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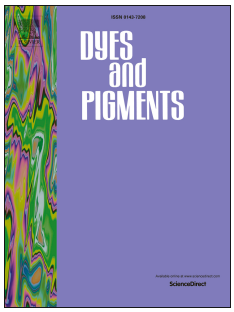

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Development of MRSA and MRAB detection kit employing loop–mediated isothermal amplification and colorimetric indicator dye

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Highlights

- Novel LAMP detecting kits against both MRSA and MRAB were developed.
- The positive reaction would be easily confirmed by the colorimetric change in naked eye.
- Colorimetric Mg^{2+} ion indicator dye **D-649** was synthesized.
- New sets of primer for both MRSA and MRAB were designed.
- The detection limits for DNA of both pathogens are 1 pg.

Abstract

Methicillin-resistant Staphylococcus aureus (MRSA) and *Multidrug-resistant Acinetobacter baumannii* (MRAB) are serious life-threating pathogens. The rapid detection of those pathogens is a challenge for the most clinical microbiology laboratories. A nucleic acid amplification technique, loop-mediated isothermal amplification (LAMP) that enables to amplify target DNA sequences in a short time period, is an excellent technique to detect the pathogens with high efficiency. We developed LAMP detecting kits against both MRSA and MRAB that utilizing a newly developed colorimetric Mg^{2+} ion indicator dye **D-649** and novel sets of primers. An aqueous soluble dye **D-649** is stable over physiological pH range and displays clear color change upon binding to Mg^{2+} ion. The addition of 0.12 mM of **D-649** to LAMP solution did not disturb the amplification process, and the positive reaction would be easily confirmed by the color change of reaction mixture from ruby to purple in naked eye under ambient light. Each primer set was designed to recognize *mecA*/*femB* gene of MRSA and *bla*OXA-23/*bla*OXA51–like gene of MRAB, and the detection limits for both DNAs were shown as 1 pg. These LAMP detection kits will be beneficial for the detection of MRSA and MRAB in clinical laboratories.

Keywords

Methicillin-resistant Staphylococcus aureus, *Multidrug-resistant Acinetobacter baumannii*, Loop-mediated isothermal amplification method, LAMP detecting kits, Colorimetric Mg^{2+} ion indicator dye

1. Introduction

Methicillin-resistant Staphylococcus aureus (MRSA)[1] and *Multidrug-resistant Acinetobacter baumannii* (MRAB)[2] are serious life-threating pathogens due to their environmental resilience, antimicrobial resistance, and outbreak potential.[3] Epidemic spread of MRSA and MRAB is common among people in hospitals[4,5], nursing homes[6] and prisons[7]. The detection of those pathogens, which requires implementation of strict protocols for active screening,[8] is a challenge for the most clinical microbiology laboratories. The rapid identification of antimicrobial susceptibility patterns of bacterial isolates from positive blood cultures is an imperative issue.

Whilst costly molecular techniques offer high sensitivity and rapid results, time-consuming culture methods also provide reliable outcomes with lower price.[9,10] Traditional cultural techniques, including growth-based assays, colony morphology, and microdilution resistance tests,[11] take up to 48 h to confirm pathogens, thus they are mainly utilized as confirming methods for molecular techniques.[12] Polymerase chain reaction (PCR)-based assays have been successfully established and utilized for the rapid detection of MRSA and MRAB directly from various clinical specimens.[13,14] However, these methods also have some complications such as complex protocols, false positive and negative results, and the necessity to run multiplex PCR to obtain reliable results.[15] Loop-mediated isothermal amplification method (LAMP) is another effective technique for rapid detection of target DNA sequences and is increasingly utilized in biomedical field.[16]

LAMP method enables to rapidly amplify DNA strand under isothermal conditions (60–65 °C) with high efficiency and sensitivity.[17] Moreover, the specificity of LAMP is considered extremely high because the primers must bind six distinct regions on the target DNA in order to amplify. The sensitivity of LAMP does not appear to be affected by the presence of non-target DNA in sample, and the method is well tolerant to known PCR inhibitors such as blood, serum and food ingredients.[18] Owing excellent features, LAMP has been utilized in various fields of genetic analysis, including the detection of MRSA and MRAB.[19,20]

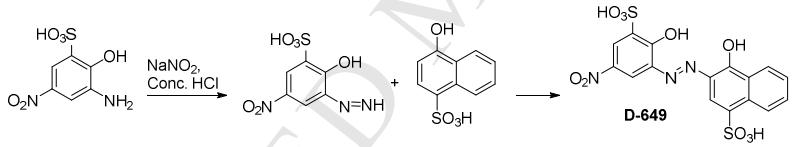
To obtain accurate analysis results, LAMP requires an efficient method to monitor the amplification progress. A number of LAMP amplification monitoring methods have been developed to date, including turbidity monitoring,[21] gel electrophoresis,[16] electrochemical methods,[22] lateral flow dipstick,[23] naked eye monitoring with DNA binding dyes such as SYBR green,[24] using colorimetric indicators such as calcein or hydroxylnaphthol blue (HNB),[25] and so forth.[26] In this work, we developed LAMP detecting kits against both MRSA and MRAB that utilizing a newly developed colorimetric Mg^{2+} ion indicator dye and novel primer sets. Herein, we disclose our effort to synthesize a novel indicator dye, to design sensitive primer sets, and to consolidate them into MRSA and MRSA detection kits.

2. Materials and methods

2.1. Materials and instruments

Reagents and building blocks for dye synthesis were purchased from Sigma-Aldrich and Tokyo chemical industry (TCI) and used without further purification. Synthesis of aryl azo dyes were followed from literature procedures.[28,29] Oligonucleotide primers were designed using the Primer Explorer V4 software (Fujitsu System Solutions, Ltd., Japan). Large fragment of *Bst* DNA polymerase was purchased from New England Biolabs (Ipswich, MA, USA). Electrophoresis was performed using Mupid-exu (Advance Co. Ltd., Japan). TLC analysis was conducted on silica gel plates. Fluorescence spectra were recorded on a bioimaging instrument (FOBI, NeoScience Co. Ltd., Korea). ^{1}H NMR and ^{13}C NMR spectra were measured on ECZR 500 MHz FT NMR spectrometer (JEOL Ltd., Tokyo, Japan) using tetetramethylsilane (TMS) as an internal reference. The pH values were measured using Seven ComPact pH meter S 220 (Mettler Toledo, Ohio, USA). Genomic DNA strand of MRSA, ATCC® 33591, was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Genomic DNA strand of MRAB strain was obtained from Konyang University Hospital. Concentrations of DNA strands were analyzed with Nanodrop 2000 spectrophotometer (Thermo Fisher scientific, USA). Theoretical pKa values were calculated with ChemBioDraw program. High resolution mass spectra were measured on a Q Exactive mass spectrometer (Thermo Fisher Scientific, USA).

2.2 Synthesis of D-649



Scheme 1. **Synthetic scheme for D-649**

A solution of 4-nitro-2-aminophenol-6-sulfonic acid (6.6 g, 25 mmol) in deionized water (100 mL) at 0 □ was slowly treated with concentrated HCl (7.5 mL) followed by a solution of sodium nitrite (1.9 g, 26 mmol) in deionized water (1 mL). The reaction mixture was stirred at 0 ℃ for 1 h and treated with a solution of 1-naphthol-4-sulfonic acid (5.5 g, 25 mmol) in deionized water (100 mL). The reaction mixture was stirred at room temperature for 2 h whilst pH of the reaction mixture was adjusted to 7 with aqueous 2 N NaOH. The resulting mixture was filtered through a fritted filter, and the filter cake was dried under reduced pressure to provide the crude product. Flash chromatography (SiO_2, *iso*-butanol/*n*-propanol/EtOAc/water: 2/4/1/3, *rf* 0.4) afforded 4-hydroxy-3-((2-hydroxy-5-nitro-3-sulfophenyl)diazenyl) naphthalene-1-sulfonic acid (**D-649**) as a pink solid (4.6 g, 38%): ^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.55 (d, J = 8.6 Hz, 1H), 8.35–8.28 (m, 3H), 7.70–7.64 (m, 1H), 7.61 (s, 1H), 7.50–7.44 (m, 1H), (O-H protons were not shown); ^{13}C NMR (125 MHz, DMSO-*d6*) δ 175.5, 166.7, 135.5, 134.6, 133.2, 132.0, 131.6, 131.0, 130.7, 128.4, 128.2, 126.5, 126.2, 125.9, 123.6, 110.7; HRMS ESI-TOF *m/z* $C_{18}H_{11}N_3O_{10}S_2$, found: $[M + H]^+$ 469.99564, requires: 469.99586.

2.3 LAMP and electrophoresis processes

LAMP assays were carried out in 25 μL reaction mixture containing serially 10-fold diluted MRSA or MRAB DNA, 0.2 μM each of F3 and B3, 1.6 μM each of FIP and BIP, 0.8 μM each of LF and LB, 20 mM Tris-HCl (pH 8.8), 10 mM each of KCl, $(NH_4)_2SO_4$ and 0.1% Triton X-100, 8 mM $MgSO_4$, 1.4 mM of each dNTP, 8 U of the large fragment of *Bst* DNA polymerase (New England Biolabs, Ipswich, MA, USA), 0.12 mM of **D-649** in LAMP buffer (for reaction with dye only). Reaction mixtures without DNA were also prepared as negative controls. All reaction mixtures were incubated at 63°C for 30 min and then heated up at 80°C for 5 min to terminate the reaction in a heating block. Electrophoresis analysis was performed in 2% agarose gels, and the visualization was realized with ethidium bromide staining followed by imaging under 365 nm UV.

3. Results and discussion

3.1 Mg^{2+} ion indicator dye design and synthesis

For efficient monitoring of LAMP progress, we first decided to develop a novel colorimetric Mg^{2+} ion indicator dye at the first. Several criteria for the ideal indicator were set: the dye would has a clear color for naked-eye monitoring, would be aqueous soluble, would not be fluorescent or luminescent under normal lighting, and displaying distinguishable color change upon binding to Mg^{2+} ion. Due to their unique photochemical properties related to the facile *cis-trans* isomerization of Ar–N=N–Ar moiety upon UV–vis irradiation that acts as an efficient fluorescence quencher or molecular switcher, bis-aryl azo moiety was selected as dye scaffold.[27] Mono- and di-sulfonic acid were chosen as main Mg^{2+} ion chelating groups due to their weakly acidic, non-nucleophilic nature and bidentate binding ability. More than 70 aryl-azo dyes having aromatic sulfonic acid were synthesized and subjected to aqueous solubility test.[28,29] Among them, eight compounds displayed good solubility (Figure 1).

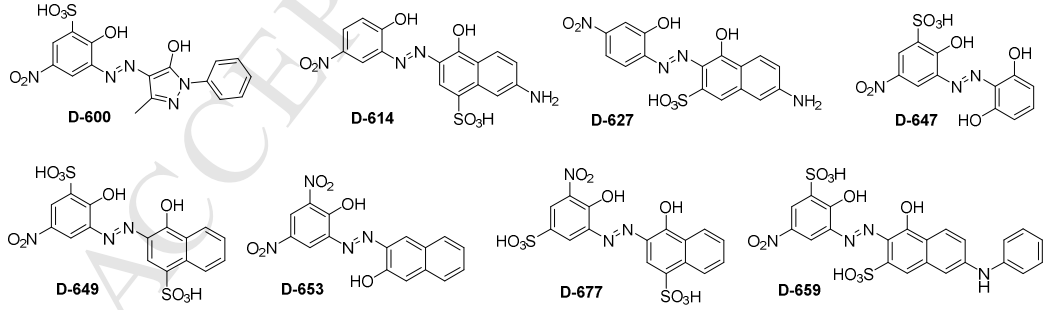


Figure 1. Eight aqueous soluble azo-dyes.

3.2 Mg^{2+} ion binding test of eight aqueous soluble dyes

To find the colorimetric utility, eight aqueous soluble dyes were dissolved in deionized water in two different concentrations (50 μM and 100 μM) and treated with varying amount of Mg^{2+} ion in comparison with known Mg^{2+} indicator **HNB** (Figure 2).[25] The sufficient concentration of

dyes for efficient colorimetric monitoring was shown as 100 μM. We expected that Mg^{2+} ion would stoichiometrically react with a dye to cause color change, however excess amount of Mg^{2+} ion was required to achieve colorimetric change in deionized water. Five dyes, **D-600**, **D-614**, **D-649**, **D-653** and **D-659**, displayed distinguishable color change when treated with Mg^{2+} ion. Theoretical pKa value of five dyes varied from 4 to 7 (**D-653**, 4.1; **D-659**, 4.2; **D-600**, 4.9; **D-649**, 6.0; **D-614**, 6.9), thus weak acidic nature of dyes might be a culprit for the excess amount of dye input. These five dyes were evaluated for their stability in physiological pH range in order to check their robustness.

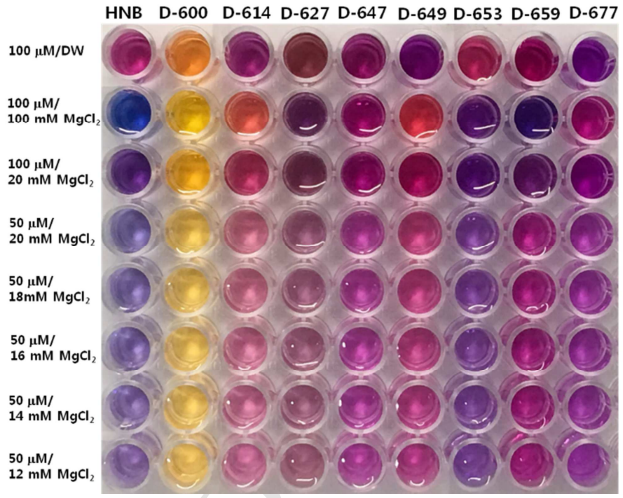


Figure 2. Treatment of excess amount of Mg^{2+} ion to HNB and eight water-soluble compounds.

3.3 Stability test of five dyes in physiological pH

Due to the basicity (pH 8.8) of our LAMP reaction mixture, stability of the indicator dye in the basic media is a critical issue. However, considering for the extendable usage, we would like to develop the dye that would be stable over a wide range of pH. Thus, five Mg^{2+} ion-responsive dyes were evaluated their color variation over physiological pH range in aqueous sodium phosphate buffer (Figure 3). Two different stock solutions (100 mM in deionized water and DMSO) were 2-fold diluted with deionized water, and pH values were adjusted with sodium phosphate buffer. Results from both stock solutions were not clearly distinguishable except **D-653**, which showed different color change patterns depending on initial stock solutions.

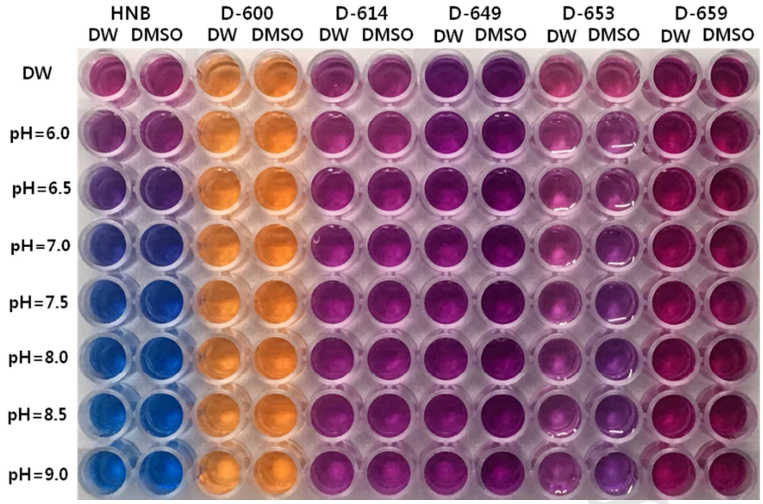


Figure 3. Stability test of 5 dyes and HNB in physiological pH range.

**pH was adjusted with sodium phosphate mono- and dibasic buffer solution. Concentration of dye solutions was 50 mM.*

Surprisingly, the results indicated that HNB, which has been utilized as an indicator for Ca^{2+} and Mg^{2+} ion and as a colorimetric reagent for alkaline earth metal ions,[25] was reacting with Na^{+} ion at pH above 6.5: it displayed different colors in deionized water and in pH 7.0 sodium phosphate buffer. The color of HNB in sodium phosphate buffer above pH 7.0 was same as when it was treated with excess of Mg^{2+} ion in deionized water as shown in figure 2. Since Na^{+} ion is a common contaminant and an important buffer constituent in biological assays, using HNB in LAMP process as an Mg^{2+} ion indicator might require caution. Three dyes, **D-614**, **D-649** and **D-659**, were maintained constant color over the range and not reacting with sodium ion.

3.4 Selection of D-649 as the Mg^{2+} ion indicator candidate

After contemplation of above test results, **D-614** and **D-649** were selected as final two candidates and subjected to further experiments. Two dyes and HNB were dissolved in both deionized water and pH 9 sodium phosphate buffer and treated with variable amount of magnesium chloride, and then their colorimetric change was monitored (Figure 4, left). After monitoring the color change, resulting mixtures were treated with sodium pyrophosphate (equal molar amount to $MgCl_2$) in order to check the recovery of original color (Figure 4, right).

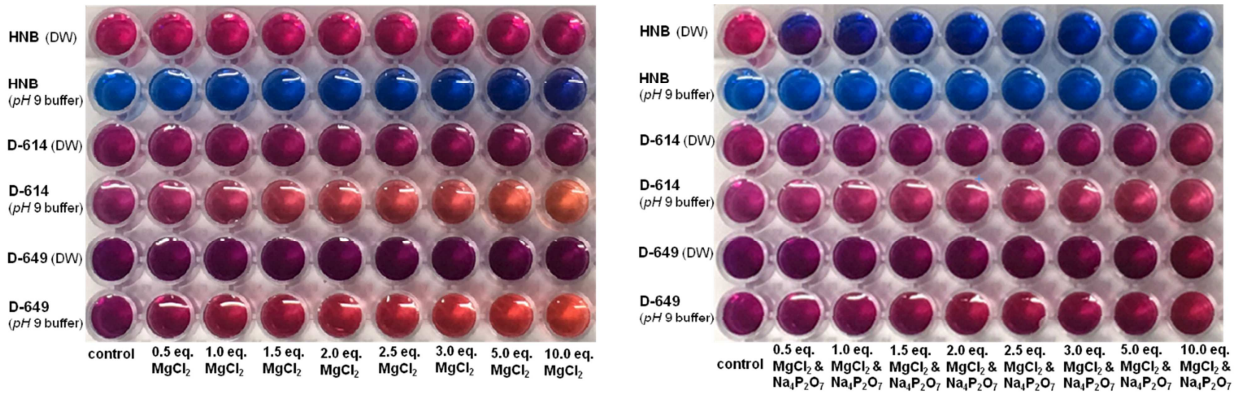


Figure 4. (left) Treatment of varying equivalent of $MgCl_2$ to dye solutions; (right) addition of $Na_4P_2O_7$ [equivalent to $MgCl_2$] to left wells. *Concentrations of dye solution were 100 μM. pH was adjusted with sodium phosphate mono- and dibasic buffer solution.*

HNB did not react with up to 10-fold excess of $MgCl_2$ in deionized water, but it changed the color from pink to sky blue in presence of Na^+ ion regardless of Mg^{2+} ion input, witnessing its relative selectivity for Na^+ ion over Mg^{2+} ion. **D-619** and **D-649** both did not respond to Mg^{2+} ion in deionized water, however, they gradually changed their color from dark pink to orange with respect to increasing amount of Mg^{2+} ion in pH 9 buffer (Figure 4, left). When sodium pyrophosphate (equal molar ratio to $MgCl_2$) was added to Mg^{2+} ion–treated wells, the original colors of **D-619** and **D-649** were recovered (Figure 4, right). The LAMP reaction liberates excess amount of pyrophosphate ion byproduct that reacts with Mg^{2+} ion to form insoluble magnesium pyrophosphate salt, thus monitoring of LAMP amplification with colorimetric Mg^{2+} indicator dye relies on the observation of color change from dye–Mg^{2+} ion complex to free dye. Therefore, recovery of original color upon treating with pyrophosphate ion is an essential issue. **D-649** more clearly recovered the original color than **D-614** when treated by sodium pyrophosphate, thus it was selected as the optimal indicator dye and incorporated into LAMP amplification process (Figure 5).

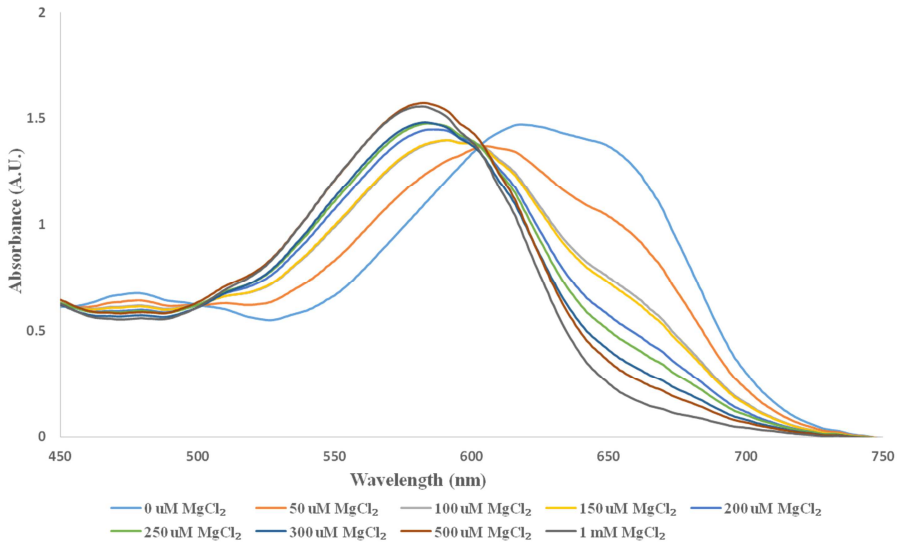


Figure 5. Effect of Mg^{2+} ion concentration on the absorption spectra of **D-649** in pH 9 buffer.
*Concentration of **D-649** is 100 µM. More than 2 eq. of Mg^{2+} ion was required for clear colorimetric observation.*

3.5 Design of novel primer sets for both MRSA and MRAB

We decided to develop new sets of LAMP primers against both MRSA and MRAB, accordingly several primer sets of both MRSA and MRAB were designed and tested for LAMP amplification process. After repeated trials, novel primer sets displaying the optimal efficiency and sensitivity were selected (Table 1). Our oligonucleotide primers sets recognize *mecA*/*femB* gene (Genbank No. AB505630.1/CP010300.1) of MRSA and *bla*OXA-23/*bla*OXA51-like gene (Genbank No. KF305669.1/DQ385606.1) of MRAB. The primer sets consisted of forward outer primer (F3), backward outer primer (B3), forward inner primer (FIP) backward inner primer (BIP), loop forward primer (LF) and loop backward primer (LB). Both MRSA and MRAB primer sets could afford successful isothermal amplifications with high efficiency and sensitivity.

MRSA primer set

Primer	Oligonucleotide sequence
F3	5'–ATGATTATGGCTCAGGTACTG–3'
B3	5'–AACCCAATCATTGCTGTTAATATT–3'
FIP	5'–TACATAAATGGATAGACGTCATCTATCCACCCTCAAACAGGTG–3'
BIP	5'–GGCATGAGTAACGAAGAATATAATCCTGGTGAAGTTGTAATCTGGAAC–3'
LF	5'–ATGAAGGTGTGCTTACAAGTGC–3'
LB	5'–CCGAAGATAAAAAAGAACCTCTGCT–3'

MRAB primer set

Primer	Oligonucleotide sequence
F3	5'–AGAATATGTGCCAGCCTCTAC–3'

B3	5'–CAGAAATTATCAACCTGCTGTCC–3'
FIP	5'–TGTCATGTCTTTTTCCCAAGCGGGGATTGGAGAACCAGAAAACGG–3'
BIP	5'–CCCAGTCTATCAGGAACTTGCGTCAGCATTACCGAAACCAATACG–3'
LF	5'–ACCTTTTCTCGCCCTTCCATTT–3'
LB	5'–GACGTATCG GTCTTGATCTCATGC–3'

Table 1. Primer sets for MRSA (up) and MRAB (bottom).

3.6 Effect of D-649 in LAMP experiments

For checking the influence of **D-649** in LAMP reaction, we prepared two sets of LAMP mixtures having serially diluted MRSA and MRAB DNA. One set of reaction mixtures were premixed with 0.12 mM of **D-649** and other set consisted of reaction mixtures without the dye (Figure 6).

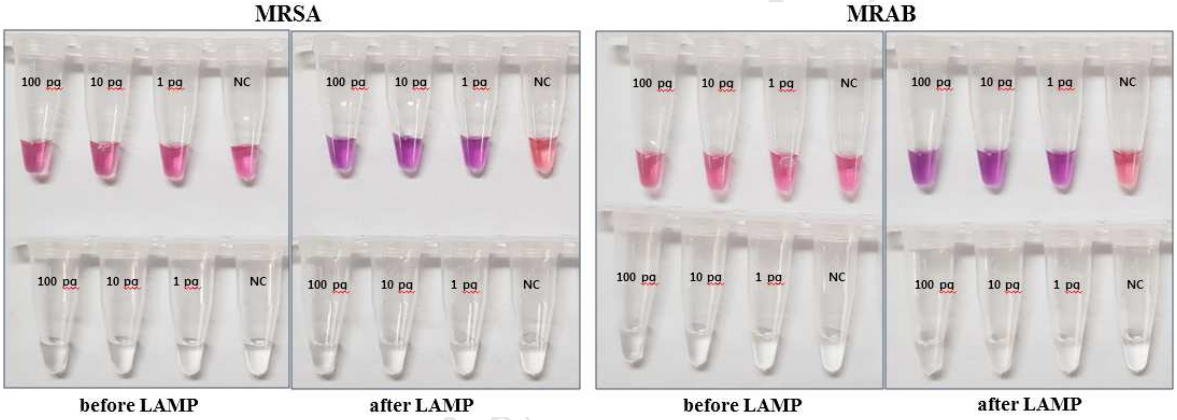


Figure 6. Comparative sensitivity of LAMP assay with D-649 (left, MRSA; right, MRAB).
upper: D-649 contained LAMP mixture, bottom: LAMP mixture without dye.

After LAMP reaction, clear color changes were observed in both MRSA and MRAB reaction mixture tubes containing **D-649** except the negative controls. The positive LAMP reactions were easily confirmed by naked eye under ambient light. The color of reaction tubes excluding negative controls was changed from initial ruby to purple, witnessing the successful separation of Mg^{2+} ion from the dye. Our novel primer sets were able to effectively amplify target DNA sequence, and the detection limits for both bacteria were shown as 1 pg. The LAMP reaction liberates pyrophosphate ion as a byproduct, which forms insoluble $Mg_2P_2O_7$ salt, causing turbidity in the reaction tube proportional to the amount of amplified DNA. Hence, we could observe a slight turbidity in the reaction tubes after amplification except negative control. For further confirming the effect of the dye in LAMP results, amplified products from both reaction mixtures containing the dye and mixtures lacking the dye were analyzed using electrophoresis in 2% agarose gels, followed by the visualization under UV irradiation. (Figure 7).

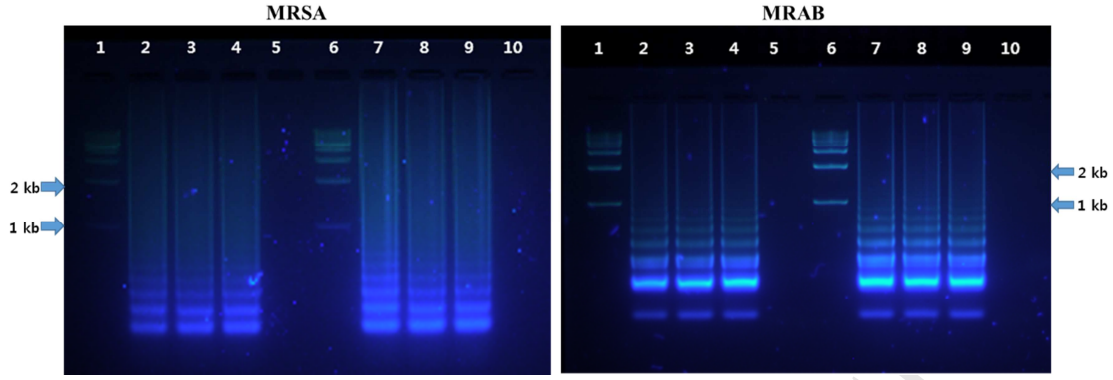


Figure 7. Electrophoretic analysis of LAMP products (left: MRSA; right: MRAB).
lane 1, marker; lane 2, dye + 100 pg DNA; lane 3, dye + 10 pg DNA; lane 4, dye + 1 pg DNA; lane 5, dye in negative control; lane 6, marker; lane 7, 100 pg DNA; lane 8, 10 pg DNA; lane 9, 1 pg DNA; lane 10, negative control.

Ethidium bromide-stained gel demonstrated the typical banding patterns observed with MRSA and MRAB, indicating the production of both pathogens' DNA with inverted repeats of the target sequence. Comparison of outcomes from LAMP mixtures with premixed **D-649** (lanes 2, 3, 4) and without the dye (lanes 7, 8, 9) showed the presence of **D-649** did not disturb LAMP reaction.

4. Conclusion

We developed new LAMP detecting kits, which utilizing a newly developed colorimetric Mg^{2+} ion indicator dye **D-649** and novel primer sets, for both MRSA and MRAB. An aqueous soluble colorimetric Mg^{2+} indicator **D-649** was synthesized, and novel sets of primer for both MRSA and MRAB were designed for effective amplification. The positive LAMP reactions for both MRSA and MRAB were easily confirmed by naked eye under ambient light. By analyzing LAMP products with electrophoresis, the existence of **D-649** did not disturb the results, and the detection limits for both DNAs were shown as 1 pg. These LAMP detection kits would be beneficial for the detection of MRSA and MRAB in clinical laboratories. Currently, MRSA detection kit is being evaluated for clinical application.

Acknowledgement

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Declaration of interest

The authors are employee and shareholder of BioActs Co. Ltd., and M monitor, Inc.
BioActs Co. Ltd. holds on patents: MRSA, KP10-2017-0179022; MRAB, KP 10-2017-0179023.

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