LITERATURE REVIEW

1. Mulberry silkworm, *Bombyx mori*

1.1 Biology of *B. mori*

B. mori was originally in a wild state. As a result of being tamed over a long period by human beings and also due to artificial selection and rejection, it is now a domestic animal. This species of insect belongs to the phylum Arthropoda, class Insecta, order Lepidoptera, family Bombycidae. *B. mori* is monophagous insect that feed on only mulberry leaves. Its life cycle is completed by passing through four different stages; egg, larva, pupa and moth (Figure 1). During the period of pupation, the body is defenseless against external enemies and hence, the silkworm spins a protective shell around the pupa and this shell is known as the cocoon, the origin of silk tread.

Silkworm is an insect which undergoes complete metamorphosis. The duration of the whole life cycle is about 55-60 days at temperatures of 23-25°C (Tazima, 1962). The egg has an outer layer, the shell or chorion. Shape of the egg varies according to strain but in general it is unevenly elliptical. Colour of the egg after oviposition is usually pale yellow. After few days, the egg appears light brown in colour which is gradually darkens (Aruga, 1994). There are two types of egg; diapause and non-diapause. Silkworm races indigenous to temperate regions usually lay diapause eggs, while those of tropical regions lay mostly non-diapause eggs (Tazima, 1978). Once the embryonic growth inside the egg is complete, the young larva hatches. During hatching the young larvae eat up a part of the egg shell and emerge through the hole thus made. The newly hatched larvae are usually dark brown with fairly setae on all segments. Young larvae, soon after hatching so called the first instar, start feeding on mulberry leaves and grow. After feeding for a little over three days, they reach the end of their first instar, the larvae stop feeding completely and undergo ecdysis within 24 hours afterward. This period of ecdysis is called the first moult. After moulting they resume feeding, grow and repeat the moulting process. In the common strains of silkworm, this process repeated four times. There are four moults and

between two successive moults lies an instar. There are thus five instars in the life cycle of silkworm. About half of the whole life cycle is the larval stage, the only stage at which they take food. The range of food selection of this insect is very narrow, almost limited to mulberry leaves. Hence, the silkworm is classified as a monophagous insect (Aruga, 1994). In the fifth instar, the silkworms feed for six to eight days after which they stop feeding and are ready to spin cocoons. These, so called “ripe” or “mature” larvae, become very restless, raising their heads in search of a place to spin cocoon. In mature larvae, the silk glands are so large and weight about 40% of the body weight. The larvae are transferred to a spinning nest, which is usually made of straw or cardboard. The colour of the cocoon in commonly reared silkworm strains is mostly white. Besides white cocoons, there are cocoons of different colour such as golden yellow, flesh colour, crimson, bamboo leaf colour, and green (Tazima, 1978). It takes the larvae about 4 or 5 days after moulting to pupate and 9 to 14 days more until the emergence of the moths. Soon after that male and female moths mate. A male moth can mate with two or three females in one day, but the ability to mate deteriorate very quickly. The female moth begins to lay eggs shortly after the separation from the male and lays continuously for several hours until all eggs are deposited (Tazima, 1962).

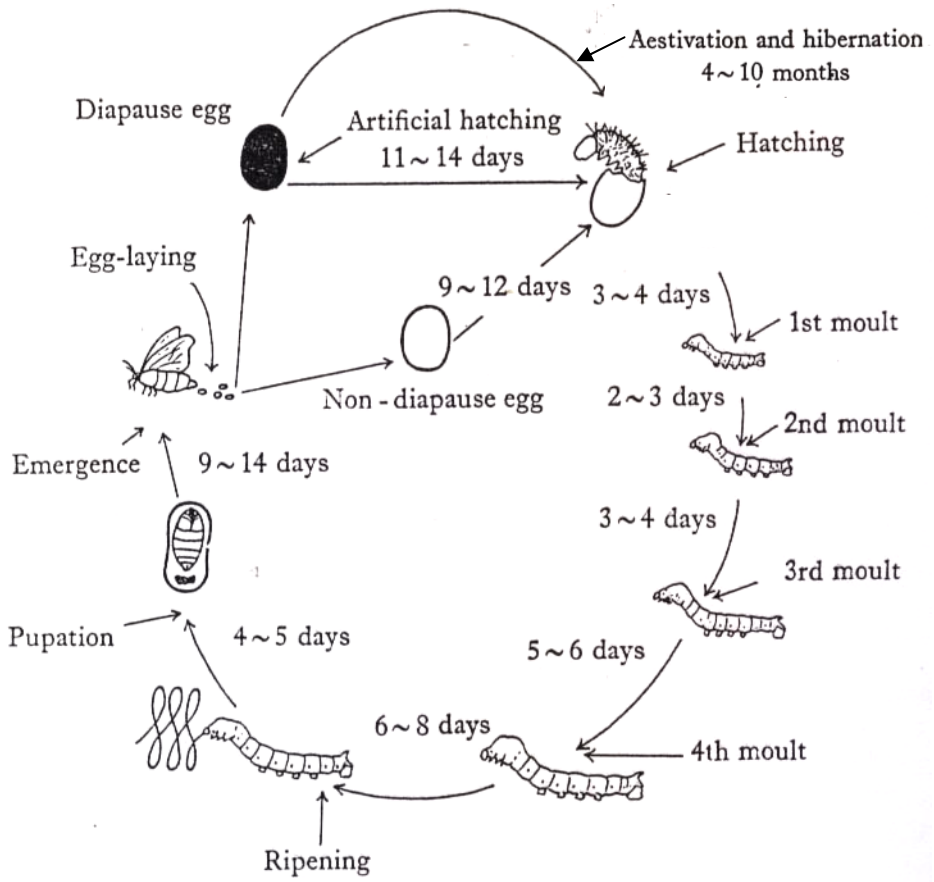

Figure 1 Life cycle of the mulberry silkworm, *Bombyx mori* which undergoes 4 developmental stages; egg, larva, pupa and moth.

Source: Tazima (1962)

1.2 Classification of *B. mori*

Mulberry silkworms are classified in different ways. Classification may be based on the native regions, the number of larval moult, the rearing period, body marking or pattern, body colour of mature larva, colour of cocoon and colour of egg (Department of Agriculture, 1980; Aruga, 1994).

1.2.1 Classification based on voltinism

Silkworms can be classified as univoltine, bivoltine and polyvoltine. Univoltine types have only one generation in a year and usually reared in spring. They have a long life cycle and their larvae and cocoons are large. Bivoltine types have two generations under natural climatic condition in any specific temperature. Compared to univoltine, the bivoltine silkworms are generally strong and healthy and are reared in spring and autumn. Their life cycle is shorter than that of univoltine silkworms. Polyvoltine or multivoltine types have many generations in a year. Larvae are generally strong and healthy with a short life cycle. Their cocoons are quite inferior to those of the univoltine and bivoltine silkworms. They are intermediate between univoltine and bivoltine rather than being pure bivoltine.

1.2.2 Classification based on native regions

On the basis of native regions, the silkworms are classified into Japanese, Chinese, European and Tropical races.

The Japanese race has univoltine and bivoltine silkworms. The larvae are usually strong and healthy, the cocoon mostly white and barrel-shaped. The cocoon fiber length is short and the reeling property is generally poor.

The Chinese race has univoltine, bivoltine and polyvoltine silkworms. They grow rapidly by feeding actively on mulberry leaves but are susceptible to high humidity. The cocoons of polyvoltine silkworm are spindle-shaped. The silk fibre is thin and its reeling property is good.

The European race has only univoltine silkworms. Generally, the eggs, larvae and cocoons are larger than those of the Japanese and Chinese races. The larval period is longer and the larvae grow fat by feeding actively on mulberry leaves. Cocoons are white and buff coloured and the fibre is longer with good reeling property.

The tropical race has polyvoltine silkworms. The larvae are strong and healthy and their laval period is short. Cocoons are yellow and the reeling property is poor.

1.2.3 Classification based on moulting

A few times during the larval stage, silkworms stop feeding, become inactive and shed their skin. This period is called moulting period and classification can be based on the number of larval moults which can be 3, 4, 5 moults or more. Silkworms with four larval moults are the ones which are widely reared. Those with two or six moults have a shorter life cycle and produce thin fibres whereas those with five moults have longer life cycle and their cocoons composed of thicker fibres.

1.2.4 Classification based on cocoon colour

The cocoon are broadly classified into white and coloured. The white ones are of two types; inferior white and superior white. Genetically some of these are inferior to yellow cocoons and others are superior. Among the white cocoons are pure white and somewhat dirty white cocoon. Coloured cocoons are of different colours; yellow, buff, straw and green.

1.2.5 Classification based on rearing period

According to the rearing period, silkworms are broadly classified into spring and summer-autumn silkworms. The summer-autumn silkworms are further categorized as summer, early autumn and very late autumn silkworms. Those reared even later, that is, after the very late autumn is referred to as early winter silkworms.

1.2.6 Classification based on larval marking

The commonly reared silkworms may appear to be plain or show pattern so called patterned or marked silkworms. Different types of larval marking such as stripes, dark colour, zebra bands, brown spots, quail marks, multistar, etc., have been known for a long time.

1. 3 *B. mori* in Thailand

In Thailand, there are three district silkworm strains, recommended by the sericulture research institute for sericulturists to rear in different parts of the country. The first is multivoltine strains or Thai strains, the second is bivoltine hybrid (Japanese x Chinese) and the third is multivoltine x bivoltine strains. As Thailand is in tropical region, temperature is quite high, over 30^{o}C in hot season, hence the bivoltine hybrids are usually difficult to rear but they produce high yield or cocoon whereas, the multivoltine strains are vice versa. However, the cross-breeds between multivoltine and bivoltine are conducted to combine the good characters from the two strains (Noppaseney, 2002). Multivoltine strain has the shorter life cycle with larval period of 18-23 days compare to 20-30 days of the bivoltine strain. A female multivoltine strain lays an average of 300 eggs while the bivoltine strain lays 400-500 eggs (Sungkompitak, 1989).

2. The causative agent of grasserie disease

2.1 Baculoviruses

2.1.1 Classification of baculoviruses

The causative agent of grasserie disease is *Bombyx mori* nucleopolyhedrovirus (BmNPV). This virus is classified in the genus Nucleopolyhedrovirus, family Baculoviridae. The family Baculoviridae is classified by the basic character as an enveloped, rod-shaped virion (approximately 50 x 250) containing a circular double-stranded DNA genome ranging from 50 to 100 million Daltons. They were previously divided into three subgenera based on their

morphological properties (figure 2). Subgenus A consists of the nuclear polyhedrosis viruses (NPVs). The characteristic feature of this group is that several virions are occluded in a polyhedral, proteinaceous inclusion body (IB). There are two morphological subgroups within the NPVs. The single-nucleocapsid NPV (SNPV) in which only one nucleocapsid is found per envelope and the multinucleocapsid NPVs (MNPVs) in which several nucleocapsids are packaged per envelope. Subgenus B consists of the granulosis viruses (GVs). In this group virions containing single nucleocapsid and only one virion is occluded in one inclusion body which is oval in shape. Subgenus C consists of virions which are not packaged into IBs at any stage of their life cycle, they are nonoccluded viruses (NOVs) (Bilimoria, 1986).

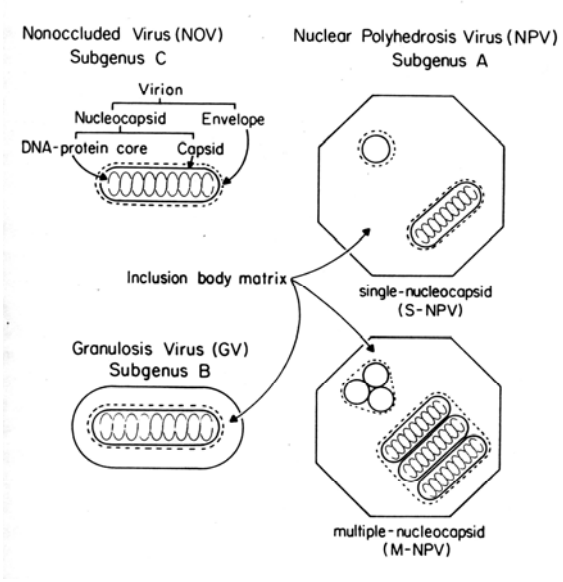

Figure 2 Structure diagram of insect viruses in the family Baculoviridae. It comprised three subgenera; subgenus A, B and C. The S- and M-NPV contain numerous virions per inclusion body

Source : Bilimoria (1986)

Currently, Baculoviridae comprises only two genera, nucleopolyhedrovirus (NPVs) and granulovirus (GVs). The terms nuclear polyhedrosis virus and granulosis virus have been replaced by nucleopolyhedrovirus and granulovirus, respectively (van Regenmortel *et al*., 2000). Baculoviruses have only been found in over 600 species of arthropod hosts. The majority

of baculovirus host are within the order Lepidoptera. They have also been isolated from the insects in orders Diptera, Hymenoptera, Coleoptera, Neuroptera, Thysanura and Tricoptera and the crustacean in order Decapoda (shrimp) (Hong *et al.*, 2000).

Morphology of NPVs, either multiply or singly enveloped does not appear to be crucial in taxonomy. For example, *Bombyx mori* SNPV (BmSNPV) and *Autographa californica* MNPV (AcMNPV) are far more closely related to each other than they are to most other NPVs whether SNPVs or MNPVs (Hunter-Fujita *et al*., 1998). The abbreviations commonly used for baculovirus are based on the first letter of the insect species and the viral subgroup, e.g. *Bombyx mori* SNPV as BmSNPV and *Pieris brassicae* GV as PbGV. Although baculoviruses are usually named after the host species from which they were isolated, this does not provide a satisfactory form of identification. In several instances, different baculoviruses have been isolated from the same insect species while a single virus, or a group of closely related strains, can occur in more than one insect species, e.g. *Autographa californica* MNPV (Hunter-Fujita *et al*., 1998).

2.1.2 Biology of baculoviruses

Baculoviruses are diverse group of viruses found mostly in insects. The baculo portion of the name refers to the rod-shaped capsids of the virus particles. The virus particle is composed of a protein shell (capsid) that surrounds the nucleic acid. Baculovirus capsids are usually 40-50 nm in diameter and 200-400 nm in length. Within the capsid, the DNA is condensed into a nucleoprotein structure known as the core. The capsid plus the core are collectively refered to as the nucleocapsid (O'Reilly *et al*., 1992). Nucleocapsids contain a single molecule of circular supercoil is double-stranded DNA. The length of baculoviral DNA is between 80-200 kb (van Regenmortel *et al*., 2000). Nucleocapsids are made in the nucleus of infected cells and are subsequently enveloped by one of two processes. Nuclocapsids can bud through the plasma membrane of the infected cell; such budded virus particles are releases into the extracellular fluid with a loosely fitting membrane envelope. Nucleocapsids may also acquire an envelope within the nucleus where they are produced. The envelope of virions embedded in

occlusion bodies fits closely around the nucleocapsids, and additional nuclear material may be compressed between the membrane and the nucleocapsids during the occlusion process. Viral occlusion bodies are formed in the nucleus and comprise enveloped nucleocapsids embedded in a crystalline protein matrix. Polyhedral occlusion bodies of NPVs are known as polyhedra, occlusion viruses, or polyhedral inclusion bodies. The protein making up the crystalline matrix of an NPV occlusion body is known as polyhedrin (O'Reilly *et al*., 1992).

A unique feature of the NPV life cycle is the production of two virion phenotypes. Those virions found within polyhedra are termed "polyhedra-derived virus" (PDV) and the other form, found in the hemocoel of the infected host insect, is termed "budded virus" (BV). Historically, a number of terms have been used for the two virion phenotypes. The PDV phenotype has also been called "occluded virus" (OV). Alternate names for the BV phenotype include "nonoccluded virus" (NOV) and "extracellular virus" (ECV or EV) (Blissard and Rohrmann, 1990). The composition of the envelopes of the two forms is different, PDV envelopes are synthesized within the nucleus during occlusion body morphogenesis, BV envelopes are acquired when newly synthesized nucleocapsids bud through the host cell cytoplasmic membrane and are released into the haemolymph. Spikes of a virus-coded glycoprotein (budded virus envelope fusion protein) protrude through the end of virus particle (Hunter-Fujita *et al.*, 1998).

The main features of the biology of NPVs are illustrated in Figure 3. Polyhedra are ingested by a susceptible insect and solubilized in the alkaline condition of the midgut. Virions of the polyhedra derived virus (PDV) phenotype are released and enter midgut epithelial cells by fusion with microvilli. Nucleocapsids are transported to the nucleus where uncoat of the viral DNA occurs. Progeny nucleocapsids bud through the nuclear membrane and are transported to plasma memrane but apparently lose the nuclear derived envelope in the cytoplasm. The nucleocapsids then bud through the cytoplasmic membrane into the hemocoel acquired the budded virus (BV) specific envelope that contains the virus-encoded envelope glycoprotein. These virions (of the BV phynotype) appear to be specialized for secondary infection of other host cells. A second group of progeny nucleocapsid becomes enveloped within

the nucleus by *de novo* assembled envelope. These virions are subsequently occluded within polyhedrin protein. Maturation of the polyhedra includes the addition of polyhedral envelope around the periphery of the forming occlusion bodies. Upon insect death and cell lysis, the polyhedra are released into the environment (Blissard and Rohrmann, 1990).

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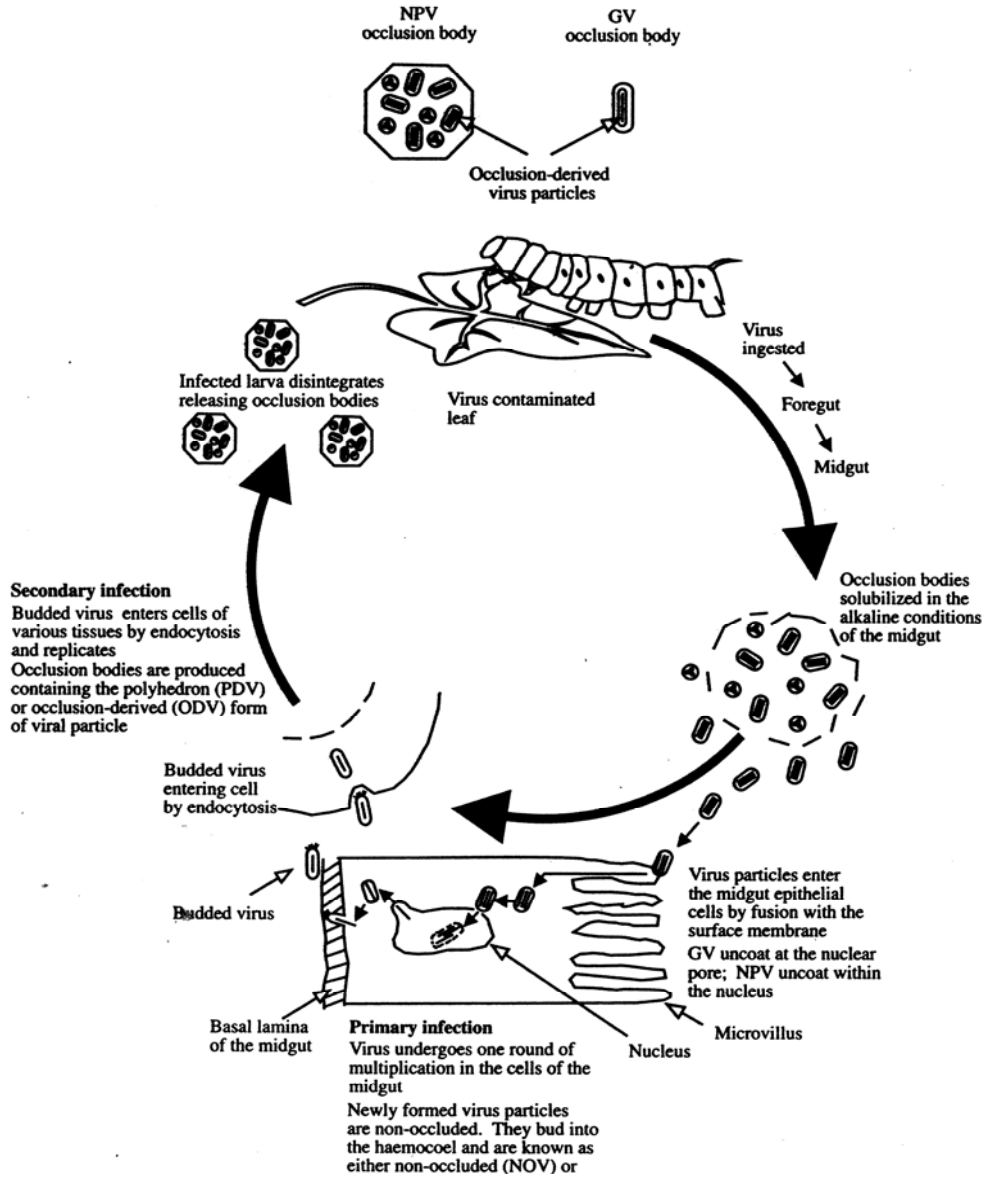


Figure 3 Typical cellular infection cycle of the nucleopolyhedrovirus. Polyhedra are ingested and are dissolved in the alkaline condition of the midgut, releasing infectious virions. Primary infection begins in which the virions infect midgut cell and budded virions (BV) are produced within the nucleus. The budded virions disseminate the virus within the insect host and start the secondary infection in which the pre-occludded virions (PDV) are produced. Subsequently, the PDV become embedded in the polyhedrin matrix and polyhedra are formed. The polyhedra are released into the environment when the insect dies and disintegrates.

Source: Hunter-Fujita *et al*. (1998)

2.2 *Bombyx mori* nucleopolyhedrovirus

Grasserie disease caused by *Bombyx mori* nucleopolyhedrovirus (BmNPV), is known under a variety of names. It is called “jaundice” in America, “grasserie” in France, “giallume” in Italy, “nobyo” in Japan and “gelbsucht” or “Fettsucht” in Germany and Austria (Tanada and Kaya, 1993). In Thailand, it is called “ rok tur”, “ rok taubeum”, “rok tauluang”, or “rok kati” (Kaewwises, 2000). There are two types of BmNPV particle, thick rod-shaped of 80 x 330 nm and slender rod-shaped with the size of 45 x 340 nm (Khosaka *et al*., 1971). In Thailand, the local strain BmNPV composed of rod-shaped nucleocapsids with the size of 45x340 nm, embedded within the polyhedral inclusion bodies (PIBs). The PIBs were 2-7 μm in diameter, and tetragonal shape with rather round angles. However, the globular and hexagonal PIBs were observed but unfrequently (Attathom and Sinchaisri, 1987).

Francki *et al.*(1991) had classified *Autographa californica* MNPV (AcMNPV) as the type species of the MNPV morphotype and *Bombyx mori* SNPV as the type species of the SNPV morphotype. The significance, however, of the SNPV classification of BmNPV is not clear since BmNPV can produce both MNPV and SNPV type virions depending upon the host or tissue in which it replicates. In general, NPVs produce two types of progeny, the budded virus (BV) and the occluded virus (OV), during its life cycle (Blissard and Rohrmann, 1990). BVs are predominantly produced during an early phase of infection and acquire their envelopes as they bud through the plasma membrane. BVs are highly infectious *in vitro* and are responsible for the systemic spread of the virus in the infected insect. OVs are thought to obtain their envelopes by *de novo* synthesis in the nucleus during a late phase of infection, and are subsequently occluded in polyhedra. In the field, polyhedra protect the occluded virions from the environment and are required for horizontal transmission (Rohrmann, 1992).

Infection mostly takes place through feeding of polyhedra-contaminated mulberry leaf, rarely through wounding. The factors influencing the outbreak of this disease are high temperature and humidity, or the sudden fluctuations of these two parameters, poor ventilation in the rearing room, and ineffective disinfection of rearing room and equipments and feeding of

tender mulberry leaves during late instars. Inadequate larval spacing, starvation and excessive moisture in the rearing bed also have been known to contribute towards the outbreak and spread of the disease (United Nation, 1990).

2.3 Symptomatology and pathology of *Bombyx mori* nucleopolyhedrovirus

Symptoms of grasserie disease caused by BmNPV were described as followed; during the early stage of infection, no symptoms are noticed except for the worms being slightly sluggish. Initially the skin shows oily and shining appearance. As the disease progresses, the skin becomes thin and fragile and the body becomes milky white with intersegmental swelling. The rupture of the fragile skin liberates the liquefied internal organs containing numerous numbers of polyhedra which become source of inoculum to the new host. The characteristic symptom of this disease is that the larvae become restless and crawl aimlessly along the ridges or rims of rearing trays, and subsequently falling on the ground and die. Death takes place after infection in about 4-5 days in the young larvae and 5-7 days in the grown up larvae (United Nation, 1990).

Johansen *et al.*(1986) reported that the infection of BmNPV was restricted to the nuclei of infected tissues. The evidence of NPV infection was the hypertrophied nuclei, almost filled the cells with numerous polyhedra. The fat body, tracheal matrix and hypodermis were heavily infected. Moderate infection was found in the nuclei of silk glands and the epithelial sheath of testes. There were no polyhedra in mid gut cells, muscle cells and malpighian tubules of silkworms. The follicular cells possess a relatively thick basement membrane at their basal surface which probably acts as a barrier to greatly retard the invasion of virus particle from the haemolymph. Attathom and Sinchaisri (1987) observed pathological changes occurred in NPV infected cells of silkworm. The hypertrophied nucleus undergoes chromatin and nucleolus disappearance. Nucleocapsids in group were observed in the diffuse mass of chromatin, the virogenic stroma, in the central area of the nucleus. As disease developed, the virions were membrane enveloped singly and occluded in the polyhedra. The polyhedra located randomly in the nucleus or in line along periphery of the nucleus. They also observed several ultrastructural

changes in associated with the BmNPV development, for example, the curved and vesicular membrane profiles appearing randomly in the infected nuclei.

2.4 Genes of *Bombyx mori* nucleopolyhedrovirus

BmNPV genome was 128,413 nucleotides long and contained 136 open reading frames (ORFs) encoding predicted proteins of over 60 amino acids (Figure 4) (Acharya *et al*., 2002). Gene expression pattern following baculovirus infection is regulated in a cascade fashion, where the activation of each set of genes relies on the synthesis of proteins from previous class. Based on this temporal regulation, the baculovirus genes are grouped into three classes; early, late and very late. Although most baculovirus genes can be placed into one of the above classes, some are transcribed in more than one phase. Early genes may be defined as those genes that are transcribed in the absence of any viral genes expression. The late and very late genes are dependent on early viral gene expression and on DNA replication. The transcription of very late genes, those expresses primarily during the occlusion phase, is activated approximately 10 to 12 hr after the initiation of late gene transcription (O'Reilly *et al*., 1992).

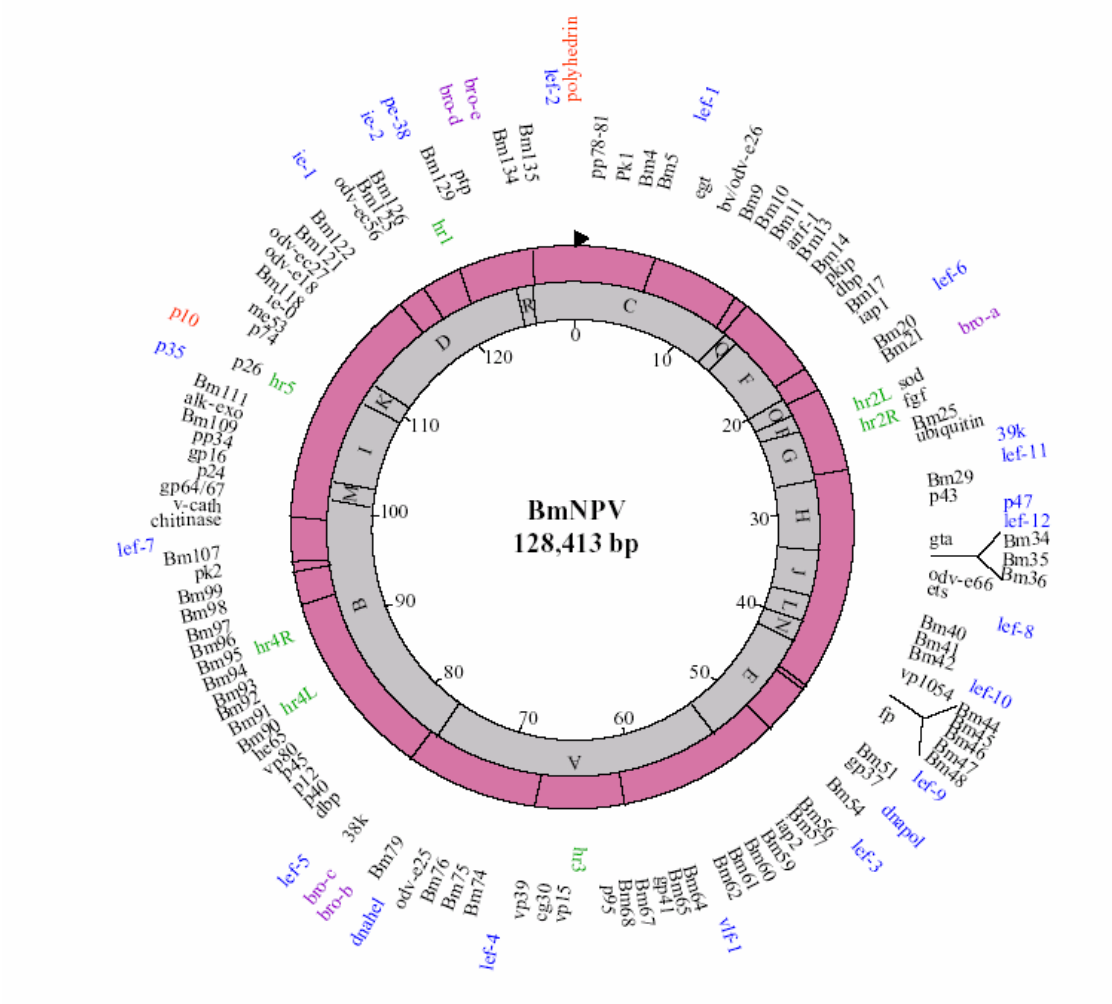
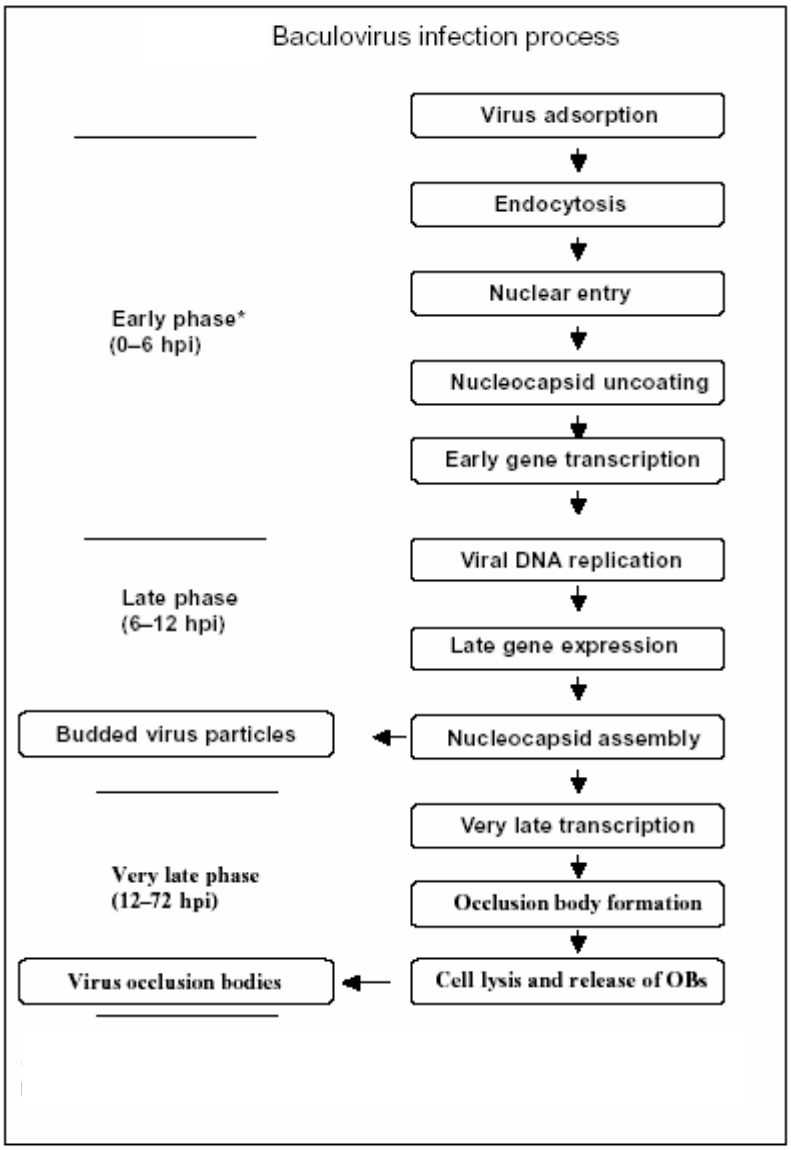


Figure 4 Genomic organization of BmNPV. BmNPV genome is a covalently closed circular double stranded DNA of 128,413 bp.

Source : Acharya *et al*. (2002)

There are three phases of gene transcription of baculovirus as illustrated in Figure 5 (O'Reilly *et al.*, 1992; Boucias and Penland; 1998, Acharya *et al.*, 2002).



Figture 5 Baculovirus infection process contains three phases; early, late and very late phase. The timing are based on AcNPV infection. The kinetics of BmNPV infection is a little slower; hpi = hours post infection. OBs = occlusion bodies.

Source : Acharya *et al*. (2002)

2.4.1 Early phase of gene transcription

In a permissive cell line, the baculovirus displays a temporally regulated biphasic life cycle. Phase I is marked by the production of the budded phenotype and requires the early and late gene transcription, whereas phase II results in the formation of the OV phenotype and the occlusion production.

The release and unfolding of viral DNA in the nucleus mark the initiation of gene transcription. Historically, the early transcription phase has been differentiated into the immediate-early and the delayed-early stages. Immediate early (IE) genes are those genes that can be transcribed by uninfected insect cells and require no viral gene products for their expression. Delayed early (DE) genes, on the other hand, require other viral gene products for their transcription. Early phase transcription, around 0-6 hrs post infection (h pi) results in the production of a series of structural and nonstructural proteins. These include the regulatory proteins (immediate-early, IE; late expression factors, Lefs), the cell and host modulator proteins (anti-apoptotic p35 and ecdysone UDP-glycosyl transferase), biosynthetic enzymes (viral RNA and viral DNA polymerase complexs), and various structural proteins. Early genes are transcribed by RNA polymerase II. Many early promoters contain a functional TATA box typical of most RNA polymerase II promoters of higher eukaryotes (Boucias and Pendland, 1998).

2.4.2 Late phase of gene transcription

The transition from early to late phase (>6 h pi) transcription is marked by two events; the replication of the viral DNA and the switch from early to late promoters. Late gene transcription is inhibited by aphidocolin, a DNA synthesis inhibitor, and cycloheximide, a protein synthesis inhibitor. All late genes possess the highly conserved late promoter (ATAAG) motif. which unlike the early promoters, is recognized by a novel viral RNA polymerase complex resistant to α-amanitin, an antibiotic known to bind to and inhibit RNA polymerase II. The viral RNA polymerse activity, extracted from infected cells by glycerol gradient ultracentrifugation , contains an array of peptides having sizes corresponding to various baculovirus-encoded late

expression factor (Lef) (Boucias and Penland, 1998). Many of late gene products are proteins that in concern with the early proteins produce the BV phenotype. Morphogenesis of the nucleocapsids takes place in the nucleus. Component capsid proteins, synthesizes on the ribosomes in the cytoplasm, are preferentially transported back into the nuclear region (Boucias and Penland, 1998).

The late phase extends from 6 h pi to approximately 20 to 24 h pi. During this phase, a distinct electron-dense structure known as the virogenic stroma forms in the nucleus. Capsid sheaths appear to assembly in pockets at the edge of the virogenic stroma and are filled, while associated end-on with the stroma, with a nucleoprotein core to form nucleocapsids. Progeny nucleocapsids leave the nucleus, possibly a variety of routes, and travel to the cytoplasm through the cytoplasmic membrane where they interact end-on with regions of this membrane having peplomerlike structures. Nucleocapsids usually bud individually from the cytoplasmic membrane although occasionally more than one nuclecapsid is observed in a budded virion (O'Reilly *et al*, 1992).

2.4.3 Very late phase of gene transcription

The very late phase or occlusion-specific phase begins around 20 h pi. Electron microscopy reveals the elaboration of membrane envelope segments within the nucleus during this occlusion process. Nucleocapsids interact end-on with membranes and eventually become enveloped, either individually (SNPVs) or in groups (MNPVs). Envelopment of the nucleocapsids appears to be an essential prior to embodiment within the polyhedrin matrix of an occlusion body. During the occlusion phase, the production of budded virions is greatly reduced, if not terminated (O'Reilly *et al*., 1992).

The first light microscopic indication of this occlusion phase is the formation of several polyhedral inclusion bodies (PIBs) in the nucleus. As they accumulate, the PIBs often form a ring around the inside of the nuclear membrane, eventually, the nucleus becomes virtually filled with occlusion bodies. The second phase of baculovirus replication involves the synthesis

and release of the viral occlusions. The polyhedrin and p10 are the two dominant very late genes. The very late promoters lack of the TATA and CATG motifs of the early gene but it possesses a group of 7 nucleotides upstream of the TAAG and AT-rich untranslated downstream region that mediates the hyper-expression of the very late genes (Boucias and Pendland, 1998).

The one important gene of the very late genes is polyhedrin gene. Polyhedrin is a phosphorylated molecule of 28.8 kDa (Gomi *et al*., 1999) which constitutes the matrix of baculovirus occlusion bodies so called polyhedra. Polyhedrin plays a significant role in the replicative cycle of baculovirus. It is a late protein which is synthesized in the last infection step in large amounts and this in turn reflects a corresponding abundance in the amount of accumulated polyhedrin mRNA, probably achieved through high rates of mRNA synthesis. This suggests that heterologous genes can be introduced into the genomes of baculoviruses and highly expressed effectively under the control of the polyhedrin promoter (Iatrou *et al.*, 1985). This high level of expression from polyhedrin gene promoter makes recombinant baculovirus expression vector system very useful in foreign protein production (Patil and Dandin, 1990). The occlusion body facilitates virus survival and dispersal in the environment and, in the context of biological control, is a convenient, safe, and simply manipulated product (Burand *et al*., 1992). Studies of occlusion body protein suggest that the numerous different lepidopteran NPVs have evolved from a lepidopteran NPV rather than cross-infecting from other orders of arthopods. Gene sequence data relating to occlusion body proteins (polyhedrin or granulin) are proving to be an important tool for discerning phylogenetic relationships, allowing lepidopteran NPV evolution to be divided into two distinct branches, namely group I and II (Zanotto *et al*., 1993).

3. Grasserie disease of mulberry silkworm

3.1 Factors affecting the grasserie disease

There were many factors that accelerate outbreak of grasserie disease in Thailand (Attathom and Sinchaisri, 1987). First, many of Thai farmers raise multivoltine type silkworm, the more frequency of the rearing times per year, the less thorough disinfection of the rearing

places will be practiced. Therefore, inadequate sanitation is of fundamental reason for outbreak of the disease in those poor-management farms. Second, in the remote area where it is inconvenient to obtain silkworm eggs from the Government Experiment Station, the farmers maintain their own seed stocks. Such seeds apparently have no guarantee on the quality and disease. Continuous cultivation of silkworms from these seed stocks creates the problems of inbreeding as well as the outbreak of the diseases. Third, unsuitable rearing condition, for example inappropriate rearing places and tools, poor ventilation, overcrowding, poor quality of mulberry leaves and improper rearing techniques will all weaken the silkworms to the infection of viral disease. The well-management sericultural farms are practically unaffected by the viral disease.

3.2 The prevention and control of silkworm disease

3.2.1 The use of anti-disease varieties

In view of the inadequate disinfection and prevalence of unhygienic conditions in the rearing areas, the use of disease resistant silkworm varieties can be the better option.

Eguchi *et al* (1998) had succeeded in evolving a silkworm hybrid which is completely resistant to *Bombyx mori* densonucleosis virus type 1 (BmDNV 1). Since the resistance to BmDNV 1 is controlled by a single major gene. It is possible to introduce the resistance into a susceptible breed easily by cross breeding.

On the contrary, resistance of the silkworm to nuclear and cytoplasmic polyhedrosis virus (NPV and CPV) and also infectious flacherie (IFV) is controlled by polygenes (Watanabe,1986). Therefore, introduce of complete resistance to these viruses is practically impossible in the silkworm breeds. However, breeding of strains with comparatively more resistance to these viruses is possible by selection of survivors after virus exposure (Uzgawa and Aruga, 1966).

In India, attempts had been made to induce resistance to BmNPV into a susceptible silkworm stock Ka by mating with a relatively more resistant stock, g133 followed by the exposure of progenies to BmNPV and selecting the survivors. The F1 population was found to have a survival rate of 41.66% in which the average of resistant stock was 68.84% and susceptible stock was 20.55%. F2 generation was comprised of segregating population so a wide range of survival was observed ranging from 50-86% (Sen *et al.*, 1999).

In Poland, Kremky and Michalska (1988) studied on resistance of silkworm line to BmNPV. The studies were carried out during four sericultural seasons on six silkworm lines inoculated with BmNPV and submitted to a low temperature treatment. Survival rates were estimate on the 9^{th} day after the treatment, and before pupation. Survival rate of the group inoculated with the virus in every generation was higher than the group exposed to a single inoculation. Low temperature treatment to the larvae activated latent form of the virus. Survival rate in different tested lines was similar, but reaction to inoculation and low temperature was different.

The utilization of disease-resistant varieties to prevent different kinds of disease has many advantages. No specific measures and chemical reagents are needed. The method saves labour power, material and expenses, reduces the environmental contamination, and obtains an obvious effect for disease prevention. With these advantages, it can easily be accepted, be used and be popularized. In recent years, the research on breeding of disease-resistant varieties has received much attention and shows more and more significant advantages in the field of agriculture. Up to now some disease-resistant varieties to different kinds of diseases have been bred by some silkworm breeding units in Japan. According to the epidemic situations in different areas and seasons, they use different varieties which are able to resist diseases correspondingly so that the epidemic disease are under control (Liu and Zhong, 1989).

3.2.2 Chemical method

In the past, it was considered as a non-realistic way to control the diseases of silkworm by chemical treatment, however, with the development of silkworm pathology in recent years, these methods have been used in silkworm production scale, and some of them have shown a gratifying prospect. Nevertheless, the chemical treatment needs to be improved. The major reason is that the time of these chemicals remaining in the silkworm's body is very short, so that the reagent needs to be applied day by day, and this affects silkworm health and brings a lot of troubles to the sericultural management.

The chemicals affect the metabolism of pathogens through oxidation of tissue or by denaturation and precipitation of protein of causal organisms. Disinfection of the rearing house is done 8-10 days before starting the rearing operation. The effectiveness of chemical disinfectant depends upon three factors i.e., the concentration, the duration and ambient temperature. Some of the chemicals commonly used as disinfectant are chlorine compounds, formalin, paraformadehyde and lime power (Pallavi and Kamble, 1997).

3.2.3 Physical methods

The physical methods include simple and effective physical treatment such as sunlight, streaming, burning, burying. Virus diseases are the most disastrous of silkworms' disease which could be brought under control, through integrated several control measures suitable to the existing conditions, such as destruction of pathogens through disinfection, breaking the transmission cycle, segregation of moulting worms, improvement of feeding and management to increase vigor and disease resistance, and selection of disease resistant varieties. To prevent the spread of virus, disinfection is rigorously implemented immediately after harvesting the cocoons as dead worms are the most concentrated source of infection. (Liu and Zhong, 1989).

3.2.4 The quarantine of silkworm disease

The grasserie disese predictive inspection have been developed to provide an early and accurate diagnosis method for this vial disease such as the enzyme-link immunosorbent assay (ELISA) (Vanapruk *et al.*, 1992; Shamim *et al.*, 1994), DNA hybridization (Attathom *et al.*, 1994), colloidal textile dye-based dipstick immunoassay (Nataraju *et al.*, 1994), monoclonal antibody (Nagamine and Kobayashi, 1991) and western blot analysis (Chaeychomsri *et al.*, 1995).

For pebrine disease of silkworm, the quarantine of silkworm diseases is an essential step to prevent the embryonic infection. Several predictive inspection and early diagnosis of pebrine disease have been developed, such as monoclonal antibody-sensitizes latex for pebrine spore diagnosis (Shi and Jin, 1997), multiprimer PCR for the early and simultaneous detection of several kinds of microsporidia that cause silkworm pebrine (Hatakeyama and Hayasaka, 2003). The predictive inspection of pebrine disease can help to adopt a corresponding measure for disease prevention.

During the current decade, with the permeation and enhancement of other outstanding techniques, the predictive diagnosis of viral disease and pebrine disease has made great progress. The methods mentioned above are only some examples, these methods can be widely used in correct diagnosis of pathogens and to know the possibility of cross infection among different kinds of insects or animals, it will help the research of silkworm infection disease. However, many of these methods are just research measures suitable for laboratory diagnosis. The ordinary method is still need for large scale inspection of the production line. For example, the ordinary microscopy still can not be discarded in prebrine spore's inspection (Liu and Zhong, 1989). A method which is more convenient and useful for quarantine in large-scale production still requires a greater effort in research endeavor.

3.3 Detection of nucleopolyhedrovirus

Viral disease especially that caused by the nucleopolyhedrovirus is of fundamental importance disease of silkworm. It occurred prevalently in rural areas where the Thai local varieties of silkworm were cultivated, therefore, imposed serious economic damage to the Thai

sericulture. In some farms, the viral disease outbreak can destroy the whole colony of silkworm. This phenonema often occurred in the fifth instar larvae which caused serious loss in both economy and labour intensity to the farmers. Therefore, several detection methods have been employed to early detect the present of grasserie disease in order to stop spread of the disease within the rearing colony and solve the disease problem.

3.3.1 Light microscope

Although virion of BmNPV cannot be seen by the light microscope, the occlusion bodies which covered the virions can be seen. This method is convenient and cheap. However, it is a long procedure and is not suitable for many samples. At present, light microscope is still used at Sericulture Research Centres and Sericulture Experiment Stations for grasserie disease detection.

3.3.2 Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) is the most commonly applied 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. Vanapruk *et al.* (1992) showed that viral detection by ELISA was more efficient than the light microscope. 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.

3.3.3 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 (Nataraju *et al*., 1994).

3.3.4 Monoclonal antibody-based sandwich ELISA

Shamim *et al.*, (1994) 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.*

Murine monoclonal antibodies (MCAs) were produced against *Borrelina bombycis* nuclear polyhedra. In ELISA all five MCAs reacted with nuclear polyhedra of *B. bombycis* and to a variable extent with other strains of nuclear polyhedra, namely *Amsaeta olbistriga, Heliothis armigera and Spodoptera litura*. In western blots, these recognized antigens of 31 (polyhedron protein) and 67 kDa of nuclear polyhedra (MA-321 showed reduced binding). In sandwich enzyme immunoassay employing purified rabbit polyclonal antibodies, MA-321 as low as 100 nuclear polyhedra could be detected. The results obtained by sandwich ELISA on haemolymph samples collected from rearing places revealed 100% correlation with results by microscopic examination. Haemolymph samples collected on various days from second instar larvae infected in the laboratory revealed that the sandwich ELISA could detect infection as early as 96 hr after infection.

3.3.6 Agar diffusion technique

Krywienczyn and Bergold (1961) reported that upon comparing the serological findings with the taxonomic classification of the host insects, considerable differences existed among nine systems of Lepidoptera classification available to them. The comparison suggested that the polyhedron inclusion-body proteins isolated from Tortricidae and Pieridae were more

closely related to the inclusion-body protein from Bombycidae, Saturniidae and Geometridae than to those from Galleriidae, Noctuidae, or Lymantriidae.

3.3.7 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). 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.

3.3.8 PCR technique

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). PCR-based method was developed for the detection of viral DNA in infected insect and shrimp (Faktor and Raviv, 1996; Otta *et al.*, 2003). 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). Nowadays real-time PCR was used to determine Baculovirus quantitatively (Rosinski *et al.*, 2002; Lo and Chao, 2004).