

TECHNICAL REPORT

Development of isothermal amplification assay for detection of *Nosema bombycis* infection in silkworm *Bombyx mori* targeting polar tube protein 1 gene**VG Esvaran, T Gupta, A Mohanasundaram, KM Ponnuvel****Genomics Division, Seribiotech Research Laboratory, Carmelaram – Post, Kodathi, Bangalore – 560035, INDIA**Accepted September 26, 2018***Abstract**

Microsporidiosis of the silkworm *Bombyx mori* is caused by the highly virulent parasite *Nosema bombycis* (Nageli). The infection can be deleterious due to horizontal and vertical transmission, causing heavy damage to the sericulture industry. In recent years, molecular diagnostics has revolutionized the possibility to detect diseases in terms of rapidity and simplicity, however, most of them are time consuming, require sophisticated instruments and skilled personnel. In this study, the polar tube protein 1 gene of *N. bombycis* (Indian isolate) was cloned, characterized and utilized for the development of rapid and simple loop mediated isothermal amplification assay (LAMP) for detection of microsporidiosis in silkworm *B. mori*. The LAMP reaction conditions were optimized to 65 °C for 60 min. The developed method demonstrated a higher sensitivity and the detection limit was found to be 2-fold higher than conventional PCR. This is the first report on loop mediated isothermal amplification assay that could be used to diagnose microsporidiosis at various developmental stages of the silkworm. This method serves as a robust alternative technique to conventional PCR and aids in the rapid diagnosis of *N. bombycis* infecting silkworm *B. mori*.

Key Words: microsporidiosis, *Nosema bombycis*, loop mediated isothermal amplification, polar tube protein 1**Introduction**

Sericulture is a labour intensive, important agro based industry that employs the rural poor and tribals. Silk production is the major milestone for the sericulture industry which is affected by various silkworm pathogens that has a devastating effect on silk production, leading to heavy crop loss. Among these pathogens, *Nosema bombycis* (Nageli) is a devastating microsporidian which causes microsporidiosis and is commonly known as pebrine disease in silkworm *Bombyx mori*. These microsporidians are obligate intracellular spore forming parasites that infect numerous vertebrates and invertebrates (Didier *et al.*, 1998). They infect the host through a unique invasion apparatus the polar tube, a coiled structure connected to the anterior end of the spore. On appropriate stimuli, the polar tube everts out of the spore and ejects the sporoplasm into the host cell. Three polar tube proteins have been identified so far and among them polar tube protein 1 (*PTP 1*) was predicted to

be involved in host cell adherence during penetration (Keohane *et al.*, 1998). It can be transmitted to silkworm off-springs through eggs laid by the infected mother moth by transovarial transmission. The diseased and dead larvae are also the sources of secondary contamination which spreads at a faster rate (Bhat *et al.*, 2009).

The gold standard followed for pebrine detection is mother moth examination test through a microscope, which is laborious and time consuming. A rapid and sensitive method for detection would help the rearers to escape from crop loss. Molecular diagnostics involving the detection of nucleic acid sequence or the antigen specific to the pathogen are widely used for disease identification and control (Kaya Ghosh and Weiss, 2009). Polymerase chain reaction has been used for pebrine detection using the normal conventional PCR with multiple primers targeting the eggs, and the quantitative real-time PCR for the detection of pebrine in eggs as well as hatched larvae (Hatakeyama and Hayasaka, 2003). Even though PCR has been found to be the novel diagnostic technique contributing to the success of molecular diagnostics there have been few disadvantages that need to be overcome which include time, expertise and specialized equipments. Hence, to fill these gaps in the field of molecular diagnostics a novel and new method was developed

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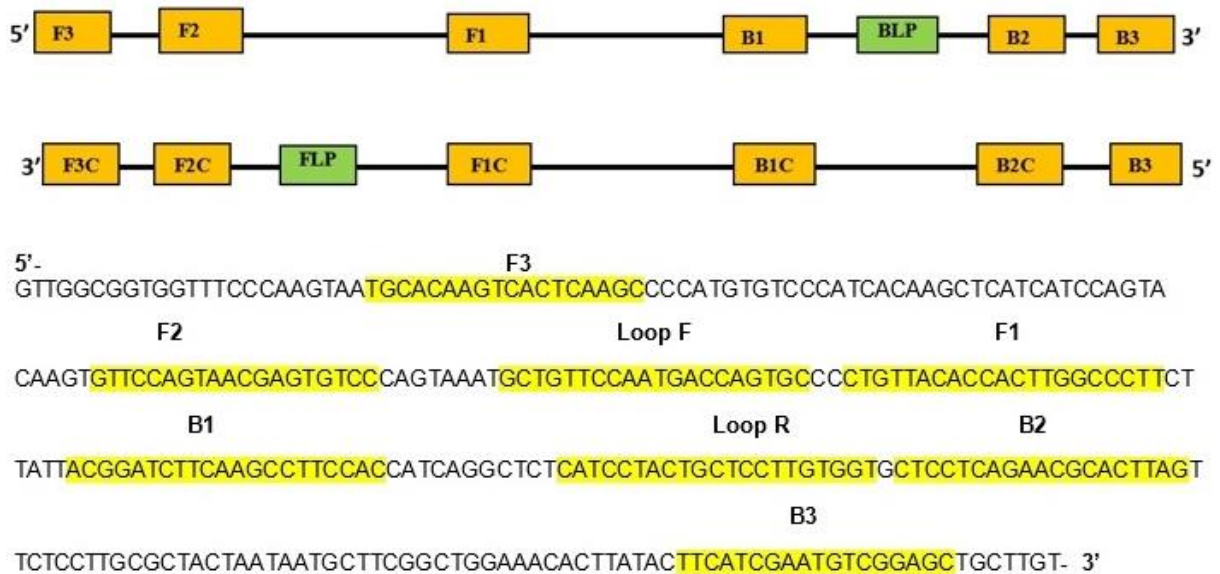


Fig. 1 Primer designing chart and the location of PTP1 primer in the partial DNA sequence (accession No. KY636450) of Polar tube protein 1 gene of *N. bombycis* was used to construct the inner and outer primers. Inner primers FIP and BIP comprise complementary sequence to F1 and sense sequence of F2, and sense sequence of B1 and complementary sequence of B2, respectively. Outer primers F3 and B3 include the sequence of F3 and B3 respectively

termed loop mediated isothermal amplification (LAMP) (Fu *et al.*, 2016). In this method, the target DNA sequence is amplified by strand displacement DNA polymerase without 5'-3' exonuclease activity that leads to the production of single stranded DNA (Notomi *et al.*, 2000).

It specifically detects genomic DNA by using a set of two or three oligonucleotide primers specific to different regions of a target gene. The LAMP method is found to be sensitive and can amplify even from few to 10 copies of target DNA in less than 30 min (Mori *et al.*, 2001; Nagamine *et al.*, 2002). The simplicity and ease of detection make this technique more innovative. The results can be detected visually by turbidity caused by the white precipitate of magnesium pyrophosphate or by the use of fluorescent dyes like calcein, hydroxy naphthol blue (HNB) and the strand displacement activity of *Bst* polymerase makes the DNA denaturation step

negligible (Tomito *et al.*, 2008). LAMP is being extensively used to amplify DNA for detection and for diagnostic purposes like examination of pathogens such as viruses, fungi, bacteria, and parasites (Huang, 2010; Chen *et al.*, 2016; Lee *et al.*, 2017). This method has been widely applied in fields for on-site detection because of its low cost, high specificity, efficiency, simplicity of operation, rapidity and its major impact has been on effective disease diagnosis and food safety testing. In this study, LAMP was employed to detect the microsporidian infection in silkworm *B. mori* using six distinct primers targeting the *PTP1* gene region of the pathogen *N. bombycis* (Indian isolate). The specificity and detection limit of the assay was evaluated using real-time PCR. The developed diagnostic method was compared with the conventional PCR and the method proved to be rapid, sensitive and specific for screening pebrine disease in *B. mori*.

Table 1 Primer sequence for LAMP and PCR

F3	5'-TGCACAAGTCACTCAAGC-3'
B3	5'-GCTCCGACATTTCGATGAA-3'
FIP	5'-AAGGGCCAAGTGGTGTAAACAGTTTTGTTCCAGTAACGAGTGTCC-3'
BIP	5'-ACGGATCTTCAAGCCTTCCACTTTTCTAAGTGCCTTCTGAGGAG-3'
Loop F	5'-GCACTGGTCATTGGAACAGC-3'
Loop R	5'-CATCCTACTGCTCCTTGTGGT-3'

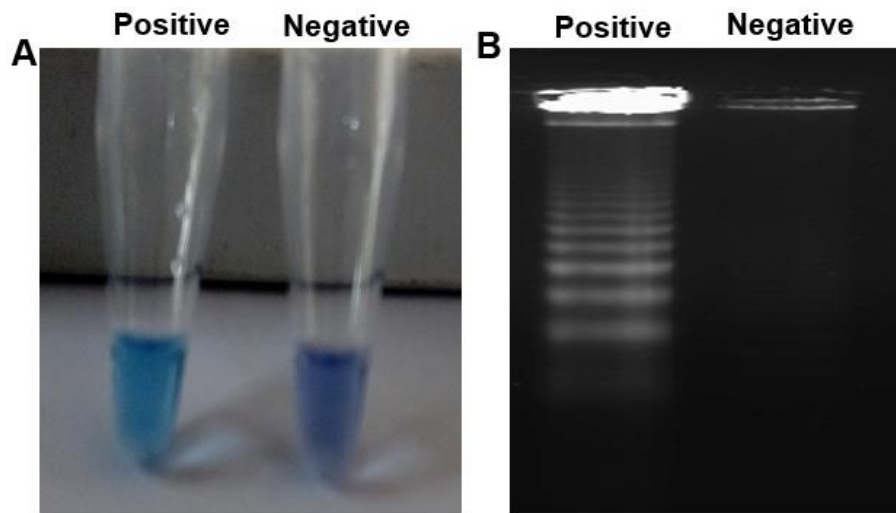


Fig. 2 Detection of *N. bombycis* infection through Loop mediated isothermal amplification assay. A) Visual detection of LAMP reaction using HNB dye. B) Agarose electrophoresis based detection of LAMP products

Materials and methods

Inoculation and multiplication of pebrine spores

The *Nosema bombycis* spores (*NIK-1*) were obtained from Central Sericultural Training and Research Institute (CSR&TI), Mysore. The purified spores were counted using hemocytometer and diluted to a concentration of 1×10^6 spores/mL (Shimanuki and Knox, 2000). The 4th instar first day silkworms were fed with mulberry leaves smeared with *N. bombycis* spores at a concentration of 1×10^6 spores/mL while the control larvae were administered with water and subsequently fed with clean mulberry leaves. The DNA was extracted from midgut tissues collected at different hours post infection (hpi): 12, 24, 48, 72, 96, 120 hpi from the artificially infected larvae and total DNA was extracted from the water treated control larvae. Apart from these tissues, egg, little tissue from the abdomen of moth were also collected and percoll purified spore was also used for DNA extraction.

DNA extraction

A small amount of tissue was excised from the silkworm moth and around 20 eggs were used for fast DNA extraction (modified at the laboratory). The excised tissue or egg was placed in a microfuge tube and homogenized by addition of lysis buffer containing 0.1 M Tris-Cl, 1M NaCl, 0.01M EDTA of (500 μ l) and crushed using micro pestle. The crushed tissue was briefly vortexed and centrifuged at 13000 rpm for 5 min and the aqueous layer was transferred to a new microfuge tube. An equal volume of freshly prepared phenol: chloroform: isoamyl alcohol (24:24:1) were added and the contents of the tube were mixed gently. The tube was centrifuged at 13000 rpm for 5 min and the supernatant was transferred to a microfuge tube. An equal volume of isopropanol was added and the tubes were incubated at room temperature for 20 min followed by centrifugation at 13000 rpm for 5

min to pelletize the DNA. The pelleted DNA was washed with 70% ethanol and air dried. The dried pellet was dissolved in 50 μ l of sterile distilled water and stored at -20 °C for further use. The percoll-purified spores were disrupted using glass beads and the DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen GmbH, Hilden, Germany).

Detection of pathogen using conventional PCR

The pathogen multiplication was analyzed through conventional PCR using the gene specific primer designed for targeting the *PTP1* gene region. The amplification was performed using the following conditions: Initial denaturation at 94 °C for 3 min, 30 cycles of 94 °C for 1 min, 55 °C for 30 sec, 72 °C for 30 sec and a final extension of 72 °C for 10 min. The amplified product was analyzed using agarose gel electrophoresis.

Cloning and sequencing of *PTP1*

The *PTP1* gene sequence (Gene accession No. KB909850) was BLAST searched in *N. bombycis* genome database and a sequence coding for *N. bombycis* was retrieved from the database. The retrieved sequence was used to design forward and reverse primers using Primer3 programme (<http://frodo.wi.mit.edu/cgi-bin/primer3/>). Two pairs of primers specific for *PTP1* gene of *N. bombycis* (forward 5'-GATGAGAATTAGATCCTTCAAAC-3' and reverse 5'-GCTCAGTTTAGCACACATGGATTATTGCC-3') were used for the study. The presence of *PTP1* gene in *N. bombycis* infected samples was confirmed by cloning the purified PCR fragments using pGEM-T easy vector. The plasmid DNA was isolated from PCR verified positive clones and purified through DNA purification column (Promega, Madison, USA) for sequencing with M13 primers in Eurofins Genomics India Pvt. Ltd., Bangalore. The sequence was submitted to NCBI database (accession No. KY636450).

Primer Designing

The conventional as well as LAMP primers were designed using the *PTP1* region of *N. bombycis* (accession No. KY636450) using the Primer 3.0 and LAMP designer software, respectively. Six primers for LAMP consisting of two outer primers, two inner primers and two optional loop primers were designed. The primer design was performed by using the online tool LAMP designer version 1.15. (<http://premierbiosoft.com/crm/jsp/com/pbi/crm/client/side/ProductList.jsp>) and were checked for cross homology. All the parameters were set by default. The primer design chart and sequence are presented in Fig. 1 and Table 1, respectively.

Optimization of LAMP reaction

Lamp was carried out in a 25 µl reaction consisting of 2.5 µl of 10X isothermal buffer (20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 150 mM KCl, 2 mM MgSO₄, 0.1% Tween 20), 2 mM MgSO₄, 3.5 mM dNTP's, 0.1% Triton-100, 1M Betaine, 120 mM of HNB (), 1.6 µM of inner primers (FIP and BIP), 0.4 µM of loop primers 0.1 µM of outer primers, 8U of *Bst* polymerase, 1µl of template DNA and finally sterile water was added to make the final reaction volume to 25 µl. The mixture was incubated at 65 °C for different time periods (15, 30, 60, 90 min) and the reaction was terminated at 80 °C for 5 min.

Detection of LAMP products

In our study HNB is used for visual detection of LAMP products. HNB is used as the metal-ion indicator which monitors the change in the Mg²⁺ ion concentration during LAMP reaction and allows the positive reaction to be detected in sky blue color and the negative in violet which can be detected easily by the naked eyes. The amplified LAMP product can also be resolved on agarose gel, which produces a ladder like banding pattern.

Copy number calculation of PTP1 gene using qPCR

Quantitative PCR was performed to calculate the copy number using SYBR green I technology on Agilent Stratagene *Mx3005P* qPCR instrument. The concentration was determined using the spectrophotometer and 10-fold serially diluted *PTP1* plasmid samples were used as template to obtain the standard curve. PCR cocktail was made with 1 µl of template DNA followed by addition of 1X SYBR green master mix and 10 pmol of each primer. The reaction was performed for 40 cycles under the following reaction conditions: denaturation at 95 °C for 5 min followed by annealing at 55 °C for 30 sec and elongation at 72 °C for 1 min. Fluorescence was detected at the end of each elongation phase. Standard curve was established by plotting the threshold cycle (Ct) on the Y-axis and the natural log of concentration on the X-axis and the total copy number (Xt) of the *PTP1* gene was calculated by relating the Ct value (Yt) to the standard curve and then the single cell copy number of the *PTP1* gene in the *Nosema* infected silkworm samples was calculated by the following formula:

Plasmid copy number = Pcn

$$Pcn = \frac{6.023 \times 10^{23} \text{ copy/mol} \times \text{concentration of DNA (g)}}{\text{DNA length (bp)} \times 660 \text{ g/mol/}\mu\text{l}}$$

Where 6.023 X 10²³ is the avagadro number and 660 Daltons is the average weight of DNA . The concentration of DNA was measured using the UV spectrophotometer.

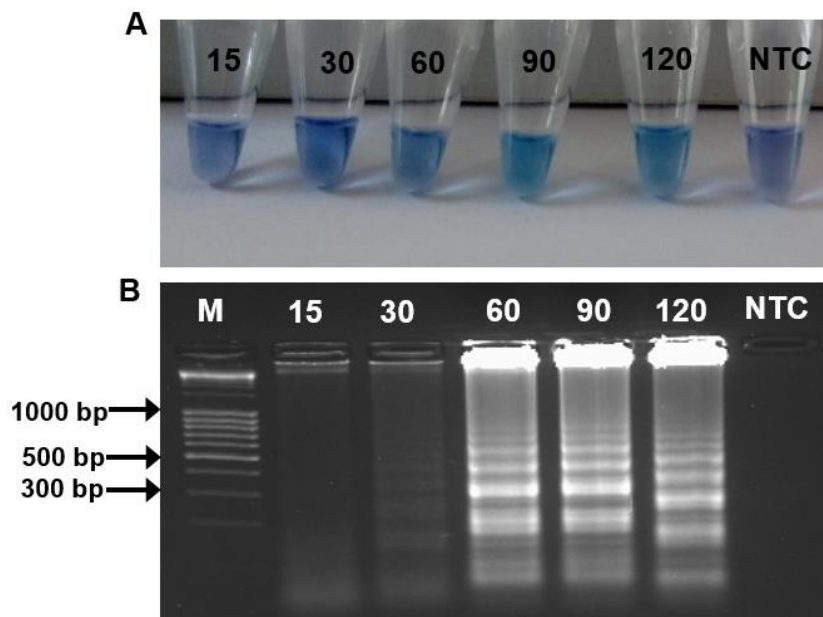


Fig. 3 Optimization of LAMP reaction time for detection of *N. bombycis* at 65 °C for 15, 30, 60, 90, 120 min along with non template control (NTC). A) Visual detection using HNB dye. B) Agarose gel based detection of LAMP products

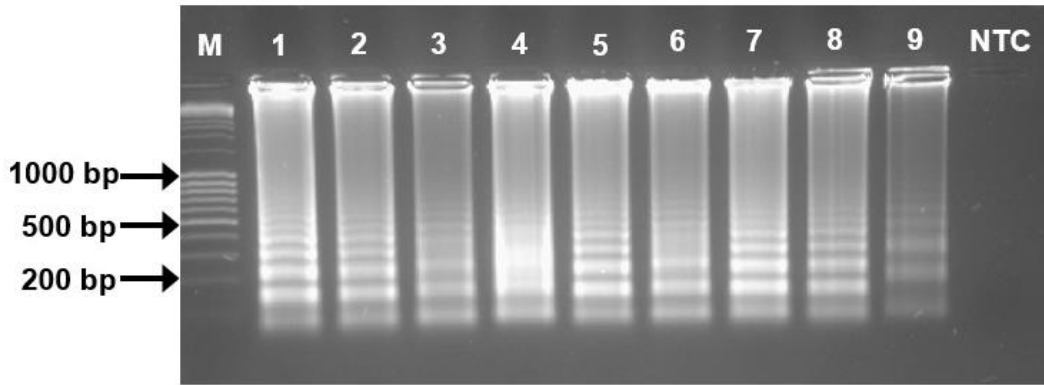


Fig. 4 Validation of LAMP reaction targeting PTP 1 gene in detecting *Nosema* in different infected moth samples

Sensitivity of LAMP

To analyze the sensitivity of LAMP reaction the spore DNA was used as the template. The sensitivity of LAMP was determined by 10-fold serial dilution of the spore DNA. The conditions for conventional and qPCR was followed as mentioned above and LAMP was performed at 65 °C for 60 min and terminated at 80 °C for 5 min. The normal LAMP reaction was carried out with *Bst* 3.0 polymerase was also compared with the warm start master mix (NEB#E1700S) using the DNA of infected egg, larvae and moth and visualized using HNB dye at a concentration of 120 mM.

Results

The multiplication of *Nosema* spore was found to be sequential starting from midgut followed by trachea, fat body, malpighian tubules and finally infecting the whole body. The larvae were found to

be susceptible immediately after molt and hence the infection was given immediately after the third molt. The multiplication was detected after 24 hours of post infection in the midgut using conventional PCR. The DNA extraction was performed by modifying the usual method and this took approximately 30 min. This method is neither laborious nor requires any further purification steps and the DNA can be directly used as a template for the conventional as well as LAMP assay.

Cloning and sequencing of *N. bombycis* *PTP1* gene revealed the presence of an open reading frame of 1230 bp, coding a polypeptide of 409 amino acids. Its overall GC content was found to be 46.50%. The molecular weight (40.4 kDa) and the isoelectric point (5.82) of the putative ORF was predicted using the Prot Param tool. The coiled structure of polar tube is a distinctive invasion apparatus of microsporidia, plays a vital role in ejection of sporoplasm into the host cell. The major

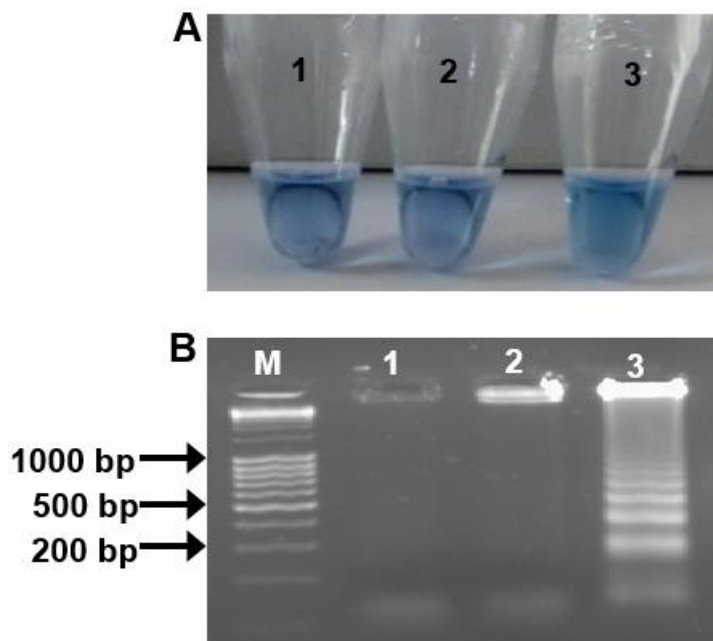


Fig. 5 Specificity of developed LAMP assay targeting *PTP1* gene in detecting the pathogen *N. bombycis*. Lane 1, 2 and 3: NPV, DNV-2, *Nosema* infected genomic DNA samples

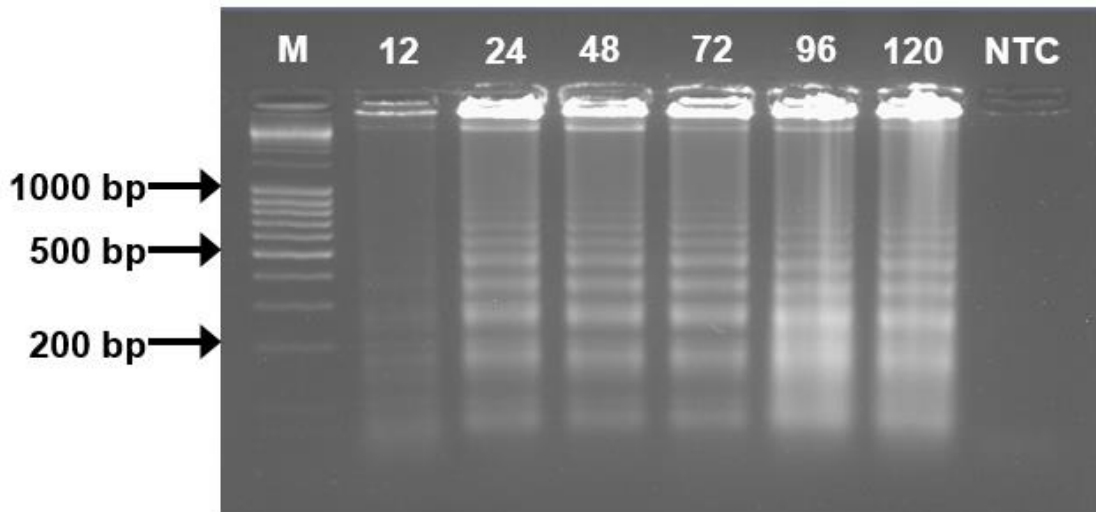


Fig. 6 Agarose electrophoresis profile of LAMP assay targeting the polar tube protein 1 gene of *Nosema* infected silkworms at different hours post infection under laboratory conditions

aminoacid coded by PTP- 1 was found to be proline (17.1%) with 10 positively and 14 negatively charged residues. Hence, the *N. bombycis* PTP-1 sequence (KY636450) was chosen as the target region for designing the LAMP. The positions of the primers within the (position 774 to 1043) PTP1 sequence are shown in Fig. 1. The predicted size of the PCR product (F3/B3) was found to be 270 bp. The sequence of the LAMP and conventional PCR primers are listed in Table 1. To confirm the specificity of the LAMP products, the 270 bp product was cloned into pJET vector and sequenced. The results showed that the 270 bp targeted region of the sequence was 100% homologous with the reported PTP1 sequence. The conventional PCR and microscopic examination was initially used to validate the pathogen multiplication.

The LAMP assay was performed using the infected moth as a template and the best results were obtained in 25 µl reaction with 2.5 µl of primer mix (40 pmol FIP and BIP, 20 pmol of loop forward and reverse, 10 pmol of F3 and B3 primers), 2.5 µl of 10X isothermal buffer, 3.5 mM dNTPs, 2 mM MgSO₄, 0.1% Triton X-100, 1M betaine, 120 mM of HNB and 1µl of *Bst* polymerase 3.0 with strand displacement activity. The reaction was performed in 0.2 mL tubes and kept in water bath at 65 °C for 60 min followed by termination at 80 °C for 5 min. The positive sample was observed in sky-blue color while the negative sample displayed the violet color (Fig. 2A). After assessing the color change, the result was reconfirmed using a 1.5% agarose gel electrophoresis and as expected, the typical ladder-like pattern was observed on gel for the positive sample (Fig. 2B).

Optimization of LAMP reaction conditions

The optimization of LAMP reaction was achieved by performing the reactions at different temperatures and time points with the optimized

components described above. Positive results were obtained at 65 °C within the time interval of 30 to 90 min and prominent bands were found on agarose gel, in case of the samples kept at 65 °C for 60 min. LAMP reaction at 65 °C for 60 min was optimized for PTP1 primer (Fig. 3A,B). When the reaction time reached 30 min, the color of LAMP products slightly changed and the intensity of ladder-like pattern on gel electrophoresis was low, indicating that the amplification efficiency of LAMP was low at 30 min. Therefore, the appropriate reaction condition of the established LAMP method was set at 65 °C for 60 min.

Validation of the LAMP products

To further confirm the LAMP products, the product was cloned in pJET vector and the PCR verified positive clones were forwarded for sequence analysis. The sequencing results showed that the 270 bp target fragment was 100% homologous to the *N. bombycis* PTP1 gene that was used for designing of the primers. These results indicated that the LAMP products were specifically amplified from the PTP1 gene of *N.bombycis*.

Specificity of LAMP

The specificity of the LAMP primers for the detection of *N. bombycis* was analyzed by using DNA samples from different infected moths. A sky-blue color and a ladder-like pattern was observed on agarose gel, in case of the positive samples, whereas the negative sample remained violet in color. The sky-blue color and ladder-like pattern of bands were generated by the DNA of *N. bombycis* (Fig. 4). The PTP1 primers were validated and used to test the cross reactivity with the samples infected with BmBDV and BmNPV. The ladder like banding pattern was observed only in the *Nosema* infected samples while the silkworm *B. mori* infected with bidenso virus and nucleopolyhedrovirus (BmBDV

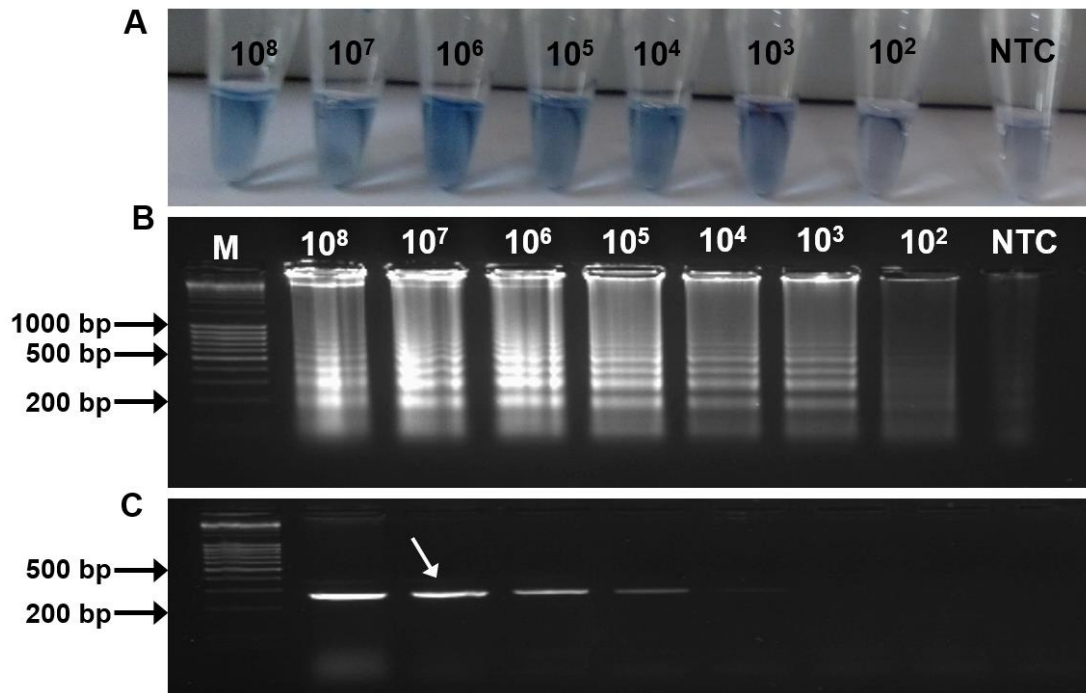


Fig. 7 Sensitivity comparison of LAMP reaction using serially diluted Spore DNA of *N.bombycis*. A) Visual detection of LAMP products using HNB dye. B) Agarose based detection of LAMP products. C) Agarose electrophoresis profile of conventional PCR product and the white arrow indicates the amplified *PTP1* gene with a product size of 270 bp

and BmNPV) showed negative results under similar conditions (Fig. 5A,B). Conventional PCR with *PTP1* specific primers (*PTP1* F3/B3) also showed good specificity. This result indicated that the LAMP primers can be used to specifically detect *N. bombycis*. The sensitivity was also tested on artificially inoculated silkworms wherein the pathogen was detected at 24 hpi using the LAMP assay under laboratory conditions (Fig. 6).

Sensitivity of LAMP and PCR

Absolute quantification was done using real-time PCR for calculating the exact copy number of the plasmid cloned with *PTP1* gene. To determine the detection limit, the number of *PTP1* gene copies in 100 ng/ μ l of spore DNA was calculated from their respective C_T (Cycle threshold) using the linear equation of *PTP1* standard curve. The conventional PCR and LAMP reactions were performed with tenfold serially diluted spore DNA. PCR products were resolved through agarose gel electrophoresis while the products of LAMP assay were detected by HNB-visualization as well as through agarose gel electrophoresis. A 270 bp band was specifically amplified and successfully detected by PCR when the spore DNA template was diluted to 1×10^5 copies/mL and the ladder-like patterns of the LAMP products were observed from 1×10^8 copies/mL to 1×10^3 copies/mL indicating that the detection limit was 1×10^3 copies/mL (Fig. 7A,B,C). Thus, LAMP was 2-fold more sensitive than PCR. According to HNB-visualization and gel electrophoresis all the

samples tested for reproducibility were positive thereby indicating that the established LAMP had good robustness and reproducibility. Further, the optimized LAMP reaction with *Bst* 3.0 polymerase was compared with the commercial master mix (Warm start master mix, New England Biolabs, Cat No. E1700) using the infected egg, larvae and moth which also revealed similar results (Fig. 8A,B,C).

Discussion

Microsporidiosis is a deadly disease caused by the microsporidia *N. bombycis* in silkworm *B. mori*. The pathogen is unique and can spread from mother moth to egg through transovarian mode of transmission and by the consumption of infected leaf (horizontal transmission) or contaminated egg surface (transovum transmission). The early detection of pathogen prevents the spread of the disease and crop loss. The *PTP1* gene of microsporidia was found to be one of the essential genes involved in host cell invasion. The *PTP1* gene was cloned, characterized and was found to be rich in proline content, a common feature of polar tube proteins found in the other microsporidians. It was already reported that the amino acid proline accounts for the essential component of polar tube proteins. It forms a fixed kink with polypeptide resulting in chain rigidity which is important for the polar tube protein during the discharge and passage of sporoplasm (Keohane *et al.*, 1996). Many molecular-based methods have been developed for

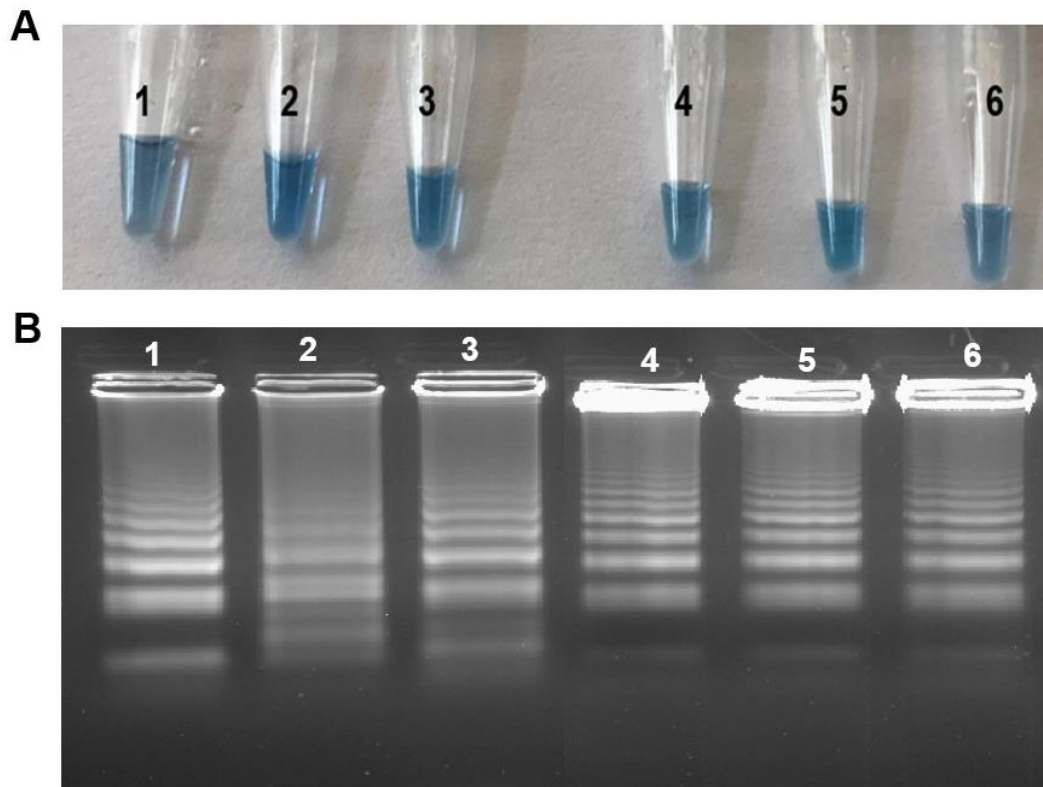


Fig. 8 Comparison of LAMP assay using *Bst* 3.0 polymerase with warm start master mix. A) Visual detection of LAMP products using HNB dye. B) Agarose based detection of LAMP products amplified using *Bst* 3.0 polymerase (Lane 1 - 3) and commercial warm start polymerase (Lane 4 - 6). Lane 1 and 4: *Nosema* infected tissue from the abdomen of silkworm. Lane 2 and 5: *Nosema* infected Eggs. Lane 3 and 6: *Nosema* infected larvae

the detection of *N. bombycis*, however, such techniques were found to be very expensive and time consuming. LAMP is a new on-site diagnostic method with straight-forward principles, and comprehensive protocol that allows a semi-skilled person to perform the assay without any sophisticated instrument (Notomi *et al.*, 2000; Abdullahi *et al.*, 2015). In LAMP reaction, the specific region of the DNA sequence was amplified using *Bst* DNA polymerase and the reaction takes place at a high speed, under constant temperature conditions (60-65 °C) with high accuracy for the specific region (Nagamine *et al.*, 2002). In this study, we have developed and analyzed the LAMP assay for detection of microsporidiosis in *B. mori*. It is considered as an effective gene amplification method to be used for pen side diagnostic tests, that can be performed at the field level without any technical expertise or instrument (Parida *et al.*, 2008). This method allows naked eye detection by addition of DNA intercalating dyes like SYBR green, Picogreen, propidium iodide or metal-ion indicators such as calcein or HNB (Hill *et al.*, 2008). In 2014, LAMP was developed for *N. bombycis* detection only in *B. mori* eggs using filter paper based card known as FTA (Flinders Technology Associates)

card as template by targeting the large and small subunit of rRNA using 4 primers and the EB1 gene was used as the target for detection of pathogen in the infected eggs (Yan *et al.*, 2014; Ping *et al.*, 2015). In our study, for the first time, we have used the LAMP for detecting microsporidian *N. bombycis* at all stages of the silkworm i.e., egg, larvae and moth with the *PTP1* gene as the target sequence for the detection of microsporidian infection. A new DNA extraction method was followed to extract DNA from midgut of the infected larvae, egg and moth which is advantageous in two different ways. Firstly, when compared with the conventional method, the new method requires only minimal amount of tissue without the requirement of further purification. Secondly, the new method allows extraction within 30 min when compared to the conventional method which involves longer incubation time to act as a rate limiting step (Singh *et al.*, 2011). Hence, this method was employed for DNA extraction and further used for conventional PCR, LAMP and qPCR. The metal ion indicator was added prior to the reaction to avoid non-template amplification which is one of the major constraints of LAMP. The LAMP reaction was optimized using six different primers targeting eight regions of the *PTP1*

sequence of *N. bombycis*. Nagamine *et al.* (2002), reported that the reaction time involved for LAMP could be shortened using loop primers, however, it is not mandatory for the LAMP reaction to take place. Even though the two loop primers were optional, they were included in our study to accelerate the reaction and the optimization of LAMP reaction conditions was achieved by performing the reaction at different time intervals, temperatures and primer concentrations. In few experiments, a higher level of detection limit was reported for the LAMP when compared to the PCR while in other experiments, the detection limit result of LAMP was equivalent to the PCR (Bakheit *et al.*, 2008; Zhao *et al.*, 2010; Tang *et al.*, 2011). The difference in outcome of these experiments is probably related to the characteristics of the primers and the sequence of the specific region in DNA that can affect the sensitivity and specificity of the molecular technique (White *et al.*, 2006). The final LAMP conditions comprised of 40 pmol of FIP and BIP, 10 pmol of F3 and B3, 20 pmol of Loop F and R and 8U of *Bst* 3.0 polymerase (New England Biolabs, Ipswich, MA). In the 25 μ l of LAMP reaction, even though 1M Betaine (Sigma-Aldrich, Bangalore, INDIA) and 0.1% Triton X-100 were optional, they were added to the reaction to prevent secondary structure formations in the GC rich sequence regions. Since conventional PCR is usually used in the detection of disease, due to its simplicity and cost when compared to real-time PCR, the sensitivity of LAMP was compared with conventional PCR.

In this study, the plasmid containing *PTP1* gene was serially diluted and used to compare the sensitivity of the LAMP. Results revealed that, the LAMP had 2-fold higher sensitivity than the conventional PCR. However, the higher specificity and sensitivity has a disadvantage as it may lead to cross reactivity, which can cause non-template amplification during the reaction (Mori *et al.*, 2001). Hence, filtered tips were used to prevent aerosol contamination and closed reaction tubes and proper disinfection of the pipettes with ethanol are recommended (Wang *et al.*, 2009). Yan *et al.*, (2014) has reported the detection of *N. bombycis* in single silkworm egg, however, our study showed positive results when DNA was extracted from ten eggs and this may be either due to the spore load in the eggs or the differential DNA extraction procedure. The comparison of our optimized LAMP assay using *Bst* 3.0 polymerase cocktail with that of the commercial LAMP kit (New England Biolabs, Ipswich, MA) revealed that the developed method would be economical, reliable and field based assay sensitive in detecting the pathogen in all the developmental stages of silkworm. The previously reported electrochemical and pH based detection of LAMP targeting different region of polar tube protein gene requires technical expertise and detection was found to be tedious compared to the developed LAMP assay (Xie *et al.*, 2014; 2015). In conclusion, the developed LAMP method was found to identify the infection of *N. bombycis* in all the stages of silkworm *B. mori*. It has a better sensitivity than conventional PCR. Furthermore, the presence of the pathogen can be detected with the naked eye

immediately after the LAMP reaction by using HNB. This technique can thus be a preferable choice over the conventional standard PCR for detection of *N. bombycis* in silkworm seed centers.

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