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Rapid and sensitive detection of *Nosema bombycis* using loop-mediated isothermal amplification and colorimetric nanogold

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ABSTRACT: Salt-induced self-aggregation of gold nanoparticles (AuNP) carrying the ssDNA probes can be prevented specifically by complementary DNA. Loop-mediated isothermal amplification (LAMP) can amplify DNA rapidly. In the present study, we established a LAMP-AuNP method for detection of the microsporidium *Nosema bombycis* by combining the two techniques. The DNA templates of microsporidia were extracted by FTA card. The amplified product can be visually detected via hybridization at 63 °C for 5 min with a ssDNA-labelled nanogold probe, followed by salt-induced AuNP aggregation. The LAMP-AuNP assay needs approximately 65 min. This method can detect 10 spores/ml and is specific for *N. bombycis*. When it was used to detect pebrine disease of the silkworm eggs, the maximum sample size for silkworm eggs was 700, in which only one egg was infected with *N. bombycis*. The new LAMP-AuNP is a simple, sensitive and specific detection assay for *N. bombycis*.

KEYWORDS: FTA card, gold nanoparticle, LAMP, microsporidia, silkworm eggs

INTRODUCTION

Microsporidia are a group of obligate intracellular eukaryotes that infect almost all of vertebrates and invertebrates[1–3]. Silkworm pebrine disease is a devastating disease in sericulture. *N. bombycis* cannot only parasitize in the silkworm larva, pupa, and moth, but can also be transmitted to the next generation through the silkworm eggs, which leads to a decline in the yield and quality of cocoons, and causes great losses in sericulture[4]. Because it is a kind of infectious disease and can be transmitted to the progeny through the eggs, silkworm pebrine disease is listed as the only inspection and quarantine target in silkworm eggs production.

Spot check of the female silkworm moths is the most important part of the pebrine inspection work, but its accuracy is affected by many factors, which may cause unqualified silkworm eggs to flow into the market and would make the users suffer unnecessary economic losses[4,5]. Many methods such as identification by naked-eyes, microscopic examination, serological and molecular biology detection techniques, were established to detect the *N. bombycis*[6–8]. To improve the detection of *N. bombycis*, China also has introduced the eggs testing for silkworm pebrine disease as a reserved inspection project[4]. However, the inspection for pebrine disease of silkworm eggs under microscopy is a time-consuming, labor-intensive work due to the huge amount of silkworm eggs. Molecular biology diagnostic methods with high specificity and sensitivity is needed urgently for silkworm pebrine disease[9,10]. To apply molecular biology detection techniques for inspection of *N. bombycis* of silkworm eggs, it is necessary to establish a simple and sensitive method. Loop-mediated isothermal amplification (LAMP) was established by Japanese scholar Tsugunori Notomi, it can detect only a few copies of target nucleic acid under isothermal conditions utilizing self-recurring strand-displacement DNA synthesis initiated by specially designed primer sets[11]. This technology has been widely used for the detection of pathogenic microorganisms[12,13].

LAMP products could be detected by agarose gel electrophoresis and may also be assessed indirectly by the amount of white magnesium pyrophosphate precipitate or fluorescence formed[14,15].

Gold nanoparticles (AuNP) have been used widely as tools to detect target nucleic acid sequence of pathogenic microorganisms[16,17]. It has been reported that salt-induced self-aggregation of AuNP carrying the ssDNA probes can be prevented specifically by complementary DNA[18]. To date, method combining AuNP with LAMP has not been used for *N. bombycis* detection. In this study, we established a rapid, simple, efficient method for detection of *N. bombycis* by using glass beads and FTA card to extract the DNA, performing DNA amplification by LAMP and visualizing amplification products by the AuNP colorimetric assay.

MATERIALS AND METHODS

Microsporidia, bacteria, fungi and virus

The *N. bombycis*, *Enterococcus*, *Beauveria bassiana*, *Bombyx mori* Nuclear Polyhedrosis Virus (BmNPV) and *B. mori* Cytoplasmic Polyhedrosis Virus (BmCPV) were provided by the pathology laboratory of the Sericultural Research Institute of Chinese Academy of Agricultural Sciences.

Silkworm eggs

Silkworm eggs for microsporidian inspection which had been tested as pebrine positive under microscope were provided by the pathology laboratory of the Sericultural Research Institute of Chinese Academy of Agricultural Sciences.

Extraction of microsporidian DNA

Purified microsporidian spores or silkworm eggs were placed in a 2 ml tube, and glass beads (Sigma Aldrich (Shanghai) Trading Co., Ltd., China) (1/2 of the volume of the tube) and 1 ml TE (10 mM tris-HCl and 1 mM EDTA, pH 8.0) buffer were added into the tube[4]. The sample was crushed by a Mini-Bead Beater (BioSpec, USA) at 4800*g* for 5 min with glass beads and buffer, and 100 µl of the resulting solution of broken spores was added to the FTA card (Whatman, UK). The FTA card was dried at room temperature in dark, and a small round hole (diameter of 2 mm) on the FTA card was clipped. The card pieces were washed three times with a special FTA buffer and then washed twice with TE-1 (10 mM tris-HCl and 0.1 mM EDTA, pH 8.0) buffer. After drying at 56 °C, the FTA card pieces could be used as DNA templates for PCR and LAMP reactions.

Primers and LAMP assay optimization

LAMP primers were designed by using the PRIMER EXPLORER 4 (Eiken Chemical, Tokyo) according to the small subunit ribosomal RNA (SSU rRNA) gene of *N. bombycis* (GenBank Accession No. AY259631). The details of the primers are listed in Table 1. For optimization of the temperature and time conditions, LAMP reactions were carried out at 60, 63, or 65 °C for 45 or 60 min, respectively. LAMP reactions were performed in 25 µl of total reaction mixture containing 1.6 µM each forward inner primer (FIP) and backward inner primer (BIP), 0.2 µM each forward outer primer (F3) and backward outer primer (B3), 1.4 mM of dNTP mix (Sangon Biotech, Shanghai, China), 6 mM $MgSO_4$, 0.4 M betaine (USB Corporation, OH, USA), 8 U *Bst* 2.0 WarmStart DNA polymerase (large fragment; New England Biolabs Ltd., Beijing, China), and 1x thermopol-supplied reaction buffer. LAMP products were analysed by 2% agarose gel electrophoresis.

PCR primers and reaction conditions

PCR primers were designed according to the SSU rRNA gene of *N. bombycis*: forward primer, 5′-TCCAATGGATGCTGTGAA-3′; reverse primer: 5′-AAGAACAGGGACTCATTCA-3′. The amplification was performed using the following conditions: initial DNA denaturation at 98 °C for 2 min, 30 cycles of 98 °C for 10 s, 50 °C for 15 s, and 72 °C for 20 s.

Preparation of gold nanoparticles (AuNP)

Before AuNP preparation, all glasswares were treated with chromic acid. An AuNP colloid comprised of approximately 15-nm-diameter particles was prepared[18]. Briefly, 100 ml of 0.01% $HAuCl_4$ solution (in Milli-Q purified water) in a 500 ml conical beaker was boiled thoroughly, and then 3.5 ml of 1% trisodium citrate solution was added under constant stirring. After the color of the solution changed to red about 45 s, it was boiled for another 5 min, then the heating source was removed, and the colloidal gold solution was stirred for another 10 min. The solution was stored in a dark bottle at 4 °C.

Gold nanoparticles (AuNP) probe preparation

A monosense ssDNA probe for the AuNP-based hybridization step was designed to the sequence spanned by the F1c–B1c region of the LAMP amplicon and labelled with a thiol group at the 5′ end (Table 1). The ssDNA-labelled AuNP probe was prepared by modification of previous methods[16,17].

Table 1 Primers used for loop-mediated isothermal amplification-AuNP assay for the detection of *N. bombycis*.

Primers	Position	Sequence (5′–3′)
F3	858–875	TCCAATGGATGCTGTGAA
B3	1085–1103	AAGAACAGGGACTCATTCA
FIP	934–958/884–906	GACCTGTTTTAATCCTCTCCTTCAT–TAATTTCAACAAGATGTGAGACC
BIP	982–1004/1042–1064	GTTGCACGCGCAATACAATAATA–TTTACTAGCAATTCCATGTTCAA
N. bombycis thiol probe	SH–A10/960–980	SH–AAAAAAAAAA–GTTATGCCCTAAGATAATCTG

In brief, 2 nM (20 µl of 100 µM stock) ssDNA probe was added to 5 ml colloidal AuNP (15 nm diameter) and incubated at 50 °C with shaking at 150*g* for 24 h. Then, 500 µl of the buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl and 15 mM $MgCl_2$) was gradually added to the mixture followed by further incubation at 50 °C with shaking at 150*g* for 24 h. After centrifugation at 12 000*g* at 4 °C for 45 min, the pellet was washed with 500 µl wash buffer (10 mM Tris-HCl, 50 mM KCl and 1.5 mM $MgCl_2$) before resuspended in 500 µl of wash buffer for storage at 4 °C.

Optimization of the AuNP probe hybridization for detection of LAMP amplicons

The hybridization for the detection of LAMP amplicons was conducted in a total volume of 15 µl by mixing the LAMP amplicon solution and AuNP probe at different ratios (7:3–3:7 µl) at 63 °C for 5 min. Following addition of 5 µl 30 mM $MgSO_4$ solution, the mixtures were examined visually for any color change. As the salt concentration induced aggregation, the effect of different $MgSO_4$ concentrations on AuNP probe was further tested.

Specificity of LAMP-AuNP test

Specificity was tested using DNA extracted from *N. bombycis*, bacteria, fungi and virus. The resulting DNA products were visualized by the AuNP colorimetric assay and agarose gel electrophoresis (AGE). We also used AuNP probe to detect 0.5 nM noncomplementary DNA (ACTGCGCGTGGCAAATGCGGA).

Comparison of sensitivity between LAMP-AuNP and PCR assays

The sensitivity of the two assays was determined using DNA templates extracted from different spore concentrations (10–10^4 spores/ml). The resulting DNA products visualized by the AuNP colorimetric assay (analysed by naked-eyes observation) and AGE were compared.

Evaluation of LAMP-AuNP assay with infected silkworm eggs

Different numbers of uninfected silkworm eggs (99, 199, 299, 399, 499, 599, 699, 799, 899, and 999) mixed with one infected egg were immersed in 30% KOH at 37 °C for 10 min, rinsed twice with ddH_2O and once with 1 mol/l HCl, washed twice with ddH_2O again, and sufficiently broken with a sterile tooth pick, respectively. Then 1 ml of TE buffer was added to the mixture of eggs. The mixed eggs were then treated according to steps delineated in extraction of microsporidian DNA section to extract the DNA. The samples were analyzed using LAMP-AuNP and PCR assays as described above and the results were compared.

RESULTS

LAMP conditions and LAMP primer specificity

LAMP reactions at 60, 63, and 65 °C using 500 pg of *N. bombycis* DNA template revealed that 63 °C yielded the highest amounts of DNA products (Fig. 1a). For LAMP reactions performed at 63 °C for 45 and 60 min, DNA quantity at 60 min was higher (Fig. 1b). A reaction time of 60 min was thus selected as optimal. For primer specificity testing at these optimal conditions, reactions were performed using DNA from other important pathogens of silkworm, however, no cross-reactions displayed (Fig. 1c), indicating that the primers were highly specific to amplify *N. bombycis*.

Effects of the ratios of AuNP probe to LAMP DNA product

AuNP probe was hybridized in different ratios (7:3–3:7 µl) to LAMP DNA products amplified from *N. bombycis* DNA and BmNPV DNA (negative control). Adding 5 µl of 30 mM $MgSO_4$ to the mixture with ratios of AuNP probe to LAMP product from 7:3–3:7 µl resulted in significant difference of color between positive samples (red) and negative control (blue) (Fig. 2a). And we also found that the color difference between positive and negative in the

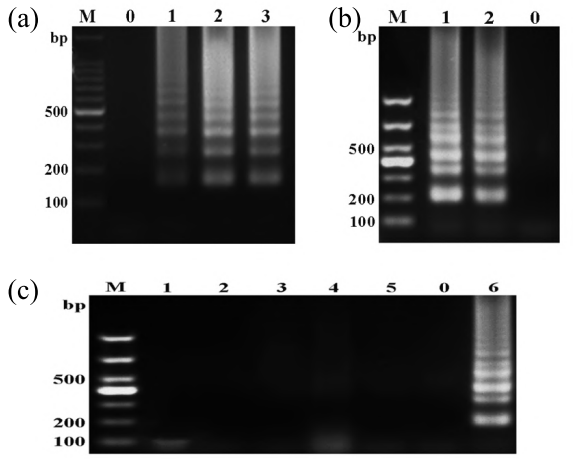

Fig. 1 Optimization of the loop-mediated isothermal amplification (LAMP) assay for the detection of *N. bombycis*. (a) Optimization of temperature for LAMP reactions using 500 pg total DNA extracted from *N. bombycis*. Lane M: 100 bp DNA ladder marker. Lane 0: negative control, lanes 1–3: LAMP amplification from reactions carried out at 60, 63, and 65 °C, respectively. (b) Optimization of LAMP reactions for various time. Lane M: 1000 bp DNA marker, lane 0: negative control, lanes 1–2: the reaction was carried out for 60 and 45 min, respectively. (c) Evaluation of LAMP primer specificity using nucleic acid templates extracted from different pathogenic microorganism and *B. mori*. Lane M: 1000 bp DNA marker, lane 0: negative control, lanes 1–6: *Enterococcus*, *B. bassiana*, *B. mori* Nuclear Polyhedrosis virus (BmNPV), *B. mori* Cytoplasmic Polyhedrosis virus (BmCPV), *B. mori*, and *N. bombycis*.

group of 5:5 µl ratio was obvious and steady, so the 5:5 µl ratio was chosen for subsequent experiments.

Effect of salt concentration

The effect of salt concentration on aggregation was tested by addition of 5 µl $MgSO_4$ at different concentrations (20, 30, and 40 mM). Color changes were compared between LAMP amplicons from *N. bombycis* target DNA and noncomplementary target DNA (Fig. 2b). Addition of 20 mM $MgSO_4$ to mixtures containing non-target DNA did not give the change from red to blue, and addition of 30 mM or 40 mM $MgSO_4$ to mixtures containing *N. bombycis* amplicons remained red or purple red, whereas addition of 30 mM $MgSO_4$ or 40 mM $MgSO_4$ to mixtures containing non-target DNA make the color change from red to blue. Based on these data, the 30 mM salt concentration was chosen in this study.

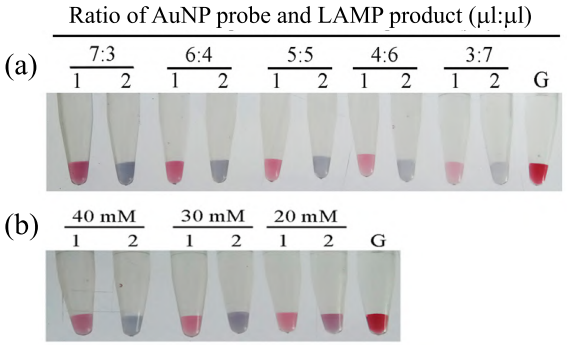

Fig. 2 Optimization of the AuNP hybridization assay. (a) Effect of ratios of AuNP probe to LAMP amplification. Tubes containing either (1) *N. bombycis*-LAMP amplification or (2) BmNPV-LAMP amplification (noncomplementary DNA) were mixed with the probe at ratios of 7:3–3:7 µl followed by addition of 5 µl of 30 mM $MgSO_4$. The positive control (G) consisted of untreated AuNP probe. (b) Effect of salt concentration. Three sets of tubes containing 5 µl of (1) *N. bombycis*-LAMP amplification or (2) BmNPV-LAMP amplification (noncomplementary DNA) were mixed with 5 µl of AuNP probe solution followed by addition of $MgSO_4$ (5 µl fixed volume) at concentrations between 20 and 40 mmol/l. The positive control (G) consisted of untreated AuNP probe.

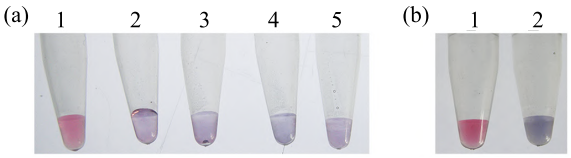

Fig. 3 Colorimetric results for the specificity of LAMP-AuNP detection of *N. bombycis*. (a) LAMP-AuNP assay by naked-eyes using the DNA template extracted from different pathogenic microorganism. Lanes 1–5: *N. bombycis*, *Enterococcus*, *B. bassiana*, BmNPV, and BmCPV, respectively. (b) Hybridization between the AuNP probe and nucleotide sequence. Lane 1: *N. bombycis*-LAMP amplification, lane 2: 0.5 nM noncomplementary DNA.

Specificity of LAMP-AuNP detection

AGE analysis of LAMP DNA amplified from *N. bombycis*, bacteria, fungi, virus is consistent with the results of LAMP-AuNP assay (Figs. 1c, 3a), indicating that the LAMP-AuNP assay was specific to *N. bombycis*. Using 0.5 nM noncomplementary DNA for AuNP hybridization assay, the color of its mixture changed from red to blue (Fig. 3b), suggesting that LAMP-AuNP assay can distinguish target DNA and noncomplementary DNA.

Comparison sensitivity between LAMP-AuNP and PCR assay

The LAMP-AuNP method was able to detect 10 spores/ml, and this result was consistent with the sensitivity of LAMP followed by gel electrophoresis (Fig. 4ab). The PCR assay can detect the minimum spore concentration of 102 spores/ml, and the PCR amplification product of 10 spores/ml is too low to be detected easily (Fig. 4e).

Detection of silkworm eggs infected with *N. bombycis* using LAMP-AuNP

Different numbers of silkworm eggs (100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000), in which only one egg is infected with *N. bombycis*, were tested by LAMP-AuNP and PCR assays, respectively. AGE analysis of LAMP DNA amplified from different samples is consistent with the results of LAMP-AuNP assay. The maximum sample size for LAMP-AuNP is 700 silkworm eggs (Fig. 4cd), while that for PCR assay is 200 silkworm eggs (Fig. 4f).

DISCUSSION

LAMP and PCR have been widely used in the detection of microsporidia[4,17,19]. However, it is difficult to apply PCR or LAMP techniques for inspection of the silkworm pebrine disease of silkworm eggs. According to previous studies[19,20], Liu et al found that there may be some inhibitory factor in silkworm eggs that interfered with PCR amplification of *N. bombycis* DNA. In this study, we used glass beads and FTA card to extract DNA, with which proteins and other impurities in the samples can be removed by simple operations on FTA card[4,21]. The recovery of genomic DNA is an important factor affecting the sensitivity of *N. bombycis* detection. The DNA recovery efficiency of the conventional method[22] to extract genomic DNA from microsporidia is too low. In contrast, treatment with acid-washed glass beads can extract most of microsporidian nucleic acids because the broken rate of spores can reach 90%[23]. In this study, the maximum sample size of LAMP-AuNP is 700 silkworm eggs, in which only one egg was infected with *N. bombycis*, while that of PCR assay is 200 silkworm eggs.

Using agarose gel electrophoresis to visualize the amplicons from LAMP is not convenient for workers in silkworm eggs producing farm. Due to their ability to interact with disulfide modified DNA and the unique optical properties, AuNP show great promise for use in rapid field diagnosis methods[17,24]. Because LAMP DNA products carry abundant negative charges on their phosphate backbones, interaction with the AuNP probe particles would increase the negative charges of the amplicon-probe complexes, thus enhancing electrostatic and steric repulsion between amplicons[16]. LAMP and AuNP hybridization assay have been used in detection of shrimp yellow head virus and *Enterocytozoon hepatopenaei*[16,17]. However, whether the LAMP-AuNP method could be used to detect *N. bombycis* in silkworm eggs, each test condition should be optimized separately.

In this study, 63 °C for 60 min was found as optimal for LAMP with high sensitivity and specificity. By using *Bst* 2.0 WarmStart DNA polymerase, the reaction of LAMP could be performed at room temperature which makes the LAMP assay more convenient. For AuNP hybridization step, we found that the ratio between LAMP amplicons and AuNP probe was essential for valid detection of target DNA. Too little or too much of LAMP amplicons or AuNP probe could result in a false negative result so the optimal ratio was set at 5:5 µl since it provided reproducible color differences between positive and negative test results (Fig. 2a). Salt-induced self-aggregation of AuNP carrying ssDNA probes can be prevented specifically by complementary DNA[18]. However, it was found that in the presence of LAMP amplicons, a high salt concentration could yet induce color change of AuNP probe, resulting in a false negative result, suggesting that the optimal salt concentration was critical to LAMP-AuNP method[17]. In this study, the optimal salt concentration was 30 mmol/l $MgSO_4$, because in the presence of LAMP amplicons, it was insufficient to induce AuNP self-aggregation (Fig. 2b).

Noncomplementary DNA or no DNA were used as templates (negative control) to confirm that the specific hybridization between the AuNP probe and its target DNA prevented its salt-induced aggregation (Figs. 1c, 2b, 3a). In this study, it is also found that large amounts of noncomplementary DNA could not prevent the salt-induced aggregation of AuNP probes (Fig. 3b).

In conclusion, AuNP have unique optical properties that make them suitable for the design of labelled AuNP probes. Salt-induced self-aggregation of gold nanoparticles carrying unisense ssDNA probes can be prevented specifically by complementary DNA. They offer the advantages of low cost and visual detection over most conventional methods of detection such as those used in fluorescence or radioactivity-based assays. LAMP can amplify DNA rapidly. The combined LAMP-AuNP method re-

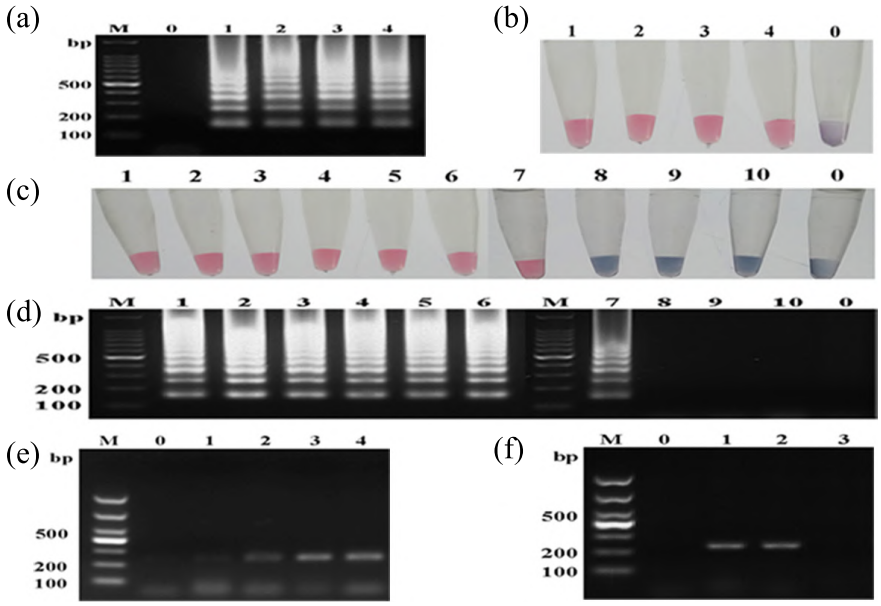

Fig. 4 Detection of *N. bombycis* using LAMP-AuNP or PCR. (a) The sensitivity of LAMP for the detection of *N. bombycis* spores/ml. Lane M: 100 bp DNA ladder marker, lane 0: negative control, lanes 1–4: the DNA template extracted from 104, 103, 102, and 10 spores/ml, respectively. (b) LAMP-AuNP assay by naked-eyes using the same template sets as in (a). lane 0: negative control, lanes 1–4: 104, 103, 102, and 10 spores/ml, respectively. (c) LAMP-AuNP results of LAMP reactions performed using the DNA templates extracted from different numbers of silkworm eggs. Lane 0: negative control, lanes 1–10: different numbers of silkworm eggs, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, respectively. (d) AGE results of LAMP reactions performed using the same template sets as in (c). Lane 0: negative control, lanes 1–10: different numbers of silkworm eggs, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, respectively. (e) The sensitivity of PCR for the detection of *N. bombycis* spores/ml. Lane M: 1000 bp DNA marker, lane 0: negative control, lanes 1–4: the DNA template extracted from 101, 102, 103, and 104 spores/ml, respectively. (f) The sensitivity of PCR for the detection of different numbers of silkworm eggs. Lane M: 1000 bp DNA marker, lane 0: negative control, lanes 1–3: different numbers of silkworm eggs, 100, 200, and 300, respectively.

quired 60 min for LAMP and 5 min for hybridization of LAMP products to an AuNP-labelled ssDNA probe followed by salt induced probe-particle aggregation to visualize color development. Such method employs simple, inexpensive equipment and involves simple steps, making it applicable for the detection of *N. bombycis* in small laboratories. More importantly, it serves as a model platform that could be adapted easily to detect other microsporidia using simple equipment.

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