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Development and optimization of a TaqMan assay for *Nosema bombycis*, causative agent of pébrine disease in *Bombyx mori* silkworm, based on the *β-tubulin* gene

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ABSTRACT

"Pébrine" is a devastating disease of *Bombyx mori* silkworms that is highly contagious and can completely destroy an entire crop of silkworms and is thus a serious threat for the viability and profitability of sericulture. The disease is most commonly attributed to microsporidians of the genus *Nosema*, which are obligate intracellular parasites that are transmitted through spores. *Nosema* infections in silkworms are diagnosed primarily through light microscopy, which is labour intensive and less reliable, sensitive, and specific than PCR-based techniques. Here, we present the development and optimization of a new TaqMan based assay targeting the *β-tubulin* gene in the pébrine disease causing agent *Nosema bombycis* in silkworms. The assay displayed excellent quantification linearity over multiple orders of magnitude of target amounts and a limit of detection (LOD) of 6.9×10^2 copies of target per reaction. The method is highly specific to *N. bombycis* with no cross-reactivity to other *Nosema* species commonly infecting wild silkworms. This specificity was due to three nucleotides in the probe-binding region unique to *N. bombycis*. The assay demonstrated a high reliability with a Coefficient of variation (CV) <5% for both intra-assay and inter-assay variability. The assay was used to trace experimental *N. bombycis* infection of silkworm larvae, in the fat body, midgut and ovary tissues, through pupation and metamorphosis to the emerging female moth, and her larval off-spring, confirming the vertical transmission of *N. bombycis* in silkworms. The TaqMan assay revealed a gradual increase in infection levels in the post-infection samples. The assay is reliable and simple to implement and can be a suitable complement to microscopy for routine diagnostics and surveillance in silkworm egg production centres with appropriate infrastructure.

1. Introduction

Pébrine disease of the silkworm *Bombyx mori* was first identified in France in 1845 and was the reason for the collapse of the French sericulture industry by 1865 (James and Li, 2012). The disease is highly contagious and can transmit horizontally and vertically. The eukaryotic microsporidian *Nosema* responsible for the disease is a highly specialised eukaryotic intracellular parasite, highly adapted to the silkworm's gut environment, where it germinates and penetrates the gut epithelium; acquiring host proteins for its survival and propagation (Li et al., 2018). Microsporidians are an early divergent clade of fungi splitting from the main fungi after the divergence of the chytrids (Capella-Gutiérrez et al., 2012; Keeling et al., 2000). Microsporidians generally infect the larval silkworms horizontally, through feeding, and if sufficient care is not taken the disease can be transmitted vertically from infected mother moth to its offspring (Hukuhara, 2018). Hence, it becomes increasingly important to screen the mother moths for the presence of *N. bombycis* spores.

The current method to screen for *Nosema bombycis* is to crush mother moths after egg-laying and examine the tissues for the presence of

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Nosema spores with light microscopy. Depending on the results, the corresponding eggs are then quarantined or destroyed. This method is very labour intensive, highly prone to false positive results (Chakrabarty et al., 2013) and cannot distinguish between *N. bombycis* and non-pathogenic species of *Nosema* that co-circulate in wild and cultivated silkworms (Dong et al., 2010). Several molecular methods have been developed for the detection of *N. bombycis*, based on the conserved Small Subunit (SSU) rRNA gene (Kampliw and Monthatong, 2019; Klee et al., 2006). An added advantage is that the variable regions of the SSU gene also can be used for studying the evolutionary relationships between *Nosema* species. However, the SSU rRNA gene is present in multiple copies in the genome making it less than ideal for *Nosema* quantification (Bateman et al., 2016; Liu et al., 2013). Hatakeyama and Hayasaka developed a multiplex PCR method for detecting in a single reaction the presence of multiple species of microsporidia in silkworm eggs (Hatakeyama and Hayasaka, 2002). However, in multiplex PCR the different co-amplifying templates all have to reach detection level before the reaction reaches saturation, which is determined by the most abundant template and normally proceeds along an S curve within 4–5 cycles from the detection threshold (SantaLucia, 2007). This means that for any one *Nosema* species to be detected in a multiplex reaction, its template must be present at >2% of the most abundant *Nosema* species (i.e. 4–5 amplification cycles), making the method inherently unreliable for absolute diagnoses of templates present under this threshold (Herrmann et al., 2004). Additionally, the multiple competing targets in multiplex PCR makes it impossible to accurately quantify any one target. We recently developed a sensitive Loop-Mediated Isothermal Amplification (LAMP) assay targeting the *Nosema* polar tube protein (Esvaran et al., 2018). However, LAMP assays use multiple primer pairs for each target and furthermore operates within the annealing temperature range of these primers, making it inherently susceptible to illegitimate primer-primer interactions. These affect the legitimate interactions, leading to an increased likelihood of both false positive and false negative results, especially if multiple targets are assayed simultaneously (Sahoo et al., 2016). LAMP is furthermore also rather sensitive to inhibitors in the template while the reaction kinetics are complex and unpredictable, making it unsuitable for accurate target quantification. The most recent approach to detecting *N. bombycis* has been the development of a nucleic-acid-based lateral-flow strip (NALFS) that combines PCR amplification of the Large Subunit (LSU) rRNA coupled with biotin and carboxyfluorescein labels to visualize the reaction products in the capture zone (He et al., 2019). But these assays are yet to be commercialized and the assays have inherent problems where inappropriate application of the sample using the dropper is not easy and may result in test errors.

The aim of this study was to develop and optimize a sensitive, specific and reliable method to detect and quantify *N. bombycis* in silkworms. The approach was to develop a TaqMan assay targeting the *N. bombycis β-tubulin* gene, which has been suggested to be a single-copy gene in most microsporidians (Franzen and Müller, 1999) and is also a useful gene for phylogenetic studies, similar to the SSU rRNA gene. We recently developed a broad-range real-time quantitative PCR (qPCR) assay for the *Nosema β-tubulin* gene based on the SYBR-green detection chemistry, which has been used successfully to detect generic *Nosema* in wild and commercial silkworms (Esvaran et al., 2019), although without being able to distinguish between different species of *Nosema*. Such additional species-specificity can be incorporated into the assay by adding a species-specific TaqMan probe as the detection chemistry. The protocol can furthermore be expanded into a duplex or multiplex assay, if desired, by including additional probes specific for other targets (Holland et al., 1991). The method can be used as a stand-alone method or as a complement to other diagnostic methods, as long as the appropriate infrastructure is available for its use. The assay was used to trace *N. bombycis* infection from orally inoculated silkworms through pupation and vertical transmission by the mother moths to the following generation of young larvae. These assays revealed a gradual post-inoculation increase in *N. bombycis* levels throughout development, as well as significant differences in infection between different types of tissue.

Table 1
Primer and probe sequences of the β-tubulin gene (amplicon size 268 bp).

Oligonucleotide	Sequence (5′ → 3′)	Reference
Forward Primer	5′GGCTGTTCTTATCGATTTAG 3′	This study
Reverse Primer	5′GAGAAGGGTTCCCATTCCTG 3′	Esvaran et al., 2019
Probe	5′[FAM]ACGCCATTAGACAAGGACCATAC [TAMRA] 3′	This study

2. Materials and methods

2.1. Assay design

A forward primer was designed by Eurogentec based on the available gene sequence on NCBI and used together with a previously reported reverse primer (Esvaran et al., 2019) for conventional PCR and for the new TaqMan assay (Table 1). A TaqMan probe was designed to fit the *N. bombycis β-tubulin* gene sequence (GenBank accession DQ663475) by Eurogentec (Eurogentec, Bangalore, India) with a FAM (6-carboxy-fluorescein) fluorophore at the 5′ end and TAMRA (6-carboxy-tetra-methyl-rhodamine) quencher at the 3′ end (Table 1).

2.2. Optimisation of assay conditions

To determine the optimal primer and probe concentrations, several concentrations were tested and determined to 100 nM for the primers and to 250 nM for the probe. Several PCR runs were performed to find the annealing temperatures for both the primers and the probe, and the optimal annealing temperature was determined to 60 °C. The optimized concentrations and annealing temperatures were used to generate standard curves.

2.2.1. Conventional PCR conditions

For PCR analysis, the Emerald master mix (EmeraldAmp® GT PCR Master Mix, TaKaRa), was used according to the manufacturer's recommendation using an annealing temperature of 60 °C (initial denaturation at 94 °C for 2 min followed by 30 cycles of [94 °C for 30 s, 60 °C for 30 s, 72 °C for 35 s]). A non-template control (NTC) was included in all runs. Amplification was carried out in a MJ Research PTC-200 Peltier Thermal Cycler. The amplified products were visualised on a 1.5% agarose gel with 3 μl ethidium bromide per 80 ml gel.

2.2.2. TaqMan assay conditions

For the TaqMan assay, a reaction mixture consisting of 5 μl of Takyon™ Low Rox Probe Master Mix dTTP Blue (Eurogentec), 100 nM of each primer, 250 nM of the probe, 0.75 μl distilled water and 2 μl of template was used. A positive control containing the plasmid with the *β-tubulin* gene sequence and a non-template control (NTC) were included in all PCR runs. The TaqMan assay was run-in Real-Time PCR machine Agilent Technologies Stratagene Mx3005P'as follows; initial denaturation at 95 °C for 2 min followed by 40 cycles of [95 °C for 30 s, 60 °C for 30 s, and 72 °C for 35 s].

2.3. Cloning the β-tubulin gene fragment from several silkworm-infecting Nosema species

To obtain a positive control and standard for the assay, the *N. bombycis β-tubulin* gene from infected *B. mori* was amplified using the same primers as for the TaqMan assay and the PCR product was cloned into pJET 1.2 Blunt vector using the Clone JET PCR Cloning Kit (Thermo Scientific, Waltham, MA, USA) and transformed into *Escherichia coli* DH5α cells (Bangalore Genei). A plasmid containing the *β-tubulin* gene

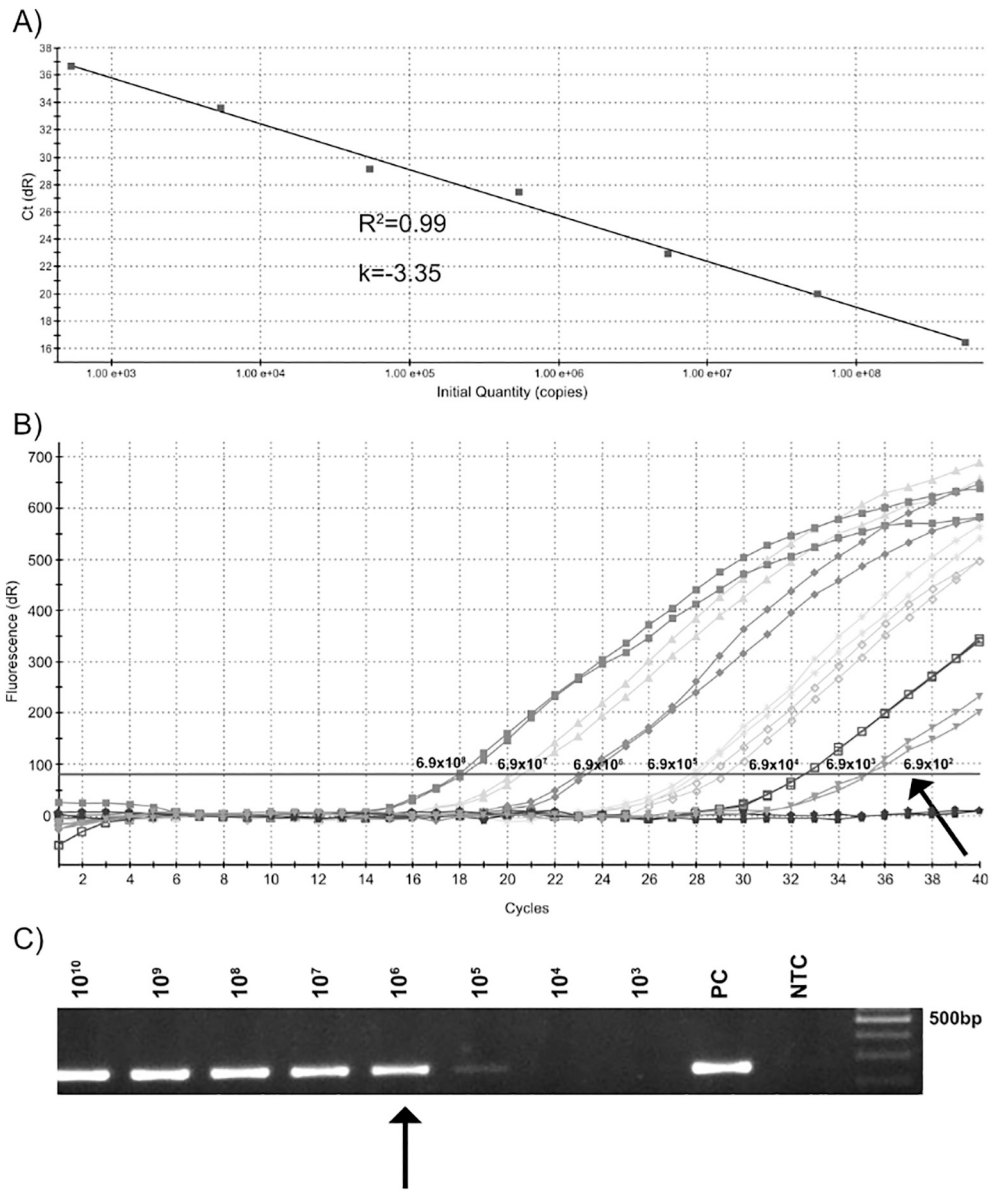

Fig. 1. A) Standard curve with tenfold serial dilutions of the plasmid containing the *β-tubulin* gene of *N. bombycis*. B) Amplification of tenfold serial dilution of the plasmid containing the *β-tubulin* gene of *N. bombycis* using the TaqMan assay. Each dilution was run in duplicate. Copy numbers per reaction are shown next to the corresponding curve and the limit of detection is shown with an arrow. C) Amplification of tenfold serial dilution of the plasmid containing the *β-tubulin* gene of *N. bombycis* using conventional PCR. The PCR products were visualised on 1.5% agarose gel with 3 μl/ 80 ml of ethidium bromide to determine the limit of detection. Copy numbers per reaction are shown next to the corresponding band and the limit of detection is shown with an arrow. PC-Positive Control- *N. bombycis β-tubulin* gene cloned in pJET plasmid, NTC-Non-template Control.

insert was extracted using the FavorPrep ™ Plasmid Extraction Mini kit (Favorgen). The sequence of the cloned *β-tubulin* gene was confirmed, and the concentration and purity of the plasmid was measured using Nanodrop 2000C Spectrophotometer (Thermo Scientific). The plasmid copy number concentration was calculated as follows:

$$\text{Number of copies} = \frac{\text{concentration of plasmid in ng}/\mu\text{l x } 6.022 \text{ x} 10^{23}}{\text{length x 1 x } 10^{9} \text{x } 660}$$

To evaluate the specificity of the assays, the *β-tubulin* gene from other *Nosema* spp. (*Nosema mylitta, Nosema assamensis* and *Nosema ricini*) infecting wild silkworms (*Antheraea mylitta, Antheraea assamensis* and *Samia cynthia ricini*) were also cloned.

2.4. Sensitivity and specificity

A tenfold dilution series of the plasmid containing the *β-tubulin* gene ranging from 10^8 to 10^2 copies was used to generate a standard curve for calculating the efficiency of the real-time PCR using the formula: % efficiency $= 10^{(-1/\text{slope})} * 100$.

The sensitivity of the TaqMan assay was assessed by comparing the results of the conventional PCR. To determine the specificity, the assay was tested against DNA samples from silkworms infected with *B. mori* bidensovirus (BmBDV), *B. mori* nucleopolyhedrosis virus (BmNPV), *Antheraea proylei* nucleopolyhedrovirus (AnprNPV) and *Beauveria bassiana* (*B. mori* muscardine). The DNA samples from silkworms infected with BmBDV, BmNPV, AnprNPV, *B. bassiana* were prepared in previous studies (Esvaran et al., 2019; Naik et al., n.d.; Shantibala et al., 2018). To check the assay for cross-reactivity, samples containing plasmids with a *β-tubulin* gene insert from *N. assamensis, N. mylitta or N. ricini* were analysed.

2.5. Infection studies

The silkworms (maintained inhouse) were infected with *N. bombycis* spores by feeding them with leaves inoculated with *N. bombycis* spore suspension. A spore concentration of 10^6 spore/ml was used based on the LD_{50} value of *N. bombycis* from a previous study (Esvaran et al., 2019). Before feeding, the silkworms were starved for 3–5 h after the 3rd moulting stage to ensure that all inoculated leaves were consumed. ~100 mg of tissue samples of infected and uninfected silkworms was collected from the midgut, fat bodies and ovaries days 1–7 post-

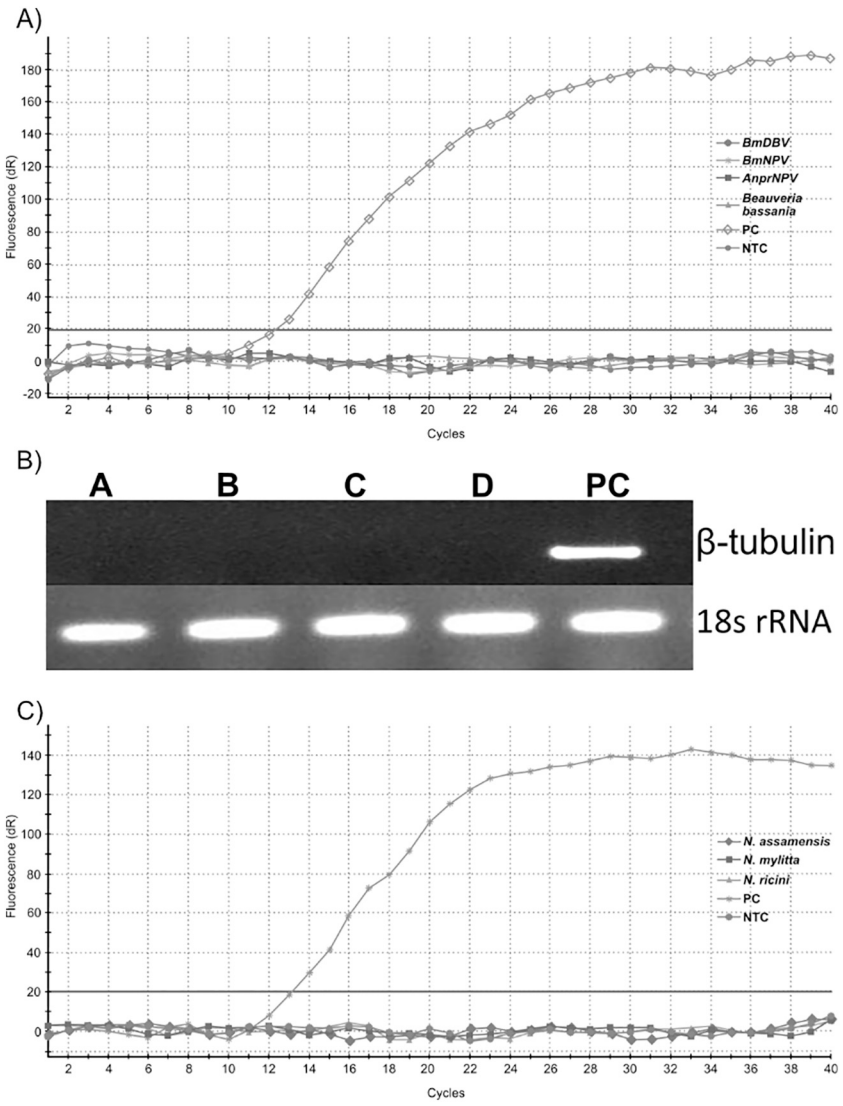

Fig. 2. A) Specificity of the primers and probes against the *N. bombycis β-tubulin.* Samples containing DNA extracted from silkworms infected with BmBDV, BmNPV AnprNPV and *B. bassiana* fungi were analysed together with positive control containing a plasmid with *N. bombycis β-tubulin* gene insert (PC) and a non-template control (NTC). B) Specificity against the *N. bombycis β-tubulin* gene was tested using conventional PCR. DNA samples from silkworms infected with BmBDV (A), BmNPV (B), AnprNPV (C) and B. bassiana fungi (D), were analysed together with positive control containing a plasmid with *N. bombycis β-tubulin* gene insert (PC). C) Test for cross-reactivity with other *Nosema* species. Samples containing plasmids with *N. assamensis, N. mylitta and N. ricini β-tubulin* gene inserts were analysed together with positive control containing a plasmid with *N. bombycis β-tubulin* gene insert (PC) and a non-template control (NTC).

infection. This was done to check the tissue penetration of the infective spores. For comparison, samples of infected mother moths and hatched larvae were collected from commercial egg production centres and analysed with the TaqMan assay.

2.6. DNA extraction

The DNA was isolated from the samples using an in-house Phenol: Chloroform: Isoamyl alcohol (PCI) method. First, the tissue samples were crushed using a micro pestle in a 2PK buffer (200 mM Tris, 300 mM NaCl, 25 mM EDTA with SDS) followed by a PCI treatment. Following centrifugation of the mixture, the upper aqueous layer was carefully transferred to a new tube with an equal volume of isopropanol added in order to precipitate the DNA. After centrifugation, the pellet was washed with an equal volume of 70% ethanol to ensure the obtained pellet was not contaminated. All centrifugation steps were performed at 13,000 rpm for 10 min at 4 °C. The pellet was air dried and dissolved in nuclease-free water before storage at −20 °C.

3. Results

3.1. Sensitivity

The initial plasmid concentration was calculated to be 6.9×10^{11} copies/μl. A standard curve of the plasmid was generated from a tenfold serial dilution and displayed a linearity 0.99 (R^2 value) with a slope value of −3.35. The efficiency of the assay was determined to be 98.8% (Fig. 1). The TaqMan assay had a higher sensitivity than conventional PCR with limit of detection at 10^2 and 10^6 copies per reaction, respectively (Fig. 1B and C).

3.2. Specificity and repeatability

The specificity of the TaqMan assay was tested by analysing samples containing three other silkworm pathogens: BmBDV, BmNPV, and *B. bassiana* (*B. mori* muscardine). The assay was negative for all three pathogens (Fig. 2), the same results was also seen with conventional PCR (Fig. 2B). To test the cross-reactivity of the TaqMan assay, samples of wild silkworms infected with various *Nosema* spp. were analysed. The TaqMan assay did not detect any of the other *Nosema* spp., indicating that the assay is specific to *N. bombycis* (Fig. 2C). This was further confirmed by an alignment of the *β-tubulin* gene sequences of *N. bombycis, N. assamensis, N. mylitta* and *N. ricini* which revealed variations at the nucleotide positions 5, 20 and 23 of the probe binding sites (Fig. 3).

Repeatability of the assay was analysed by looking at the intra-assay and inter-assay variability. The intra-assay variability was calculated from standards run in duplicate within the same run and the Coefficient

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CLUSTAL 2.1 multiple sequence alignment

Nosema_ricini          GGCTGTTCTTATCGATTTAGAACCAGG-AACAATGGACGCTATTAGACAAGGACCTTATG
Nosema_assamensis      GGCTGTTCTTATCGATTTAGAACCAGG-AACAATGGACGCTATTAGACAAGGACCTTATG
Nosema_mylitta         GGCTGTTCTTATCGATTTAGAACCAGG-AACAATGGACGCTATTAGACAAGGACCTTATG
Nosema_bombycis        GGCTGTTCTTATCGATTTAGAACCAGGGGACAATGGACGCCATTAGACAAGGACCATACG
                       *************************** .*********** **************:** *

Nosema_ricini          GTGAATTATTTAGACCTGATAATTTTGTCTTTGGGCAATCTGGTGCTGGTAATAATTGGG
Nosema_assamensis      GTGAATTATTTAGACCTGATAATTTTGTCTTTGGGCAATCTGGTGCTGGTAATAATTGGG
Nosema_mylitta         GTGAATTATTTAGACCTGATAATTTTGTCTTTGGGCAATCTGGTGCTGGTAATAATTGGG
Nosema_bombycis        GTGAACTATTTAGACCTGATAACTTTGTCTTTGGTCAATCTGGTGCTGGTAATAATTGGG
                       ***** ***************** *********** ************************

Nosema_ricini          CTAAAGGTCATTACACTGAAGGGGCTGAATTAATTGATAGTGTAATGGATGTAGTAAGAA
Nosema_assamensis      CTAAAGGTCATTACACTGAAGGGGCTGAATTAATTGATAGTGTAATGGATGTAGTAAGAA
Nosema_mylitta         CTAAAGGTCATTACACTGAAGGGGCTGAATTAATTGATAGTGTAATGGATGTAGTAAGAA
Nosema_bombycis        CTAAAGGTCATTACACTGAAGGGGCTGAATTAATTGATAGTGTAATGGATGTAGTAAGAA
                       ************************************************************

Nosema_ricini          AAGAAGCTGAATCTTCGGATTGTCTTCAGGGATTTCAGATTACACACTCTTTGGGTGGGG
Nosema_assamensis      AAGAAGCTGAATCTTCGGATTGTCTTCAGGGATTTCAGATTACACACTCTTTGGGTGGGG
Nosema_mylitta         AAGAAGCTGAATCTTCGGATTGTCTTCAGGGATTTCAGATTACACACTCTTTGGGTGGGG
Nosema_bombycis        AAGAAGCTGAATCTTCGGATTGTCTTCAGGGATTTCAAATTACACACTCTTTGGGTGGGG
                       ************************************.***********************

Nosema_ricini          GTACTGGTGCAGGAATGGGAACCCTTCTC
Nosema_assamensis      GTACTGGTGCAGGAATGGGAACCCTTCTC
Nosema_mylitta         GTACTGGTGCAGGAATGGGAACCCTTCTC
Nosema_bombycis        GTACTGGTGCAGGAATGGGAACCCTTCTC
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Fig. 3. Alignment of the *β-tubulin* gene sequence (cloned sequences) from *N. bombycis, N. assamensis, N. ricini* and *N. mylitta* in CLUSTALW. Primer and probe sequences are highlighted in the alignment.

Table 2
Intra-assay (within the runs) and inter-assay (between the runs) variability of the standards analysed with the TaqMan assay.

Copies per reaction	Intra-assay variability		Inter-assay variability	
	Mean ± SD	CV (%)	Mean ± SD	CV (%)
6.9×10^2	35.7 ± 0.1	0.3	35.2 ± 0.7	2.0
6.9×10^3	32.9 ± 0.0	0.0	32.9 ± 0.1	0.3
6.9×10^4	29.2 ± 0.5	1.8	29.1 ± 0.1	0.4
6.9×10^5	28.2 ± 0.1	0.5	28.0 ± 0.2	0.8
6.9×10^6	23.5 ± 0.2	1.0	23.5 ± 0.1	0.5
6.9×10^7	20.8 ± 0.2	1.1	20.8 ± 0.1	0.3
6.9×10^8	18.4 ± 0.2	1.0	18.2 ± 0.3	1.8

SD, Standard deviation; CV, Coefficient of variation.

of variation (CV) value was found to be less than 5% (Table 2). The inter-assay variability was analysed by comparing the standards between two runs and the CV was also found to be less than 5% indicating a high degree of repeatability for the developed assay (Table 2).

3.3. Sample analysis

Samples collected from the silkworms infected with *N. bombycis* spores on days 1–7 post-infection were analysed with the new TaqMan assay and with conventional PCR. Both assays revealed a gradual increase of the pathogen over the seven days following infection (Fig. 4A and B). *N. bombycis* was identified with both the TaqMan assay and conventional PCR in tissue samples from midgut, fat body and ovary of infected silkworms. The TaqMan assay also revealed that the highest copy number was found in the midgut samples followed by the ovary and then fat body samples. Two sets of samples were collected from *N. bombycis*-infected mother moths and her offspring and analysed using the TaqMan assay. All samples were positive for *N. bombycis*, confirming vertical transmission (Fig. 5).

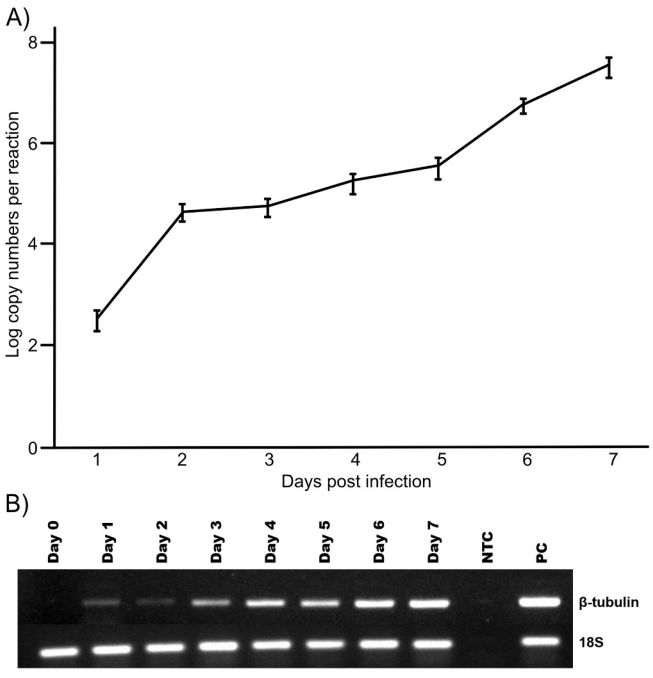

Fig. 4. *B. mori* samples collected days 1–7 post-infection and analysed with A) the TaqMan and B) conventional PCR. The error bars represent the standard deviation of the samples.

4. Discussion

Pébrine is a major disease infecting silkworm in India with occasional crop losses. The disease primarily spreads through infected eggs, but also through contaminated rearing houses, feed etc. When the

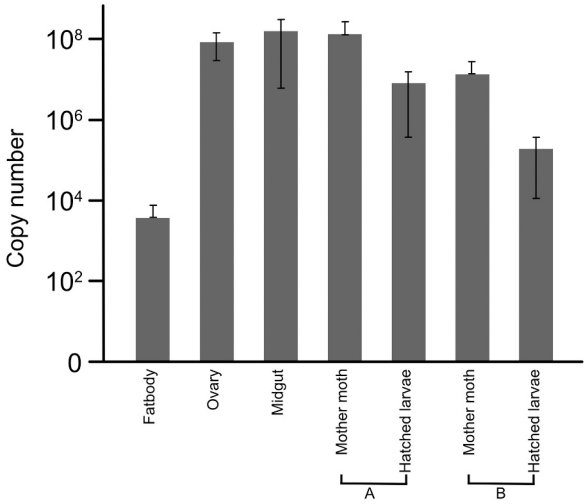

Fig. 5. Pathogen load detected with the TaqMan assay in samples from the midgut, fat body, ovary, infected mother moth and hatched larvae. Two sets of mother moth and hatched larvae were analysed and indicated in the figure as set A and B. The error bars represent the standard deviation of the samples.

typical disease signs are observed, the usual approach is to examine mother moths at various seed production centres with light microscopy to find *N. bombycis* spores (Pasteur, 1870). However, these types of methods have been found to not be reliable and prone to give false-positive results. Molecular methods, such as real-time PCR, are often highly reliable, and a probe-based PCR assay, such as a TaqMan assay, would be a more suitable diagnostic tool than microscopy. A comparison of the TaqMan assay with conventional PCR showed that both of these methods can be used for detecting *N. bombycis* in silkworms, but the TaqMan can also be used to quantify the amount of *N. bombycis* in the samples. Although the primers used here are capable of binding to several *Nosema* species, the probe is designed to only target *N. bombycis* making the assay specific. This makes it possible in the future to design and include probes for other *Nosema* spp. for a multiplex TaqMan assay. TaqMan assays are used for detecting a wide range of targets including viral and bacterial pathogens in humans, animals and plant specimens (Nagy et al., 2017). They are also used for genotyping, quantifications of mutations and for gene expression profiling (Alía et al., 2020; Tajadini et al., 2014; Wang et al., 2020). Several TaqMan assays, which have been proven to be highly sensitive, have been developed to detect microsporidians in different types of samples, including assays for targeting a microsporidian *β-tubulin* gene in shrimp (Piamsomboon et al., 2019) and for detection of microsporidians in aquaculture (Frenette et al., 2020).

A previous study showed that the pébrine disease-causing agent *N. bombycis* is capable of tissue penetration with a high infection level in midgut followed by the ovary, malpighian tubules, eggs and fat body (Esvaran et al., 2019). The same was seen in this study, where we sampled infected silkworms and analysed three of the tissue types; midgut, ovary and fat body. Although not all the microsporidian species displays transovarial vertical transmission, *N. bombycis* has been reported to display this type of transmission and it has been attributed to its higher penetration and virulence compared to other microsporidia (Fine, 1984). The pathogen is transmitted from adult moths to larvae via contaminated egg surfaces. To test this, we analysed mother moths and their offspring with our new TaqMan assay and detected *N. bombycis* in both groups, with a higher pathogen load in mother moths, indicating a vertical mode of transmission. The TaqMan assay developed in this study, showed a high sensitivity and was capable of detecting as few as 10^2 copies of the target gene, compared to the conventional PCR LOD of 10^6 copies. It also displayed a high specificity for *N. bombycis*, as no cross-reactivity with other *Nosema* spp. were seen in this study. This assay is a good candidate to replace the existing tedious mother-moth examination with light microscope providing greater reliability, ease and elimination of the false positives. We also showed that the assay is suitable for quantification of the pathogen by analysing experimentally infected silkworms. The method can support routine analysis in centres for mass production of silkworm eggs providing they have appropriate infrastructure to support the analysis.

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Author contribution

AJ designed, executed the research, wrote the manuscript; DK cloning; MT Data analysis and reviewed the manuscript; JRM, AN, provided technical advice and reviewed the manuscript; OT technical advice and financial support; HD provided technical advice and scientific inputs; RKM technical advice and scientific inputs; KMP technical advice and scientific inputs; all authors edited the paper. All author(s) read and approved the final manuscript.

Declarations of interest

None.

Data availability

Data will be made available upon reasonable request to the senior author K. M. Ponnuvel.

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