for the target pathogens following 1.5% gel electrophoresis (**Figure 5a**), and the change in color intensity was negligible for nontarget pathogens (**Figure 5b**). This indicates that the PCR products generated during the gene-specific

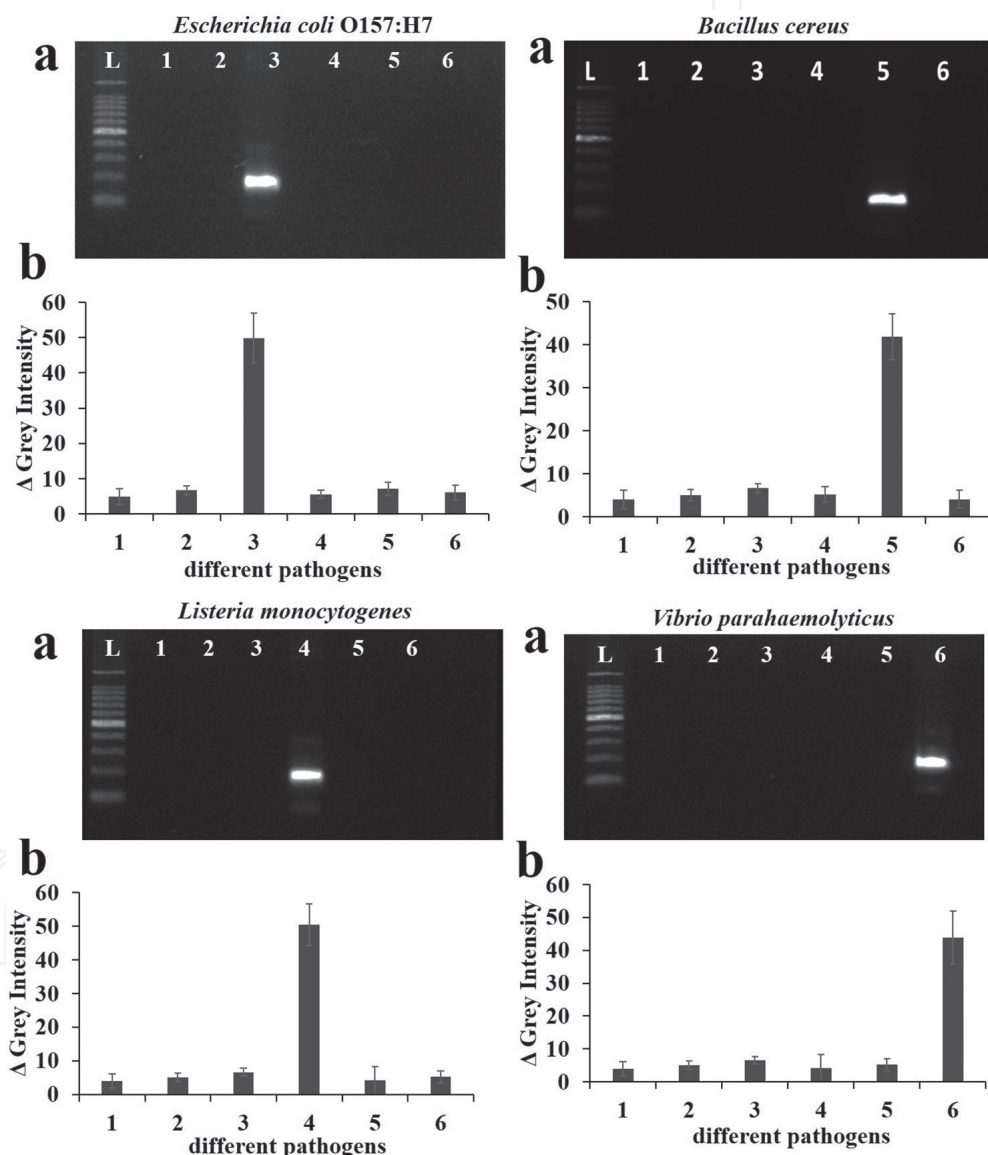


Figure 5. Specificity study of HRPzyme-integrated PCR performed in PBS containing different food pathogens (*E. coli* O157:H7, *B. cereus*, *L. monocytogenes*, and *V. parahaemolyticus*). (a) Agarose gel (1.5%) electrophoresis-based analysis. (b) Δ gray intensities derived from ImageJ analysis. The different pathogens used in this study were *S. enterica* Typhimurium (1), *C. sakazakii* (2), *E. coli* O157:H7 (3), *L. monocytogenes* (4), *B. cereus* (5), and *V. parahaemolyticus* (6).

PCR amplification process successfully blocked the folding of the HRPzyme sequence. In this assay procedure, color generation is dependent upon the application of gene-specific primers integrated with the HRPzyme sequence for the detection of the 16S rRNA sequence, enabling the differentiation of foodborne pathogens. The results in **Figure 5b** show that the presence of the target pathogen results in an intense color intensity signal, while the presence of other food pathogens at the same concentration generates only weak signal. This confirms the high selectivity of the proposed HRPzyme-integrated PCR-based colorimetric strategy for food pathogen detection. Further, in **Table 2**, we have compared our proposed methods with previously reported methods.

3.5. Detection of pathogens from different spiked food samples

A variety of agricultural products have been linked to human illness worldwide. Some agricultural commodities such as fruits and vegetables are more vulnerable to pathogenic bacterial contamination [51]. Therefore, the ability of detection methods to detect pathogens from fresh fruits and vegetables is critical. In this study, we demonstrated the ability of the proposed strategy to detect pathogens among spiked food samples such as apple, chicory, water dropwort, and white radish samples purchased from the local market. Such fruit and vegetable samples contain various biological components [52] such as proteins, phenols, and polysaccharides that may reduce the reliability of the method. **Figure 6** shows the analysis of various food samples spiked with *E. coli*. Based on these results, our method showed good linearity across various concentrations of *E. coli*, indicating the high reliability of the proposed method.

Detection methods	Detection limit (cfu mL ⁻¹)	Detection range (cfu mL ⁻¹)	Detection time	Reference
Real-time PCR assay based on immunomagnetic separation	1.0×10^1 – 1.0×10^4	10	1.2 hrs	[42]
Immunosensors	1.0×10^4 – 1.0×10^8	1.0×10^5	1.5 hrs	[43]
Antibody-aptamer Sandwich ELISA	1.0×10^3 – 1.0×10^8	1.0×10^3	<3 hrs	[44]
Selective filtration technique combined with antibody-magnetic nanoparticle nanocomposites	2.0×10^1 – 2.0×10^4	20	45 min	[45]
Aptasensor, gold-nanoparticles aggregation	1.0×10^2 – 1.0×10^7	56	1 hr	[46]
Immuno-PCR	1.0×10^3 – 1.0×10^5	1.0×10^3	4 hrs	[47]
Antibody-conjugated magnetic nanoparticles (MNPs)	1.0×10^2 – 1.0×10^8	100	1 hr	[48]
Aptasensor, peroxidase mimics magnetic nanoparticles	Not given	7.5×10^5	1 hr	[49]
Aptasensor, gold-nanoparticles aggregation	1.0×10^1 – 1.0×10^6	10	4 hrs	[50]
HRPzyme-Integrated Polymerase Chain Reaction	1.0×10^0 – 1.0×10^6	1	~1.2 hrs	This study

Table 2. Comparative study of the proposed method with previously reported methods of foodborne pathogen detection.

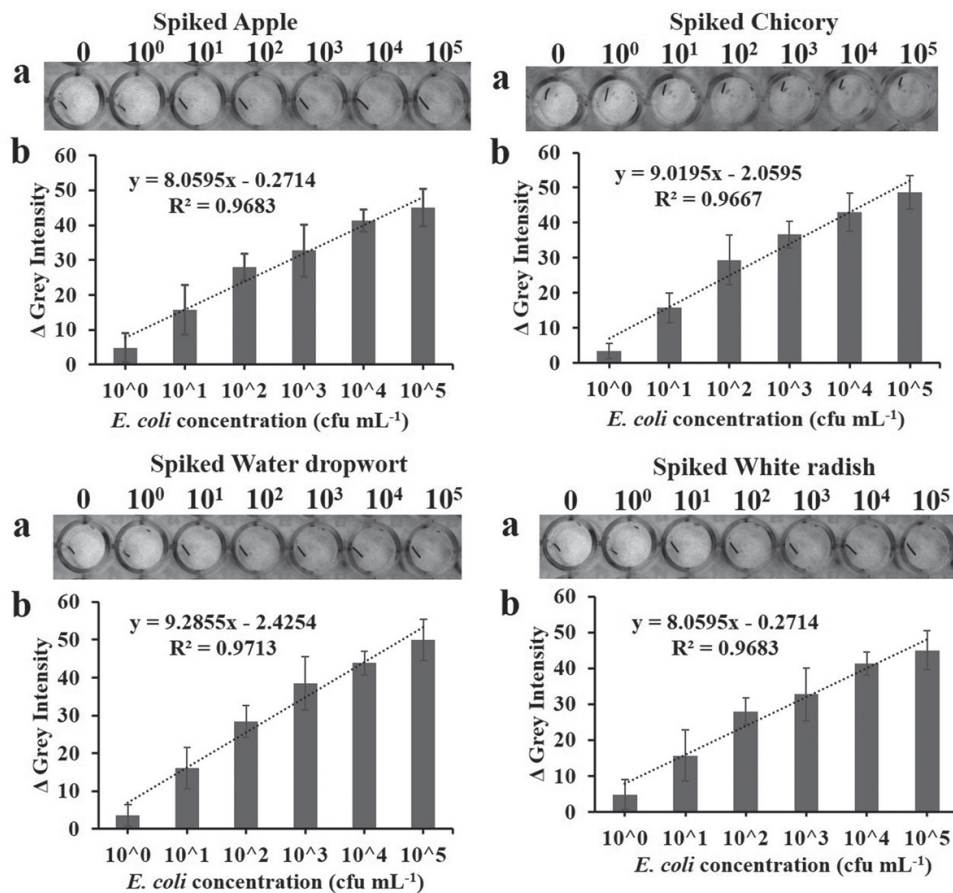


Figure 6. Linear relationship between Δ gray intensity and bacterial concentrations from 0 to 10⁵ cfu mL⁻¹ in spiked samples such as apple, chicory, water dropwort, and white radish. (a) Grayscale image. (b) Δ gray intensities of different concentrations, derived from ImageJ analysis.

4. Conclusions

In this study, we demonstrated the performance of the HRPzyme-integrated PCR-based colorimetric method for the simple, sensitive, and selective detection of 16S rRNA sequences from various food pathogens. The proposed strategy showed several advantages such as label-free, simple and easy procedure, ultrasensitive (detection limit up to single cfu mL⁻¹), highly selective, detection within 70 min, affordable at remote areas, easily integrated into smartphone-based image processor. PCR amplification is carried out with 16S rRNA-specific primers modified at the 5'-end with HRPzyme sequences. After PCR, in the presence of hemin, unamplified primers fold into a G-quadruplex structure, and a colorimetric signal is then generated in the presence of a chromogenic substrate. We successfully detected the presence of foodborne bacteria, including *E. coli* O157:H7, *B. cereus*, *L. monocytogenes*, and *V. parahaemolyticus* at levels as low as a single cfu mL⁻¹ in buffer as well as in spiked fruit and vegetable samples. We believe that this method could be employed in the detection of pathogenic bacteria from biological samples by simply modifying existing primers with the HRPzyme sequence at the 5' end. Further, this method could be integrated with a field-portable PCR instrument for the on-site detection of pathogens in resource-limited areas.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

Author details

Bhagwan S. Batule¹, Seong U. Kim², Hyoyoung Mun¹, Won-Bo Shim³ and Min-Gon Kim^{1,2*}

*Address all correspondence to: mkim@gist.ac.kr

1 Department of Chemistry, School of Physics and Chemistry, Gwangju Institute of Science and Technology, Gwangju, Republic of Korea

2 Advanced Photonics Research Institute, Gwangju Institute of Science and Technology, Gwangju, Republic of Korea

3 Department of Agricultural Chemistry and Food Science and Technology, Gyeongsang National University, Republic of Korea

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