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Detection of Grasserie Virus, BmNPV in the Fifth Instar Larvae of Silkworm, *Bombyx mori* (L) (Race: PM x CSR2) Through Polymerase Chain Reaction

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A B S T R A C T

The mulberry silkworm, *Bombyx mori* L is a Lepidopteran insect, life cycle of which include: Egg, Larval Instars, Pupa within silky cocoon and adult moth. It is purely domesticated insect since long, which make it a quite delicate venture, easily susceptible to viral and other diseases. The viral diseases are difficult to manage due to a very short life cycle of silkworm. One of the most effective solutions is a timely detection of such infection so that to stop spread of the disease. The present attempt is concerned with studies on a polymerase chain reaction (PCR) with a set of specific primers to the Grasserie virus gene region was used to diagnose *B. morinucleopolyhedro virus* (BmNPV) infection which were made available from Eurofins Genomics India Pvt Ltd Bangalore. The nucleic acid DNA was extracted from the mid gut tissue of the fifth instar larvae of silkworms and was subjected for amplification. After the amplification the samples were loaded on 1% Agarose gel and electrophoresis was run at 65 volts. The gel was stained using stain (ethidium bromide) and used to visualize under UV illuminator. The results of the amplification of the polymerase chain reaction were utilized for the detection of infection of Grasserie BmBPV.

Introduction

The silkworm *Bombyx mori* L has been domesticated for sericulture in the past 5,000 years. From the beginning of the nineteenth century, the silkworm has been used for basic science studies, such as genetics, physiology, and pathology, because of its large body size, its importance in sericulture, easy rearing, and a large number of described mutants (Willis, *et al.*, 1995). Insects possess an efficient and potent innate immune system to discriminate and eliminate invading pathogens and parasites, but lack acquired immunity or immunological memory similar to that present in vertebrates (Lemaitre and Hoffmann, 2007). Drosophila melanogaster, the type model of insects, has been particularly extensively. Since 4,500 years, silkworm, *Bombyx mori* has become a purely domesticated insect. Like other domesticated animals, it is a quite delicate venture easily susceptible to a number of seasonal diseases, (Govindan *et al.*, 1998 and Prasad, 1999).

Occurrence of seasonal disorders and diseases is a periodic surge in disease incidence, corresponding to seasons or other calendar periods (Rane, 1911). In tropical countries Grasserie also known as the hanging disease is one of the most destructive diseases of silkworms. The causative agent is Borrelina bombycis virus, of the family Baculoviridae. The Baculoviridae comprises only 2 genera nucleorpolyhedorsis virus (NPVs) and granulovirus (GVs). In this infection the virus multiplies and forms polyhedra in the nucleus of infected cells. Infection mainly takes place through wounds and feeding of polyhedral contaminated mulberry leaves.

The high temperature, humidity and their sudden fluctuation, bad ventilation, ineffective disinfection of rearing house and rearing appliances, starvation and inadequate larval spaces as well excessive moisture in the rearing bed affect spreading of the disease. The majority of baculovirus host are within the order Lepidoptera. They have also been isolated from orders Diptera, Hymenoptera, Coleoptera and some crustaceans, (Hong *et al.,* 2000).

According to Mallika (2006) the Grasserie infected silkworm shows disease symptom during the final stage of larval development and die without cocoon production resulting in the waste of expense, time and labour work therefore accountable for considerable economic losses in the Indian silk industry. The incidence of Grasserie is reported in the silkworm rearing areas of the entire district of Akola from Vidarbha region of Maharashtra, throughout the year. This infection is difficult to cure due to a very short life cycle of silkworm. The greatest way to manage Grasserie disease is to prevent disease infection. However, the presumable most effective solution for the control of Grasserie disease is to detect viral infection as early as possible in order to stop spread of the disease in rearing units. Lack of rapid and accurate disease detection technique causes severe spread of Grasserie disease seasonally (Mallika, 2006). Earlier, techniques have been developed to detect this viral disease such as the enzyme-link immunosorbent assay (ELISA) (Vanapruk *et al.,* 1992), DNA hybridization (Attathom *et al.,* 1994), colloidal textile dye-based dipstick immunoassay (Nataraju *et al.,* 1994), and western blot analysis, (Chaeychomsri *et al.,* 1995).

PCR is an extremely sensitive technique which amplifies target DNA sequences and PCR amplification of conserved fragment enabled the detection of low level of viral DNA (Mallika, 2006). It has been employed for the detection of viral DNA such as human virus (Umlauft *et al.,* 1996), aminal virus (Peng *et al.,* 1998) and plant virus (Levesque, 2001). No such detection study so far has been carried out for Grasserie virus in silkworms from, Maharasthra. So in the present study we used PCR technique and polyhedrin gene (polh) to detect early infection of Grasserie virus (BmNPV) in silkworm *Bombyx mori*. This study will help to prevent the spread of the Grasserie, and to eradicate this viral disease during silkworm rearing.

Materials and Methods

The experimental silkworms were collected from the farmers in Baramati Taluka Dist. Pune 413 115 Maharashtra, India. They were dissected for the midgut tissue. The identification of diseased worms infected with Grasserie in the fields initially was made on the basis of gross pathology. Initially the skin shows oily and shining appearance with progress of infection, skin becomes thin and fragile and the midgut appeared milky white with inter-segmental swelling (Photo plate I). The larvae infected with Grasserie in the rearing centers were found to be slightly sluggish. For reliable and distinct PCR

product in rapid detection, a set of specific primers procured from Eurofins Genomics India pvt. Ltd., Bangalore, which is the cloned nucleotide sequence within BmNPV polyhedrin gene. Primers – (bp -424 bp) Forward primer: 5' AATTCGCAGTGAAA CCCG 3'Reverse primer: 5' AGAGTC TGTGCCGATGT 3' (Mallika, 2006). The oligonucleotide sequences of forward primer began from position 221-240 of polhORF and reverse primer began from 616-644 of polhORF.

These primers amplified a 424bp PCR product. Using these primers PCR was performed on the basis of studies by Mallika (2006) and using the prescribed protocol for DNA extraction (Insect DNA extraction kit Nucleopore, Genetix ltd.). DNA extracted from the midgut tissue of the non infected healthy and infected fifthinstar larvae of silkworms are amplified with primers by specific polhBmNPV isolates PCR Protocol: 1µl DNA sample (~50µl); Sterile water: 31µl; Buffer: 5µl; $MgCl_2$: 2µl; Template DNA: 1µl; Forward primer: 1µl; Reverse primer: 1µl; Taq DNA: 1µl.

After amplification the samples were loaded on 1% Agarose gel and electrophoresis was run at 65 volts. The gel was then stained with ethidium bromide and visualized under UV illuminator (Gel Doc Machine). The work was repeated for three times for consistency in the results.

Results and Discussion

The specific pathogens that are difficult to culture in vitro or require a long cultivation period present in the infected silkworms, was diagnosed by PCR. Similar method was earlier used for detection of Lymantria dispar NPV (LdNPV) on the surface of an egg in Gypsy moth, by Burand *et al.,* (1992). It was preceded, with extraction of DNA from experimental silkworms, PCR amplification, followed by detection of amplicons by visualization. Mid gut tissues of infected silkworm moths were used to illustrate the Grasserie disease detection by PCR. On Visualization the Gel, (Photo plate-II)it is reported that DNA extracted from Grasserie BmNPV infected silkworm yielded the amplification product of ~424bps (Lane 1, 2, 3, 4, 5, *i.e.* BmNPV polhgene confirmed presence of Grasserie BmNPV infection but not in lane 6 and 7 indicating infection other than grasserie. The lane 8 loaded with DNA extracted from healthy non infected control larvae no PCR amplification product was found. The PCR products obtained was ~424bps for Grasserie as expected and were in accordance to that obtained from the DNA extracted from BmNPV (polhgene), in Lane M. As PCR products were specific to the virus used as the DNA template therefore no nonspecific sequences were observed. Strong intensity of PCR product bands were clearly visualized on the gel. These studies provide proof that PCR is a competent tool for detecting virus of Grasserie disease in silkworm.

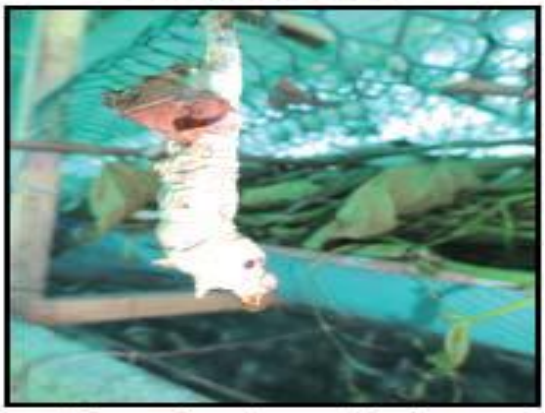
The silkworm is agriculturally very important for silk production, so their pathological and genetic studies on diseases have been very significantly and extensively carried on. Severe economic losses caused by the pathologies, such as virus, bacterium and fungus so on, so the comprehensive understanding the innate immunity pathway and host-pathogen interaction will attribute for us defending economic losses and benefit from the silk industry. In the past several years, the studies on the innate immunity of *B. mori* have gained significant results, such as much recognition, modulation, signaling, effectors and other immune molecules (Vitthalrao Khyade, 2016.

Photo plate -1 Grasserie Infected fifth instar larvae of silkworm, *Bombyx mori* (L) (Race: PM x CSR2)

1. Infected larva with oily and shiny skin

2. Larva with swell intersegments and sluggish appearance

3. Larva with thin and fragile integument

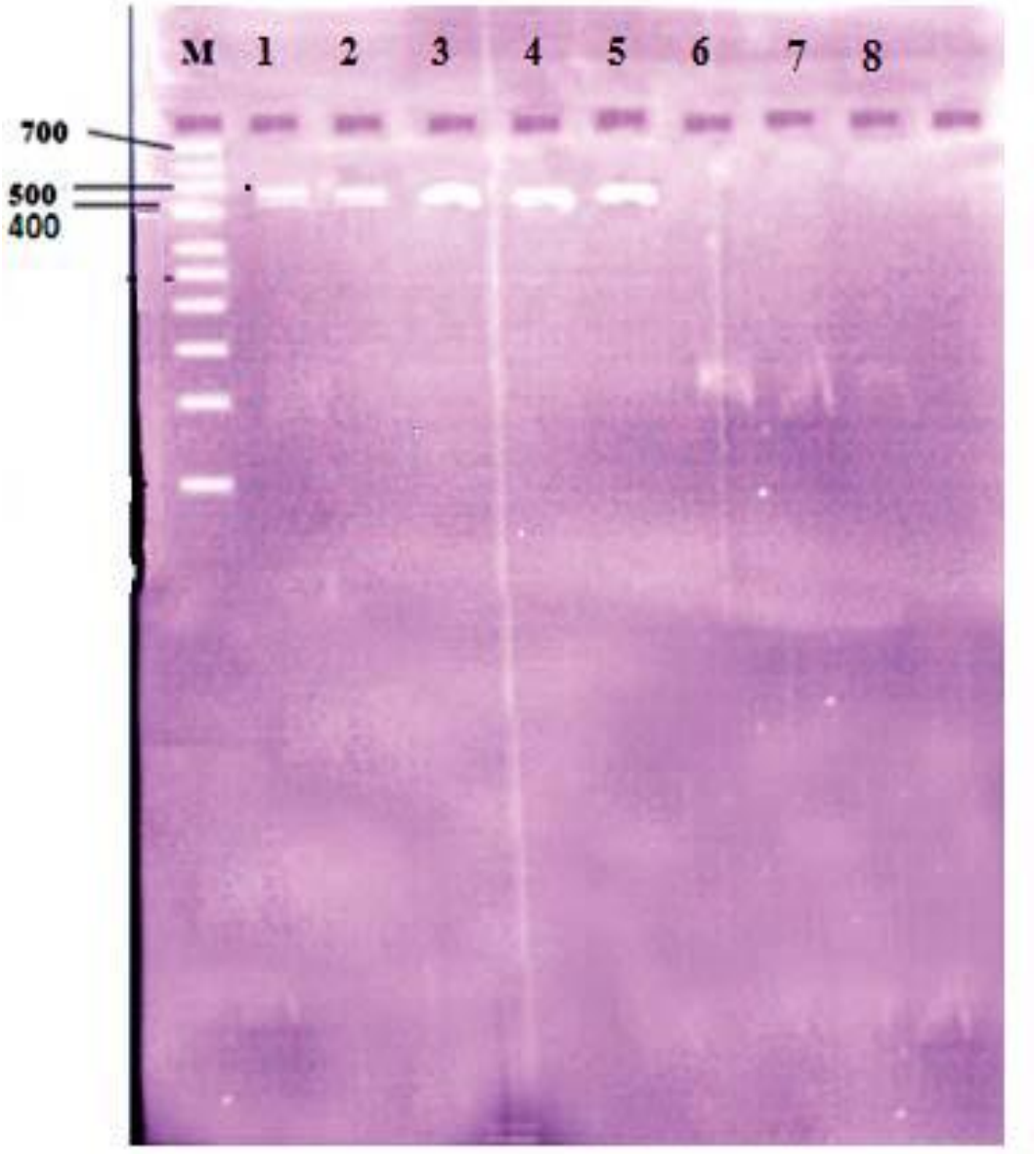


4. Larva hanging upside down

5. Milky white integument ruptured skin

6. Polyhedral bodies oozing out of midgu

Photo plate.2 The Gel plate showing Polymerase Chain Reaction Amplification of DNA from the fifth instar larvae of silkworm, *Bombyx mori* (L) (Race: PM x CSR2) infected with grasserie causing BmNPV



Lane – M: DNA Marker; Lane – 1: BmNPV Detected; Lane – 2: BmNPV Detected; Lane – 3: BmNPV Detected; Lane – 4: BmNPV Detected; Lane -5: BmNPV Detected; Lane – 6: BmNPV Not Detected; Lane – 7: BmNPV Not Detected; Lane – 8: Control Healthy (BmNPV Absent)

The comparison between Toll and Imd pathway enable us to further understand the mechanisms of innate immune responses. Recent years, phylogenitic analysis that many immunity members in the invertebrate immunity have very similar defending genes to the mammals, this result illustrate the similarities between some of the strategies used both by insects and mammals to sense infection and amplify the information.

Even though, some paper published that the structural and function similarities between the Toll and the TLR dependent activation of NF-κB has been interpreted as evidence for the existence of a common ancestor and shared mechanisms between the vertebrate and invertebrate innate immune systems. Many reports revealed that stem cell is involved in the regeneration by destroy and in maintain the homeostasis in the Drosophila intestinal. So, in the present study speculated that some kinds of stem cell are also involved in the intestinal homeostasis of silkworm.

Recently, a genome-wide analysis of immune-related genes of *B. mori* revealed that the factors associated with the signal transduction pathways are conserved in *B. mori* and non-lepidopteran insects. However, the function of most genes encoding recognition proteins in *B. mori* is still unknown. Nonetheless, in the near future, the immune-related genes can be elucidated by the development of functional analyses such as RNA interference, transgenic technology, GAL4/UAS system and zinc-finger nuclease technique.

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