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Probe-specific loop-mediated isothermal amplification magnetogenosensor assay for rapid and specific detection of pathogenic *Leptospira*

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ABSTRACT

This study highlighted the performance of the developed integrated loop-mediated isothermal amplification (LAMP) coupled with a colorimetric DNA-based magnetogenosensor. The biosensor operates through a DNA hybridization system in which a specific designed probe captures the target LAMP amplicons. We demonstrated the magnetogenosensor assay by detecting pathogenic *Leptospira*, which causes leptospirosis. The color change of the assay from brown to blue indicated a positive result, whereas a negative result was indicated by the assay maintaining its brown color. The DNA biosensor was able to detect DNA at a concentration as low as 200 fg/μl, which is equivalent to 80 genomes/reaction. The specificity of the biosensor assay was 100% when it was evaluated with 172 bacterial strains. An integrated LAMP and probe-specific magnetogenosensor was successfully developed, promising simple and rapid visual detection in clinical diagnostics and service as a point-of-care device.

1. Introduction

The development of diagnostic tools for infectious disease detection is mainly directed toward accurate, rapid and easy-to-use tests which allow early treatment. This is important for the improvement of management and control of infectious diseases. Currently, various isothermal amplification techniques are employed to produce numerous copies of DNA without the utilization of thermocycler equipment. With the presence of various accessory proteins for mimicking *in vivo* mechanisms, isothermal amplification can be applied in the identification of infectious agents in small-scale hospital laboratories, as reviewed by Karami, Gill, Motamedi and Saghafinia [1]. Several isothermal amplification techniques have been developed, including transcription-mediated amplification or self-sustained sequence replication, nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), rolling circle amplification (RCA), loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA) and helicase-dependent amplification (HDA).

LAMP has been widely used in clinical diagnostics, especially in developing countries. The presence of two primer sets makes it highly specific, which allows it to recognize six distinct regions on the target DNA. Moreover, the simplicity and rapidity of LAMP are acknowledged because the reaction can be completed within 1 h under a single temperature. Currently, researchers are enthusiastic about the development of a detection method to be integrated with LAMP that will assuredly be successful in a point-of-care setting. Detection methods for LAMP have expanded from simple visual observation of turbidity to turbidimeters [2], fluorescent agents [3,4], colorimetric agents [5,6], lateral flow dipsticks [7,8] and lab-on-a-chip devices [9].

To the best of our knowledge, no study has been done on the application of probe-based magnetic beads in the detection of LAMP amplicons, although efforts to enhance the detection of LAMP amplicons have been ongoing. In some present studies, magnetic beads were applied during the sample preparation phase, in which the magnetic beads were coated with the detector agent to capture the sample before being processed and undergoing the LAMP method [10,11]. A genosensor offers highly sensitive and specific detection of genomic DNA. Its wide application to clinical analysis has been reviewed by D'Orazio [12], including cardiac markers, cancer biomarkers, DNA analysis, blood glucose measurement, and markers for renal disease, infectious

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disease, and autoimmune diseases, among others.

The advent of magnetic beads in biosensing, particularly in DNA hybridization sensors, provides versatile tools for environmental evaluation and clinical diagnostics, due to the presence of ample surface area and their high dispersion capability of the beads [13]. The immobilization of specific DNA probes on the beads allows the detection of complementary sequences of DNA strands through hybridization events. High DNA hybridization efficiency can be achieved by taking into account several factors, including surface probe density, the density of the charge on the surface, confirmation of probe strands, and the curvature of the solid surface [14].

In the present study, we applied magnetic beads for the detection of LAMP products where *Leptospira* was used as the model organism. *Leptospira* is the causative agent of an emerging disease, leptospirosis. The disease is difficult to diagnose because its clinical symptoms are often confused with other diseases, such as influenza, hepatitis, dengue fever, or hemorrhagic fever [15]. Hence, swift and specific detection of the presence of this causative agent is crucial for proper and rapid treatment. Previously demonstrated laboratory diagnosis methods include cultivation, DNA detection, antigen detection, antibody detection, and microscopic demonstration of the bacteria [16]. However, these methods do not permit early definitive diagnosis; they lack sensitivity, they are laborious, and they require highly skilled personnel to operate the tests [16]. Hence, we developed LAMP with magnetogenosensor detection assay to detect pathogenic *Leptospira*. Additionally, the application of a portable magnetic separator in the present method allows it to be applied in a point-of-care setting.

2. Material and methods

2.1. Reference strains and environmental isolates

A total of 172 bacterial strains which included reference strains ($N = 80$) and environmental isolates ($N = 92$) were used for analytical specificity evaluation. All reference strains were acquired from the Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM); the Institute for Medical Research (IMR), Malaysia; the Hospital Universiti Sains Malaysia (HUSM), the Belgian Co-ordinated Collections of Microorganisms (BCCM), Belgium; the National Autonomous University of Mexico (UNAM), Mexico; and the London School of Hygiene & Tropical Medicine (LSHTM), England. Additionally, environmental isolates were acquired from the Leptospirosis Team of the Department of Medical Microbiology & Parasitology, Universiti Sains Malaysia (USM).

2.2. Amplification of specific genes using LAMP assay

Specific primer pairs for pathogenic *Leptospira* targeted the *lipL32* gene. The forward inner primer (FIP), backward inner primer (BIP), forward outer primer (F3), backward outer primer (B3), backward loop primer (LB), and probe that were used in the study are listed in Table 1.

The LAMP assay was performed using a Loopamp DNA Amplification Kit (Eiken Chemical Co. Ltd., Japan) in a total volume of 25 µl of reaction mixture that contained 1 × reaction mix; 8 U *Bst* DNA polymerase; 5 µM each of F3 and B3; 40 µM each of FIP and BIP; 20 µM of LB; 2 µl DNA template (20 ng); and deionized water. The reaction mixture was incubated at 63 °C for 1 h, followed by a termination step at 80 °C for 10 min.

2.3. Immobilization of probes

The immobilization of the biotinylated capture probe on the magnetic bead was carried out based on a procedure recommended by the supplier, with some modifications. Briefly, 2 mg/ml of Dynabeads MyOne Streptavidin T1 (Invitrogen, Norway) were washed three times with 400 µl of binding and washing (B&W) buffer and resuspended in 800 µl of B&W buffer containing 1 µM of the biotinylated probe. The biotinylated probe was captured onto the beads for 20 min at room temperature with gentle rotation of the tube. Subsequently, the probe-coated beads were washed three times with 800 µl B&W buffer. The beads were finally resuspended in 200 µl phosphate-buffered saline (PBS) buffer to achieve a final concentration of 10 mg/ml and kept at 4 °C for further use.

2.4. Magnetogenosensor assay detection

The hybridization step was conducted by resuspending LAMP amplicons and a solution of 0.05% bovine serum albumin (BSA) in PBS, into the probe-coated beads. Hybridization between the probe-coated beads and fluorescein isothiocyanate (FITC)-labeled LAMP amplicons took place during incubation for 20 min at room temperature with gentle mixing. Then the tube mixture was placed on the portable magnetic separator to wash away the non-hybridized LAMP amplicons, using 50 µl PBS. Subsequently, 10 µl anti-fluorescein horseradish peroxidase (anti-fluorescein-POD) (Roche, Switzerland) was added to the hybridized capture probe-target amplicon magnetic beads and allowed to stand at room temperature for 10 min with gentle mixing. The tube mixture was placed again on the portable magnetic separator for the washing step. Finally, 20 µl of TMB One solution (Promega, USA) was added to the tube mixture, and color change from brown to blue was observed.

2.5. Analytical performance of LAMP-colorimetric magnetogenosensor assay

The analytical sensitivity of the LAMP-colorimetric magnetogenosensor assay was determined by diluting the purified genomic DNA of pathogenic *Leptospira* to final concentrations of 20 ng, 2 ng, 200 pg, 20 pg, 2 pg, 200 fg, 20 fg, 2 fg, 200 ag, 20 ag, and 2 ag. The amplified DNA was detected by 2% agarose gel electrophoresis with RedSafe staining solution (iNtRON Biotechnology, Korea) and the magnetogenosensor assay. The analytical specificity of the assay was evaluated

Table 1
Oligonucleotide sequence of primers and capture probe.

Oligonucleotide	Sequence (5' →3')	Length (mer)	Source
Primers			
F3	GTAAGCGACGCTTTCAAAG	19	[17]
FIP	6-FAM-CCGACATTCTTTCTACACGGATCGAATTCCCCCAGAAGAAAAATCAATGC	44	[17]
B3	TGTCTCTCTTCTTTATAAGTATCG	24	[17]
BIP	TTATGCCTGACCAAATCGCCGAATTCTCACCATCATCATCATCGT	39	[17]
LB	CTGCGAAAGCAAAACCAGTTC	21	[17]
Capture probe	Bio-CACCATCATCATCATCGTCC	20	This study

F3, forward outer primer; FIP, forward inner primer; B3, backward outer primer; BIP, backward inner primer; LB, loop backward primer; 6-FAM, carboxyfluorescein; Bio, biotin.

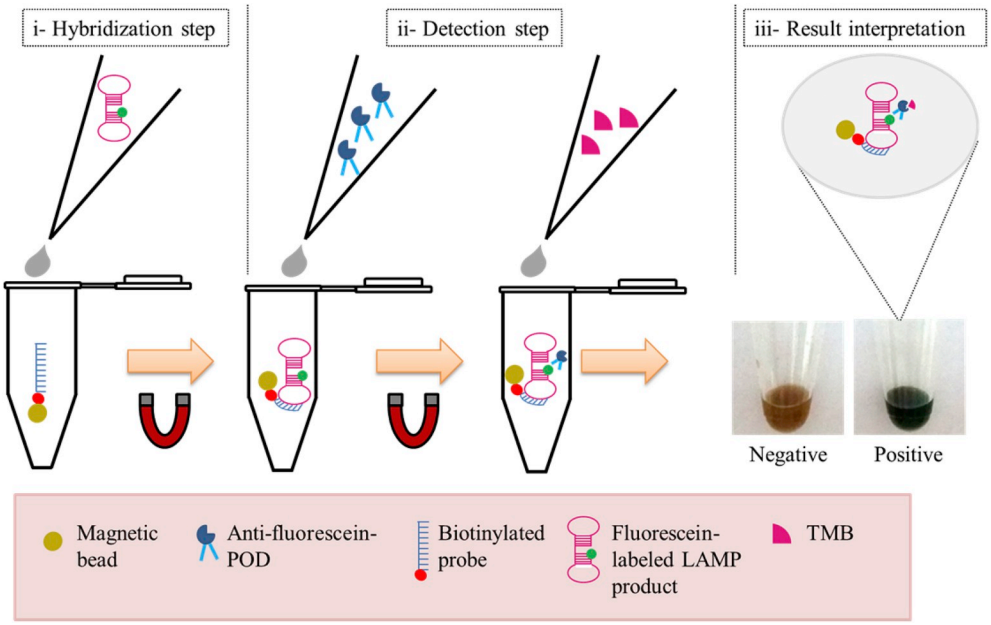

Fig. 1. Principle illustration of colorimetric magnetogenosensor assay. LAMP-magnetogenosensor assay involves hybridization and detection steps. During hybridization step, fluorescein-labeled LAMP amplicon and specific probe coated on magnetic beads will be hybridized. The non-hybridized LAMP amplicon will be washed away during washing step under portable magnetic separator. Subsequently, detection step involves recognition of hybridized fluorescein-labeled LAMP amplicon/specific probe magnetic beads by anti-fluorescein-POD. The non-hybridized particle will be washed away during another washing step under portable magnetic separator. Addition of TMB as the substrate of POD will cause brown assay color change to blue color, which indicates positive result. The inset photo shows the interpretation of the LAMP-colorimetic magnetigenosensor assay for positive and negative test results. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

using purified genomic DNA of various reference strains.

3. Results and discussion

3.1. Assay principle

The LAMP amplicon detection system is depicted in Fig. 1. The specific capture probe was designed to target the 3′ end region of the expected target amplicon due to its high hybridization efficiency [18], whereas the 5′ ends of the probe were labeled with a biotin tag. The probe was selected using several criteria, including its complementary sequence, length, guanine-cytosine (GC) base content, and formation of hairpins or loops. The FIP was modified and labeled with a fluorescein tag at the 5′ end. The FIP was modified instead of the BIP because the capturing probe was designed based on an FIP-linked complementary strand. Consequently, the hybridized capture probe-target amplicons could be detected using anti-fluorescein-POD.

Colorimetric detection of the assay was achieved using 3,3′,5,5′-tetramethylbenzidine (TMB) as a substrate for the POD. A blue color assay was produced when TMB was oxidized, which typically occurs as a result of the production of oxygen radicals by hydrolysis of hydrogen peroxide by POD. Subsequently, the assay passed through a green stage, and finally it became yellow. The green color resulted from its partial conversion from a blue to a yellow product. The assay color changed to yellow with the addition of equimolar hydrogen peroxide.

There are several previous studies that have applied colorimetric detection for LAMP assay that targeting *Leptospira* sp. Lin, Chen, Lu, Yan and Yan [19] has described the application of SYBR Green I-based LAMP for the detection of pathogenic *Leptospira*. SYBR Green is a fluorescent double-stranded DNA-specific intercalating dye. Fluorescence is enhanced through association with or dissociation from DNA. Thus, positive LAMP result will fluoresce under UV light. In another study, Chen, Weissenberger, Atkins, Chao, Suputtamongkol and Ching [20] applied hydroxy naphthol blue (HNB) for detection *Leptospira* LAMP products. HNB was used as a metal indicator for calcium and a colorimetric reagent for alkaline earth metal ions [6]. The positive signal is indicated by violet color of the assay turns to sky blue color. Those colorimetric detection methods are useful in the point-of-care setting. However, the developed colorimetric magnetogenosensor assay in this study increases the specificity of the detection, by including the specific probes that will only hybridized to the accurate amplified target. Thus, false positive result during LAMP amplification can be screened when using the developed colorimetric magnetogenosensor assay.

A high concentration of salt, 0.15 M NaCl in buffer, was used to minimize the electrostatic repulsion between the probe-coated beads. Despite the reduction of electrostatic repulsion, the target strands were easily hybridized with the probes, and they enhanced the hybridization efficiency. The hybridization efficiency of the capture probes and target amplicons was also facilitated using a solution with an optimum pH (pH 7.4). A decrease in surface steric hindrance can be attained by avoiding an extreme pH in the solution, below pH 4.5 or above pH 8.5, as reported by Zhang, Lang, Yoshikawa and Gerber [21].

3.2. Performance of the biosensor

The results of the analytical specificity test are shown in Table 2. The biosensor assay was positive when tested with all pathogenic *Leptospira* strains ($N = 31$), by which the brown color of the assay was turned to blue after being tested. However, the biosensor assay gave a negative result by maintaining its brown color when tested with intermediate *Leptospira* strains ($N = 43$), non-pathogenic *Leptospira* strains ($N = 38$), and other microorganisms ($N = 60$). The representative analytical specificity results are shown in Fig. 2 and reveal that the designed probe that coated the magnetic beads was specific to the intended target. The probe could clearly discriminate between negative and positive samples. The assay also showed 100% specific when being tested with environmental isolates (Table 3). The sensitivity of the biosensor was compared with gel electrophoresis detection, and the result showed the equal sensitivity of both methods (Fig. 3). The sensitivity of both detection methods was 200 fg/μl of target DNA, which was equivalent to 80 genomes/reaction. Hence, the present method can be applied to real sample diagnosis since its sensitivity enabled it to detect the estimated density of *Leptospira* in blood samples, which is $8.0–3.9 \times 10^4$ leptospires/ml in blood [17].

Due to the high sensitivity of the LAMP method, the Loopamp DNA Amplification Kit manufacturer (Eiken Chemical) has reported that carryover contamination of LAMP is quite significant, and opening the post-LAMP tube should be prevented. As the use of the biosensor developed in this study involves opening the post-LAMP tube mixture, we highly recommend taking extra precaution during its handling. Contamination can be prevented by performing the detection step in

Table 2
Analytical specificity of LAMP-colorimetric magnetogenosensor assay.

Strains	Serogroup or species	No. of isolates	Analytical specificity of LAMP-colorimetric magnetogenosensor assay
Pathogenic *Leptospira* (n = 18)	*L. interrogans* serovar Canicola	1	+
	L. interrogans serovar Pomona	2	+
	L. interrogans serovar Australis	1	+
	L. interrogans serovar Bataviae	2	+
	L. interrogans serovar Pyrogenes	2	+
	L. interrogans serovar Icterohaemorrhagiae RGA	1	+
	L. interrogans serovar Hebdamadis	1	+
	L. borgpetersenii serovar Ballum	1	+
	L. borgpetersenii Celledoni	1	+
	L. interrogans serovar Autumnalis	1	+
	L. interrogans serovar Tarassovi	1	+
	L. interrogans serovar Copenhageni	1	+
	L. interrogans serovar Javanica	1	+
	L. interrogans serovar Djasiman	1	+
	L. interrogans serovar Hardjoprajitno	1	+
Intermediate *Leptospira* (n = 5)	*L. fainei* serovar Hurtsbridge	2	–
	L. licerasiae serovar Varillal	2	–
	L. wolfii	1	–
			–
Non-pathogenic *Leptospira* (n = 4)	*L. biflexa* serovar Patoc	2	–
	L. meyeri serovar Semaranga	2	–
Other pathogens (n = 53)	*Acinetobacter baumanii*	1	–
	Aeromonas hydrophilia	1	–
	Bacillus subtilis	1	–
	Burkholderia cepacia	1	–
	Burkholderia pseudomallei	1	–
	Burkholderia thailandesis	1	–
	Citrobacter freundii	1	–
	Entamoeba dispar	1	–
	Entamoeba histolytica	1	–
	Entamoeba moshkovskii	1	–
	Enterococcus avium (LMG 10744)	1	–
	Enterococcus casseliflavus (LMG 10744)	1	–
	Enterococcus faecalis (LMG 17122)	1	–
	Enterococcus faecalis (LMG 8222 & LMG 16216)	2	–
	Enterococcus faecium (LMG 16200)	1	–
	Enterococcus faecium (LMG 16192)	1	–
	Enterococcus gallinarum	1	–
	Enterococcus hirae (LMG 6399)	1	–
	Enterohaemorrhagic *E. coli* (EHEC)	1	–
	Enteroinvasive *E. coli* (EIEC)	1	–
	Enteropathogenic *E. coli* (EPEC)	1	–
	Enterotoxigenic *E. coli* (ETEC)	1	–
	Group A *Streptococcus*	1	–
	Group B *Streptococcus*	1	–
	Group F *Streptococcus*	1	–
	Group G *Streptococcus*	1	–
	Haemophilus influenza	1	–
	Klebsiella pneumoniae	1	–
	Listeria monocytes	1	–
	Proteus mirabilis	1	–
	Proteus vulgaris	1	–
	Providencia stuartii	1	–
	Salmonella Paratyphi A	1	–
	Salmonella Paratyphi B	1	–
	Salmonella Paratyphi C	1	–
	Salmonella Typhi	1	–
	Serratia marcescens	1	–
	Shigella boydii	1	–
	Shigella dysentery	1	–
	Shigella flexneri	1	–
	Shigella sonnei	1	–
	Staphylococcus aureus	1	–
	Staphylococcus epidermidis	1	–

(*continued on next page*)

Table 2 (*continued*)

Strains	Serogroup or species	No. of isolates	Analytical specificity of LAMP-colorimetric magnetogenosensor assay
	Vibrio cholerae non-O1/non-O139	1	–
	Vibrio cholerae O1	2	–
	Vibrio cincinnatiensis	1	–
	Vibrio fluvialis	1	–
	Vibrio mimicus	1	–
	Vibrio parahaemolyticus	1	–
	Vibrio vulnificus	1	–
	Yersenia enterocolitica	1	–
Total		80	

+, positive result; —, negative result.

separate rooms, using discrete equipment.

1, 20 ng/μl; 2, 2 ng/μl; 3, 200 pg/μl; 4, 20 pg/μl; 5, 2 pg/μl; 6, 200 fg/μl; 7, 20 fg/μl; 8, 2 fg/μl; 9, 200 ag/μl; 20 ag/μl; 10, ag/μl; 11, ag/μl, M, DNA ladder marker; N, Non-target genomic DNA.

4. Conclusions

We developed a rapid and specific assay for detection of LAMP amplicons by employing sequence-specific DNA probe capture. The high specificity, simplicity, and rapidity of the LAMP method, together with the application of a portable magnetic separator, allow this assay to serve as a method for point-of-care nucleic acid testing. The utilization of magnetic beads in the LAMP method increases the specificity of the detection for various intended target DNA, particularly in pathogen identification and clinical diagnosis. Furthermore, the assay developed in this study offers an advantage in the simplicity of discriminating between positive and negative results.

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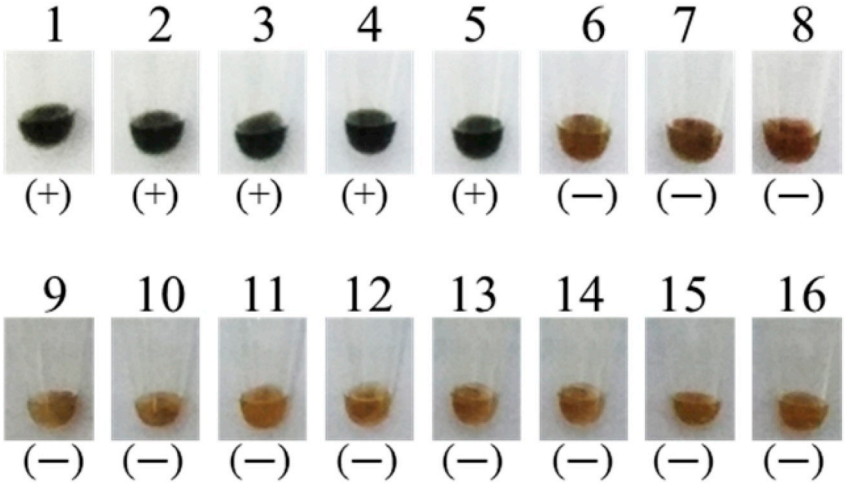

Fig. 2. Representative data for analytical specificity of LAMP-colorimetric magnetogenosensor assay. 1, *L. interrogans* serovar Australis; 2, *L. interrogans* serovar Bataviae; 3, *L. interrogans* serovar Pyrogenes; 4, *L. interrogans* serovar Icterohaemorhagiae RGA; 5, *L. interrogans* serovar Hebdomadis; 6, *L. fainei* serovar Hurtsbridge; 7, *L. licerasiae* serovar Varillal; 8, *L.biflexa* serovar Patoc; 9, *Staphylococcus aureus*; 10, *Enterococcus faecalis*; 11, *Bacillus subtilis*; 12, *Staphylococcus epidermis*; 13, *Enterococcus faecium*; 14, *Salmonella* Typhi; 15, *Klebsiella pneumonia*; 16, *Shigella flexneri*. Minus sign (−) denotes negative result while plus sign (+) denotes positive result.

Table 3
Analytical specificity of LAMP-colorimetric magnetogenosensor assay compared with 16S rRNA PCR method using environmental isolates.

Strains	Species	No. of isolates	Analytical performance of LAMP-colorimetric magnetogenosensor assay	16S rRNA PCR method
Pathogenic *Leptospira* (n = 13)	*L. kmetyi*	13	+	+
Intermediate *Leptospira* (n = 38)	*L. fainei*	2	–	–
	L. licerasiae	3	–	–
	L. wolfii	31	–	–
	L. inadai	2	–	–
Non-pathogenic *Leptospira* (n = 34)	*L. meyeri*	33	–	–
	L. idonii	1	–	–
Non-*Leptospira* spirochaetes	*Leptonema illini*	7	–	–
Total		**92**		

+, positive result; —, negative result.

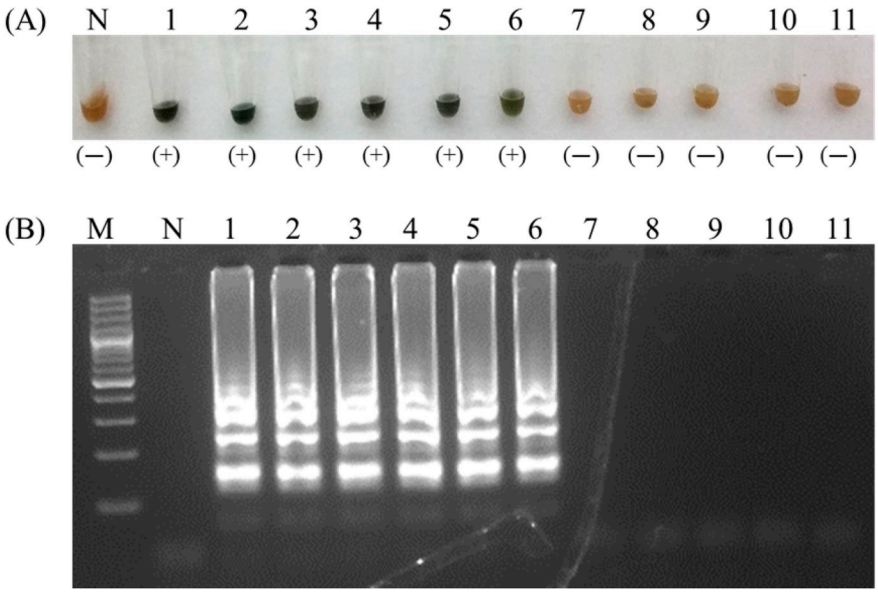

Fig. 3. Comparison of analytical sensitivity results by using two different LAMP detection methods. (A) Colorimetric detection by using magnetogenosensor assay. (B) 2% gel electrophoresis detection.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mcp.2019.03.001.

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