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Curcumin – a natural colorant-based pH indicator for molecular diagnostics†

Rajamanickam Sivakumar, Nahyung Lim, Seung Kyun Park and Nae Yoon Lee *

Loop-mediated isothermal amplification (LAMP) provides highly selective and sensitive DNA amplification and generates hydrogen ions as a byproduct under weakly buffered conditions, causing the solutions' pH to decrease from the initial basic to an acidic environment. This distinctive feature allows the color of the amplified DNA solution to change readily when suitable pH indicators are employed. In this study, curcumin, a biodegradable, non-toxic, and natural colorant, was used as a pH indicator to visually identify LAMP-amplified *Staphylococcus aureus* (*S. aureus*) and *Streptococcus pneumoniae* (*S. pneumoniae*). Curcumin (10 mM) displayed a unique color difference between negative (red) and positive (yellow) samples, and the detection process was completed within 30 s, demonstrating the effectiveness of using curcumin for on-site diagnostics. Under optimum conditions, curcumin enabled *S. aureus* and *S. pneumoniae* detection as low as 10 fg μL^{-1} and 1 pg μL^{-1}, respectively, due to its unique halochromic properties. Owing to its adaptability, ease of use, and rapid visual detection, the introduced colorimetric pH-based LAMP method can be employed as a practical alternative to conventional colorimetry for infectious pathogen identification in both laboratory and field settings.

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1. Introduction

Infectious diseases seriously threaten public health and financial stability, placing a considerable burden on human communities.[1,2] Notably, an estimated 14.9 million deaths occur annually worldwide due to infectious pathogens, accounting for almost 25% of all mortality.[3] A wide range of factors such as biological and environmental conditions, globalization, and social settings influence the development of a pandemic situation. Bacteria are responsible for a large proportion of deaths caused by infectious diseases. Over the last 40 years, more than 50 emerging contagious diseases have been confirmed, and approximately 10% of these diseases are caused by bacteria. Currently, at least 25 bacterial stains are highly suspected to be harmful to humans.[4] Remarkably, *Staphylococcus aureus* (*S. aureus*) is considered one of the deadliest pathogens due to its ability to cause a range of illnesses including sepsis, endocarditis, pneumonia, and osteomyelitis.[5,6] Moreover, *S. aureus* has many virulence factors and the ability to develop multidrug resistance (MDR), which significantly increases the risk of infection. Over the next 30 years, approximately 10 million people are expected to die from diseases caused by MDR bacteria, resulting in a loss of over 100 trillion USD worldwide.[7] Importantly, the *KatA* gene of *S. aureus* is located in the extracellular environment and is relatively stable compared to other bacterial catalases.[8] Therefore, robust techniques are highly mandatory for *S. aureus* detection for rapid diagnosis and effective treatment.[9,10]

Streptococcus pneumoniae (*S. pneumoniae*) is an example of a Gram-positive bacterium that commonly colonizes the human respiratory system and causes community-acquired pneumonia.[11] According to the World Health Organization, approximately 1 million children under the age of 5 die due to pneumonia every year, accounting for nearly 16% of all pediatric casualties.[12] Indeed, *S. pneumoniae* is regarded as a substantial pathogen that generates infectious diseases because it was responsible for 95% of pneumonia infections prior to the development of antibiotics. Typically, healthy carriers colonize *S. pneumoniae* in the upper respiratory tract. However, in susceptible persons such as kids, the elderly, and immune-defective individuals, *S. pneumoniae* may spread deeper into the lungs, middle ear, central nervous system, and sinus, leading to lethal conditions such as bacteremia, sinusitis, meningitis, and otitis media, respectively.[13] The *ply* gene is one of the primary virulence factors which generates all types of pneumococci.[14] Therefore, to control pandemics and reduce financial loss, it is essential to develop a simple, rapid, sensitive, and affordable method for the recognition of infectious diseases.[15,16]

Recently, nucleic acid amplification based techniques have been used as unique diagnostic tools for the identification of infectious diseases, where early diagnosis is essential.[2,17]

Department of BioNano Technology, Gachon University, 1342 Seongnam-daero, Sujeong-gu, Seongnam-si, Gyeonggi-do, 13120, Korea. E-mail: nylee@gachon.ac.kr

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Although various nucleic acid amplification techniques are currently employed for disease monitoring, isothermal-based methods, especially loop-mediated isothermal amplification (LAMP), have been extensively used for infectious disease detection because of their quickness, high sensitivity, and selectivity.[18,19] Furthermore, the detection process is a significant step in LAMP and falls into two categories: direct (based on DNA detection) and indirect (based on byproduct detection). In direct methods, numerous DNA-intercalating dyes and metal nanoparticles have been introduced to differentiate between negative and positive samples.[20,21] In indirect methods, hydrogen ions (H^+) and pyrophosphate molecules are generated as byproducts when a *Bst* polymerase incorporates dNTPs into the target gene sequence under weakly buffered LAMP conditions. Indeed, the initial alkaline pH of the LAMP assay shifts to an acidic pH because of H^+ production. Many pH-sensitive organic dyes have been used to visualize pH variations.[22]

Recently, a commercially available phenol red has been effectively used in weakly buffered LAMP amplicon detection.[23] Similarly, other organic dyes including neutral red, xylenol orange, and methyl green have been utilized for diverse pathogen detection.[24–26] Although these synthetic organic dyes are readily able to visualize LAMP amplicons, most are non-biodegradable, which cause adverse environmental effects. Furthermore, there is a growing need to introduce novel pH indicators for laboratory and industrial pH-based LAMP amplicon detection. Natural pigments have drawn increased attention because they are safe, non-toxic, and biocompatible, making them ideal candidates for use as color indicators in a variety of applications.[27] Anthocyanins, shikonin, betalains, and alizarin are a few examples of natural colorants that have been successfully employed as efficient indicators to measure freshness of food in retail settings.[28–30] More importantly, the longevity and pH tolerance of natural colorants must be considered in order to use them as effective pH indicators.

Curcumin is a natural bioactive substance found in the turmeric plant root. Turmeric contains approximately 2%–5% curcuminoid, which gives a characteristic yellow color. Curcumin has been extensively studied for its unique anti-inflammatory and antioxidant properties.[31] Significantly, it appears yellow at a pH between 1 and 7 and reddish-orange at a pH higher than 8.[32] Because of its unique pH-responsive color shift, curcumin has recently attracted attention in the production of composite films for use in food packaging.[33] Curcumin's color-changing properties at different pH levels contribute to real-time food quality monitoring.[34] Consequently, adding curcumin to the packaging process enables customers to quickly verify the quality of food *via* the naked eye without the need for expensive equipment. Given these characteristics and applications, curcumin is an effective pH indicator. Curcumin has polyphenolic components in its structure, which deliver several benefits such as antibacterial, antioxidant, and anticancer properties. Besides its physiological characteristics, curcumin also exhibits a discernible pH-responsive color transition. Despite the effective use of pH-responsive curcumin in the fields of drug delivery and food safety,[33,35] to the best of our knowledge, no reports have been published on its use in LAMP amplicon colorimetric detection. Curcumin is a complex compound consisting of two methoxy-phenolic moieties attached with α,β-unsaturated carbonyl group.[36] In fact, the α,β-unsaturated carbonyl (diketone) group plays a crucial role in curcumin's color change. In acidic media (pH < 7), the phenolic hydroxyl groups are not ionized, and the diketone group remains unchanged. Therefore, curcumin retains its original (yellow) color. As the pH increases (pH > 8), the diketone moiety of curcumin serves as a hydrogen donor, causing hydrolysis and curcumin degradation. Moreover, intramolecular hydrogen transfer takes place in the diketone, leading to keto–enol tautomer development, as well as a conjugated system,[37] which changes curcumin's yellow color to an orange-reddish color (Scheme 1a). Under weakly buffered conditions, the unamplified LAMP sample has an alkaline pH (~8.2), which changes to an acidic pH (~7.1) due to H^+ formation during the amplification. Therefore, we introduced curcumin as a colorant to monitor this pH variation and distinguish between negative and positive samples *via* the naked eye (Scheme 1b). Two common infectious pathogens, *S. aureus* and *S. pneumoniae*, were selected for proof-of-concept analyses, and the sensitivity and specificity of the newly developed method were investigated.

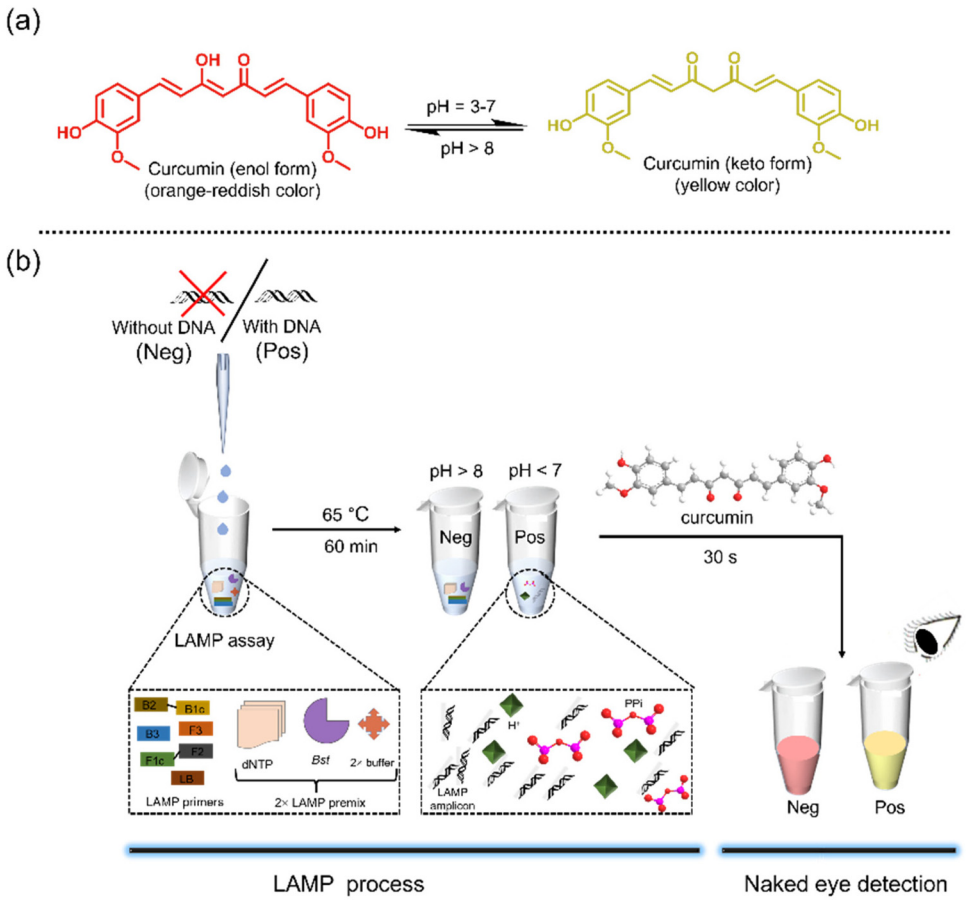
2. Experimental section

2.1. Materials and reagents

Curcumin ($C_{21}H_{20}O_6$, 98%) was purchased from the Tokyo Chemical Industry (Chuo-ku, Japan). The 2× LAMP master mix, which included an isothermal amplification buffer, dNTPs, Mg^{2+} salts, and *Bst* DNA polymerase was purchased from NanoHelix Co., Ltd (Daejeon, South Korea). The 100 bp DNA marker was acquired from Takara (Shiga, Japan). Agarose powder was purchased from BioShop (Burlington, Canada) and used for gel electrophoresis. Ethidium bromide was purchased from Dynebio (Seongnam, South Korea). The sequences of *katA* of *S. aureus* and *ply* genes of *S. pneumoniae* were synthesized and cloned into a plasmid by Cosmo Genetech Co., Ltd (Seongdong-gu, South Korea), and stored at −20 °C. Five sets of LAMP primers were purchased from Cosmo Genetech Co. Ltd (Seongdong-gu, South Korea). An Orion Star™ A211 pH benchtop meter was purchased from Thermo Fisher Scientific (Seoul, South Korea). A UV transilluminator was purchased from Korea Labtech (Gyeonggi-do, South Korea) and used for agarose gel electrophoresis. The actual *Acinetobacter baumannii* (*A. baumannii*) sample was taken from hospitalized patients in Thailand, and kept on the FTA card.

2.2. Primer design

katA (GenBank accession number AJ000472) and *ply* (GenBank accession number EF490446) genes were selected as specific targets for *S. aureus* and *S. pneumoniae* detection. A set of five



Scheme 1 (a) Color change of curcumin based on its keto–enol tautomer form under different pH conditions. (b) Schematic showing the weakly buffered LAMP process and naked eye detection process.

primers comprising two inner primers (FIP and BIP), two outer primers (F3 and B3), and one loop primer (LB) were designed employing Primer Explorer V5 software (**https://primerexplorer.jp/lampv5e/index.html**) for *katA* and *ply* gene amplification. Table S1† lists the target genes and the corresponding primer sequences.

2.3. LAMP reaction

Initially, a working primer mix containing 0.8 μM LB, 1.6 μM of each FIP and BIP, and 0.2 μM of each F3 and B3 was prepared. In a microtube, a 25-μL solution including 11.5 μL of 2× LAMP master mix, 3 μL primer mix, 1 μL DNA control (*S. pneumoniae*), and 9.5 μL of nuclease-free water was mixed to perform LAMP. A heat block was used to perform the LAMP assay at 65 °C for 60 min. Subsequently, the amplified products were visualized on a 1.5% agarose gel stained with ethidium bromide to confirm the LAMP results. To prove reproducibility, three sets of experiments were conducted.

2.4. Optimization of the volume of the LAMP primer mix

Various primer mix volumes were used to identify the ideal volume for efficient LAMP amplification. To obtain a total volume of 25 μL, different primer mix volumes (2.5, 3.0, and 3.5 μL) were first added to the LAMP mixture containing target DNA in three microtubes. For negative samples, water was used instead of the target DNA. Then, the LAMP assay was performed at 65 °C for 60 min.

2.5. Optimization of the volume of the 2× LAMP master mix

The commercially available 2× LAMP master mix volume may influence the amplification process. Thus, the LAMP mixture was mixed with different 2× LAMP master mix volumes (11.5, 12.0, and 12.5 μL), and allowed to heat at 65 °C for 60 min.

2.6. Optimization of the amplification time

In three microtubes, the 2× LAMP master mix (11.5 μL), primer mix (3 μL), target DNA (1 μL), and nuclease-free water (9.5 μL) were mixed properly. Then, the mixture was heated to 65 °C for 30, 45, and 60 min.

2.7. LAMP amplicon colorimetric detection using curcumin

Scheme 1a shows the general colorimetric approach for LAMP amplicon detection using the introduced method. To identify the LAMP amplicons based on their pH differences, the negative and positive samples (each 5 μL) and 10 mM curcumin (5 μL) were appropriately mixed in a microtube.

2.8. Grayscale analysis

The microtubes were placed against a white background after the amplification, and to avoid color fading, photos were taken immediately using a smartphone camera. The analysis involved the conversion of the RGB images to grayscale images, followed by grayscale intensity calculations using the ImageJ software. The regions of interest (ROIs) were manually selected for analysis. Specifically, rectangular ROIs measuring 50 × 50 pixels were selected using a toolbar. Three consecutive measurements were performed to confirm consistency.

2.9. Sensitivity test

Plasmid DNA of *S. pneumoniae* and *S. aureus* were used to evaluate the sensitivity of the introduced method under optimized conditions. The plasmids, at a concentration of 1 ng μL^{-1}, were serially diluted 10-fold using water as low as 1 fg μL^{-1}. The limit of detection (LOD) was determined *via* agarose gel electrophoresis and the pH-responsive curcumin-based visual method. Each experiment was repeated three times to check the reproducibility of the introduced method.

3. Results and discussion

3.1. Optimization conditions for the LAMP process

The DNA quantity produced in weakly buffered LAMP directly correlates with byproduct H^+ generation. Therefore, a high pH difference between negative and positive samples can be obtained by adjusting different parameters. Three LAMP assays were primarily prepared employing different primer mix volumes (2.5, 3.0, and 3.5 μL) to confirm the impact of primers on the amplification (Fig. 1a–c). Notably, the 3.0 μL primer mix (Fig. 1b) resulted in a considerable pH difference when compared to the other volumes, despite the ladder-like band (agarose gel electrophoresis) appearing in all primer mix volumes. Therefore, we selected 3.0 μL of primer mix as the optimum volume and used it for all subsequent experiments.

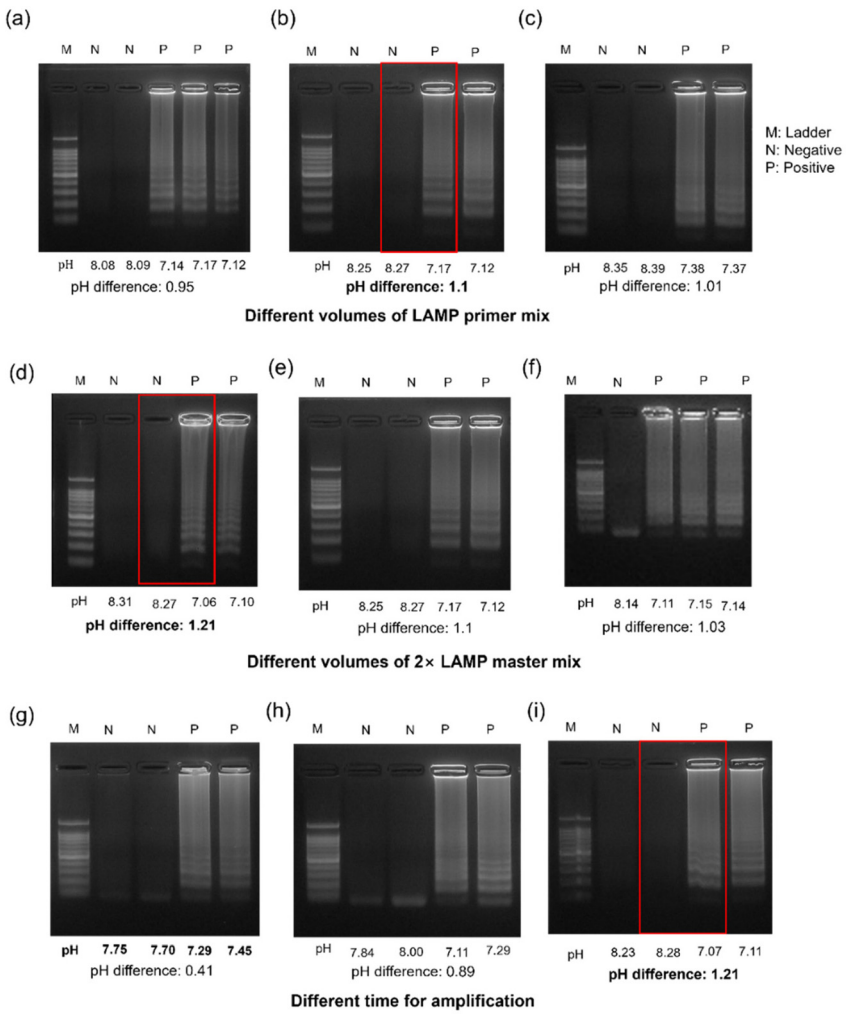


Fig. 1 (a–c) Agarose gel results obtained after optimizing the volume of the primer-mix in the ranges of 2.5–3.5 μL. (d–f) Agarose gel results obtained after optimizing the volume of the 2× LAMP master mix in the ranges of 11.5–12.5 μL. (g–i) Agarose gel results obtained after optimizing the amplification time in the ranges of 30–60 min.

Next, the 2× LAMP master mix volume was optimized to determine the amplification (Fig. 1d–f). In this case, the 11.5 μL 2× LAMP master mix contained in the 25 μL LAMP assay produced a discernible pH variation between the negative and positive samples (Fig. 1d). Therefore, 11.5 μL was determined to be the ideal volume for further experiments. In addition to optimizing the reagent volumes, the reaction time is another important factor for successful LAMP. Notably, the pH difference increased progressively as the reaction time was increased from 30 to 60 min (Fig. 1g–i). Based on these observations, the primer mix (3 μL), 2× LAMP master mix (11.5 μL), and amplification time (60 min) were fixed in the LAMP assay to amplify the *ply* and *katA* genes.

3.2. Solvent selection for LAMP amplicon detection

Curcumin often has poor solubility in protic solvents such as methanol, ethanol, and water. However, it is soluble in aprotic solvents such as ethyl acetate, dimethylformamide (DMF), dimethyl sulfoxide (DMF), and acetone. Therefore, a 50 mM curcumin solution was prepared using these aprotic solvents (Fig. 2a). Notably, 50 mM of curcumin in ethyl acetate and DMSO failed to distinguish between negative and positive samples; thus, these solvents were excluded. Curcumin in acetone clearly developed the color in negative (red) and positive (yellow) as compared to DMF. The color change principle depends on curcumin's halochromic effect, that is, its ability to change color in response to pH variations. This behavior is typically observed for pH indicators, which change color substantially when placed in acidic or alkaline settings. The pH of the LAMP assay should be acidic in positive samples and alkaline in negative samples because of H^+ generation during amplification in weakly buffered conditions. Therefore, in this study, curcumin was introduced as a pH indicator for LAMP amplicon detection based on its halochromic effect. As the human eye resolution is limited to discerning color shifts due to various factors such as color blindness, age, and sex, a grayscale-based image-processing approach was employed to validate the reliability of the curcumin-mediated colorimetric examination (Fig. 2b). The images of the positive and negative samples (Fig. 2a) were first converted to their respective color intensities using the ImageJ software. For ethyl acetate, the color intensities of the negative (144.2) and positive (143.9) samples were almost equal, whereas significant differences in color intensities between these samples were observed in DMF (17.13), DMSO (7.25), and acetone (31.27). Based on the findings obtained using ImageJ, acetone produced a larger color intensity difference than the other solvents; therefore, it was employed for further experiments. Owing to the numerical formats of the color shift, the grayscale-based image processing technique clearly indicated whether amplification had occurred when naked-eye detection was not feasible.

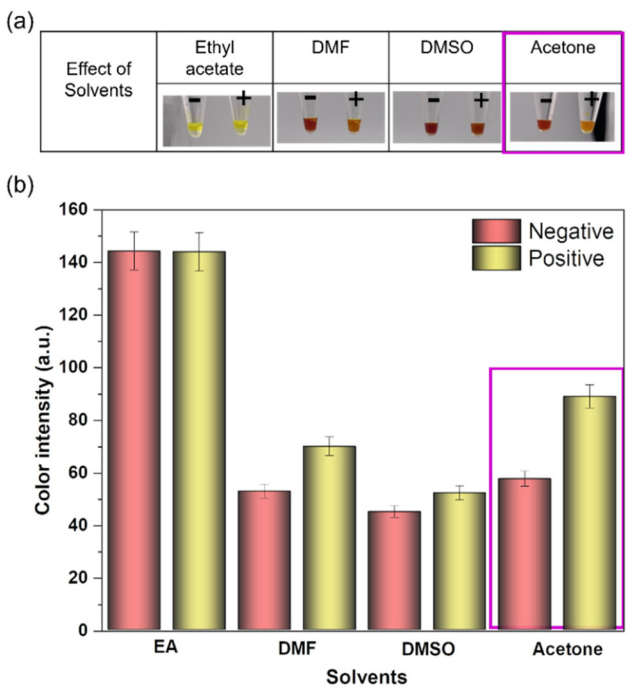


Fig. 2 Solvent selection for LAMP amplicon detection based on (a) colorimetry and (b) grayscale analysis.

3.3. Concentration and volume ratio optimization for LAMP amplicon detection

Initially, various curcumin concentrations were prepared in the range of 10–100 mM using acetone as the solvent. A significant color difference was observed between the negative and positive samples at nearly all curcumin concentrations, as shown in Fig. 3a. Based on the colorimetric outcomes, any of these concentrations can be used. Moreover, the grayscale analysis produced numerical differences of 28.1, 20.5, 19.5, and 18.7 for concentrations of 10, 25, 50, and 100 mM, respectively (Fig. 3b). Based on these results, 10 mM of curcumin produced a sizable numerical difference between the negative and positive samples compared to the other concentrations; therefore, we selected this concentration for further experiments.

Next, different volume ratios of 10 mM curcumin and LAMP amplicons were examined to optimize the detection process (Fig. 3c). The total volume was fixed at 10 μL, which contained the LAMP product and 10 mM curcumin to carry out the colorimetry experiment. A high LAMP product volume was mixed with a low curcumin volume to achieve a volume ratio of 7 : 3. Similarly, ratios of 6 : 4, 5 : 5, and 4 : 6 were prepared using LAMP amplicon and curcumin. Remarkably, a distinguishable color was generated within a few seconds for all ratios (Fig. 3c). Grayscale analysis was performed (Fig. 3d), and a volume ratio of 5 : 5 (LAMP product : 10 mM curcumin) was selected based on the color intensity difference. Statistical analysis data showed a value of $p \leq 0.0001$ for all the concentrations of the curcumin and volume ratios tested, which indicated that the introduced method is highly reliable and robust (Fig. S1†).

3.4. Sensitivity test

S. aureus is one of the most frequent causative agents of foodborne diseases and hospital-acquired infections. Virulence

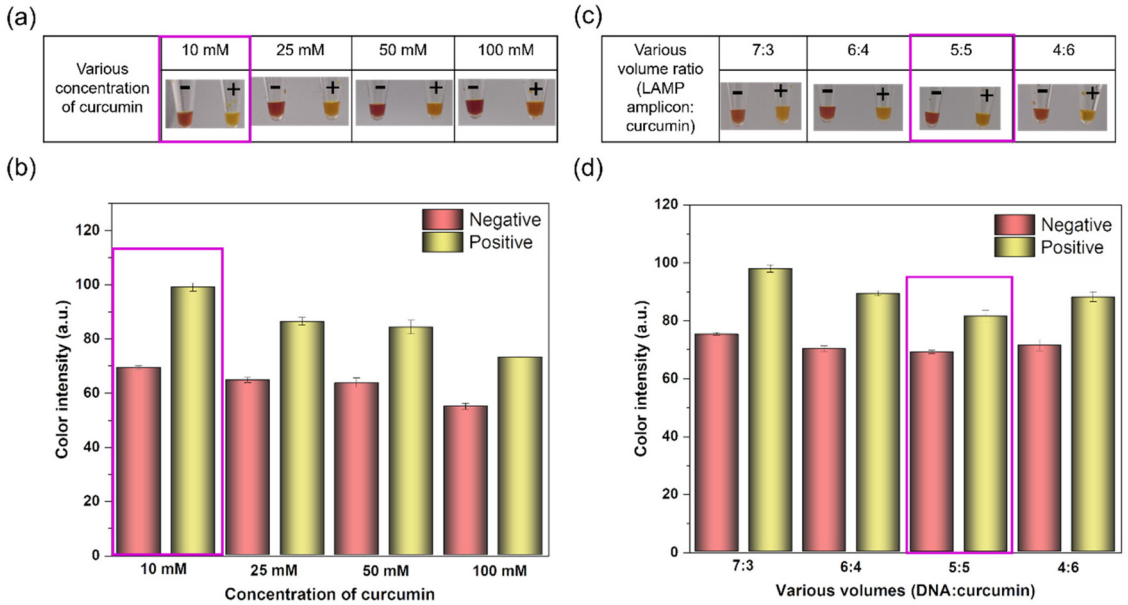


Fig. 3 Concentration and volume ratio optimization for LAMP amplicon detection based on (a and c) colorimetry and (b and d) grayscale analysis.

proteases and *S. aureus* toxins can easily permeate the human bloodstream, resulting in fatalities. It is desirable to develop a technique that can accurately identify extremely low *S. aureus* concentrations. Therefore, the ability of the proposed method towards the detection limit of *S. aureus* was examined. Initially, LAMP was performed using the proposed method and employing 10-fold serially diluted *S. aureus* (from 1 ng μL^{-1} to 1 fg μL^{-1}) as a template. The pH of the LAMP mixture was altered during amplification because of H^+ production; these pH variations can be visually differentiated by curcumin, which has never been involved in LAMP amplicon detection to date.

The designed LAMP primers were used to amplify different quantities of the *katA* gene of *S. aureus* DNA, ranging from 1 ng μL^{-1} to 10 fg μL^{-1}. Fig. 4a shows the sensitivity of the reaction when employing curcumin. The negative sample (0 ng μL^{-1} of target DNA) and 1 fg μL^{-1} of *S. aureus* DNA exhibited a red color when 10 mM curcumin was added. This six-order-of-magnitude dynamic range demonstrates the efficiency of the introduced method. Furthermore, samples with as low as 10 fg μL^{-1} of the target DNA were clearly visible to the naked eye, indicating the robustness of the introduced colorimetry technique. The curcumin-based visual outcomes matched the 1.5% agarose gel electrophoresis results (Fig. 4b). Furthermore, a DNA concentration down to 10 fg μL^{-1} produced a good color intensity difference from the negative sample based on the grayscale analysis (Fig. S2†).

Recently, infectious *S. aureus* was successfully detected using various techniques, including immunoassays, aptasensors, electrochemical sensors, and metal nanoparticle-based colorimetric assays.[38–41] However, most of these techniques pose significant challenges owing to the use of expensive antibodies, bulky analytical equipment, and longer detection times. Furthermore, the proposed method is highly desirable and user-friendly, as all existing techniques use synthetic pH indicator dyes for the colorimetric detection of LAMP products based on pH variations.[23–26] By contrast, curcumin, a natural and biodegradable colorant, was employed in this study. A visual color change was observed within 30 s after successful amplification of the *katA* gene in the curcumin-based LAMP assay. Therefore, the proposed colorimetric method can serve as a practical alternative to advanced methods and expand their applicability to resource-limited environments.

Another pathogen, *S. pneumonia*, was selected to examine the performance of the curcumin-based visual method. This sensitivity experiment was repeated in triplicate, confirming a *S. pneumonia* LOD as low as 1 pg μL^{-1} in both curcumin-based colorimetry and agarose gel electrophoresis (Fig. 4c and d). Table S2† presents a comparative analysis between the proposed method and other relevant techniques for the identification of infectious microorganism.

3.5. Specificity test

To illustrate the specificity, the target DNA was subjected to LAMP using matched or non-matched primer pairs (Fig. 5a). The curcumin-based colorimetric LAMP assay developed a yellow color when *S. aureus* was amplified using matching primers. Conversely, when *S. aureus* was reacted with non-matched primers, the reaction displayed the same red color as the negative control, indicating that amplification was unsuccessful (Fig. 5a). These colorimetric results corresponded with those of the agarose gel electrophoresis (Fig. 5b). Similarly, *S. pneumoniae* was successfully amplified in the microtube when matching primers were used (Fig. 5c and d). Furthermore, other infectious pathogens, such as *Listeria monocytogenes*, *Enterococcus faecium*, and the SARS-CoV-2

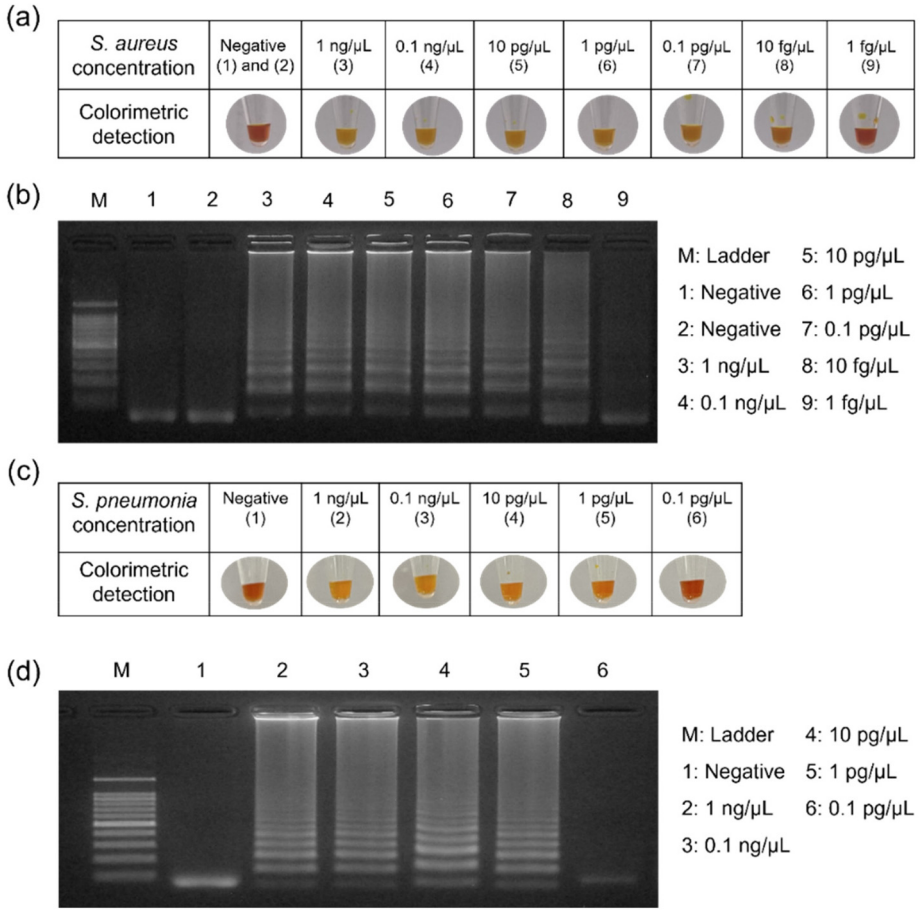


Fig. 4 Sensitivity results of the (a and c) curcumin-based colorimetry and (b and d) agarose gel electrophoresis for *S. aureus* and *S. pneumonia* detection.

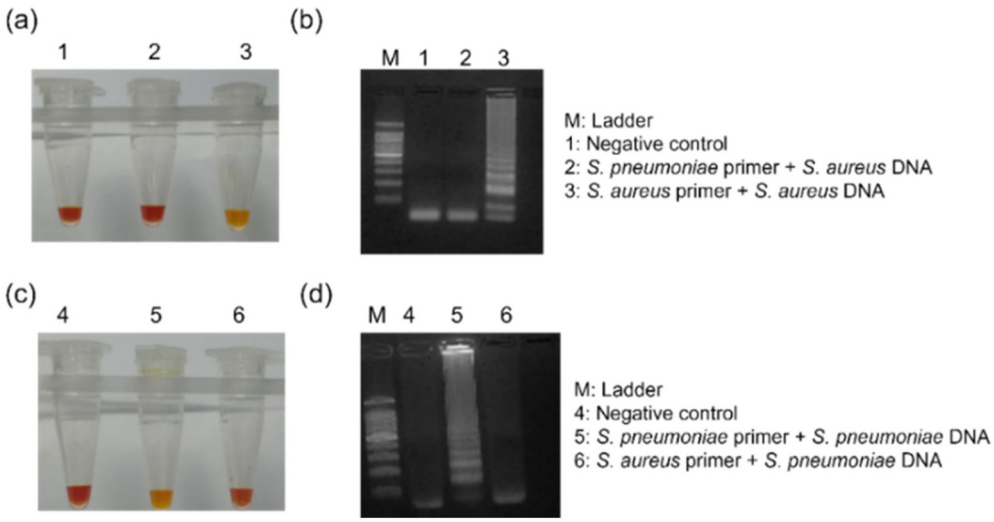


Fig. 5 Specificity results of the (a and c) curcumin-based colorimetry and (b and d) agarose gel electrophoresis for the detection of *S. aureus* and *S. pneumonia*.

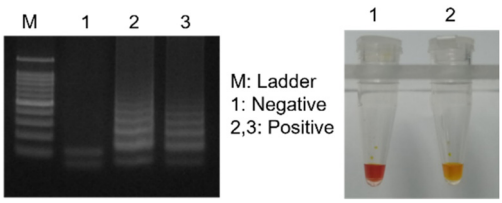


Fig. 6 Application of curcumin-mediated pH-based colorimetric LAMP for *A. baumannii* detection in a real sample.

plasmid were not detected when the *katA* primers were used (Fig. S3†). This implies that the curcumin-based colorimetric LAMP method is not only rapid and sensitive but also highly specific for *S. aureus* and *S. pneumoniae*.

3.6. Real sample analysis

To confirm the effectiveness of the introduced method, a real sample was analyzed. MDR *Acinetobacter baumannii* (*A. baumannii*) is a Gram-negative bacterium that causes hospital-acquired infections in humans (informed consent was obtained). Thus, we investigated the use of the introduced technique for the rapid identification of *A. baumannii* in real samples. Initially, under ideal conditions, the *bla*OXA-23-like carbapenemase gene of *A. baumannii* preserved on an FTA card was used for LAMP. The LAMP amplicon was detected within 30 s using a curcumin-mediated pH-based colorimetric strategy, demonstrating the significance of the introduced method (Fig. 6).

4. Conclusions

In this study, we successfully introduced curcumin as a pH indicator for the first time for weakly buffered LAMP amplicon visualization. Various parameters, such as the primer mix and 2× LAMP master mix volumes, and the amplification time were optimized to identify an effective LAMP process. The negative and positive samples produced red and yellow colors, respectively, within 30 s owing to curcumin's halochromic effect. The proposed pH-dependent visual detection technique efficiently discriminated *S. aureus* and *S. pneumoniae* at concentrations as low as 10 fg μL^{-1} and 1 pg μL^{-1}, respectively, indicating that curcumin, a natural colorant, could be a useful option to combine with the LAMP process for the visual detection of infectious pathogens. The introduced curcumin-based colorimetric LAMP technique can be applied in a safe and environmentally-friendly manner to monitor infectious diseases in the fields of food safety and healthcare as they are biocompatible, cost-effective, rapid, and highly sensitive.

Author contributions

Rajamanickam Sivakumar: writing-original draft preparation, investigation, methodology, data curation, conceptualization, and editing. Nahyung Lim: methodology, and data curation. Seung Kyun Park: methodology, and data curation. Nae Yoon Lee: fund acquisition, supervision, methodology, conceptualization, writing-review, and editing.

Institutional review board statement

This study was approved by the Institutional Review Board (IRB) of the Gachon University (1044396-202409-HR-161-01).

Data availability

The data supporting this article have been included as part of the ESI.†

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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