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RESEARCH REPORT

A Field-Friendly Loop-Mediated Isothermal Amplification (FF-LAMP) method for rapid detection of *Nosema bombycis* in silkworm, *Bombyx mori*

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Abstract

Pebrine is a destructive disease that exhibits horizontal and vertical transmission and therefore it is the only mandatory quarantine item in sericulture. Here, a field-friendly loop-mediated isothermal amplification (FF-LAMP) method has been developed and validated for the rapid detection of *Nosema bombycis*, a causative agent of pebrine disease in silkworm, *Bombyx mori*. FF-LAMP primers were selected and designed for small ribosomal subunit gene and the assay was performed to detect the *N. bombycis* infection in silkworm. The FF-LAMP reaction was effective at 6 mM $MgSO_4$, 1.4 mM dNTPs at 63 °C. The detection range of LAMP assay was found to be 10^1 dilutions of *N. bombycis* spores. Specificity of the primers was tested using DNA isolated from pebrine infected silkworm, pebrine free silkworm and pure *N. bombycis* by conventional PCR and FF-LAMP assay. Results revealed that the primers were specific to *N. bombycis* DNA. The FF-LAMP assay was validated in different basic silkworm seed farms with simultaneous microscopic examination of *N. bombycis* infection. This newly developed method is highly effective, specific, sensitive and rapid in detecting *N. bombycis* infection, eliminating the DNA purification steps and usage of sophisticated equipment. This method can be used in testing large number of samples making it field friendly method in sericulture industry.

Key Words: *Bombyx mori*; *Nosema bombycis*; pebrine; Loop-Mediated Isothermal Amplification; NCBI

Introduction

Sericulture is a main source of livelihood for subsistence with farmers engaged in silk production. Like other economically important insects, silkworm is also prone to many diseases caused by pathogens. These pathogens are responsible for significant economic loss in the sericulture industry. Among them, *Nosema bombycis*, a microsporidian causes pebrine disease in silkworms, *Bombyx mori*; not only horizontally transmitted in the silkworm larva, pupa, and moth, but also vertically to the next generation through the silkworm eggs; adversely affecting silk yield and quality of cocoons resulting in huge losses to sericulture. Pebrine disease is the only microbial infection, listed under quarantine items with regard to silkworm egg production (Fu *et al*., 2016).

Pasteur (1870) discovered that the parasite (*N. bombycis*) could transmit from mother moths to progenies *via* transovarial transmission. Based on this observation, the mother moth microscopic examination method to eliminate eggs laid by infected moths and produce *N. bombycis*-free eggs for the sericultural industry was developed. From then, sericultural countries have adapted light microscopy examination for the detection of *N. bombycis* spores (Fujiwara, 1984). Even though, microscopy is inexpensive and easy to use, it has several limitations such as late detection (microsporidian spores are visible only after 3-8 days of onset of infection), detects only spore stage and only infections leading to a spore production above 1 x 10^6 spores/g tissue (Yan *et al*., 2014). Therefore, a low intensity infection could easily be missed as the infection remains undetected and spores represent only a fraction of an ongoing infection; the other life stages remain undetectable. Advancement in PCR-based methods could overcome these drawbacks as they can detect parasitic DNA of all life-cycle stages and allow discrimination of parasites *via* specific primers (Refardt and Ebert, 2006).

Mother moth and hatched larvae examination for pebrine does not completely prevent the development of pebrine disease. But, the crop

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should be monitored strictly for pebrine at each stage of silkworm rearing to prevent the infection of *N. bombycis* by external sources like mulberry leaf, contaminated rearing materials *etc*. This is possible only by adapting the detection methods which are field applicable. Even though, advancement in the molecular diagnostic approaches like conventional PCR has made great progress in enhancing the sensitivity and specificity in the detection of *N. bombycis* compared to visual identification by microscopic examination, these techniques are not suitable for conducting tests in the field conditions as they require sophisticated instruments, expertise and longer duration. Therefore, there is a demand to innovate specific, accurate and early detection method which could be utilized at the field level for effective Pebrine disease management among sericulture farmers and silkworm seed production centres. This method still demands for costly instrumental set-up like thermal cycler and UV transilluminator for the detection of PCR products and yet might not be feasible for field conditions. An approach combining low-cost DNA extraction process and PCR detection for high-throughput screening could be the ultimate goal for pebrine detection in sericulture. Notomi (2000) discovered loop-mediated isothermal amplification (LAMP) technique in 2000. Since then, LAMP has been improved constantly and widely used for the detection of pathogenic microorganisms (Njiru *et al*., 2008; Jiang *et al*., 2012; Singh *et al*., 2013). Yan *et al*. (2014) found the main hindering factor affecting the sensitivity of microsporidian inspection is the recovery of genomic DNA. Furthermore, he has successfully applied the LAMP method for detection of *N. bombycis* using FTA Cards for genomic DNA extraction.

To make the current invention, a practically effective and field-oriented diagnostic system for silkworm pebrine disease, LAMP detection method was reoriented using special lysis buffer to detect *N. bombycis* infection; which requires less sophisticated instruments for amplification of the target gene and the product could be detected by chromogenic system. To our knowledge, it is the first attempt of its kind to simplify the DNA extraction method from microsporidia infected tissues and combine it with LAMP assay to develop field-friendly diagnostic method (FF-LAMP assay) for the detection of Pebrine in silkworms.

Materials and Methods

Silkworm race and microsporidia

The bivoltine silkworm race, CSR2 and purified spores of *Nosema bombycis* (NIK-1S) maintained at Central Sericultural Research and Training Institute, Mysuru, India were used for this experiment. *N. bombycis* spores were propagated through periodical inoculation of spores to silkworms (II instar 1st day) followed by collection of infected moths. The *N. bombycis* spores were isolated following tissue homogenization and purified by percoll centrifugation.

Preparation of N. bombycis *infected silkworm larvae/tissue*

Silkworm larvae after hatching and after each moult were fed with mulberry leaves that were uniformly smeared with *N. bombycis* spores at different doses (10^1-10^8) separately. From there, the onset of infection was examined every day by homogenizing the host tissues in physiological saline and observing under phase-contrast microscope (600X). Similarly, the infections at different developmental stages of silkworm *viz*. egg, pupa and moth were also examined. The experiment was performed in 5 replications containing 100 silkworm larvae /replication /dose of spores. Samples were collected from each replication on daily basis and were preserved at -20 °C until use.

Five batches of silkworms were inoculated with different concentrations of *N. bombycis* spores from 10^1 to 10^8 immediately after brushing and after each moult, respectively. The inoculated silkworm samples were collected every 24 h intervals. Samples were collected from 1st instar 2nd day to moth emergence and eggs. Samples were stored at -20 °C for further use.

DNA extraction from N. bombycis

The spores of 10^7 were suspended in equal volume of solution containing 100 mM NaCl, 200 mM Sucrose, 10 mM EDTA and 30 mM Tris-HCl (pH 8.0) along with 0.45-0.50 mm glass beads for disruption of spores. The suspension was vortexed followed by centrifugation at 5000 rpm for 10 min. Supernatant was added with ¼$_{th}$ volume of 2.5 % SDS, 250 mM EDTA and 500 mM Tris-HCl (pH 9.2) along with 100 µg/ml of proteinase K followed by

Table 1 List of primers used for conventional PCR and FF-LAMP assay

Primer	Sequence (5'-3')	Length	Amplicons (bp)	Method	Reference
SSU-F	ACCAGGTTGATTCTGCCTGA	20	794	PCR	Ravikumar *et al* 2011
SSU-R	GTTGAGTCAAATTAAGCCG	19	794		
F3	GCGGCTTAATTTGACTCAA	19	124	PCR & FF-LAMP	Yan *et al* 2014
B3	ACCTGTTTTAATCCTCTCCT	20	124		
FIP	GCCATGCACCACTATCATGATCGCGGGGTAATTTACCAG	39	-	FF-LAMP	
BIP	GTTTCCAATGGATGCTGTGAAGTTCATATGTATCACTACATCTGTCT	47	-		

Table 2 Comparison of Light Microscopy and FF-LAMP results in the detection of *N. bombycis*

Sampling place	No. of Samples Tested	Presence of *N. bombycis* spores			
		Microscopy		FF-LAMP	
		+	-	+	-
P4- BSF (Hassan)	451	1	450	6	445
P3- BSF (Mysuru)	607	70	537	138	469
P2- BSF (Ambuga)	4	0	4	0	4
P2- BSF (Dharmapura)	11	0	11	0	11
P2- BSF (Horsely Hills)	6	0	6	0	6
P2- BSF (Krishnagiri)	28	0	28	1	27
P2- BSF (Madakasira)	4	0	4	0	0
P2- BSF (Palakkad)	10	0	10	0	0
SSPC-Mysuru	2	0	2	0	0
SSPC-KR Nagar	2	0	2	0	0
SSPC- Dharmapuri	145	1	144	1	144
CSRTI-Mysuru	12	12	0	12	0
SSBS-Coonoor	66	0	66	0	66
SSPC-Tumkur (DoS-KA)	25	0	25	0	25
Total	**1373**	**84**	**1289**	**158**	**1197**

incubation at 55 °C for 1 h. The proteins and SDS were removed by precipitation with 1M potassium acetate by incubating for 1h at 4 °C. After centrifugation at 5000 rpm for 20 min, the DNA was precipitated by 2 volumes of cold ethanol. The DNA pellet was dissolved in the MilliQ water and stored at -20 °C for further use. The DNA was quantified using spectrophotometer (Amersham BioSciences, Hongkong, China).

Direct-PCR (dPCR)

Ethanol pre-soaked (30 min) *N. bombycis* infected host tissues were homogenized in 200 µL of lysis buffer containing 10 mM Tris-HCl, 1 mM EDTA buffer (pH 8) and 10 mM NaCl (pH 8.0). The homogenized tissues were centrifuged at 10000 rpm for 10 minutes. Proteinase K (200 µg/mL) was added to the supernatants and mixed gently. Then the supernatants were incubated at 55 °C for 30 min followed by incubation at 95 °C for 5 min. The resulting supernatant was diluted in 1:25 ratio and used as template for performing PCR reactions. Before optimization of the lysis buffer, the chemical ingredients with varying concentrations were tested. As the detergent inhibits PCR reaction, the same were not utilized in formulating the lysis buffers.

The PCR reaction was performed to amplify the 16S small subunit ribosomal RNA (SSU-rRNA) of *N. bombycis* by using the PCR cycle conditions as described by Ravikumar *et al*. (2011). The reaction was carried out in a total volume of 10 µL containing 1X Taq buffer, 0.25 mM dNTPs, 0.50 µM of each primer, 2.5 mM $MgCl_2$, 1U *Taq* DNA polymerase and 2 µL of pre-diluted extract/ template. All the PCR components were from Thermo Fisher Scientific, USA. The amplified products were resolved on 1.3 % agarose gel and visualized by ethidium bromide staining under UV transillumination. All the reactions were performed on PTC 100 (MJ Research, USA).

Polymerase Chain Reaction (PCR)

Primers previously designed and successfully used by Yan *et al*. (2014) for *N. bombycis's* small ribosomal subunit gene (Accession No. AY259631) were used for this study. For optimising the LAMP assay, conventional PCR was performed using F3 and B3 primers (Table 1). Initially, the PCR conditions were optimized for annealing temperature (45 °C and 47 °C) and different $MgCl_2$ concentrations (1 mM to 6 mM). At optimal PCR conditions, the F3 and B3 primers were tested for non-specific amplifications against pebrine free silkworm DNA along with *N. bombycis*'s DNA and a negative control. The PCR conditions constituted initial denaturation step at 94 °C for 5 min followed by 35 cycles comprising of 94 °C for 30 s of denaturation, 47 °C for 30 s of primer annealing and 72 °C for 1 min of extension with final extension step at 72 °C for 10 min.

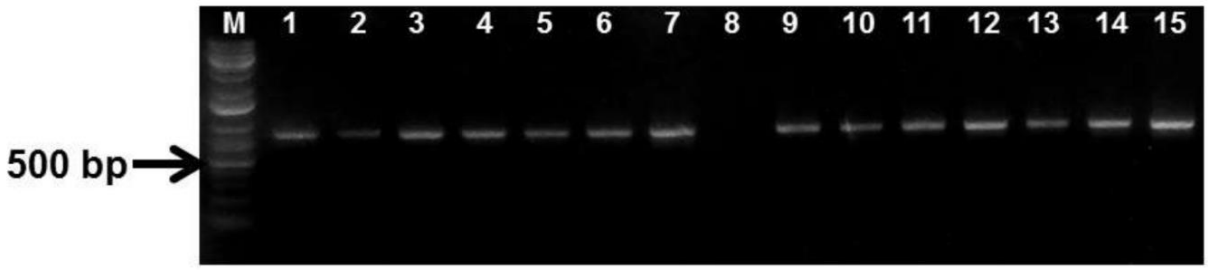

Fig. 1 Conventional PCR amplification of DNA extracted from pebrine infected silkworms using lysis buffer. M 100 bp ladder, lane 1 to 3 and 9 to 11 Proteinase K treatment for 30 min, lane 4 to 6 and 12 to 14 Proteinase K treatment for 60 min, lane 7 and 15 positive control, lane 8 Blank without DNA

The PCR reaction was performed in a 25 µL reaction mix containing template DNA, 1X PCR buffer, 4.0 mM $MgCl_2$, 250 µM dNTP mix, 10 µM of each primers and 1U Taq polymerase). The PCR product was analysed on 2 % Metaphor agarose gel electrophoresis stained with ethidium bromide and photographed under UV transilluminator. The PCR product was sequenced by Sanger dideoxy method and the data was submitted to NCBI.

Extraction of genomic DNA from N. bombycis *infected tissues for LAMP assay*

N. bombycis infected silkworms were briefly homogenized in mortar and pestle using lysis buffer (pH 8.0), the mixture was heated for 10 min at 95 °C, centrifuged for 10 min at moderate speed. 2 µl of the supernatant was directly used as the DNA template for the LAMP assay.

Optimization of LAMP assay

The LAMP reaction was optimized for $MgSO_4$ and dNTPs concentration, and temperature; $MgSO_4$ concentrations (1-6 mM), dNTPs at 1.4 mM and 2.8 mM, the inner (FIP and BIP) to outer primers (F3 and B3) concentration at 2:1, 4:1, 6:1 and 8:1, and reaction temperature at 60-65 °C.

FF-LAMP reaction system

The FF-LAMP reaction for the detection of *N. bombycis* was performed in a total volume of 25 µL containing 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 6 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1 % Triton X-100, 1.4 mM dNTPs, 1.6 µM (each) FIP and BIP primers, 0.2 µM (each) F3 and B3 primers, 0.4 % glycerol, 8U *Bst* DNA polymerase, template DNA (crude homogenate 2µl), 120 µM Hydroxy Naphthol Blue (HNB) and distilled water. The mixture was incubated at 63 °C for 45 min in a water bath.

Detection of FF-LAMP amplification

FF-LAMP amplification was detected by chromogenic assay by adding HNB dye prior to the reaction. The initial color of HNB at pH 8.8 will be maroon/violet. After the reaction, the color would turn to sky blue (in presence *N. bombycis* DNA), if the result is positive; otherwise, there would be no change in colour indicating negative result (in presence of no template or silkworm DNA). These results can be observed by naked eye under daylight without using any instruments. Amplified products were also confirmed by electrophoresis in ethidium bromide stained 2 % agarose gels under UV light.

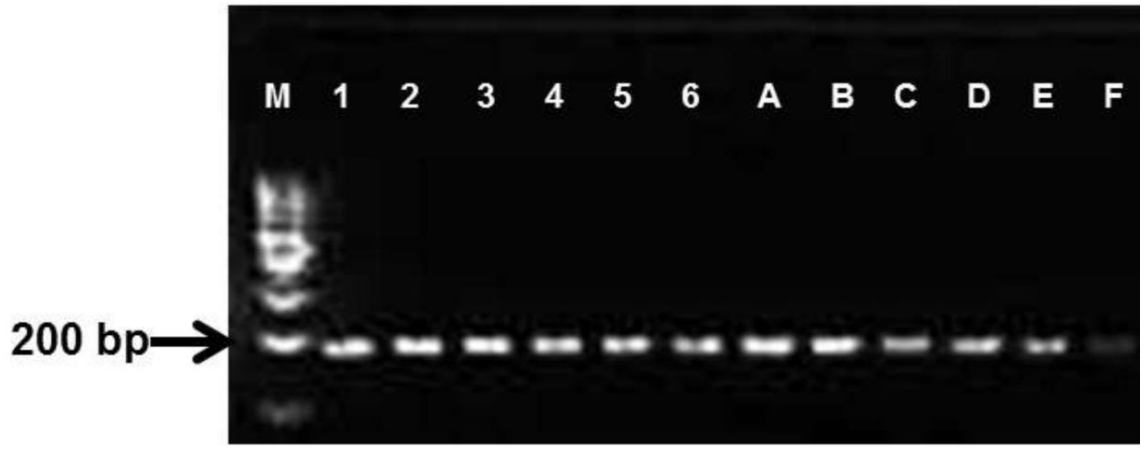

Fig. 2 Optimization of PCR conditions for detection of *N. bombycis* using F3 and B3 primers. M 100 bp ladder, lane 1-6 1 mM to 6 mM $MgCl_2$ and annealing temperature of 45 °C, lane A-F 1 mM to 6 mM $MgCl_2$ and annealing temperature of 47 °C

Validation of the FF-LAMP assay

A total of 1373 samples collected from different sericulture organizations of Central Silk Board, India as listed in Table 2 were subjected to FF-LAMP assay. All the test samples were also inspected simultaneously for *N. bombycis* infection by regular LAMP and light microscopy method. Independent sample t test was applied to understand the effectiveness of microscopy and FF-LAMP assay in pebrine detection. All statistical analyses were performed using SPSS 11.5 statistical package.

Results and Discussion

Genomic DNA was isolated from pure *N. bombycis* spore, pebrine-free silkworm, and also from *N. bombycis* infected silkworm using different isolation methods. DNA isolated from Pebrine infected silkworm using different buffers was utilized for direct PCR. DNA extracted from the lysis buffer was amplified by dPCR and the amplification product (794 bp) for SSU-F and SSU-R primers, that targets the small ribosomal subunit of *N. bombycis* was observed in agarose gel electrophoresis (Fig. 1). DNA isolated from pure *N. bombycis* spore was utilized to check the efficiency of the selected primers (F3 and B3) using conventional PCR. The F3 and B3 primers form the outer primers in LAMP reaction could be utilized for conventional PCR. The amplification product (Fig. 2) could be seen in all the tested conditions of $MgCl_2$ (1-6 mM) and annealing temperatures (45 °C and 47 °C). Pebrine-free silkworm DNA was utilized as negative control. Non-specific amplification was observed with silkworm DNA and thus the PCR product (~120 bp) was subjected to sequencing (Fig. 3). The analysis of sequencing showed hits to an extent of 120 bp of the PCR product. The sequencing of *N. bombycis* spore PCR product and nucleotide BLAST showed 100 % identity with *Nosema* species; whereas the sequenced product of non-specifically amplified pebrine-free silkworm DNA didn't show any identity with any organism. These results indicate that F3 and B3 primers are specific to *N. bombycis* SSU rDNA and the sequence of *N. bombycis* F3 and B3 region was submitted to NCBI (Accession No. MG831719). The pair-wise alignment of *N. bombycis* SSU rDNA

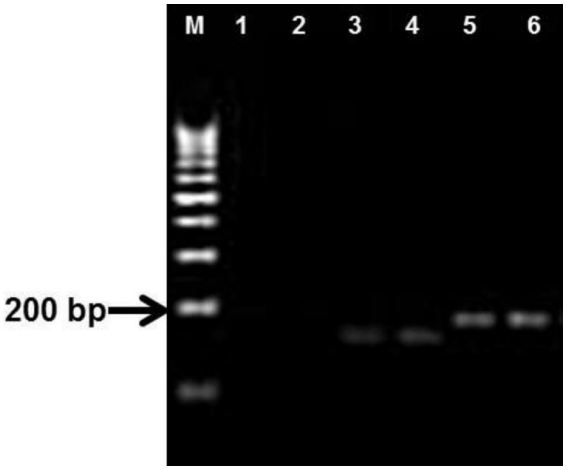

Fig. 3 Agarose gel electrophoresis of PCR products for detecting *N. bombycis*. M 100 bp ladder, lane 1 and 2 blank (no DNA), lane 3 and 4 Pebrine-free silkworm DNA, lane 5 and 6 *N. bombycis* DNA

(Accession No. AY259631) as the query sequence with that of F3 and B3 primer amplified product as subject sequence had 100 % identity (Fig. 4). Further, the DNA isolated from *N. bombycis* infected silkworm was utilized for LAMP assay using inner primers FIP and BIP along with F3 and B3. The sequence, amplicon size of all the primers employed in this study is detailed in Table 1. The loci of each LAMP primer in the small ribosomal subunit of *N. bombycis* DNA (Accession No.AY259631) are shown in Fig. 5.

FF-LAMP Assay

The tissue samples (egg, larvae, pupae, and moth) were homogenized in lysis buffer for breaking the chitin layered *N. bombycis* spores and heated at 95 °C to facilitate release of DNA into the buffer system. Then the sample was centrifuged and 2 µl of supernatant that contains DNA was sampled as template for the FF-LAMP assay in PCR tubes (0.2 ml tubes or 96 well plates). The optimization of FF-LAMP assay for different parameters showed that

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Query  61   TCATGATAGTGGTGCATGGCCGTTTCCAATGGATGCTGTGAAGTAATGATTAATTTCAAC  120
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Sbjct  1    TCATGATAGTGGTGCATGGCCGTTTCCAATGGATGCTGTGAAGTAATGATTAATTTCAAC  60

Query  121  AAGATGTGAGACCCTCATTTAGACAGATGTAGTGATACATATGAAGGAGAGGATTAAAAC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   AAGATGTGAGACCCTCATTTAGACAGATGTAGTGATACATATGAAGGAGAGGATTAAAAC  120

Query  181  AGGT  184
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Sbjct  121  AGGT  124
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Fig. 4 Pairwise alignment of *N. bombycis* SSU rRNA (query) and PCR amplified F3 and B3 region of Pebrine infected sample (subject)

GCGGCTTAATTTGACTCAACGCGGGGTAATTTACCAGGTATAACATGGTATAA
F3 F2

TATTTTATCATGATAGTGGTGCATGGCCGTTTCCAATGGATGCTGTGAAGTAA
F1 B1

TGATTAATTTCAACAAGATGTGAGACCCTCATTTAGACAGATGTAGTGATACAT
B2

ATGAAGGAGAGGATTAAAACAGGT
B3

Fig. 5 The locus of each primer in the DNA sequence of *N. bombycis* small ribosomal subunit

the effective target amplification could be obtained with 6 mM $MgSO_4$ concentration, dNTPs concentration at 1.4 mM, the inner to outer primer ratio at 16:1 and reaction temperature at 63 °C. The optimal combination of $MgSO_4$, dNTPs and inner and outer primers concentrations not only achieves the target amplification, but helps in maintaining the maroon/violet color at the start of the reaction (pH 8.8) with HNB. The optimization of LAMP reaction helps to distinguish easily between the positive and negative reactions.

HNB showed more effective color contrast between the positive and negative samples as compared to the other dyes used in this study (data not shown). HNB at pH 8.8, at the start of the reaction will be in violet color and turns sky blue color, if the samples are positive for *N. bombycis* infection. To summarize, no colour change (remains violet) is observed in blank and negative samples; while the colour changes to sky blue in the samples positive for *N. bombycis* infection by the FF-LAMP assay. The non-specific amplification observed does not affect the results of LAMP assay as non-specific amplified product from silkworm DNA was less and in turn could not alter the color of the reaction. In the LAMP assay, as the nucleic acid polymerize, the solution becomes acidic by the release of protons (H^+) which is detected by chromogenic assay by adding HNB dye prior to the reaction (Shunbi *et al*., 2014; Yan *et al*., 2014). Therefore, non-specifically amplified product from silkworm DNA is not a reliable readout enough to change the initial color of HNB dye. To further confirm the obtained results, agarose gel electrophoresis was run to find the ladder like band appearance (Fig. 6).

Silkworm larvae fed on *N. bombycis* were detected 12, 24, 48, and 72 h post-inoculation by the FF-LAMP method. At 48 h post-inoculation, the results showed that the amplification reaction was positive by the FF-LAMP method and negative by microscopy (Fig. 7). In contrast, at 72 h post-inoculation, both the methods detected a positive amplification i.e., *N. bombycis* infection. This experiment illustrates that the FF-LAMP technology shifts the diagnosis to 24 h earlier than that obtained through conventional microscopic method, even before the proper spore formation.

Specificity of FF-LAMP assay

The *N. bombycis* and silkworm DNA was tested utilizing the FF-LAMP method established in this study. The results showed that amplification of *N. bombycis* DNA occurred only; whereas all the amplifications were negative for silkworm DNA (Fig. 8), indicating that the LAMP primers used in this experiment cannot amplify the genome of the silkworm and is very specific for *N. bombycis.* The primers used in this study have no specificity to silkworm genes as evidenced by Primer BLAST.

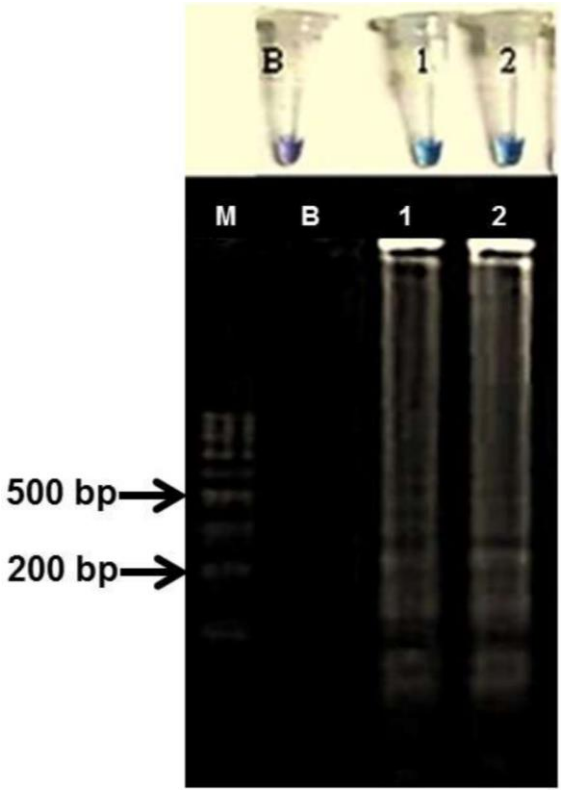

Fig. 6 Agarose gel electrophoresis of FF-LAMP products. M 100 bp ladder, B no DNA template, lane 1 2 µl of *N. bombycis* DNA, lane 2 2 µl of crude extract of Pebrine infected sample

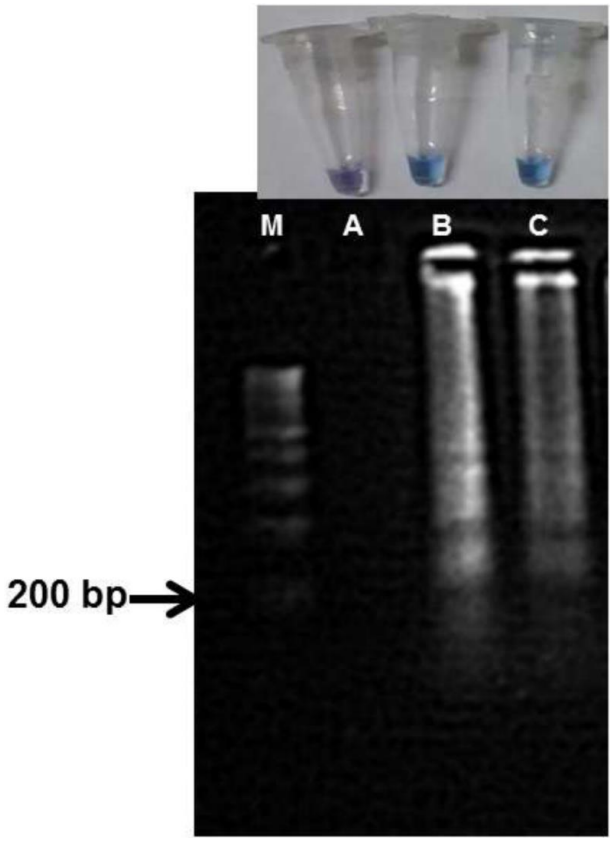

Fig. 7 Agarose gel electrophoresis of FF-LAMP products at different intervals of post-inoculation. M 100 bp ladder, lane A 24 h, lane B 48 h and lane C 72 h

Validation of FF-LAMP assay

Samples collected from various basic seed farms and silkworm seed production units of Central Silk Board and other agencies were tested for *N. bombycis* infection by using FF-LAMP assay and microscopic method in coordination with scientific personnel from respective units. Totally, 1373 samples were subjected for Pebrine detection (Table 2), of which 84 (6.11 %) samples were positive and 1289 samples were negative for *N. bombycis* infection by microscopy method; whereas, 158 (11.51 %) samples were positive and 1197 samples were negative for *N. bombycis* infection by LAMP method. Independent sample t test performed on the field samples tested by microscopy and FF-LAMP assay resulted in non-significant differences [t(54) = 0.019, p = 0.985] indicating that FF-LAMP assay is as effective as the traditional microscopy method. In addition to its superiority in terms of specificity and early detection of *N. bombycis*, FF-LAMP assay showed increased number of positive samples (5.4 %) than microscopy; which could not detect due to lower sensitivity of microscopy. The samples tested positive for infection by FF-LAMP assay was further confirmed by running agarose gel electrophoresis, where the typical ladder-like bands appeared.

There are many other methods such as normal PCR and loop mediated isothermal amplification (LAMP) that are targeting the amplification of ssu-rRNA gene sequence for early diagnosis and identification of silkworm microsporidia (Kawakami *et al*., 1995, 2001; Hatakeyama and Hayasaka, 2001, 2003). Though, PCR based methods can diagnose *N. bombycis* even at low spore concentration than microscopic method, but the methods are not being used for screening pebrine in seed production centres and field level as these modern techniques require highly sophisticated instruments and involves extensive processing of samples and technological interventions. These methods majorly involve DNA extraction and targeted gene amplification. Pan *et al*. (2005) reported an extraction method for microsporidian DNA from infected eggs, which had been treated with 30% KOH at 37 °C. A couple of researchers (Ravikumar *et al*., 2011; Gourab *et al*., 2017) used commercially manufactured kits or standard protocols (Sambrook, 1989) for extraction of DNA and agarose gel for detecting PCR products. The Liu *et al*. (2004a and 2004b) optimized the PCR conditions to inspect simulated ‘‘infected’’ silkworm eggs with the minimum detection of 299 spores, and suggested that silkworm egg contains some substances that may inhibit the PCR amplification. FF-LAMP method achieved sensitivity equal to conventional PCR with lesser steps and resources. Yan *et al*. (2014) applied LAMP assay for the

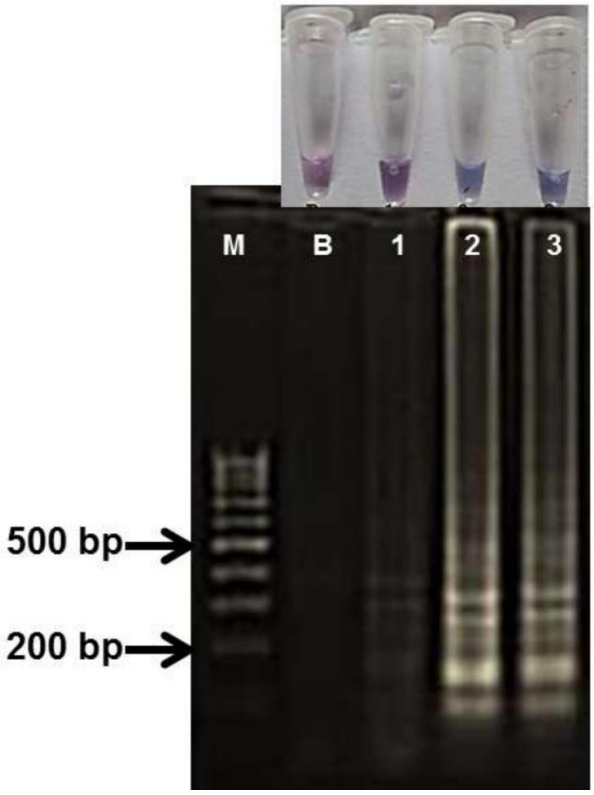

Fig. 8 Agarose gel electrophoresis of FF-LAMP products. M 100 bp ladder, B no DNA template, lane 1 Pebrine-free silkworm DNA (negative control), lane 2 and 3 Pebrine infected samples

detection of *N. bombycis* using FTA cards and glass beads method to extract microsporidian DNA, but it is not cost effective as compared to FF-LAMP assay. Further, attempts were made by researchers to simplify the LAMP technique with several detection measures like in LAMP-pH and LAMP-gold nanoparticle (LAMP-AuNP) methods. The former involves amplification of *N. bombycis* polar tube protein 1 gene and direct detection by measuring the released hydrogen ions during LAMP reaction using a pH meter (Shunbi *et al*., 2014); whereas, the LAMP-AuNP assay involves extraction of *N. bombycis* DNA by FTA card FTA card method followed by visualization of the amplified product via hybridization at 63 °C for 5 min with a ssDNA-labelled nanogold probe (Weijiang *et al*., 2019). Esvaran *et al*. (2018) employed phenol chloroform method for extraction of *N. bombycis* DNA followed by amplification of polar tube protein 1 gene through LAMP method and detection of amplification by colour change. These methods are time consuming, require sophisticated instruments and skilled personnel. Extraction of DNA for PCR amplification requires DNA with high purity, involves time-consuming processes and other factors such as salt ionic concentration etc. in the final sample interfere with PCR amplifications. In this study, LAMP assay was combined with single step DNA extraction method using special lysis buffer formulated to isolate *N. bombycis* DNA, which could be utilized for *N. bombycis*-SSU rRNA gene amplification successfully like direct-PCR (dPCR) method in the crude homogenate by FF-LAMP method. Moreover, this study shows that *N. bombycis* infection could be diagnosed 24 hours prior to that of light microscopy with limited resources; therefore, the LAMP assay described could be an effective alternative for light microscopy; which is simple, less time consuming, cost-effective and amenable to high throughput for pebrine inspection in silkworm.

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