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Original paper

Targeting essential genes of *Nosema* for the diagnosis of pebrine disease in silkworms

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ABSTRACT. Pebrine is one of the devastating diseases mostly caused by notorious *Nosema* – a microsporidian infecting silkworms. Identification of novel genes associated with the pathogen plays a key role in developing a reliable diagnostic tool for the detection of disease. Targeting potential biomarkers will help in developing strategies for fast and efficient control measures, which can prevent the spread of infection. This study was to identify genes present commonly in *Nosema* such as *Nosema bombycis*, *Nosema mylitta*, *Nosema assamensis* and *Nosema ricini* infecting Indian silkworms in order to find potential gene markers for early pebrine disease diagnosis. Real time PCR was used to validate the genes active early during the infection cycle, confirming the expression of genes and their order of expression. 16S rRNA and β-tubulin were found to be expressed early in infection followed by PTP1 and PTP2, PTP3, SWP5 and MetAP2 genes. These identified molecular markers can be used in addition to conventional gene primers which are traditionally used for the detection of pebrine.

Keywords: β-tubulin, markers, silkworms, pebrine, PCR

Introduction

Silkworms are specialized organisms, which passes through various stages of life cycle to develop into well-developed fully-grown adult moths. They produce silk specifically during the metamorphosis stage of larvae to silk moth. Silkworm rearing has been the livelihood of many people from rural background including farmers. Like any other organism, silkworms are also prone to various diseases and insect infestation which impose severe threat to the silkworm rearing. *Nosema*, a Microsporidia is one among the opportunistic pathogen which causes a devastating disease called as "pebrine" (also called as microsporidiosis or pepper disease) in silkworms leading to huge crop losses (~36%), affecting sericulture economics. Microsporidians have evolved over many years and possess unique ability to steal host genes as well as nutrients, causing immunodepletion and severe crisis leading to death of host. They were initially considered to be primitive eukaryotes and currently classified as Fungi owning to their peculiar genome and biology. They are noxious to almost all the vertebrates and invertebrates including humans. *Nosema bombycis* was the first microsporidian to be identified as entomopathogen infecting silkworms. These are the only silkworm pathogens which can infect transovarially, thus infecting the newly emerging offsprings. In the year 1870, Louis Pasteur studied the source of infection, pathogenesis, transmission, diagnosis and devised remedial measure, mother moth examination for disease prevention. This examination of mother moths under light microscope is the routinely used method around the world till date. This method has many loopholes and is capable of generating false positives with unknown sensitivity and specificity. Moreover the method requires skilled personnel in order to perform the analysis. There has been immense development on the Real Time qPCR assays platform since, they are more reliable and

accurate. Hatekeyama and Hayasaka have developed a loop mediated isothermal amplification assay for detecting the pebrine disease in silkworm eggs; however quantification of spores in single reaction mixture is not addressed by the method. A Nucleic acid Lateral Flow Assay stip has been developed to detect pebrine disease with favourable specificity towards common disease isolates based on LSUrDNA sequence [1,2]. The disease becomes difficult to be identified when there is a co-infection with some other pathogen. Molecular markers are the most important tools today for disease diagnosis for both plant and animal kingdom, since they are specific to particular host.

Polymerase chain reactions (PCRs) utilise and exploits molecular markers to amplify and detect pathogen infections in host. PCR techniques are highly specific and sensitive, yields faster results with high reliability. PCRs can be used in identifying differentially expressed genes which will aid in better understanding on the mechanism underlying the pathogenesis. Till date, only 16S small ribosomal DNA primers were used as a target for the detection of microsporidians [3–5] in host infections. However, recent studies have reported that the 16S rRNA gene sequence of *Nosema/ Vairimorpha* to be an unreliable indicator for phylogenetic analysis among closely related microsporidian species [6]. The end binding protein (*EB1*), polar tube protein 1 (*PTP1*) and both 16s large and small subunit ribosomal RNA genes of *Nosema* sp. were recently exploited for development of isothermal amplification assay [7]. This method was found to be more sensitive, however this method was prone to contaminations giving rise to false positive results [7–10]. It is therefore essential to identify a better diagnostic markers preferably a conserved marker across microsporidia species, that can be used for development of more efficient diagnostic tools and also which can be used in characterization studies. Identification of early genes of *Nosema* infecting the silkworms is very essential since, the disease can be diagnosed at early stage of its occurrence, helping to take necessary actions to avoid the spread of the disease. Hence in this study, the early genes of microsporidian *Nosema* infecting Indian silkworms were studied and the expression of these genes were analysed using the real time quantitative PCR, which can provide an additional tool for disease diagnosis.

Materials and Methods

Isolation and extraction of spores from infected silkworms. The *Nosema* spores (from *Nosema bombycis*, *Nosema mylitta*, *Nosema assamensis*, *Nosema ricini*) were collected from infected *Bombyx mori*, *Antheraea mylitta*, *Antheraea assama* and *Samia ricini* silkworms respectively from different geographical regions of India such as Karnataka, Assam, Manipur and Tamil Nadu. Infected silk moths around 20–30 in number were crushed in 0.6% potassium carbonate (K_2CO_3) solution using a sterile mortar and pestle. The crushed suspension was filtered twice over an adsorbent cotton to remove the host tissue debris. The filtrate was centrifuged at 8000 rpm for 10 min. and the sediment was dissolved in sterile distilled water. The suspension was overlaid onto a 500 µl aliquot of percoll suspension and centrifuged carefully at 8000 rpm for 20 min. The pellet obtained was washed thrice with water and stored at 4°C. The different silkworms were processed separately. The tubes, pipettes, other containers used were cleaned with 2% bleaching powder and autoclaved in order to prevent contamination. The percoll purified spores were counted using haemocytometer under Zeiss AXIO Lab.A1 microscope 40× magnification.

Isolation of spore DNA and silkworm DNA. The purified spores were diluted to 1×10^8 spores/ml using sterile distilled water. The spore DNA was extracted by agitating the spore suspension with glass beads for 60 min. and the suspension was processed using Qiagen blood and tissue DNA extraction kit. Further, the spore DNA was quantified using Beckman DU®530 Spectrophotometer and stored for further analysis. The infected silkworm samples were lyophilized in liquid nitrogen and crushed using cell lysis buffer (50 mmol Tris–HCI/L, pH 8.0, 100 mmol NaCl/L, 20 mmol EDTA/L). Further, Proteinase K (100 µg/ml) was added to remove the protein debris followed by Phenol chloroform isoamyl alcohol (PCI) extraction (25:24:1). The aqueous phase containing the nucleic acid complex was precipitated using ethanol and the DNA was dissolved in 100 µl of sterile distilled water. The silkworm DNA was also extracted from infected larvae, moth, egg, and freshly hatched larvae from the eggs using PCI method as described above.

Inoculation of spores. Spores of *N. bombycis* were prepared by repeated wash (thrice) using

Table 1. List of oligonucleotide primers designed for the screening the essential and early genes of *N. bombycis* infection in Indian silkworms

Target Gene	Forward primer	Reverse primer
16S SSU RNA (16S)	5'ATAAATCGGAGGGCAAATCG3'	5'GCTTTCGCTTCTGTTCATCC3'
β-tubulin	5'CTTTGGACAATCTGGTGCTG3'	5'GAGAAGGGTTCCCATTCCTG3'
Serpin	5'ATATTCCATCATGGCAACCA3'	5'GTAGCAAAATTGGAAGAGAAGG3'
EB1	5'TTTTTCACTTGATCGAGAAGC3'	5'CCTAATTCCCCCATGATCG3'
PTP1	5'CGGTGGTTTCCCAAGTAATG3'	5'AGGATGAGAGCCTGATGGTG3'
PTP2	5'TTCCTAACCGTGCACAACAA3'	5'TGCTGTTTCAATGGCTTGAG3'
PTP3	5'CCACAAACGCCTTTACTGGT3'	5'TCGGTCATTCCAAGGCTATC3'
SWP 5	5'AATGCCGAAGCTCAAAAAGA3'	5'GCAAACACAGCAAGAATGGA3'
MetAP2	5'TAAAGGGGGAGAAGGGTGAT3'	5'TCGAATTGCGGATTAAAAGC3'
Septin	5'ATTTTCAATAATGGCTGCTGGT3'	5'TTTCCGAATCCTGGTGTATCA3'
Otubain	5'TTTTCAAGTGAGTGGATTTCCTT3'	5'TTGTGCTGAAATGGCAAGAC3'
α-tubulin	5'TCCGAATTCAGGTTGGAATG3'	5'CGACGCCTGGCTCTAAG3'

distilled water and diluted to achieve a concentration of 10^8 spores/ml. Mulberry leaves were smeared with 500 µl of the spore solution and air dried before inoculation. Spore-coated mulberry leaves were fed to the silkworms followed by feeding untreated mulberry leaves. The silkworms in the control group were fed mulberry leaves smeared with distilled water. Midgut tissues randomly dissected from four larvae at 24, 48, 72, 96, 120, 144, and 168 hours post infection (hpi) were used for RNA extraction. These samples were collected in duplicates.

Isolation of RNA and cDNA synthesis. Total RNA was extracted from the infected midgut larval tissue using TRIzol followed by chloroform extraction and homogenate was centrifuged separating the mixture into an organic phase, containing proteins and aqueous phase containing RNA. The aqueous phase containing the RNA was precipitated with equal volume of ice cold isopropanol. After centrifugation the pellet was washed with ethanol, air-dried and dissolved in RNase free water. RNA integrity was checked by electrophoresis on a 0.8% agarose gel. The yield and purity of the RNA were assessed using spectrophotometer (Beckman DU530). The traces of genomic DNA were removed by treating the RNA samples with DNase I (Invitrogen). The single-stranded cDNA was synthesized by incubating 2 µg of total RNA with 1 µl of dNTPs and 1 µl of random hexamer (Thermo Fisher Scientific) at 65°C for 10 min. The mixture was further added with 1X reverse transcriptase buffer, 1 µl of dithiothreitol (DTT) and 1 µl of M-MLV RT enzyme. The primer annealing was initiated by incubating the mixture at 25°C for 10 min., followed by extension at 50°C for 60 min. and the reaction was terminated by heating at 72°C for 15 min. The synthesized cDNA was used for further validation by using *β-actin* as reference gene.

Selection of gene target. The essential gene sequence of *N. bombycis* used for the study were retrieved from *N. bombycis* sequence deposited in the NCBI database except for the otubain gene (Acc No: XM_002996555) which was retrieved from *N. ceranae* infecting honey bee microsporidia. The gene sequence included in the study: *16S rRNA* (Acc No: GQ334399) from *N. bombycis* NA1 isolate, *Serpin* (Acc No: FJ705061), *β-tubulin* (Acc No: DQ663475), *α-tubulin* (Acc No: DQ091252), *Septin1* (Acc No: KF421133), *EB1* (Acc No: KF421134), *PTP1* (Acc No: KB909850), *PTP2* (Acc No: HQ881498), *PTP3* (Acc No: JF739554), *MetAP2* (Acc No: EOB11465), *SWP 5* (Acc No: EF683105) from *N. bombycis* CQ1 isolate.

Primer design and PCR conditions. The critical parameter for successful amplification in a PCR is the accurate design of primers as the primer designed greatly affects the yield of the product. The designed primer determines the size, location of the amplified product and melting temperature (Tm) of the amplified region. Further the target genes were selected based on their role and essentiality in the multiplication and establishment of pathogen in

Table 2.The gDNA profile showing the presence or absence (+/–) of the gene in the genome of *Nosema* spp. infecting the Indian silkworms

Gene	*N. bombycis*	*N. assamensis*	*N. mylitta*	*N. ricini*
16S SSU	+	+	+	+
β-tubulin	+	+	+	+
PTP1	+	+	+	+
PTP2	+	+	+	+
PTP3	+	+	+	+
SWP 5	+	+	+	+
MetAP2	+	+	+	+
EB1	+	+	+	+
serpin	+	+	–	–
septin	+	+	+	+
otubain	–	–	–	–
α-tubulin	–	–	–	–

the host. They were chosen from either sequenced genome or from sequenced genome of closest species using NCBI (National Centre for Biotechnology Information) blast. Gene specific primers were designed using primer 3 software and are listed in Table 1. The primers were designed in such a way that it could be used for both conventional and real time PCR. The conventional PCR was performed in a MJ Research PCR System (Bio-Rad, Hercules, CA, USA). Reaction mixture was made up to a final volume of 20 µl containing 1X Emerald master mix (Thermo Fischer Scientific), 10 pmol of each gene-specific primer and 1 µl of previously synthesized single-strand cDNA. The PCR reaction protocol was as follows: Initial denaturation at 95°C for 10 min. and then 35 cycles of 95°C for 30 s and annealing at 57°C for 60 s followed by elongation at 72°C for 60 s. The amplicons were resolved on 1.2% agarose gel with 0.1 mg/ml ethidium bromide run at 100 V for 45 min.

Real time PCR analysis. The traditional agarose gel electrophoresis of PCR amplicons can be used to detect the presence or absence of the infection, however qPCR is often more sensitive which helps in real time detection and quantification of the target gene. This technique relies on the development of two primers and the 3'-end of the primer should not have any complementarity with the other regions of the target genome and the amplicon size should not exceed 200 bp. Real-time PCR amplification to quantify the copies of DNA and RNA was performed in a 20 µl reaction mixture using the SYBR green qPCR master mix (Takara) and 10 pmol of each specific primer. The qPCR reaction was carried out in 96-well microtiter plates using STRATAGENE Mx 3005P. The amplification was programmed as follows: Initial denaturation at 95°C for 2 min. followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 95°C for 30 s and 72°C for 80 s. Fluorescence was measured repeatedly at each cycle during the annealing step. This procedure was followed by a melt-curve dissociation analysis to confirm product size. The amplification results were expressed as the threshold cycle (Ct) value, which represented the number of cycles needed to generate a fluorescent signal greater than a predefined threshold.

Results

Quality analysis of spore DNA isolated from *N. bombycis*, *N. assamensis*, *N. ricini* and *N. mylitta* carried out in 0.8% agarose indicated that the

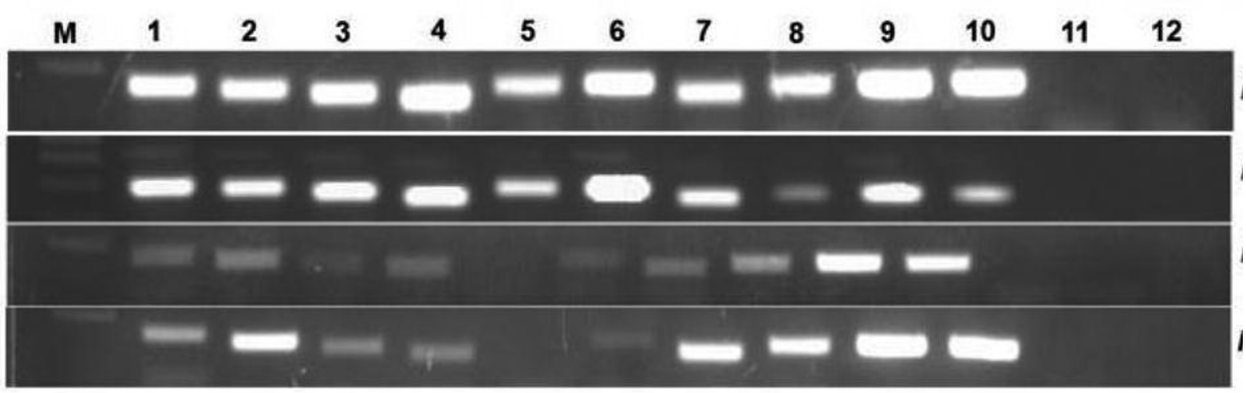

Figure 1. PCR amplification of Microsporidian spore DNA extracted from *Nosema* sp. infecting Indian silkworm races using reported *Nosema* specific primers infecting the insects
Lane: M – 1Kb mass ruler. *Nosema* specific primers: 1 – 16S SSU; 2 – β-tubulin; 3 – EB1; 4 – SWP5; 5 – Serpin; 6 – PTP1; 7 – PTP2; 8 – PTP3; 9 – Septin; 10 – MetAP2; 11 – Otubain; 12 – α-tubulin.

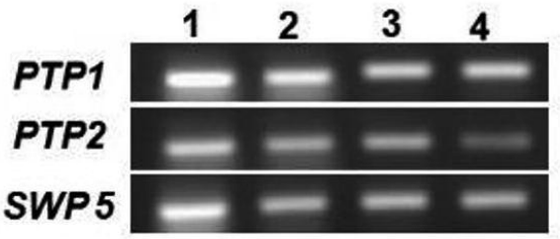

Figure 2. PCR profile of gDNA infected with *N. bombycis* confirming the vertical transmission of pathogen from mother moth to its off springs
Lanes: 1 – infected moth; 2 – respective egg; 3 – hatched larvae; 4 – infected positive control DNA.

quality of DNA was pure; without any contamination, which was further confirmed using spectrophotometry. 12 genes namely *16S rRNA*, *Serpin*, *β-tubulin*, *α-tubulin*, *Septin1*, *EB1*, *PTP1*, *PTP2*, *PTP3*, *MetAP2* and *SWP 5* were used in this study.

The presence and absence of genes mentioned above in the gDNA of infected Indian silkworms were analysed and are presented in Table 2. Out of 12 genes tested for its presence in the infected silkworm, otubain and alpha tubulin genes failed to amplify in all the Indian isolates. Remaining 10 genes amplified confirming the presence of *Nosema* infections in *N. bombycis* and *N. assamensis* 9 genes except serpin amplified for *N. mylitta* and *N. ricini* spores (Fig. 1).

The DNA extracted from moths emerging from the infected silkworms, their respective eggs and larvae were subjected to PCR with three genes

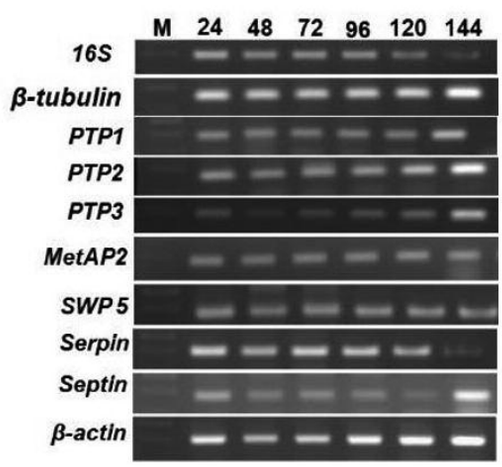

Figure 3. PCR profile of *N. bombycis* infected midgut tissue DNA with different *Nosema* specific primers. The genomic DNA of orally infected 4th instar larvae at 24, 48, 72, 96, 120, and 144 hours post infection (hpi)
Lanes 1-10: *16S SSU, β-tubulin, PTP1, PTP2, PTP3, MetAP2, SWP5, serpin, septin, β-actin* respectively.

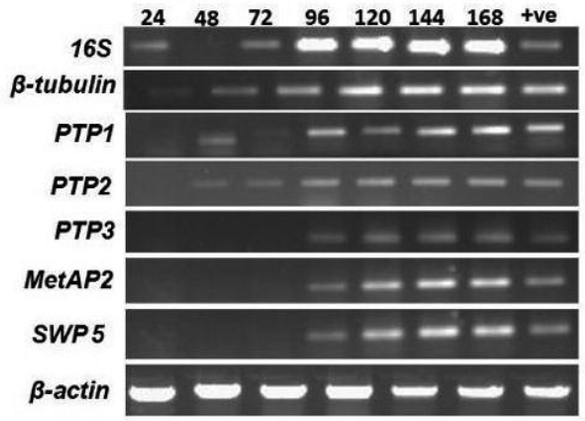

Figure 4. PCR profile of *N. bombycis* gene expression pattern in silkworm *B. mori.* The RNA from the artificially infected silkworms was isolated at 24 hours intervals till 168 hours and the single stranded cDNA synthesized from isolated RNA was amplified a products were run on 1.2% agarose gel. *β-actin* was used as internal standard.

namely *PTP1*, *PTP2*, *SWP 5*; results of which indicated the presence of pathogen specific genes, hence confirming the vertical transmission of pathogen from mother moth to off spring a very normal phenomenon observed in *Nosema* specific infections (Fig. 2).

DNA samples extracted at different hours from silkworm *B. mori* infected with *N. bombycis* were amplified for all the genes under study, except alpha tubulin and otubain (Fig. 3). The expression of the genes were tested using cDNA samples and was observed to follow a pattern in both conventional as well as RT-PCR wherein, *16S* and *β-tubulin* expressed 24 hrs post infection, followed by the expression of *PTP1* and *PTP2* after 48 hrs and *PTP3*, *SWP 5* and *MetAP2* after 72 hrs of infection (Fig. 4 and 5). It was also noted that expression of the genes *PTP3, SWP5* and *MetAP2* were all on the later days post infection to *B. mori,* indicating that these genes are required by the parasite/pathogen later in infection cycle.

Discussion

Identification of pathogen specific genes that are expressed during infection which are essential for virulence and proliferation in the host is the preliminary information required to prevent and control the diseases [11]. Inefficient, unreliable diagnostic methods and lack of proper early symptoms, pose a severe challenge in pebrine detection in silkworms. The study was attempted

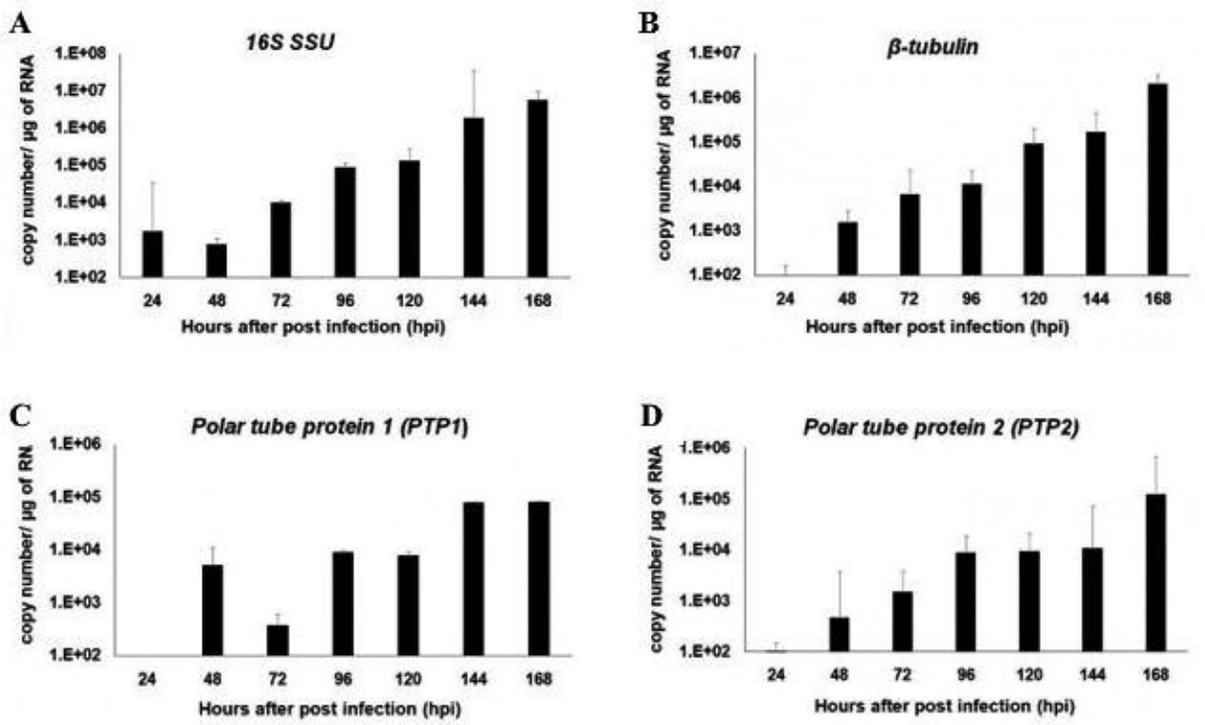

Figure 5. Histogram representing the qPCR profile of various *Nosema* specific genes expressed in the midgut tissue collected at different hours of post infection with *N. bombycis*
The experiment was performed in triplicate. The expressed gene targets namely *16s SSU, β-tubulin, PTP1, PTP2,* gene copies were quantified. The standard deviation bars are shown. A Non-template control (NTC) sample was also run to detect contamination.

mainly to screen and identify the potential genes for detection of *Nosema* sp. infecting the Indian silkworms. The oligos were designed to amplify the partial gene sequence approximately 200 bp so that it could be used for both conventional as well as real time qPCR. The acceptance of molecular diagnostics has always been slow, but threats to biological war fare and agro-terrorism have accelerated the process [12].

Over past few years there has been tremendous advancement in sample processing exclusively DNA extraction which renders increasing sensitivity and specificity in PCR reactions. The DNA extraction from spores is a tedious process as it is very difficult to break open the spores to release the content, but the commercial kits have aided in purifying what-ever little DNA we are able to isolate from the spores. The midgut tissue of insects is the major organ for nutrient absorption and digestion which also serves as an immune barrier for invading pathogens [13]. Midgut being the site of propagation for most of the silkworm pathogens, the expression and quantification of pathogen specific genes were confirmed and validated by DNA extracted from this site. Recent molecular biology methods such as PCR based assays, help in identification of *in vivo* expression and quantification of these genes. The presence of intra genomic heterogeneity and lack of universal threshold sequence identity are the major limiting factors for *16S* ribosomal RNA based sequencing [14]. Hence the essential genes of *Nosema sp.* were retrieved from the database in order to identify an alternative marker for detection of infection in silkworms apart from existing 16S rRNA gene marker. The 16S ribosomal RNA gene sequence of microsporidia has been widely used for diagnostic tests, phylogenetic studies, epidemiological investigations over wide host range as well as the geographic distribution and pathogenetic studies [15]. The 16S ribosomal RNA gene has been extensively used for microsporidian disease diagnosis in both prokaryotes and eukaryotes. The beta tubulin gene of *Nosema* sp. has also been used as an alternative marker for the detection of microsporidiosis in silkworms [16]. The essential genes, unique invasive polar tube protein genes *PTP1, PTP2, PTP3*, alpha tubulins, highly conserved cytoskeleton protein *Septin, Serpin,* a

secretory protein involved in evasion of host immune reaction were included in this study [17–20]. In addition the ubiquitously expressed end binding protein (*EB1* protein) gene and a deubiquitinating spore wall protein otubain that expresses after 3 days of infection was included for the screening [21]. A therapeutic target methionine aminopeptidase 2 which was characterised and validated for its antimicrosporidian activity was also used in this study [22,23]. Among the 12 targeted genes, most of the genes where found to be conserved among all the Indian isolates except *otubain* and *α-tubulin* genes [23].

The disease progression in case of pebrine infection was confirmed based on the gradual upregulation of targeted gene copies as well as delay in moulting- a phase during larval stage where the silkworms remain immobile, shedding their skin preparing to grow in size. Based on the gene expression studies, it is suspected that the genes *EB1*, *Serpin*, *Septin* might be tissue specific involved only in invasion process or expressed at the later stages of infection [24]. Further studies targeted at validating the complete ORF of these genes will help in gaining more insights on gene variation among the Indian and reported Chinese isolates. There was a particular pattern observed in gene expression during the study, where there was a slight decrease of gene expression at 48 hours after which there was a gradual increase in the expression of genes indicating the proliferation of *N. bombycis* inside the host *B. mori*. The *β-tubulin* gene was found to be the early gene expressed at par with the *16S* ribosomal RNA gene, used as a diagnostic marker for detection of microsporidiosis in silkworms as well as humans [16]. Our finding concluded that all the genes namely *Septin, SWP5, PTP1, PTP2, PTP3, MetAP2* can be used as potential markers and can be used in development of efficient diagnostic method for the detection and control of microsporidiosis in Indian silkworms [7,16,23,24]. Further studies on identified markers will provide more thorough understanding of the pebrine infection in silkworms.

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