J Parasit Dis (Jan-Mar 2019) 43(1):31–38
https://doi.org/10.1007/s12639-018-1053-4

ORIGINAL ARTICLE

Development and comparison of real-time and conventional PCR tools targeting β-tubulin gene for detection of *Nosema* infection in silkworms

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Received: 25 July 2018 / Accepted: 7 November 2018 / Published online: 26 November 2018

Abstract Microsporidiosis (Pebrine) caused by the microsporidian parasite is one of the important devastating disease which affect the silk production leading to an unprofitable harvest. Till date ribosomal RNA (rRNA) gene was used as a target for detection of microsporidian species. In this study, we describe conventional and SYBR green based real-time PCR techniques alternatively targeting β-tubulin gene for quantitative detection of microsporidia infecting both the mulberry and non-mulberry silkworms. The modified DNA extraction method followed in our study was found to be easy, economical and could be used for both conventional and real time PCR as template. The real time qPCR revealed the expression of β-tubulin gene in different infected tissues of the silkworm *Bombyx mori*. The sensitivity of the SYBR green based real time PCR was found to be 100 times more than the conventional PCR and PCR was found more sensitive than the microscopic examination. The developed method did not produce any false positive results with the other silkworm pathogens and healthy silkworm. The data suggest that both the developed PCR methods targeting β-tubulin gene could be used effectively in quarantine process at seed centres for early detection of microsporidian infection in silkworms.

Keywords Microsporiosis · β-tubulin · Mulberry · Non mulberry · Conventional · Real time PCR

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Introduction

Microsporidians, the unicellular fungal parasites take refuge for their existence inside wide range of hosts, from vertebrates to invertebrates, including humans. Pebrine disease in silkworms is caused mostly by the unicellular microsporidian parasite belonging to *Nosema* sp. and the infection process can be either horizontal or vertical thereby ensuring transfer of infection to the progeny. The symptoms include sluggishness, poor appetite retardation in growth as well as development and advancement leading to shrinkage in size, sometimes with small and large pepper like black spots all over the body. The clubbed wings, distorted antennae, irregular moth emergence and improper mating are also symptoms of pebrinised silk moth. These microsporidian parasites are unique in structure lacking the golgi apparatus and peroxisomes, while, the mitochondria are replaced by mitosomes (Williams et al. 2002). They are spore forming parasites having invasive organelle, the polar tube, which everts from the spore in response to environmental stimuli thereby invading the host cell for their multiplication (Wittner and Weiss 1999; Vavra and Larsson 1999). The recent advancement in molecular technologies have helped in finding new microsporidian species causing microsporidiosis in silkworms and also in differentiating different strains biochemically and morphologically (Qiu et al. 2002). Based on the molecular evidences, many of the *Nosema* species have been shifted to other genus, like *N. corneae* to *Vittaforma corneae* (Silveira and Canning 1995), *N. connori* to *Brachiola connari* (Cali et al. 1998), *N. grylli* to *Paranosema n. comb* (Sakolova et al. 2003) and *N. locustae* to *Antonospora locustae* (Slamovits et al. 2004). Microtubules are a characteristic feature of all eukaryotes and are the major components of the cytoskeleton and the mitotic spindle.

The genes for tubulins, especially β-tubulin, have been receiving increasing attention in investigations on evolutionary relationships at kingdom level phylogenetic analyses (Keeling and Doolittle 1996; Baldauf et al. 2000) and to study the species within protists, animals, fungi and plants (Mages et al. 1995; Keeling 1998; Ayliffe et al. 2001; Edgcomb et al. 2001). Microsporidians were found to have alpha-, beta- and gamma tubulin genes. Earlier reports on phylogenetic analysis based on rRNA sequence suggested for separation of *Nosema* ('true' *Nosema*) from *Nosema/Vairimorpha* group and reported that Nosema species infecting lepidoptera are closely related to each other (Baker et al. 1994; Kyei-Poku et al. 2011). The phylogenetic analysis of alpha and beta-tubulin sequence of *Nosema philosamiae* revealed that these genes could be used as a diagnostic tool to discriminate the 'true' *Nosema* species group and *Nosema/Vairimorpha* group (Zhu et al. 2013). Hence, in our study the β-tubulin gene was targeted for diagnosis of pebrine disease in mulberry and non-mulberry silkworms. The study was also aimed to develop molecular diagnostic method to detect microsporidian pathogens infecting silkworms of India at the early stage based on conventional and real-time polymerase chain reaction. The sample size was kept at n = 25 each. The symptoms and the geographic data of each infected silk moth were recorded followed by DNA extraction and polymerase chain reaction to detect the pathogen. The honey bee pathogen *Nosema ceranae* was found to be co infected with deformed wing virus (Martin et al. 2013). Similarly, most of the infected silkworms revealed symptoms of unhealthy deformed wings, while, around 75% of the silkworms were co-infected with the other viral pathogens (unpublished data) Generally, the 16S ribosomal gene of microsporidia has been always targeted for detection of microsporidial infection. However, in this study for the first time functionally essential and highly conserved eukaryotic β-tubulin gene of microsporidia has been targeted for detection of the pathogen.

Materials and methods

Primer designing of β-tubulin gene

The partial sequence of β-tubulin gene of *Nosema* sp. used for the study was retrieved from the Gene bank database (Accession No. EF151928) which was found to be 100% conserved for *Nosema* sp. infecting silkworms of India. The sequence was initially analyzed for its specificity using the in silico blast analysis with the available DNA sequences and the conserved region was utilized to design the primer using Primer 3.0. The β-tubulin gene of *Nosema* sp. was amplified using β-tubulin-F 5′CTTTGGACAATCT GGTGCTG3′ and β-tubulin-R 5′GAGAAGGGTTCCCATTCCTG 3′ primers. The estimated product size of the amplified β-tubulin gene was 181 bp.

Sample collection

The infected moth samples with disease symptoms were collected individually from different sericulture regions of India. The infected non-mulberry wild silkworms viz., *Antherea mylitta*, *A.assama and Samia ricini* were collected from State sericulture Farm, Warangal, Telangana, CMERTI, Lodhigarh, Assam and Eri Silkworm Seed Production centre, Hosur, Tamil Nadu respectively. Whereas the mulberry silkworm *Bombyx mori* inoculated with *Nosema bombycis* were reared at SBRL in a controlled condition and used for the study.

DNA extraction and spore purification

The silk moths were segregated and approximately 25 mg of abdominal tissue excluding the scales were dissected individually for DNA isolation. All the samples were microscopically examined for infection. Genomic DNA was isolated from both positive and negative samples using a modified method in which the tissue samples were crushed to release the cell contents using Tris lysis buffer (200 mM Tris, 300 mM Nacl, 25 mM EDTA) with SDS, vortexed vigorously followed by treatment with phenol: chloroform: iso amyl alcohol (25:24:1). The organic phase after centrifugation was transferred to a fresh tube to which ice–cold isopropanol was added. The insoluble nucleic acid fraction was spun at maximum speed for 5 min and the pellet was washed with 70% ethanol, air-dried and dissolved in 50 µl of sterile water. The microsporidian spores were purified from the infected tissue using percoll gradient centrifugation and the spore DNA was isolated using Qiagen Tissue DNA extraction kit. The concentration of spore DNA was quantified using the UV–Vis Spectrophotometer.

Cloning and sequencing

The β-tubulin gene primer was used to amplify the β-tubulin gene of *N. bombycis* to generate a 181 bp gene product and was cloned into the pJET vector. Plasmids from positive transformants were extracted using Qiagen plasmid DNA extraction kit and sequenced. The plasmid containing the target gene was used as positive control template for standard curve. Apart from that, a fragment of elongation factor-α was also amplified through conventional PCR using primers EF35-F and EF 957-R as reported previously (Hatakeyama and Hayasaka 2003) in a

Eppendorf master cycler (Eppendorf, Hamburg, Germany) to confirm infection of the moths with *Nosema* sp. alone and not with other microsporidian strains that infect silkworms. The amplicons were examined on 1.2% agarose gel stained with ethidium bromide for DNA visualization under UV light.

RNA and cDNA synthesis

The different tissues namely midgut, fatbody, malphigian tubules, ovary and the egg were collected from the infected mulberry and non mulberry larvae and the total RNA was extracted by crushing the tissues individually in liquid nitrogen with RNAiso Plus reagent (Takara Bio Inc.) followed by chloroform and isopropanol to precipitate the RNA. The RNA was washed with 70% ethanol and airdried RNA samples were resuspended in 50 µl of RNase free water. The quality and quantity of the RNA was evaluated using the UV–Vis spectrophotometer. The RNA was diluted to 2 µg/ml and used for the cDNA synthesis. The 2 µg/µl total RNA was treated with DNase I (Invitrogen) with the supplied buffer to remove the residual DNA contamination. The superscript III first strand synthesis kit (Invitrogen) was used for RT-PCR according to the manufacturers' protocol. Reverse transcription was performed with 20 µl reaction mixture with 1 µl of random hexamer, 10 mM dNTPs and made up to 10 µl with nuclease free water and incubated at 65 °C for 5 min. A 5X reverse transcriptase buffer (4 µl), 5 mM DTT (2 µl) and Superscript III reverse transcriptase (Invitrogen, USA) (1 µl) was added to obtain a final volume of 20 µl. The reaction mixture was incubated at 42 °C for 60 min and the reaction was terminated by heating at 75 °C for 10 min and further used for the PCR reaction.

Tissue specific expression of β-tubulin

The expression of β-tubulin gene of the pathogen *Nosema* was analysed in different tissues namely midgut, fatbody, malpighian tubules, ovary, egg of infected mulberry and non mulberry silkworms and were quantified using conventional as well as real time PCR along with 18S as internal control. One µl of first strand cDNA was used in a 20 µl SYBR green reaction mixture containing β-tubulin gene specific primers and the reaction was performed on STRATAGENE 3005P real time PCR machine.

PCR diagnosis method

An end point PCR reaction was optimized for diagnosis of microsporidian infection in the mulberry and non-mulberry silkworms. The PCR reaction mixture consisted of a total volume of 10 µl PCR mixture composed of 1 µl of spore DNA, 100 µM of each dNTP, 0.1 µM of each primer, 1 µl of 10X PCR buffer (100 mM Tris–HCl, pH 8.3 and 500 mM KCl), 1.5 mM $MgCl_2$, 0.5 µl (1.5 U/reaction) Taq DNA polymerase (Thermo scientific) and 3.5 µl of sterile water. The samples were subjected to the following thermal profile for amplification in a Eppendorf master cycler: 3 min at 94 °C, followed by 35 cycles consisting of three steps of 30 s at 94 °C, 45 s at 57 °C and 1 min at 72 °C, before a final elongation of 10 min at 72 °C. The amplicons were examined on 1.2% agarose gel and stained with ethidium bromide for visualization of the amplified product.

Real time diagnostic method

Real-time PCR was performed using 96-well plates (Bio-rad), SYBR green 2X master mix (Takara) and 0.25 µM primer concentrations on Mx3005P qPCR thermo-cycler (Agilent—Stratagene Corp.). The cycling conditions for β-tubulin primers with SYBR green were as follows: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 57 °C annealing for 30 s, and 72 °C extension for 30 s; followed by melting curve analysis of 95 °C for 1 min; and 55 °C for 30 s, 95 °C for 15 s, 60 °C for 30 s and 72 °C for 60 s. All assays were performed with three technical replications. Standard curves of the β-tubulin plasmid DNA were constructed between the cycle threshold (Ct) value and the log DNA concentration. The DNA concentration of the template was measured at 260 nm using uv–vis spectrophotometer (Thermo Scientific). In order to determine the copy number, a standard curve was plotted against plasmid concentration. A serial dilution of pJET β-tubulin plasmid, ranging from 1×10^2 to 1×10^7 copies/ml, was used to construct the standard curve. Threshold cycle (*C*t) values in each dilution were measured in triplicate and were plotted against the logarithm of their initial template copy numbers. Each standard curve was generated by a linear regression of the plotted points and the PCR amplification efficiency (*E*) was calculated from the slope of each standard curve according to the following equation.

$$E(\%) = (10^{-1/\text{slope}} - 1) \times 100\%.$$

Specificity and sensitivity of the developed diagnostic method

The conventional as well as real time PCR methods were evaluated for sensitivity and specificity to detect the microsporidiosis in the silkworms. The detection limit of spore DNA was calculated from their respective C_T (Cycle threshold) using the linear equation of β-tubulin standard curve. The highest dilution with a visible band on the agarose gel was considered to be the lowest

detectable quantity of target DNA and the equivalent copy number was calculated using Avogadro's number against the molar quantity of plasmid DNA. The specificity of the developed primers was validated by conventional PCR by using the host silkworm genomic DNA and also with the silkworm DNA infected with BmNPV, BmBDV as template for analyzing the cross reactivity of the developed primers with other microorganisms infecting the silkworms. The detection efficiency at different developmental stages was also validated.

Results

Most of the infected silkworm samples revealed symptoms of deformed wings, lack of interest for mating and the eggs produced were fragile. Majority of the infected samples were from male moths and were co-infected with viral pathogens in case of non-mulberry silkworms (data not shown). Earlier studies at this laboratory revealed β-tubulin as the early gene expressed along with the reported 16s ribosomal RNA. Hence β-tubulin was targetted to detect microsporidian infection in infected silkworms. The silkmoth having deformed wings, fail to emerge from cocoons, shrunk abdomen were tested for the presence of microsporidia. Among those samples, the microsporidian infection was found 29% in tasar, 38% in eri and 40% in muga silk moths. Among the infected samples around 30% showed positivity for both microscope and PCR, whereas, 50% were positive only for PCR (data not shown). The primers amplified 181 bp region of *Nosema* sp. consistently and hence proves to be diagnostically useful for detection of microsporidian infection in silkworms. The EF 1α primers amplify microsporidians belonging to *Nosema* sp. were used to confirm that, the microsporidian symptoms were caused by *Nosema* sp. and are not cross infected by other microsporidian strains namely V*airimorpha*, *Pleistophora* which also infects the silkworm. The 16s SSU primer were also used to validate the efficiency of the newly designed β-tubulin primers (Fig. 1). The newly designed primers were able to detect the infection consistently with a greater sensitivity from the DNA extracted from the field samples collected from different geographical regions of India. All the samples amplified the predicted product of 18S gene indicating the success of modified DNA extraction method. The PCR conditions were optimized to increase the efficiency of the newly developed of conventional and real time PCR targeting the β-tubulin gene of *Nosema* sp. (Figure 2). The PCR efficiency values are the direct reflection of baseline estimation errors in qPCR (Ruijter et al. 2009) and based on that the qPCR had a detection efficiency of 103%. The differential expression of microsporidia specific β-tubulin gene

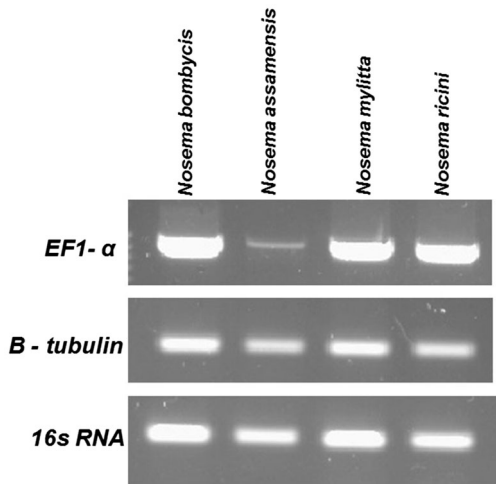

Fig. 1 Conventional PCR of newly designed β-tubulin gene primers with EF and 16S PCR primers with different spore DNA

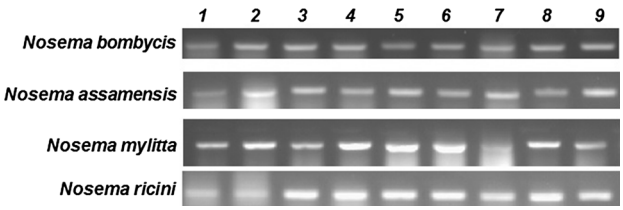

Fig. 2 Detection of microsporidian infection in field samples infecting different silkworms

in the infected tissues of mulberry and non-mulberry silkworms was analysed by conventional and real time PCR. The higher level of expression varied among the tissues according to the stage of infection. Mostly midgut was found to be high followed by ovary, malphigian tubules, egg and fat body. Since the primary route of infection (oral ingestion) of *Nosema* is being the midgut, it is obvious that it contains higher level of gene expression and ovary with a higher level of expression confirms the transovarial transmission of infection (Fig. 3a, b). The expression of the β-tubulin gene in egg revealed the transfer of infection from the mother moth to progeny. The sensitivity of the assay was validated by using the serially diluted spore DNA samples. The detection limit of conventional PCR was found to be 10 ng/μl of spore DNA, whereas the, real time PCR with SYBR green master mix was found to be 100 pg/μl of spore DNA (Fig. 4a, b). The developed method could be used to detect the infection at all the ontogenic stages of the silkworm i.e., the infection can be detected from egg to moth stage (Fig. 5). The *B. mori* silkworm larvae were found to survive till 3rd instar after hatching from the infected eggs when infected orally with 10^8 spores/larvae at fourth instar at the laboratory condition. The infection at moth egg and hatched larvae confirms the transmission of spores to the progeny. No cross reactivity of primers was

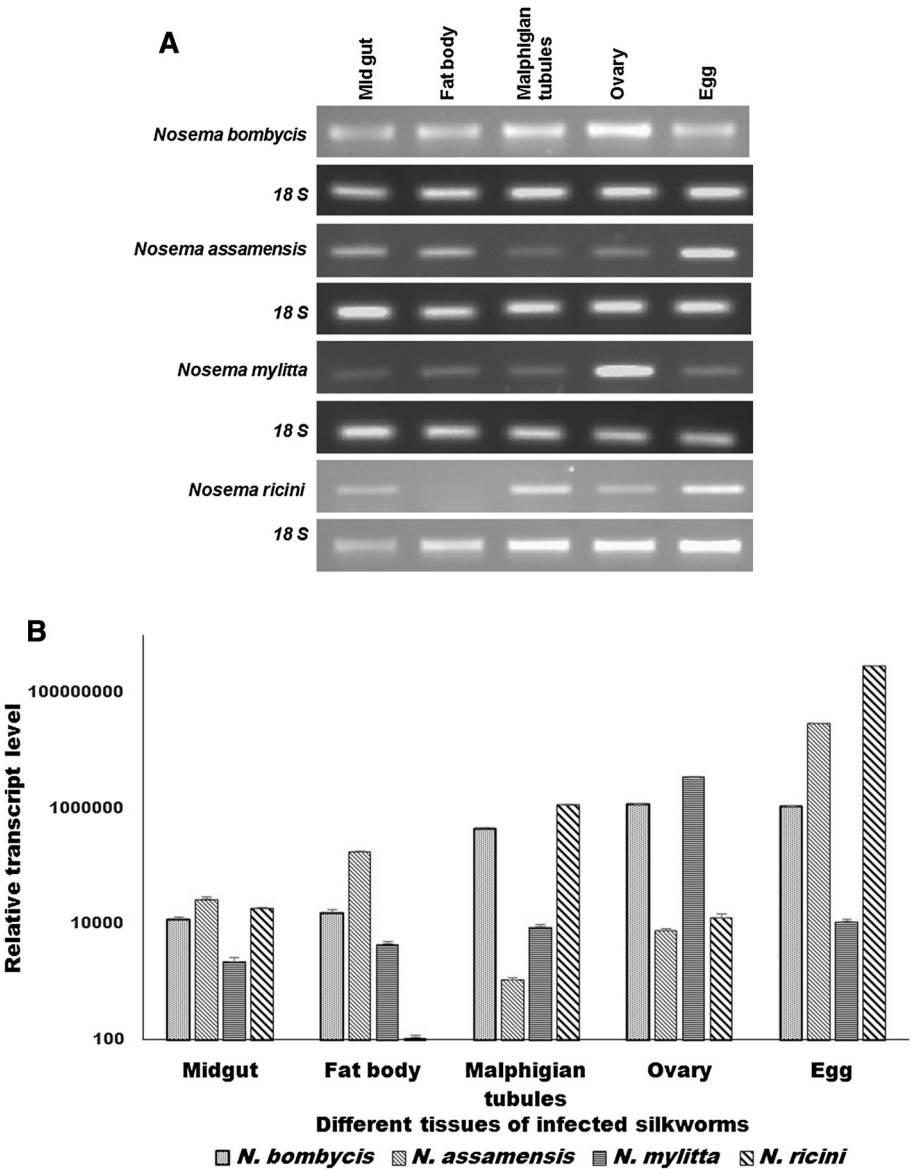

Fig. 3 Detection of β-tubulin gene expression in different tissues of infected silkworms through real time and conventional PCR. **a** Agarose gel profile of β-tubulin gene expression through conventional PCR. **b** SYBR green based real time PCR profile of β-tubulin gene expression in different tissues of infected silkworm

observed when used with the uninfected silkworm host DNA and silkworm DNA infected with BmDV/BmBDV and BmNPV (Fig. 6). Hence the developed method proves to be helpful in early detection of microsporidian infection in silkworms.

Discussion

Molecular diagnosis is always preferred over the conventional diagnostic methods for the confirmation of pathogenic infestation (Taniuchi et al. 2011). In this study we have developed a new specific PCR method for detection of microsporidiosis in mulberry and non mulberry silkworms of India. The foremost step in this technique involves DNA extraction.In this study the naturally infected samples were subjected to fast DNA extraction procedure which was used for conventional PCR and the method was found to be faster compared to reported methods and did not contain any inhibitors (Singh et al. 2011). A new set of molecular markers targeting the β-tubulin gene for detection of the pebrine disease caused by *Nosema* pathogen developed in this study could be used universally for detection of *Nosema* infection in both mulberry and non-mulberry silkworms. Till date the rRNA sequence was targeted for detection of microsporidiosis in silkworms as the microsporidian strains are classified based on their ribosomal RNA sequence (Ironside 2013). The ribosomal RNA sequence with multiple copies revealed high intra-specific mutation rate (Voigt et al. 1999). The tubulin proteins played a major role in eukaryotic cellular process (Einax and Voigt,2003) and found to be the second

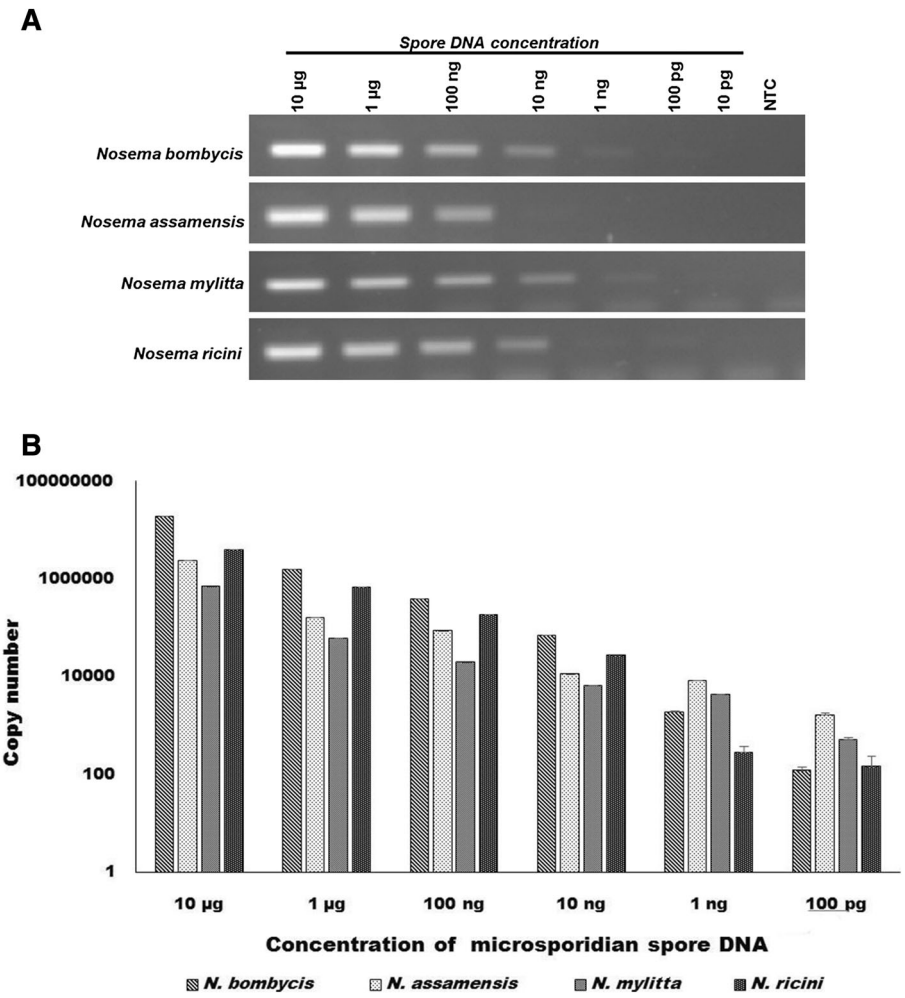

Fig. 4 Sensitivity of conventional and real time PCR in detecting the serially diluted microsporidian spore DNA. **a** Conventional PCR. **b** SYBR Green based real time PCR

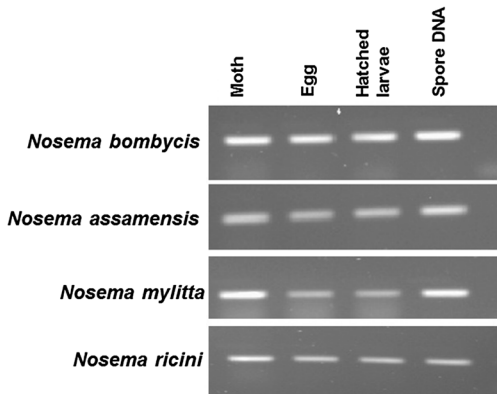

Fig. 5 Detection efficiency of β-tubulin primers at different developmental stages of silkworm

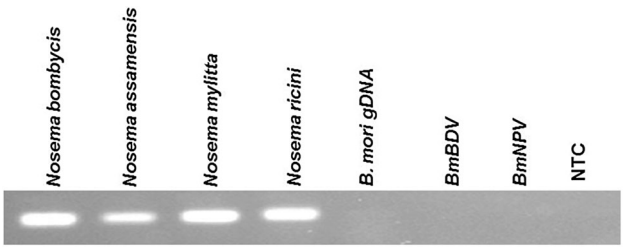

Fig. 6 Specificity of developed method in detecting the microsporidians in the infected samples

common target for molecular diagnostics in fungi (McCartney et al. 2003). Among conserved genes, the β-tubulin has been used frequently to develop diagnostics for fungi (Fraaije et al. 2001). The phylogenetic analysis of α and β-tubulin genes of *N. philosamiae* revealed a high similarity with the "true" *Nosema* species infecting Lepidoptera and hence can be used to discriminate the true *Nosema* group from the *Nosema*/Vairimorpha group. (Zhu et al. 2013). Our earlier experiments also revealed that the β-tubulin as one of the early genes expressed during infection (data not shown). Gene specific PCR is considered to be a sensitive and reliable method for diagnosis of microsporidiosis. This technique is exposed to significant risk of contamination and does not allow quantification of parasitic burden. The real time PCR was more sensitive

and less prone to contamination compared to conventional PCR and it also does not require any post amplification procedures (Idoos et al. 2009). In our study the conventional PCR was found to be less sensitive compared to SYBR green based real time PCR. But the standardized conventional PCR could be an alternative for diagnostic laboratories that do not possess the real time PCR system.

The DNA samples from the purified spores as well as infected tissues were reliable for quantification. The gene expression studies also revealed that the β-tubulin gene expression in all the tissues indicating the spread of infection from midgut to egg of the infected silkworm. In terms of routine laboratory practice, the developed conventional and real-time PCR can be performed at any of the developmental stages of the silkworm. Additionally, the use of a newly modified simple DNA extraction method eliminates the need for commercial DNA extraction kits, reduces time consumption, cost and enhances the practicability of the assay. In conclusion, the conventional and real time PCR developed in this study can be effectively utilized for detection and quantification of the *Nosema* species infecting Indian silkworm races. This method assists in quarantine process at seed centres to prevent spread of pebrine disease to other areas by rapid and early identification of pathogen infestation. The advantage of conventional PCR for routine diagnosis of microsporidiosis is that, it requires only the most basic and least expensive laboratory equipment's and less technical skill than required for real-time PCR. Although the conventional PCR assay reported here does not offer quantification, it could be very useful in presorting diagnostic samples.

Acknowledgements The authors would like to acknowledge Dr. G Subramaniyam, Dr. Rajesh Kumar CMERTI, Lahdoigarh, Assam, Dr. K. Velayudhan, ESSPC, Hosur and Dr. Wazid Hassan SBRL Bangalore for providing the infected silk moth and larval samples.

Author's contribution K.M. Ponnuvel designed the experiment, Ms. Vijaya Gowri, Ms. M. Aarthi and Ms. M. Shruthi performed the laboratory experiments. Ms. Vijaya Gowri, Dr. K.M. Ponnuvel and Ms. Tania Gupta wrote the manuscript.

Funding Funding was provided by Department of Biotechnology, Ministry of Science and Technology (BT/PR15405/TDS/121/14/2015 dt 09.02.2017).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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