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Silkworm disease diagnosis through molecular approach and their management

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INTRODUCTION

Silkworm has a greater biotic potential and the resistance is continuously offered by the environment which includes biotic and abiotic factors thus influencing the cocoon production qualitatively and quantitatively. The abiotic environmental resistance component largely includes the weather factors such as temperature, relative humidity, photoperiod etc., in respect of silkworm and unfavourable weather conditions that lead to poor harvest of mulberry. The abiotic factors usually affect the growth and development of silkworm and predispose the silkworm to the biotic causes *i.e.*, infectious diseases. The biotic factors responsible for low cocoon crop production are the silkworm diseases caused by protozoa,fungi,bacteria and viruses. Extent of crop loss due to fungal disease caused by white muscardine and green muscardine was 5-20 per cent,virus disease caused by grasserie (NPV) was 15-20 per cent, bacterial disease reduces the crop loss upto 10-15 per cent and pebrine gives 5-10 per cent damage, respectively. The history of sericulture reveals devastating impact of microsporidiosis in several sericultural countries resulting in severe damage to sericulture industry. Though, the disease is known from very remote times, it attracted the attention of sericulturists only during the 17th century. The first scientific record of the occurrence of the disease came from European countries in 1809 and the disease wiped off sericulture there. This was followed by another report from France in 1845 where the annual production of cocoons came down from 26,000 tones in 1853 to 4000 tones in 1865 due to epizootics of microsporidiosis and subsequently collapsed the French and Italian silk

industry. Later the disease spread to Spain, Syria and Romania (Steinhaus, 1949). In India, the first report of the occurrence of the disease was from Mysore in 1866 followed by an epidemic level outbreak of the disease in Kashmir in 1878 (Baig *et al.,* 1997).

Factors influencing the cause of diseases in silkworm :

Diseased silkworm extrudes pathogens into the rearing environment, which form the source for the diseases in the colony. The pathogens are extruded by infected silkworms along with gut juice (most viral diseases and pebrine disease) and faecal matter (Cytoplasmic polyhedrosis, pebrine and bacterial disease). It also enters into the rearing environment through breakage of fragile integument (nuclear polyhedrosis, septicemia) or form the body surface (muscardine and aspergillosis). The dead and the fermented larva, moth also form source of diseases. The wild insects may also form a source of for diseases especially, pebrine and muscardine . In addition, the pathogens are light, easily drift in air and have the ability to remain in active state in rearing environment, for longer period (three to several years). The pathogens contaminate the mulberry either in mulberry garden or in the rearing house it self and infect silkworms when fed on them.

The silkworm rearing practices followed in sericultural areas are not fool proof for prevention of diseases. Silkworms are reared in rearing cum dwelling houses. Most of these houses are unhygienic, badly ventilated and dark and damp helping in pathogen survival. Silkworm rearing is continuous with little time for disinfection. In fact, most sericulturists do not disinfect the rearing house at all. As the disinfectant Formaline cause discomfort to the residents. Farmers are not self sufficient with rearing appliances, such as rearing trays and chandrike and borrow them leading to contamination. Silkworm bed cleaning practices involving cleaning with hand helps in contamination. Diseased larvae are not picked and even if picked, most farmers do not discard them but rear in separate tray forming a source of infection in the rearing house. The bed refuse are transported in baskets or gunny bags that are sometimes also used for transportation of mulberry leaves to feed silkworms. While transportation, the bed refuse and diseases silkworms are dropped on the way to litter pit. In some cases the bed refuse is transported directly to the mulberry field which itself gets contaminated. In addition to unhygienic condition, the rearers do not practice rearing and personal hygiene. All these factors lead to prevalence of diseases in silkworm rearing and crop failure or low yield.

Management of diseases in silkworm rearing:

Silkworm diseases are best prevented than cured. The diseases in silkworm are prevented through proper disinfections and rearing hygiene. The disinfections aims at destruction of pathogens in the rearing house and on appliances before the start of the rearing and during rearing of those pathogens that enters into rearing area mainly from the infected larvae. Personal hygiene and rearing hygiene aims to prevention of entry of the pathogen into the rearing house and secondary contamination during rearing. Disinfection of rearing house and appliances eliminates the persistent pathogen. However, the disinfection performed at the beginning of the rearing has no effect on the pathogens that gain entry into the rearing environment during the rearing. The practical approach for the management of disease in silkworm rearing is as follows :

– Disinfection of silkworm rearing house, surroundings and rearing equipments using physical and chemical methods of disinfection.

– Rearing early instar silkworms following strict hygienic, congenial environmental and nutritional conditions.

– Rearing later instar silkworms under optimum rearing area, feeding sufficient quantity and quality mulberry under suitable environmental and hygienic.

– Prevention of entry of pathogen from outside through meticulously practice of rearing and personnel hygiene during the silkworm rearing.

– Prevention of spread of silkworm diseases by using silkworm body and rearing seat disinfectant.

In addition to the above, early diagnosis, nutrious mulberry, sufficient ventilation and rearing space adds to the prevention of diseases in silkworm rearing.

The research of the detection and diagnosis of the silkworm disease :

Since 1949, various serological techniques have been gradually and widely used in the study of silkworm diseases. Prepared NPV, CPV and DNV rabbit anti-serum and anti-DNV monoclonal antibodies. By double

diffusion and immune electrophoresis methods, it was showed that densovirus of China Zhenjiang isolate had serological affinity with Japan Saku, Yamanashi and Ina isolate (Lirong, 1981). Chiaki *et al.* (1987) conducted studies on virus identification, detection, location and early diagnosis by means of agar double diffusion, indirect enzyme antibody (IIP method) diagnostics. Latex agglutination, conjugated monoclonal antibodies to latex protein A (PALMAL) counter immuno electrophoresis, immunofluorescence, ABC2 ELISA method, reverse indirect hemagglutination reaction and immunological binding assay. At the same time, they extended the agar double diffusion, counter immuno electrophoresis, latex agglutination serological and some rural areas of early diagnosis in sericulture production. DNV has also been prepared into a diagnostic kit for the silkworm and counter immuno electrophoresis with enzyme (ELCIEP) combined the monoclonal antibodies and other immunological technologies with two-way diagnosis of silkworm densovirus. This monoclonal antibody and immunoenzyme technology or combination of fluorescent antibody technique could detect virus in the midgut of silkworm in 16 to 18 h after infection . An antigen anti-serum and monoclonal antibodies to resist *Nosema bombycis* prepared spore surface detected *N. bombycis* by indirect immunofluorescence method, latex agglutination, enzyme antibody method and carbon agglutination, construct with anti-*Nosema bombycis* monoclonal antibody immunogold silver staining (IGSS), which was used to detect *N. bombycis*. Serological techniques such as when the root is also used to study silkworm pathogenic fungi. Conidia and blastospores of silkworm white and yellow muscardines had good antigenicity, in which blastospores were better than the conidia. White muscardine blastospores and yellow muscardine blastospores had immune cross-reaction .With modern molecular biology techniques and methods of application of the silkworm pathology, some of the silkworm pathogen detection has entered into molecular level. PCR (polymerase chain reaction) has been used to diagnose *B. mori* nuclear polyhedrosis virus.The haemolymph sample of infectious Bm2NPV displayed a clear 240 bp fragment from PCR amplification. PCR could also be used to detect BmNPV and DNV from the feces of silkworm. PCR technology for *Nosema* species-specific diagnosis could distinguish the MG1and MG2 strains of *N. bombycis* from other microsporidia. It could also be used to detect *Vairimorpha necatrix* and *Pleistophora anguillarum* in tussah silkworm, with sensitivity to 1 ng level .RAPD (randomly amplified polymorphic DNA) could be used to discriminate different sources of *Nosema* from showing the genomic DNA polymorphism.

Disease management:

Grasserie and Flacherie were found more in summer as compared to other two seasons. This clearly indicated that fluctuations in temperature and humidity would lead to flacherie disease and affect the silkworm crops to a greater extent. Savanurmath *et al.(*1992) also reported that fluctuations between day and night temperature and relative humidity prevailing in rearing house were the important causes for the occurrence of flacherie and grasserie in silkworm larvae. The grasserie was high in Penukonda area and this might be due to poor hygienic rearing, huge quantity of dfls used per rearing and hiring of rearing appliances which favour multiplication of micro organisms persisted in the dwelling houses for viral flacherie (Samson *et al.,* 1990; Sivaprakasam and Robindra, 1995). Christi and Schaf (1990) also reported that silkworms reared under controlled temperature and relative humidity showed less mortality and increased build up in grasserie during summer might be due to fluctuations in temperature and humidity which are predisposing causes for viral infections (Kasi Reddy and and Krishna Rao, 2009 and Khurad *et al.,*2004).

Flacherie management :

To control flacherie, the rearing room, equipments and surroundings should be disinfected. During rearing, the rearing bed should be disinfected with recommended bed disinfectants. If the incidence is high, the rearing bed should be dusted with active lime powder before feeding. Diseased, weak larvae are to be separated from the rearing bed and disposed into a bowl containing lime water. Feeding the larvae with good quality leaves, good ventilation in the rearing room and adequate bed spacing helps to prevent the incidence of flacherie. Maintenance of recommended temperature and humidity also helps to contain flacherie.

Fungal disease management :

To control the spread and incidence of muscardine, disinfection of rearing room and appliances should be

done scientifically. Muscardine infected larvae should be immediately separated from the bed and disposed into bowl containing lime water. Maintenance of hygiene during rearing, dusting of active lime powder on the rearing bed during moulting periods, good ventilation in the rearing room and providing adequate spacing in the bed helps to prevent the occurrence of muscardine. Dusting of 1-2 per cent Dithane M45 in Kaolin or Captan in claked lime on silkworm body immediately after every moult and on the 4th day of fifth instar @ 3-5 g/sq.ft. Old news paper or paraffin paper is covered for 30 min. Feeding should be given afterwards.

Preventive measures such as disinfection and hygiene maintenance in the rearing environment is the best way to keep the disease at bay. Many studies have proved that many A. flavus strains are resistant to formaldehyde (CH_2O). Hence the use of disinfectant should be judiciously chosen. Sodium penta chloro phenoxide monohydrate as a disinfectant against Aspergillus fungi invaded into rearing tools was more effective than 3 per cent formalin (Wadee *et al.*, 1972). Benzalkonium chloride, iodine disinfectant, benzalkonium chloride + dodecyl diaminoethylglycine, didecyl dimethyl ammonium chloride, etc are proved to be effective (Kawakami, 1982). *In vitro* studies by Graham and Graham (2007) have shown that, mycelial growth and toxin production by A. parasiticus were inhibited by garlic concentration of 0.3 - 0.4 per cent. Sun drying of rearing equipments is an effective way of destroying Aspergillus pathogens to some extend. Sick worms discovered before conidification should be incinerated or placed in lime jars and never thrown around indiscriminately. The faeces and bed refuses should be disposed off properly and disinfection with anti muscardine powder should be carried out immediately.

Practice disinfection of silkworm rearing house, surroundings, appliances and silkworm egg surface. Aspergillus sp. are more tolerant to formalin hence, 3 per cent formalin solution is suggested.

Pebrine disease management :

Pebrine disease can be controlled by disinfecting the rearing room, equipments and rearing surroundings. During rearing, unequal size worms and faecal matter should be microscopically examined for the presence of pebrine spores and if observed, larvae, cocoons and layings should be collected and burnt or buried. In the grainages, scientific methods of mother moth examination should be employed. If pebrine disease is detected, effective disinfection should be undertaken before starting the next rearing or grainage operations.

Microscopic smears are to be prepared by crushing the abdominal region of the mother moth in 2ml, 0.6 per cent Potassium carbonate solution. Similarly, smears can be prepared from dead and unequal larvae, layings, faceal matter and also from the rearing room dust. The smears should be subjected to microscopic examination and if pebrine spores are detected, the crop should be destroyed followed by disinfection.

Probiotic supplementations:

Generally the early Instars of silkworm larvae (I,II, III Instars) were more susceptible to bacterial infection than the IV and V Instar stages. Studies on bacterial pathogenicity were conducted on IV Instar mulberry silkworm *Bombyx mori*. Assessment of pathogenicity and identification of bacterium of *E.coli* were isolated from flacherie infected silkworm larvae. It is most common and deadly diseases in seri field. The present study showed that LD50 dose of *E.coli* infected to IV Instar larvae was $2.4x10^6$ cells/ml and determined the LD50 value. When the inoculated worms were treated with the probionts *Bifidobacterium bifidum* and yeast at different concentrations. *Bifidobacterium bifidum* showed significant enhancement in larval weight, cocoon weight, shell weight, shell ratio, filament length, filament width, Denier and fibroin content. *Bifidobacterium bifidum* with a concentration of 6 per cent was very effective. The quantity of protein in *Bifidobacterium bifidum* (6%) 67.5 per cent. whereas the yeast treatment (6%) had 28.57 per cent over the control. Feed supplementation not only enhanced economic and nutritional parameters but also prevent bacterial infection in *B.mori* (Amala Rani *et al.*, 2011).

Table 1 : Changes in total protein content in control and probiont treated worms

Treatment	Different dosages	Protein content µg/ml
	Control	49
Bifodobacterium bifidum	6%	87 (67.5%)
Yeast	6%	63 (28.57%)

Botanicals:

Studies were carried out *in vitro* to assess the

efficacy of some herbal extracts for the containment of these microbes through turbidimetry analysis and zone of inhibition test. The observations made during this study revealed that the aqueous and alcoholic crude extracts of three herbs such as, *Acalypha indica*, *Ocimum sanctum* and *Tridax procumbens* are effective against these microbes causing flacherie and muscardine diseases in silkworm. The comparison of their effects indicated that alcoholic extracts were generally more effective than aqueous extracts and that the extracts of *Tridax procumbens* are very effective against these microbial pathogens followed by the extracts from *Ocimum sanctum* and *Acalypha indica.* Extensive studies using these extracts on the growth and cocoon production of the mulberry silkworm, *Bombyx mori* L. are likely to throw much light on the possibility of using such extracts as a prophylactic measure during silkworm rearing to improve silk production.

The circumference and area of the zone of inhibition formed in the bacterial culture plate and fungus culture plate were taken as an index for the ability of the herbal extracts to deal with the respective microbes causing diseases in the silkworm. It could be observed that alcoholic extracts are comparatively more effective than aqueous extracts and that this difference is only marginal in the case of the extracts from *Ocimum sanctum*, slightly intense for *Aclypha indica* and very intense for *Tridax procumbens.* Further, it could be noticed that both the aqueous and alcoholic extracts of *Tridax procumbens* are comparatively much effective against the bacterial and fungal pathogens causing diseases in the silkworm *B. mori.* The changes in the optical density of the nutrient broth inoculated with the bacterial culture prepared from the haemolymph of the diseased silkworm with the addition of herbal extracts were noted to find out the susceptibility of the bacterial culture to the effect of the herbal extracts both with the increase in concentration and time (Isaiarasu *et al*., 2011).

Techniques for detection of silkworm pathogens:

– Molecular techniques - PCR based
– Serological techniques – Enzyme assay based

Enzyme-linked immunosorbent assay (ELISA):

The enzyme-linked immunosorbent assay (ELISA) is the most commonly immunoassay, in which antigen-antibody complexes are absorbed into wells in plastic microtitre plates. This method is suitable for large sample numbers and can detect low concentration of virus. Moreover, results can be easily seen from colour change. ELISA has been proven to be a sensitive and reliable method for the detection of grasserie disease. Using ELISA, viruses can be detected within 1 day after inoculation, whereas, by light microscopes the virus can be detected after 3 days post-inoculation. However, this method is rather complicated and costly.

Production of monoclonal antibodies against *nosema bombycis* and their utility for detection of pebrine infection in *Bombyx mori*:

Latex agglutination assay based on monoclonal antibodies (MCAs) be useful for detection of Pebrine infection in silkworm. Four murine MCAs were produced against *Nosenu bombycis* spore. In ELISA all 4 MCAs (IgM isotype) reacted with alkali treated Nosema spores and to variable extent with acetone precipitated surface protein. However, MA-3 10 and MA-542 showed a low degree of cross reactivity with BmNPV. In contrast, MA-SO3 and MA-5 I5 were devoid of reactivity with BmNPV, *B. thuringiensis,* S. *marcescens,* Azotobactor, Rhizobium and normal hemolymph protein in ELISA. Latex beads sensitized with a combination of MA-503 and MA-51.5 (50 pg each per ml of 0.4% latex beads) could detect $1x10^5$ Nosema spores per test. Sensitization of the latex beads with the cocktail of these two MCAs through protein-A bridge further led to a 10-fold increase in the sensitivity ($1x10^4$ sporeshest) of the assay. No agglutination was observed in presence of BmNPV, *Rhizobium, Azotobactor, E. coli, B.thuringiensis, S. marcescens* and normal hemolymph protein indicating the specificity of the test. The results obtained by latex agglutination assay on haemolymph samples of infected as well as normal larvae collected from field, I1 instar larvae infected in the laboratory and from infected mother moth revealed 100 per cent correlation with results by microscopic examination (Shamim *et al*., 2006).

Colloidal textile dye-based dipstick immunoassay:

Colloidal textile dye-based dipstick immunoassay was developed for BmNPV detection. The purified anti-BmNPV IgG was used to capture the antigen on nitrocellulose membrane attached to a dipstick, and antigen was detected with colloidal textile dye labeled anti- BmNPV IgG. Dipstick textile blue dye sensitized

with 500 μg/ml of affinity purified and anti- BmNPV IgG, could detect 10 ng/ml of antigen by forming a clear blue dot in 30 min. The sensitized dye was observed to be stable for a 3 months period at 4°C. Monoclonal antibody-based sandwich ELISA, studied the development and characteristics of five hybrid cell clones secreting Murine monoclonal antibodies (MCAs) directed against BmNPV. Antibodies recognized to a variable extent four different strains of nuclear polyhedra, e.g., *Borrelina bombycis, Amsaeta olbistriga, Heliothis armigera and Spodoptera litura.*

Fluorescent antibody technique:

The fluorescent antibody technique employed was developed the first by Coons and Kaplan in 1950, and has been successfully applied in clinical diagnostic work involving bacteria, fungi, protozoa, rickettsiae and viruses. Krywienczyk (1963) found which the fluorescence in the cytoplasm of infected cells before crystallization of polyhedra in the nuclei which suggested that the inclusion-body proteins are synthesized in the cytoplasm. In the later stage they concentrate in the nuclei and their crystallization starts after the virus particles have been form there.

Nucleic acid based method:

Southern blotting :

Southern blotting is the transfer of DNA fragments from an electrophoresis gel to a membrane support. The transfer or a subsequent treatment results in immobilization of the DNA fragments, so the membrane carries a semipermanent reproduction of the banding pattern of the gel. After immobilization, the DNA can be subjected to hybridization analysis (*UNIT 2.10*), enabling bands with sequence similarity to a labeled probe to be identified. When setting up a Southern transfer, choices must be made between different types of membrane, transfer buffer, and transfer method. The most popular membranes are made of nitrocellulose, uncharged nylon, or positively charged nylon. Although these materials have different properties, the three types of membrane are virtually interchangeable for many applications. The main advantage of nylon membranes is that they are relatively robust and so can be reprobed ten or more times before falling apart. Nitrocellulose membranes are fragile and can rarely be reprobed more than three times; however, these are still extensively used, as they give lower backgrounds with some types of hybridization probe.

Northern blotting:

Northern blotting it is possible to observe cellular control over structure and function by determining the particular gene expression levels during differentiation, morphogenesis, as well as abnormal or diseased conditions. Northern blotting involves the use of electrophoresis to separate RNA samples by size and detection with a hybridization probe complementary to part of or the entire target sequence. The term 'northern blot' actually refers specifically to the capillary transfer of RNA from the electrophoresis gel to the blotting membrane. However, the entire process is commonly referred to as northern blotting. The northern blot technique was developed in 1977 by James Alwine, David Kemp, and George Stark at Stanford University. Northern blotting takes its name from its similarity to the first blotting technique, the Southern blot, named for biologist Edwin Southern. The major difference is that RNA, rather than DNA, is analyzed in the northern blot.

Isolation and identification of a pathogen of silkworm *Bombyx mori:*

In this study, the symptoms preceding some silkworms death appeared to be consistent with those of bacterial septicemia. Then a pathogenic bacterial strain, ST-1, was isolated and cultured on nutrient agar medium. The isolated strain proved to be pathogenic to healthy silkworm. The results showed that colour of the strain's colony was similar to that of a particular aurantium. It differed significantly from the colony colorus of well-known, common septicemia-causing bacteria, such as *Serratia marcescens bizio, Bacillus* spp. and *Aeromonas* spp., etc. Therefore, ST-1 was examined in detail in order to confirm its taxonomic status. Simultaneously, its pathogenicity for silkworm was investigated. A pathogenic bacterial strain, ST-1, was isolated from a naturally infected silkworm. The strain was identified on the basis of its physiological and biochemical properties and the results of sequence analysis of its 16S rRNA gene. The results of the 16S rRNA gene sequence analysis revealed that ST-1 shared the highest sequence identity (more than 99%) with *Pseudomonas chlororaphis subsp. aurantiaca*. The genomic DNA of ST-1 was extracted according to the method described in Short Protocols in Molecular Biology.

PCR Amplification and Analysis of the 16S rRNA Sequence PCR amplification of ST-1 was performed with a universal set of primers for the bacterial 16S rRNA gene. The forward primer was AGAGTTTGATCATGG CTCAG, and the reverse primer was ACGGTTACCTTG TTACGACTT . This pair of primers could amplify an approximately 1.5-kb sequence of 16S rRNA gene. The PCR protocol was as follows: an initial denaturation at 95_C for 5 min; followed by 30 cycles, each comprising heating for 20 s at 95_C, 20 s at 50_C, and 3 min at 72_C; and a final extension at 72_C for 10 min. The amplified products were subjected to 1.0 per cent agarose gel electrophoresis, cloned into pGEM-T Easy vector (Promega Corporation, Madison, WI), and then sequenced directly. The 16S rRNA gene sequences were also compared using BLASTN (NCBI). ST-1 showed high similarity with Pseudomonas (greater than 99%), and 13 sequences with which it had high homology were studied further. In addition, Palleroni had divided Pseudomonas into 5 rRNA groups on the basis of rRNA-DNA hybridization experiments. We selected 20 sequences of different strains. From these rRNA groups and compared them with the 16S rRNA gene sequence of ST-1. Escherichia coli (GeneBank Accession Number: Z83205), which is distantly related to Pseudomonas, was used as the outgroup. Pathogenicity Analysis of Strain ST-1 After the ST-1 suspension was injected into the fifth instar larvae, it was obvious that ST-1 had a lethal effect on the silkworms. The peak mortality of silkworms was concentrated between 24 and 28 h. However, with regard to the pathogenicity of S. marcescens Bizio and Bacillus thuringiensis subsp. sotto Ishiwata for silkworms, the peak mortality of silkworms was concentrated between 20 and 24 h. Therefore, the onset time for infection with ST-1 was longer by approximately 4 h than for infections with strains of the 2 control groups. The corrected mortality for silkworms was 30.0–97.5 per cent at 28 h after the ST-1 suspension of five different concentration gradient was injected into each silkworm in the experimental group. However, due to the complexity between different Pseudomonas species, ST-1 could not be identified as Ps. aurantiaca despite having 99 per cent 16S rRNA gene homologous similarity with *Ps. aurantiaca* (Anzai *et al*.,2000). Therefore, we thought it was necessary to use the way of traditional morphology, physiological and biochemical characteristics to confirm the *Pseudomonas* species. It was also worth well examining the small differences in the 16S rRNA gene sequences of different *Pseudomonas* species because these differences might lead to considerable differences in reactions to different phenomena. According to reports in the literature, DNA–DNA hybridization experiments with *Ps. aurantiaca, Ps. chlororaphis*, and *Ps. aureofaciens* yielded homology values higher than 70 per cent, confirming that these are members of the same species (Peix *et al*.,2007). However, strains belonging to *Ps. aurantiaca* differed those of Ps. chlororaphis and those of Ps. aureofaciens in terms of some phenotypic characteristics (Johnson and Palleroni, 1989 and Palleroni, 2005). Ps. *chlororaphis* tested positive for nitrate deoxidization, *Ps. aureofaciens* yielded variable results and *Ps. aurantiaca* tested negative. However, in our study, strain ST-1 was positive. Therefore, we believed that it differed slightly from other *Ps. aurantiaca* strains in terms of its biological profile . In conclusion, scientists used three methods-traditional morphology, physiological and biochemical characteristics, and 16S rRNA gene sequence analysisto finally identify ST-1 as *Ps. chlororaphis subsp. aurantiaca. ST*-1 has orange colonies on solid medium, a morphological characteristic of *Ps. aurantiaca*. Also the cell of ST-1 bacteria is short rods, 0.7–0.9 9 1.3–1.5 lm long with rounded ends. The strain is gram-negative and it can utilize sodium citrate, malonate, D-glucose, sucrose, D-fructose, D-mannose, and L-arabinose. ST-1 has a higher pathogenicity. To the best of our knowledge, this is the first strain of *Ps. aurantiaca* to be identified as a pathogen to silkworm (*B. mori*). We believe that our study will lay the foundation for the prevention and cure of this kind of disease caused by ST-1 pathogenic bacteria (Kaustubha *et al*., 2007).

Molecular cloning and characterization of *Antheraea mylitta* polyhedrosis virus genome segment 9:

Genome segment 9 of the 11-segment RNA genomes of three cytoplasmic polyhedrosis virus (CPV) isolates from *Antheraea mylitta* (AmCPV), *Antheraea assamensis* (AaCPV) and *Antheraea proylei* (ApCPV) were converted to cDNA, cloned and sequenced. In each case, this genome segment consists of 1473 nucleotides with one long ORF of 1035 bp and encodes a protein of 345 amino acids, termed NSP38, with a molecular mass

of 38 kDa. Secondary structure prediction showed the presence of nine *a*-helices in the central and terminal domains with localized similarity to RNAbinding motifs of bluetongue virus and infectious bursal disease virus RNA polymerases. Nucleotide sequences were 99±6 per cent identical between these three strains of CPVs, but no similarity was found to any other nucleotide or protein sequence in public databases. The ORF from AmCPV cDNA was expressed as a His-tagged fusion protein in *E. coli* and polyclonal antibody was raised against the purified protein. Immunoblot as well as immunofluorescence analysis with anti-NSP38 antibody showed that the protein was not present in polyhedra or uninfected cells but was present in AmCPV-infected host midgut cells. NSP38 was expressed in insect cells as soluble protein via a baculovirus expression vector and shown to possess the ability to bind poly(rI)–(rC) agarose, which was competitively removed by AmCPV viral RNA. These results indicate that NSP38 is expressed in virus-infected cells as a non-structural protein. By binding to viral RNA, it may play a role in the regulation of genomic RNA function and packaging (Heng-Ping Tao *et al*., 2011).

PCR techniques:

Recent advances in molecular biology propose the more powerful techniques for baculovirus detection. Those methods basically based on the detection of the nucleic acid of the causative agent of the disease. One of DNA-based method is polymerase chain reaction (PCR). The PCR technique exploits the ability of the enzyme, DNA polymerase to synthesize many complementary strands of DNA from a very small amount of DNA template. The DNA sequence to be amplified is identified and two short oligonucleotide sequences (primers) are constructed, each being complementary to one or other of the 3' ends of the template sequence. By subjecting the reaction mix to cycles of heating and cooling at selected temperature, the two strands separate. Following this, the temperature is reduced allowing the two primers to anneal to complementary strands and synthesis of a new copy of the DNA takes place across the region flanked by the primers, beginning at the 3' end of each primer. Each strand and the amount of target DNA to which the primers can anneal have been doubled. By cycling through the separation, annealing and synthesis (or extension) temperature *n* times, it is theoretically possible to produce 2*n* copies of the DNA region between the primers. At the end of the reaction the resulting PCR (or amplification) product can be electrophoreses and visualized on an agarose gel.

The successful amplification of a DNA fragment will be indicated by a discrete band of the same size as the target length, i.e. the sequence flanked by the primers (Hunter-Fujita *et al*., 1998). Moreover, PCR technique was employed to detect baculovirus DNA sequences from viral occlusion bodies contaminating the surface of moth eggs (Burand *et al*., 1992). PCR technique was used in combination with other methods such as ELISA, so called PCR-ELISA (Sukkhumsirichart *et al*., 2002). Now-a-days real-time PCR was used to determine Baculovirus quantitatively (Lo and Chao, 2004).

Detection of BmNPV in artificially inoculated silkworm larvae:

The appropriate number of the first instar larvae for DNA extraction and subsequent PCR amplification was determined. The expected PCR products of 424 bp were obtained and there were no different of band clearness and intensity when using DNA template extracted either from 1, 2, 3 or 4 larvae. This result suggested that only one larva of the first instar provided adequate DNA template to be amplified by PCR method. DNAs extracted from individual artificially inoculated larva of the 1st, 2nd, 3rd, 4th and 5th instar provided PCR products of expected size. No PCR products were observed when using DNA extracted from healthy larvae (Mallika kaewwises, 2006).

Development of a PCR-based method for detection of *Nosema pernyi: Nosema pernyi* is the lethal pathogen of pebrine disease in *Antheraea pernyi* :
Preparation of spores of N. pernyi:

Purified the spores of *N. pernyi* using the combined methods of differential centrifugation and percoll density gradient centrifugation. Percoll is an effective medium for cleaning microsporidian and compared the effect of percoll purification between single density gradient (30, 50 and 70%) and discontinuous density gradient (25, 50, 75% and 25, 50, 75, 100%). Tubes containing the spore-percoll mixture were centrifuged at -4 at 15,000 rpm for 30 min. The pellet, which contained purified spores, was washed with sterile NANO pure water, centrifuged again at 1500 rpm for 3 min, and resuspended in sterile NANO

pure water. A haemocytometer (Wolk *et al.*, 2000) was used to count the spores. The spore concentration was adjusted to 109 spores ml^{-1} of water and the spores were recounted to ensure that the spore density was correct and stored at 4°C until use. Two purification methods showed a similar trend that the layer closer to the tube bottom is purer and more, the middle layer less and the first layer least or not even. The purity of the strip layer near the tube bottom is from 91.8 to 95 per cent.

DNA preparation:

DNA was prepared from the frozen tissue specimens of the infected *N. pernyi* and healthy by incubation at 55°C for 4 h in TE buffer (10 $mmol•L^{-1}$ Tris-HCl, pH 8.0, 10 $mmol•L^{-1}$EDTA, pH 8.0) containing 100 $mmol•L^{-1}$ NaCl, 2 per cent SDS, 0.039 $mol•L^{-1}$ DTT and proteinase K (100 $\mu g•mL^{-1}$) followed by phenol-chloroform extraction involving RNase (100 $\mu g•mL^{-1}$) and ethanol precipitation. DNA prepared by ethanol precipitation was resuspended in TE buffer, and extracted samples were kept at -20°C until use.

PCR amplification:

Three sets of primers were used: (1) P1,5'- CACCA GGTTG ATTCT GCCTG AC -3' and P2, 5'- GCAAC CATGT TACGA CTTAT ATCAG A -3'; (2) N1, 5'-GTTGA TTCTG CCTGA GGTAG AC -3' and N2, 5'-CAATG GTATC TAATC ACCTT CG- 3'. (3) P11, 5'-CACCA GGTTG ATATT GCCTG AC- 3' and P22, 5'-GCAAC CATTA TACGA CTTAT AT- 3'. Primer P1/P2 was designed from the sequence of a gene of small subunit rRNA of *Vairimorpha necatrix* .Primer N1/N2 was designed from a partial sequence of SSU rRNA gene of *Nosema* sp. Primer P11/P22 was design from a partial sequence of SSU rRNA gene of microsporidia in this study. 25 µL PCR mixture that contained 0.2 µM of primers, 0.2 mM of dNTPs, 1×buffer, 2 mM $MgCl_2$, 1 units Taq DNA polymerase (Takara, Dalian China), appropriately diluted template DNA (approximately 50 ng) was used. The PCR procedure started for an initial 3 min at 94°C, followed by 30 cycles of 45 s at 94°C, 45 s at 55°C, 90 s at 72°C, followed by a final extension of 5 min at 72°C.

Analysis of PCR products by agarose gel electrophoresis:

Amplified PCR products were electrophoresed through 1.0 per cent agarose gel with DNA marker (DL2000), stained with ethidium bromide, and visualized using UV illumination. A digital image of each gel was analyzed using a software program by Bio-Rad. As expected, *N. pernyi*, *A. pernyi* and moth infected by *N. pernyi*, and mixture of *N. pernyi* and *A. pernyi* infected by *N. pernyi* gave clear amplicons of the size (600 bp) when primer pair N1/N2 was used (Lane 4-8). Primers P_{11} and P_{22} gave an amplification product from the DNAs of *A. pernyi* infected by *N. pernyi*, purified *N. pernyi*, moth infected by *N. Pernyi* (Lane 9, 11 and 12). The length of the products was estimated to be 1500 bp. Meanwhile, DNA sample from *A. pernyi* uninfected by *N. pernyi* and no template DNA gave any PCR products (Lane 10, 13).

All three primer pairs gave amplification products when DNAs from purified spores of *N. pernyi* and infected host tissue were used as template, and never gave PCR products from DNAs of *A. pernyi* uninfected by *N. pernyi*. Thus, the three sets of primers are expected to show high specificity to *N. pernyi*. The diagnosis technique of pebrine disease of *A. pernyi* based PCR will have broader utilization prospects in sericulture. Its application could focus on the early detection of inspection (Yi-Ren Jiang, *et al*., 2011).

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