Loop Mediated Isothermal Amplification (LAMP) for *Nosema bombycis* diagnosis by *Small subunit Ribosomal RNA (SSU rRNA)* gene

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**ABSTRACT**

In present study, Loop Mediated Isothermal Amplification (LAMP) assay was conducted for diagnosis *Nosema bombycis*. Nine isolates of *N. bombycis* were collected from infected silkworms in rearing areas in Khon Kaen province, Thailand. *N. bombycis* genomic DNAs were extracted by boiling method and used as templates in LAMP and PCR reactions. A LAMP primer set was designed specific to *N. bombycis small subunit ribosomal RNA (SSU rRNA)* gene. The results revealed that the optimal condition was constantly performed at 63°C for 1 hour. The product was directly visualized by naked eye and confirmed with agarose gel electrophoresis. LAMP assay is more sensitive than traditional PCR, since LAMP was able to detect the least 10 spores/ml while PCR needs 100 spores/ml. In addition, the present novel LAMP primer set was specific only to *N. bombycis* proven by the negative results when other *B. mori* pathogen DNAs were tested. In conclusion, the LAMP assay demonstrated a great potential alternative method in diagnosis *N. bombycis* with high sensitivity, rapidity and accuracy which can apply for pebrine disease surveillance.

**Key words:** LAMP, Microsporidia, Mulberry silkworm, Pebrine disease.

**INTRODUCTION**

Mulberry silkworm (*Bombyx mori*) is an economically important insect as it has been used for silk industry or sericulture which is the major factor in economic growth and alleviates rural poverty in several countries (Singh *et al.*, 2010; Hema *et al.*, 2011). Diseases of silkworm are one of the major factors causing the reduction of sericulture production (Sowmyashree *et al.*, 2013). Pebrine or microsporidiosis is the most serious disease in mulberry silkworm (*Bombyx mori*) caused by an obligate intracellular microsporidium of species *Nosema bombycis*. Pebrine disease was first reported in France in 1845 and later spread to several other countries worldwide for instance Spain, Syria, Romania and India (Bhat *et al.*, 2017). This disease is directly responsible for the drop of the yield and quality of cocoons. Pebrine disease is transmitted via both horizontal and vertical or transovarial transmissions. To control the disease, pebrine inspection, called “mother examination” method, has been in practice since the 1880s. After egg-laying, either fresh or dry mother moths are inspected for the presence of microsporidian spore under light microscope (Pasteur, 1870; Govindan *et al.*, 1997). However, this method is time-consuming and requires skilled technicians. Therefore, several alternative methods have been developed for *N. bombycis* detection. Molecular based techniques have been also developed such as DNA hybridization, conventional PCR, multi-primer PCR and real time quantitative PCR (Malone and McIvor, 1995; Kawakami *et al.*, 2001; Hatakeyama and Hayasaka, 2003; Fu *et al.*, 2016). Nevertheless, these techniques require sophisticated laboratory instruments and/or complex analysis procedures. Consequently, loop-mediated isothermal amplification (LAMP) was developed to address these problems (Notomi *et al.*, 2000). More than 180 reports using LAMP method have been published since the assay was originally developed (Mori and Notomi, 2009). LAMP assay was also applied for silkworm pathogens including *N. bombycis*. Wei *et al.* (2014) developed the combination of glass beads, FTA card and LAMP for *N. bombycis* detection using a primer set based on *large subunit ribosomal RNA (LSU-rRNA)* gene. They were able to detect the spores of *N. bombycis* the rate of one infected egg per 500 silkworm eggs. Meanwhile, Liu *et al.* (2015) indicated that the LAMP assay based on *end-binding protein 1 (EB1)* gene effectively detected the silkworm egg infected with *N. bombycis* with a low DNA concentration of 5 x 10^3 ng/µl. Recently, *polar tube protein 1* gene was also used in LAMP to diagnose microsporidiosis during various stages of silkworm development (Esvaran *et al.*, 2018). For our study, we selected *small subunit ribosomal RNA (SSU rRNA)* of *N. bombycis* (Genbank Accession No. AK259631) as the target gene for pebrine disease diagnosis by LAMP assay, since this gene is highly conserved within species.

**MATERIALS AND METHODS**

Microsporidian spore collection and preparation: The pebrine infection was surveyed in 62 silkworm rearing...
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Villages of 21 districts in Khon Kaen Province, Thailand. Nine isolates of microsporidia (NKK1-NKK9) were obtained. The microsporidian spores were partially purified by grinding of infected silkworms in distilled water (modified from Kawarabata and Ishihara, 1984). The spore suspension was filtered through several layers of cheesecloth. Then, the filtrate was centrifuged at 5000 rpm for 10 min at room temperature. The sediment spores were washed five times with distilled water and twice with 0.85% NaCl solution by centrifugation at 5000 rpm for 10 min. The spores were stored in distilled water at 4°C until use.

Genomic DNA extraction: The microsporidian spores from each isolate were counted by hemocytometer and the final concentration of 1x10^6 spores/ml were prepared for genomic DNA extraction by boiling method protocols. Briefly, 200 μl of spore suspension was centrifuged at 12,000 rpm for 5 minutes at room temperature. The precipitate was suspended and mixed in 200 μl of lysis buffer (50 mM Tris pH 8.0, 100 mM NaCl, 0.5 mM EDTA (pH 8.0), 10% SDS). The mixture was then incubated at 95°C for 15 min in a water bath and centrifuged at 12,000 rpm for 5 min. The supernatant was transferred to new tube and 2.5x volume of absolute ethanol was added before storage at -20°C overnight. The precipitated DNA was collected and washed with 70% cold ethanol. The pellet was dried and suspended with 50 μl TE buffer. DNA concentrations and purifications were measured and evaluated by NanoDrop Spectrophotometer (Thermo Scientific, USA). The extracted DNA was adjusted to 50-100 ng/μl and stored at -20°C.

LAMP primer design: A set of LAMP primers was designed specific to N. bombycis small subunit ribosomal RNA (SSU rRNA) gene (Accession Number: AY259631) using the Primer Explorer V4 Software (Eiken Co., Ltd., Japan). Four primers of outer forward (F3), outer backward (B3), inner forward (FIP) and inner backward (BIP) were obtained as shown in Table 1. The possible cross-reaction with heterologous N. bombycis was confirmed by BLAST software program (http: www.ncbi.nlm.nih.gov/Blast) in the NCBI database.

Conventional PCR method: To verify the correct amplicons, conventional PCR was conducted using the outer primer pair (F3 and B3) in a 25 μl reaction mixture containing 1X Bst DNA polymerase buffer, 0.2 μM of each outer primer (F3 and B3), 1.4 μM of each outer primer (FIP and BIP), 8 Unit Bst DNA polymerase, 100 ng template DNA and deionized water. To determine the optimal condition, different concentrations of dNTPs (8 and 1.4 mM), MgSO_4 (4, 6 and 8 mM) and betaine (0.5, 0.7 and 1 M) were varied in the reaction mixture. LAMP amplification was performed by incubating at different temperatures of 61, 62, 63, 64, and 65°C and different durations of incubation (15, 30, 45, and 60 min). The reaction was then terminated by heating at 80°C for 4 min. LAMP products were visualized by naked eye and gel electrophoresis on 2% agarose gel.

Specificity of LAMP: The specificity of selected LAMP primers were determined by performing PCR and LAMP reactions using DNAs from other pathogens causing various diseases in silkworm which were virus: B. mori Nucleopolyhedrovirus (BmNPV), fungi: Beauveria bassiana and Aspergillus flavus and bacteria: Bacillus thuringiensis and Staphylococcus aureus. The DNA extraction from a healthy silkworm was also used as a negative control.

Sensitivity of LAMP: To determine the lowest detectable concentration of N. bombycis spores, purified spores were diluted with distilled water in 10-fold series varying from 10^(-6)-10^6 spores/ml. Then DNA extractions were used as templates in both PCR and LAMP reactions. The reaction was conducted under the previously optimized condition. The PCR and LAMP products were analyzed by 1.5% and 2% agarose gel electrophoresis, respectively.

RESULTS AND DISCUSSION

In the present study, pebrine disease was found in nine out of 21 districts (42.86%) of Khon Kaen province, Thailand. The percentage of the disease incidence occurred lower than the previous studies. In 2003, Sirimungkarat et al. (2003) found that 13 out of 14 silkworm rearing districts in Khon Kaen presented the infection corresponding to a 92.86% infection rate. Although the present result showed a lower percentage of N. bombycis infection, it indicated that the pebrine disease was still widespread in many rearing areas in Thailand. Nine isolates of N. bombycis (NKK1-NKK9) were subjected for DNA extraction by boiling method and

| Primer | Length (bases) | Sequence (5'→3') |
|--------|---------------|------------------|
| F3     | 21            | GGAAAGAATACCAAGGAGTGG |
| B3     | 21            | GGAACCTGTTTTAATCCTCTCC |
| FIP (Flc-F2) | 45         | GCCATGCACCCACATCATGATAA AA-ATTGGCGGCTTAATATTGAC |
| BIP (Ble-B2) | 47       | GTTCTTACAATGATGCTTGAAGTCTCATGTATGATCATACATCGTCT |

Table 1: The sequences of LAMP primers.
used as DNA template in PCR and LAMP assays. The outer primers (F3 and B3) of LAMP designed specific to *N. bombycis* SSU rRNA gene allowed the amplification of expected 215 bp PCR products with all DNAs extracted from nine *N. bombycis* isolates (Fig 1). The PCR products were sequenced and aligned against GenBank nucleotide database. These nucleotide sequences turned out to be similar to *N. bombycis* (Accession No. JF443668) with 99% similarity and E-value of 0.00.

**LAMP reaction optimization:** In present study, the concentrations of dNTPs, betaine and MgSO₄ were varied to investigate the optimal LAMP reactions. As so as the reaction was performed at five different incubating temperatures and three different periods. According to the patterns on agarose gel electrophoresis and turbidity detection by naked eyes, the best amplification results were obtained with the reaction composing of 0.8 mM dNTPs, 0.7 M betaine and 6 mM MgSO₄ and performing at 63 °C for 60 min (Fig 2 and 3). Therefore, the DNAs from nine *N. bombycis* isolates were conducted under this optimal condition and all positive results were presented as shown in Fig 4. Several previous studies reported that the optimal concentration of dNTPs ranged between 0.8 -1.4 mM (Curtis *et al*., 2008; Maeda *et al*., 2009; Wei *et al*., 2014). Among all factors analyzed, MgSO₄ had the greater effect on LAMP reaction since free Mg²⁺ affects DNA polymerase activity.
primer annealing and magnesium pyrophosphate formation (Mori et al., 2001; Yeh et al., 2005).

**Specificity of LAMP:** After using DNA extracted from five species of main silkworm pathogens, the positive results of single band PCR product and LAMP ladder-like pattern on agarose gels were only presented with *N. bombycis* DNA template as shown in Fig 5. In addition, a single continuum

![Fig 3](image.png)

**Fig 3:** The positive result of the optimal LAMP reaction (+) observing by the naked eye comparing to the negative control (-).

![Fig 4](image.png)

**Fig 4:** The LAMP products from all nine *N. bombycis* isolates (NKK1-NKK9) by 2% agarose gel electrophoresis (M= 100 bp DNA marker, N= negative control).

![Fig 5](image.png)

**Fig 5:** The results of specificity test of PCR (A) and LAMP (B) assays for *N. bombycis*. 1= *Nosema bombycis* 2= Nucleopolyhedrovirus 3= Beauveria bassiana 4= Aspergillus flavus 5= Bacillus thuringiensis 6= Staphylococcus aureus 7= healthy silkworm (M= 100 bp DNA marker, N= negative control)

![Fig 6](image.png)

**Fig 6:** The results of sensitivity test of PCR (A) and LAMP (B) assays *N. bombycis*. 
of fragments was observed in gel electrophoresis for the A. flavus LAMP products, the cause of which may have something to do with unspecific amplification of primer dimers or the activity of Bst DNA polymerase that undergoes linear target isothermal multimerization (Kuboki et al., 2003).

**Sensitivity of LAMP:** In order to evaluate PCR and LAMP sensitivity, DNA were extracted from tenfold series of 10⁴ to 10⁶ spores/ml of N. bombycis dilutions. Then DNAs were directly used as templates in both PCR and LAMP reactions. The minimum concentration of N. bombycis detected by PCR assay was 10² spores/ml while LAMP assay could detect the least at 10 spores/ml (Fig 6). These results indicated that LAMP was 10-folds more sensitive than PCR assay. The results agree with previous studies that the FTA card and LAMP can detect N. bombycis with the minimum concentration of 10 spores/ml (Wei et al., 2014) However, they used higher concentrations of betaine and dNTPs with 1.6M and 2.8mM respectively. Moreover, Esvaran et al. (2018) reported that LAMP assay sensitivity was 2-fold higher than conventional PCR using polar tube protein 1 gene as a target of N. bombycis diagnosis.

**CONCLUSION**

The incidence of deadly pebrine disease caused by virulent microsporidian, Nosema bombycis, still remained the problem in sericulture in Thailand and other countries. LAMP assay using the newly designed primer set based on N. bombycis Small subunit ribosomal RNA (SSU rRNA) gene was introduced for early investigation of N. bombycis isolates found in the present survey. The optimization of the reaction was performed with the reaction containing of 8 Unit Bst DNA polymerase, 0.2 µM of each outer primer, 1.4 µM of each outer primer, 0.8mM dNTPs, 0.7 M betaine and 6mM MgSO₄ at 63°C for 60 min. The reaction with the designed primers is highly specific to N. bombycis and the cause of which may have something to do with unspecific amplification of primer dimers or the activity of Bst DNA polymerase that undergoes linear target isothermal multimerization (Kuboki et al., 2003).

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