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DEVELOPMENT OF A MOLECULAR DIAGNOSTIC TECHNIQUE FOR THE DETECTION OF BACULOVIRUS INFECTING OAK TASAR SILKWORM, *ANTHERAEA PROYLEI*

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Abstract– *Antheraea proylei*, a temperate oak tasar silkworm, is one of the economically important silkworms reared for the production of oak tasar silk. *A. proylei* is frequently infected with *A. proylei* nucleopolyhedrovirus (AnprNPV), which causes tiger band disease leading to severe economic loss in oak tasar silk production. Therefore, development of an accurate diagnostic tool may facilitate early detection of pathogen, thus preventing massive economic loss. In the current study, we have developed a real-time quantitative polymerase chain reaction (RT-qPCR) for diagnosis of AnprNPV. The primers specific to *p94* gene of AnprNPV were designed to investigate their sensitivity and specificity. The AnprNPV detection limit in RT-qPCR was found to be 2.5×10^1 copy number with 98.12% efficiency. The developed diagnostic technique is 100 times more sensitive than the conventional PCR for the detection of AnprNPV. Further, the technique was validated with filed samples wherein the AnprNPV viral loads in the oak tasar silkworms were ranged from 10^3 to 10^{10} copies/µl.

INTRODUCTION

Temperate oak tasar silkworm, *Antheraea proylei* is frequently susceptible to tiger band disease caused by *A. proylei* nucleopolyhedrovirus (AnprNPV) which belongs to Alphabaculovirus under Baculoviridae family. The AnprNPV has a double-stranded super coiled circular DNA with a genome size of 126.9 kb (Shantibala *et al.,* 2018). Baculoviruses infect insects via the oral route, which is mediated by the occlusion-derived virus (ODV). This refers to the entrapment of orally infectious baculoviruses in protein crystals known as occlusion bodies (OBs) (Slack and Arif, 2006). Tiger band diseased silkworm larvae exhibit loss of appetite, retarded growth, shrinkage and softening of the body finally leading to death. The disease is characterized by the appearance of black bands/stripes across the silkworm's body (hence the name tiger band disease). The extent of the damage due to tiger band disease has resulted up to 70–80% of oak tasar crop losses in North-eastern India. Development of an accurate and reliable diagnostic tool may facilitate early detection of viral pathogen, thus preventing massive economic loss in oak tasar sericulture. However, such type of diagnostic techniques are not available for the detection of AnprNPV in *A. proylei* silkworms. General precautionary measures are currently used to manage tiger band disease in oak tasar silkworm, but no specific control measures are available. Given the scarcity of information on tiger band disease and its causative agent, developing precise diagnostic tools for pathogen detection is critical in order to prevent disease spread and total crop loss in oak tasar sericulture.

To identify wild-type baculoviruses, a variety of techniques have been used, including DNA dot blot hybridization assays (Keating *et al.,*1989), radio-immunoassays (Knell *et al.,* 1983), serological methods (Brown *et al.,*1982), and microscopic diagnosis (Taverner and Connor, 1992). Due to their laboriousness, unreliability, or use of radioactive materials, these techniques have not been widely

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used. Due to its increased specificity and sensitivity, PCR can be used for the detection of baculovirus screening (Hewson *et al.,* 2011; Arneodo *et al.,* 2012). Real-time PCR has increased PCR acceptance due to its improved speed, sensitivity, and reproducibility, as well as the reduced risk of carry-over contamination (Mackay *et al.,* 2002). Real-time PCR has also shown to be economically advantageous (Martell *et al.,* 1999) when used in a high throughput laboratory, particularly when replacing traditional, culture-based methods of virus detection.

In the current study, a RT-qPCR technique was developed based on the viral p94 gene for the molecular detection of AnprNPV in *A. proylei* silkworms. Comparative genomics revealed the unique presence of *p94* gene in the genomes of alphabaculovirus infecting wild silkworms such as *A. proylei*, *A. pernyi*, *A. yamamai, Samiaricini, and Philosamiacynthiaricini* (Supplementary Tables S1 and S2). Since disease control is frequently met with limited success, early detection and identification of pathogens is critical in preventing disease spread and ensuring safe and sustainable sericulture (Ravikumar *et al.,* 2011).

MATERIALS AND METHODS

Primer designing

The primers were designed based on the whole genome sequence information of AnprNPV (GenBank accession no. LC375539.1). The upstream and downstream primers were designed using Primer 3.0(https://primer3.org/). The *p94* was amplified using *p94*-F 5′*GCAGCAATTTCCTTCCAAAG* 3′ and *p94*-R 5′*TCTGTTGACGCTCTTGTTGG*3′ primers. These primers were used for RT-qPCR with an expected product size of the amplicon to be 193bp. Similarly, *p94* gene was amplified in conventional PCR using *p94*-F 5′ TCTGTTGACGCTCTTGTTGG 3′ and *p94*- R 5′ TGTGGTACGTTGCCGAGATA 3′ with an expected product size 454bp.

Genomic DNA extraction

The genomic DNA of AnprNPV was extracted as per the procedure described by Khajje *et al.,* 2022.

Cloning and establishment of standard curve for RT-qPCR

To obtain a standard and positive control for the assay, the AnprNPV *p94* gene from infected *A. proylei* was amplified using the specific primers 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 *p94* gene insert was extracted using the FavorPrep ™ Plasmid Extraction Mini kit (Favorgen). The sequence of the cloned *p94* gene was confirmed and the sequence was submitted to NCBI GenBnak (https://www.ncbi.nlm.nih.gov/genbank/under accession no OP934065. The concentration and purity of the plasmid was measured using Nanodrop 2000C Spectrophotometer (Thermo Scientific). The gene copy number concentration was calculated as follows: Number of copies = concentration of plasmid ng/µl x 6.022×10^{23}/ length (bp) x $1 \times 10^{9} \times 660$.

Sensitivity and specificity

A tenfold dilution series of the plasmid containing the *p94* gene ranging from 10^8 to 10^2 copies was used to generate a standard curve for calculating the efficiency of the RT-qPCR using the formula: % efficiency = $10^{(-1/\text{slope})}$x100. The sensitivity of the RT-qPCR assay was assessed by comparing the results of the conventional PCR. To determine the specificity, conventional PCR was tested against DNA samples from silkworms infected other microbial pathogens such as *Nosemabombycis*, *Bombyxmori* nucleopolyhedrovirus (BmNPV), and *Bombyxmoribidensovirus* (BmBDV).

Conventional PCR method

For PCR analysis, the Emerald master mix (Emerald Amp® GT PCR Master Mix, TaKaRa), was used according to the manufacturer's recommendation using an annealing temperature of 58 %C (initial denaturation at 94 %C for 2 min followed by 30 cycles of [94 %C for 30 s, 58 %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 visualized on a 1.5% agarose gel.

RT-qPCR analysis

For the RT-qPCR, a reaction mixture consisting of 5 µl SYBR Premix Ex Taq II (TliRnaseH Plus) 0.1 µM of each primer, 1.9 µl distilled water, 0.1 ml ROX 1ml of the diluted DNA template was used. A positive control containing the plasmid with the *p94* gene sequence and a non-template control (NTC) were

included in all PCR runs. The RT-qPCR assay was run-in Real-Time PCR machine Insta Q96 Himedia Laboratories as follows; initial denaturation at 95 %C for 2 min followed by 40 cycles of [95 %C for 30 s, 58 %C for 30 s, and 72 %C for 35 s].

Collection and validation of disease-infected oak tasar silkworms

Disease-infected 5th instar *A. proylei* larvae were collected at different sericulture farms during spring and autumn seasons in Manipur and Nagaland states of NE region (RSRS, Imphal, Research Extension Centre Yaikangpao of Manipur state; Kikruma, Thumkhonglok, and Dolansabi in the sates of Nagaland, India).

The bioassay studies were conducted with AnprNPV as described by Khajje *et al.*, 2022. A batch of uninfected larva was used as a control. For each experiment, 50 larvae in triplicates (n = 50) were infected with AnprNPV. Three infected larvae from each batch were used for the detection purpose and rearing of the remaining larvae was continued until they revealed tiger band disease symptoms. To confirm the infection time period, DNA was extracted at 0, 12, 24, 36, 48, and 72 h intervals and used in conventional and RT-qPCR analysis with *p94* primers. Validation of the method was taken up with silkworm samples showing tiger band disease symptoms collected from 5 sericultural farms. The genomic DNA was extracted and further used in conventional and RT-qPCR analysis with *p94* primers for detection of AnprNPV infection.

RESULTS AND DISCUSSION

The initial plasmid concentration was calculated to be 2.5 × 10^{10} 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 – 3.37. The efficiency of the assay was determined to be 98.12% (Supplementary Fig. 1A). The RT-qPCR assay had a higher sensitivity than conventional PCR with limit of detection at 10^1 and 10^3 copies per reaction, respectively (Supplementary Fig. 1B and C). Dissociation curve with a single peak was obtained from *p94* fragments (melting temperature, Tm 58 %C) indicating specific amplification of desired viral gene (Supplementary Fig. 2).

The specificity of the RT-qPCR assay was tested by analysing samples containing three other silkworm pathogens: *N. bombycis*, BmNPV, and BmBDV. The assay shown negative for all three pathogens except AnprNPV (Fig. 1). Samples collected from the silkworms infected with AnprNPV at time intervals of 0, 12, 24, 36 and 48 h of post infection were analysed with both RT-qPCR assay and conventional PCR. Both assays revealed a gradual increase of the pathogen over the 24 h of incubation following infection (Fig. 2A and B). The highest AnprNPV viral copies were found at 72 hr of incubation

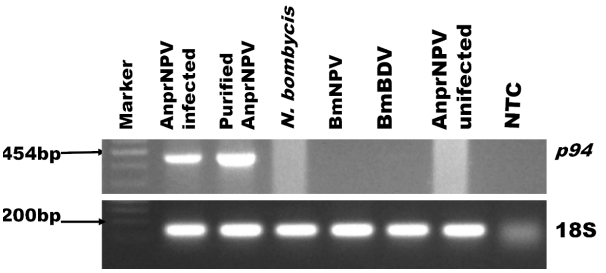


Fig. 1. Specificity of Anpr NPV*p94* primers using conventional PCR. DNA samples from silkworms infected with AnprNPV, purified AnprNPV, *N. bombycis*, BmNPV, BmBDV, AnprNPV uninfected silkworms were analysed together with non-template control (NTC). 18S (a housekeeping gene) was used as an internal control.

Field evaluation studies indicate that among 50 diseased samples collected from 5 different locations, 38 samples (76%) were found to be

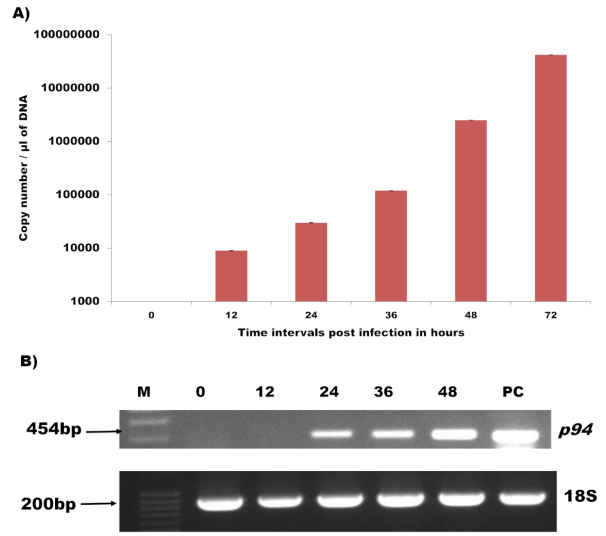


Fig. 2. A&B. Detection of efficiency of AnprNPV*p94* primer at different time intervals of 0, 12, 24, 36 and 48 h in 4th instar larvae for infection study. 18S is used as housekeeping gene. A. real-time qPCR using the *p94* primer. B. Conventional PCR using the *p94* primer

Table S1. Presence of P94 gene across genomes of Nucleopolyhedrovirus infecting wild silkworms

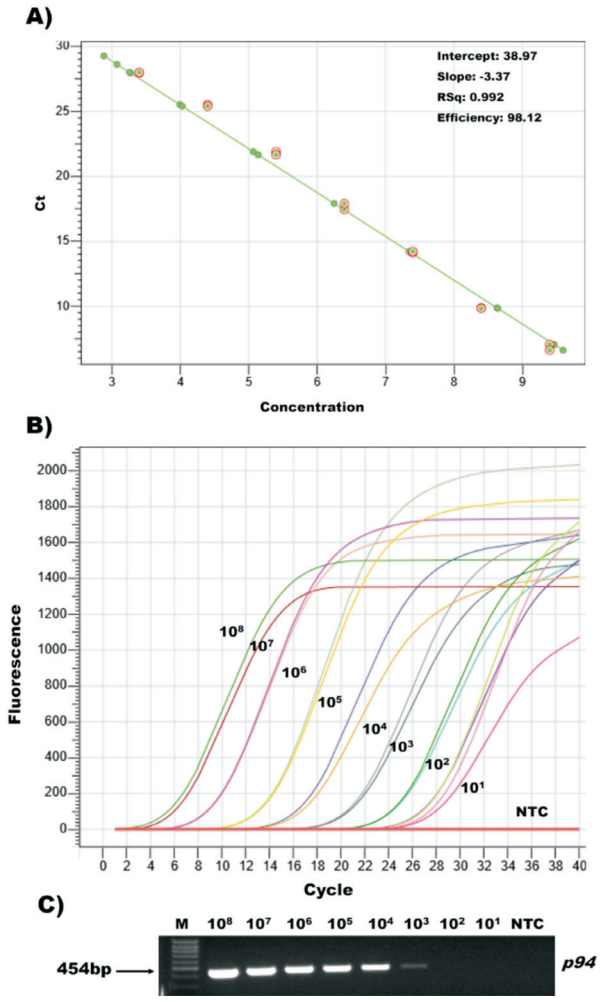
Virus and origin of report	Gen bank accession	P94 gene ORF length	No of A. acid residues	Protein molecular wt
Antheraea proylei nucleopolyhedrovirus strain Manipur isolate Anpr NPV-TS (Institute of Bioresources and Sustainable Development, Imphal, India)	KY979487.1	1491	496	56.93
Antheraea proylei nucleopolyhedrovirus strain Tkhulen IBD (Institute of Bioresources and Sustainable Development, Imphal, India)	MH797002.1	1491	496	56.93
Antheraea pernyinucleopolyhedrovirus Liaoning (Yamaguchi University)	LC375540	345	114	13
Antheraea proylei nucleopolyhedrovirus Manipur (Yamaguchi University)	LC375539.1	1524	507	58.25
Samiacynthianucleopolyhedrovirus (Yamaguchi University)	LC375538.1	1491	496	56.93
Antheraea yamamainucleopolyhedrovirus **Nagano** (Yamaguchi University)	LC375537.1	1491	496	56.93
Antheraea pernyinucleopolyhedrovirus (Yamaguchi University)	LC194889	858	285	32.71
Philosamiacynthiaricininucleopolyhedrovirus virus (Jiangsu University of Science and Technology, PR China)	JX404026.1	2388	795	90.7
***Samiaricininucleopolyhedrovirus* Son La** Yamaguchi University	LC375543.1	1368	455	51.8
Samiaricininucleopolyhedrovirus **Mondulkiri** (Yamaguchi University)	LC375542.1	1368	455	51.8
Samiaricininucleopolyhedrovirus Guangxi Yamaguchi University	LC375541.1	2388	795	90.7
Antheraea pernyinucleopolyhedrovirus (Zhejiang Sci-Tech University, PR China)	DQ486030	1527	508	58.28
Antheraea pernyinucleopolyhedrovirus isolate AnpeMNPV-L2 (Dalian Institute of Biotechnology. PR China)	EF207986.1	1479	492	56.42

Table S2. BlASTalignment of AnprNPV p94 gene

select all *13 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Antheraea proylei nucleopolyhedrovirus strain Manipur isolate AnprNPV-TS	Antheraea proylei nucleopolyhedrovirus	351	351	100%	2e-92	99.48%	126930	KY979487.1
Antheraea proylei nucleopolyhedrovirus strain TkhulenIBD	Antheraea proylei nucleopolyhedrovirus	351	351	100%	2e-92	99.48%	126930	MH797002.1
Antheraea pernyi nucleopolyhedrovirus Liaoning DNA, complete genome	Antheraea pernyi nucleopolyhedrovirus	351	351	100%	2e-92	99.48%	126646	LC375540.1
Antheraea proylei nucleopolyhedrovirus Manipur DNA, complete genome	Antheraea proylei nucleopolyhedrovirus	351	351	100%	2e-92	99.48%	126324	LC375539.1
Samia cynthia nucleopolyhedrovirus Nagano DNA, complete genome	Samia cynthia nucleopolyhedrovirus	351	351	100%	2e-92	99.48%	126094	LC375538.1
Antheraea yamamai nucleopolyhedrovirus Nagano DNA, complete genome	Antheraea yamamai nucleopolyhedrovirus	351	351	100%	2e-92	99.48%	126270	LC375537.1
Antheraea pernyi nucleopolyhedrovirus DNA, complete genome, strain: Liaoni...	Antheraea pernyi nucleopolyhedrovirus	351	351	100%	2e-92	99.48%	126593	LC194889.1
Philosamia cynthia ricini nucleopolyhedrovirus virus, complete genome	Philosamia cynthia ricini nucleopolyhedrovirus virus	340	340	100%	3e-89	98.45%	125376	JX404026.1
Samia ricini nucleopolyhedrovirus Son La DNA, complete genome	Samia ricini nucleopolyhedrovirus	335	335	100%	2e-87	97.93%	128602	LC375543.1
Samia ricini nucleopolyhedrovirus Mondulkiri DNA, complete genome	Samia ricini nucleopolyhedrovirus	335	335	100%	2e-87	97.93%	128240	LC375542.1
Samia ricini nucleopolyhedrovirus Guangxi DNA, complete genome	Samia ricini nucleopolyhedrovirus	335	335	100%	2e-87	97.93%	125921	LC375541.1
Antheraea pernyi nucleopolyhedrovirus, complete genome	Antheraea pernyi nucleopolyhedrovirus	326	326	94%	1e-84	98.90%	126629	DQ486030.3
Antheraea pernyi nucleopolyhedrovirus isolate AnpeMNPV-L2, complete geno...	Antheraea pernyi nucleopolyhedrovirus	274	274	100%	4e-69	93.26%	126246	EF207986.1

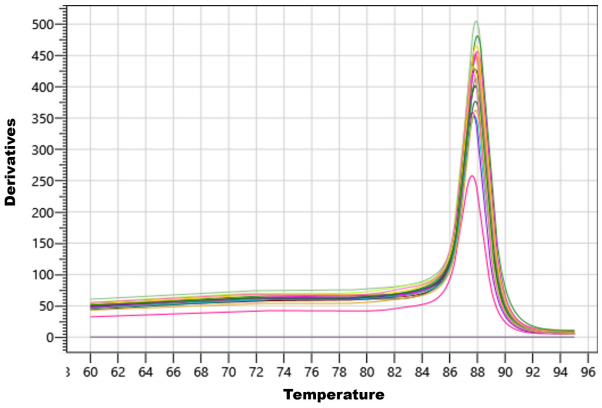
infected with AnprNPV. The conventional and real time PCR analysis results are shown in Fig. 3A&B. The results revealed high copy numbers of AnprNPV which ranged between 10^3-10^{10} copies/µl in the infected samples. The result confirms widespread infection of Indian isolate of AnprNPV in different sericulture farms of Manipur and Nagaland. This validation also confirms the prevalence of AnprNPV in the local sericulture farms.



Supplementary Fig. 1A. Standard curve with tenfold serial dilutions of the plasmid containing the *p94* gene of AnprNPV. Results obtained with 10-fold serial dilutions from (10^1–10^8 copies/µl) of plasmid showing the linear relationship between Ct values and the dilutions of the recombinant pJETAnprNPV plasmid. The figure displays the regression value of 0.992 and amplification efficiency of 98.12%. X-axis indicates the copy number and Y-axis indicates the Ct values. **B)** Amplification of tenfold serial dilution of the plasmid containing the *p94* gene of AnprNPV using the real-time qPCR assay. Each dilution was run in duplicate.

Baculoviruses generally display a horizontal mode of transmission, mainly through ingestion of occlusion bodies (OBs); however, AnprNPV shows a trans-ovum vertical mode of transmission in addition to a horizontal mode (Khajje *et al.*, 2022). Early detection of the virus aids in the prevention of disease spread and substantial crop losses. In comparison to other conventional methods, molecular diagnosis methods were deemed to be the primary detection method as they are highly specific to pathogen. PCR-based detection methods have been reported in the detection of NPV in *B. mori* silkworms Similar to this, microsporidian and DNV detection using PCR-based methods were established (Esvaran *et al.*, 2019; Hou *et al.*, 2005; Khyade and Tyagi, 2017; Ravikumar *et al.*, 2011). RT-qPCR based detection method for microsporidia *N. bombycis* was developed to save the *B. mori* crop loss (Ravikumar *et al.*, 2018). However, very few reports were established on RT-qPCR quantification of AnprNPV. Otherwise, there are no reports on early detection method for AnprNPV by qPCR that calculates high sensitivity detection.

In this study, a precise molecular-based technique for detection of AnprNPV from diseased field samples using the RT-qPCR method was demonstrated. The results show that it is highly specific for AnprNPV. The RT-qPCR analysis reveals that the sensitivity for developed method can detect up to 2.5×10^1 copies/µl. The study also shown a high efficiency rate of 98.9%, while in in conventional PCR the detection limit for AnprNPV is up to 2.5×10^3 copies/µl. Thus, it is concluded that the present RT-qPCR assay is 100 times more sensitive than the conventional PCR in detection of AnprNPV infection. On comparison with earlier



Supplementary Fig. 2. Melting curve analysis results obtained with tenfold serial dilutions from (10^1–10^8 copies/µl) of Plasmid DNA in RT-qPCR assay.

reports such as Lu *et al.,* 2019, a loop-mediated isothermal amplification with lateral flow dipstick (LAMP-LFD) method was developed which detects AnprNPV DNA with a low limit detection of 5 fg/µl (400 copy number) (Hu *et al.,* 2016). Similarly, Ravikumar *et al.,* (2011) has reported the detection of BmBDV virus at 24 h post infection by multiplex PCR assay.

The inferences of the detection method include early detection of the pathogen compared to earlier reported methods. It is possible to detect the AnprNPV infection even in 12 h post infection through the developed RT-qPCR technique (Fig. 2). The visible symptoms of AnprNPV infection were observed post 7 days of infection showing retarded growth and black bands/stripes across the body of the silkworm. These results are in accordance with earlier reported observations in which the symptoms led to the death of the larvae (Khajje *et al.,* 2022). This information would help the sericulture farmers to control the disease by discarding the infected larvae/batches and applying disinfectants and taking other prophylactic measures for better control of disease.

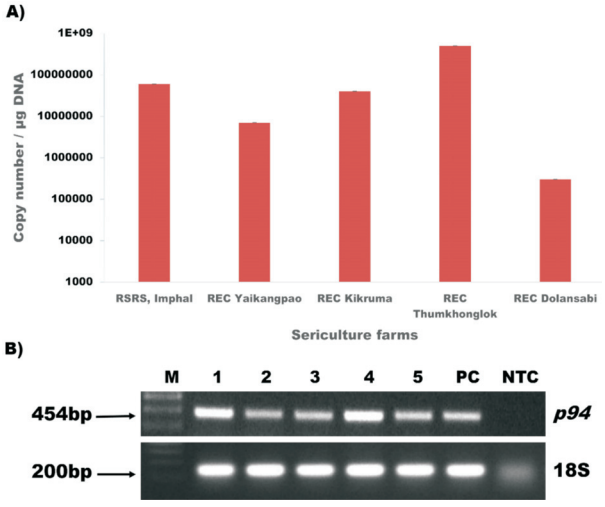


Fig. 3. A & B. Analysis of AnprNPVinfection in field samples A. RT-qPCR B. Conventional PCR using the *p94*primer.

Field validation of RT-qPCR indicated the presence of AnprNPV infection and prevalence of tiger band disease in the sericulture farms. Hence, it is decisive that the developed assay will be useful for detecting AnprNPV infection at early stages with real time quantification might provide higher sensitivity and specificity from 24 h post infection. This analysis will provide a platform to formulate appropriate disease control strategies for early detection of pathogens in order to prevent disease spread and total crop loss in oak tasar industry.

CONCLUSION

A real-time quantitative polymerase chain reaction was developed for the diagnosis of AnprNPV infection in oak tasar silkworms. The AnprNPV detection limit in RT-qPCR was found to be 2.5×10^1 copy number with 98.9% efficiency. The devoted technique is 100 times more sensitive than the conventional PCR. The AnprNPV viral load in the field oak tasar silkworm samples ranged from 10^3 to 10^{10}copies/µl as estimated by the developed RT-qPCR method.

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Conflict of Interest

The authors declare no conflict of interest.

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