THESIS

VERTICAL TRANSMISSION OF NUCLEOPOLYHEDROVIRUS IN THAI MULBERRY SILKWORM, *BOMBYX MORI* L. (LEPIDOPTERA: BOMBYCIDAE)

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GRADUATE SCHOOL, KASETSART UNIVERSITY

2007

THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Master of Science (Agricultural Biotechnology)
DEGREE

Agricultural Biotechnology
FIELD

Interdisciplinary Graduate Program
PROGRAM

TITLE: Vertical Transmission of Nucleopolyhedrovirus in Thai Mulberry Silkworm, *Bombyx mori* L. (Lepidoptera:Bombycidae)

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THESIS

VERTICAL TRANSMISSION OF NUCLEOPOLYHEDROVIRUS IN THAI MULBERRY SILKWORM, *BOMBYX MORI* L. (LEPIDOPTERA: BOMBYCIDAE)

SIRINDA KHUMNOI

A Thesis Submitted in Partial Fulfillment of

the Requirements for the Degree of

Master of Science (Agricultural Biotechnology)

Graduate School, Kasetsart University

2007

Sirinda Khumnoi 2007: Vertical Transmission of Nucleopolyhedrovirus in Thai Mulberry Silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae). Master of Science (Agricultural Biotechnology), Major Field: Agricultural Biotechnology, Interdisciplinary Graduate Program. Thesis Advisor: Associate Professor Tipvadee Attathom, Ph.D. 61 pages.

Grassery disease of silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae) caused by *Bombyx mori* nucleopolyhedrovirus (BmNPV), is considered one of the most destructive disease affecting in Thai sericulture. Infected silkworms expressed disease symptom during the final stage of larval growth and died without cocoon production. Vertical transmission of grassery disease in Thai variety of silkworm was studied. Fifth instar larvae of Nang noi variety were inoculated with 10^8 PIBs/ml of BmNPV and disease infection was observed in all further developmental stages and their F1 offspring. Egg hatching was 97.99% in the parent healthy silkmoth and 59.30% in BmNPV-infected parent silkmoths. In F1 offspring of the healthy silkmoths, mortalities of the first to fifth instar larvae were 0.37, 0.19, 0.11, 0.00 and 0.00%, respectively. In F1 offspring of the BmNPV-infected silkmoths, mortalities of the first to fifth instar larvae were 11.31, 6.81, 5.21, 5.82 and 5.12%, respectively. This study suggests the possibility of BmNPV infection in the gonadal tissues of the silkmoth resulting in reduction of silkmoth's fecundity.

Transmission electron microscopic observation of BmNPV-inoculated silkworms revealed BmNPV infection in fat tissues of the larva, pupa and adult moth, in testes and ovary tissues of the adult moths. No virus particles or polyhedra of BmNPV were observed in eggs and all larval instars of their F1 offspring. However, using PCR with primers complementary to BmNPV *polh* gene, PCR amplified *polh* regions of 424 bp were obtained from some of the fifth instar larvae, pupae and adult moths. The *polh* gene PCR amplified products were also obtained from all of the observed eggs and larval instars of the F1 offspring. Results indicated the existence of BmNPV in the F1 offspring of the infected silkworms which confirmed vertical transmission of grassery disease in Thai silkworm variety. The knowledge of BmNPV transmission not only helps to establish proper preventive control strategy for grassery disease, but also helps to prevent outbreak of grassery disease and finally to eliminate this viral disease within silkworm population.

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ACKNOWLEDGMENTS

I would like to express my sincere thanks and grateful appreciation to my advisor, Associate Professor Dr. Tipvadee Attathom for her encouragement, guidance, dedicated efforts and devoted her valuable time to advise me throughout the course of this research.

I would like to express my sincere thanks to my co-advisors, Assistant Professor Dr. Kanungnit Reanwarakoun and Assistant Professor Dr. Weerawan Amornsak for their kind advices.

Sincere appreciation is extended to Ms. Mallika Kaewwises for her help and suggestion, Mr. Wisit Fichun (Sericultural Extension Centre 9: Kanchanaburi Province), who supported samples of silkworm.

My sincere thanks go to Mrs. Sunicha Khumwilai for her kind assistant in silkworm rearing, Mrs. Apinun Sonong, Mrs. Yuphin Srihirun for their help of TEM techniques and Mr. Rungrote Sochanthuk for molecular technique advices and members of insect pathology laboratory whose names have not mentioned here for all advices, help and meaningful friendship.

This research work is partially supported by the Center for Agricultural Biotechnology through the fund from Subproject Graduate Study and the scholarship from Graduate School, Kasetsart University.

Most of all, I would like to express all my love and gratitude to my beloved parents, Mr. Somyote and Mrs. Boonma Khumnoi and my sister for their support, love, understanding and encouragement throughout my life.

Sirinda Khumnoi

October 2007

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LIST OF ABBREVIATIONS

A^{o}	=	Angstrom
AcMNPV	=	*Autographa californica* multiple-embedded nucleopolyhedrovirus
B. mori	=	*Bombyx mori*
BmNPV	=	*Bombyx mori* nucleopolyhedrovirus
BV	=	budded virion
° C	=	celsius degree
cm	=	centimeter
cm^2	=	square centimeter
DDSA	=	dodecenyl succinic anhydride
DMP-30	=	tridimethyl amino methyl phenol
DNA	=	deoxyribonucleic acid
ds DNA	=	double stranded deoxyribonucleic acid
ELISA	=	enzyme-linked immunosorbent assay
GA	=	glutaraldehyde
GV	=	granulovirus
HaSNPV-WT	=	wild-type *Helicoverpa armigera* single-embedded nucleopolyhedrovirus
HaSNPV-AalT	=	genetically modified variant *Helicoverpa armigera* single-embedded nucleopolyhedrovirus
hpi	=	hours post-infection
H. zea	=	*Heliothis zea*
kbp	=	kilo basepair
L. dispar	=	*Lymantria dispar*
μl	=	microliter
μm	=	micrometer
ml	=	milliliter

LIST OF ABBREVIATIONS (cont'd)

mm	=	millimeter
min	=	minute
M. brassicae	=	*Mamestra brassicae*
M. separata	=	*Mythimna separata*
ng	=	nanogram
nm	=	nanometer
NMA	=	nadic methyl anhydride
NPV	=	nucleopolyhedrovirus
OB	=	occlusion body
PCR	=	polymerase chain reaction
PDV	=	polyhedral-derived virion
pg	=	picogram
PIBs	=	polyhedral inclusion bodies
p.i.	=	post-inoculation
SDS-PAGE	=	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
S. littoralis	=	*Spodertera littoralis*
S. exigua	=	*Spodoptera exigua*
S. frugiperda	=	*Spodoptera frugiperda*
TEM	=	transmission electron microscope
T. ni	=	*Trichoplusia ni*
TnCPV	=	*Trichoplusia ni* cypovirus
TnNPV	=	*Trichoplusia ni* nucleopolehedrovirus

VERTICAL TRANSMISSION OF NUCLEOPOLYHEDROVIRUS IN THAI MULBERRY SILKWORM, *BOMBYX MORI* L. (LEPIDOPTERA: BOMBYCIDAE)

INTRODUCTION

Sericulture is an important industry of Thailand. Thai sericulture has been changed from farm household in the past to the agro-industry business at present. Although many attempts from the government have been made to support and promote sericulture, the silk production has not yet met the demand of the textile industry (Center for Agricultural Statistics, 1990). The major cause of this low production of silk was due to the outbreaks of diseases of the silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae). Muscadine, flacherie, pebrine and grassery were reported as diseases of the silkworm found in Thailand (Aoki, 1971).

Grassery disease caused by *Bombyx mori* nucleopolyhedrovirus (BmNPV) is considered the most destructive disease affecting silkworm. The infected silkworm expresses disease symptom during the final stage of larval development and die without cocoon production resulting in the waste of expense, time and labour work. This disease is wide-spread to all silkworm rearing areas in Thailand. The damage is estimated at 30-100% annually (Kaewwises and Niyomvit, 1995). Although disinfection of silkworm rearing places and appliances by spraying with formalin is generally practiced before each rearing season, it can not apparently prevent occurrence of grassery disease (Watanabe, 2002).

Grassery disease is known under a variety of names in different countries: named “Jaundice” in United State of America, “Grasserie” in France, “Giallume” in Italy, “Gelbsucht” in Germany, “Fettsucht” in Austria (Krassilstschik, 1896), “Nobyo” in Japan (Tanada and Kaya, 1993) and “ Rok tur”, “ Rok taubeum”, “Rok tauluang”, or “Rok kati” in Thailand (Kaewwises, 2000). The rod-shaped virus particle contains a circular double stranded DNA with the molecular size of 125 kbp (Maeda and Majima, 1990).

There are two types of BmNPV particle, thick rod-shaped with the size 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 BmNPV strain composed of rod-shaped nucleocapsids with the size of 45 x 340 nm, embedded within the polyhedral inclusion bodies (PIBs). The polyhedral inclusion bodies were 2-7 micrometer (μm) in diameter and were in tetragonal shape with rather round angles. However, globular and hexagonal PIBs were also observed but unfrequently (Attathom and Sinchaisri, 1987).

Ridhards *et al.* (1998) and Kukan (1999) reviewed that dispersal of baculoviruses in lepidopteran insects by adults may occur following vertical transmission to their progenies either inside the eggs (transovarial) or on the surface of the eggs (transovum). Hamm and Young (1974) reported that nucleopolyhedrovirus (NPV) of *Heliothis zea* was transmitted by surface contamination of the eggs with PIBs. Neelgund and Mathad (1978) found transovum and transovarial transmission of NPV to the progeny of armyworm, *Mythimna (Pseudaletia) separata*. Fuxa *et al.* (1992) reported vertical transmission of NPV in *Spodoptera frugiperda* from F1 infected larvae and pupae to the first instar larvae about 86-98% and 69-88%, respectively. Recently, Khurad *et al.* (2004) reported vertical transmission of NPV in silkworm, *B. mori* by per oral inoculation of the occlusion bodies (OBs) to fifth instars at dosage of 2,000 OBs/larva, resulted about 50% mortality of the larvae and pupae prior to adult eclosion.

The presumable most effective solution for the control of grassery disease is to detect viral infection as early as possible in order to stop spread of the disease in rearing house. Therefore technique that permits detection of grassery disease at the earliest stage of infection is strongly needed. Several diagnostic methods are described for detection of nucleopolyhedroviruses (NPVs) infection for examples agarose gel double diffusion (Young *et al*., 1975), a dot-blot hybridisation assay (Ward *et al.*, 1987), DNA hybridization (Keating *et al.*, 1989; Ebling *et al*., 2001), the enzyme-linked immunosorbent assay (ELISA) (Langridge *et al*., 1981; Vanapruk *et al*., 1992), monoclonal antibody-based sandwich ELISA (Shamim *et al*., 1994), colloidal textile dye-based dipstick immunoassay (Nataraju *et al*., 1994) and polymerase chain reaction (PCR)-based analysis (Burand *et al*., 1992; Hughes *et al.,* 1993; Faktor and Raviv, 1996; Woo, 2001; Charpentier *et al.,* 2003; Kaewwises *et al*., 2004).

The vertical transmission of BmNPV in Thai mulberry silkworm has not been thoroughly study. This study is aimed at investigation the possibility of vertical transmission of the NPV in Thai variety of *B. mori*. The knowledge of BmNPV transmission not only helps to establish proper preventive control strategy for grassery disease, but also helps to prevent outbreak of grassery disease and finally to eliminate this viral disease within silkworm population. Transmission electron microscope (TEM) observation and polymerase chain reaction (PCR)-based method are used to clarify mode of transmission of BmNPV in Thai mulberry silkworm variety.

OBJECTIVE

To investigate the possibility of vertical transmission of BmNPV in Thai variety of silkworm, *Bombyx mori*

LITERATURE REVIEW

1. Biology of silkworm, *Bombyx mori* L.

The silkworm, *B. mori*, is an insect in the order Lepidoptera, family Bombycidae. The races of *B. mori* reared at present, are believed to be derived from the original Mandarina silkworm, namely *B. mandarina* Moore (Krishnaswami *et al.*, 1973).

Biology of silkworm had been studied by Sakaguchi (1978). The silkworm is holometabolous insect which complete its life cycle of four different metamorphosing phases: egg, larva, pupa and adult (moth) in about 50 days at temperatures of 23-25° C. Of this life cycle, about half 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 (Moraceae, genus *Morus*). Hence, the silkworm is classified as a monophagous insect.

The larval stage is very important period for silk-protein synthesis and egg formation. The larva just after hatching has many setae on the body surface. The young larva is about 3 millimeters (mm) in length. At about 2 days after hatching, the setae become less conspicuous, the body length reaches about 7 mm and the surface of skin becomes glossy. Silkworm molts four times during the larval period of its fifth instars. The duration of molting is rather constant among different races, while the length of each feeding period varies among different instars and also different races. During this time, the larva produces a new cuticle and sheds the old one. This phenomenon of shedding the old skin is called ecdysis. In the fifth instar, the larva attains a maximum length about 70 mm and feeds voraciously. When the larva develops fully and stops eating for 24 hours, the skin becomes transparent. This mature larva is usually placed in a spinning nest for the process called “pupation”, around 20-25 days after hatching. The stage from which the larva ceases spinning silk thread to the pupal ecdysis is called the pharate pupa and the duration is about 24 hours. When the larva transforms to a pupa, drastic changes occur in various tissues and organs. The cuticle is soft and in light yellow color, but after two or three hours the cuticle becomes hard and brown. Female and male pupae can easily be distinguished by their morphological features. In the female, there is a vertical line across center of the ventral side of

the eighth abdominal segment and a genital aperture at the ninth abdominal segment, while in the male, there is only a genital aperture which is situated at the ninth abdominal segment.
The female pupa is usually larger than the male pupa. The pupa stage lasts for 9-14 days.
The pupal stage is closed system, but the metabolic activity is very high due to histolysis and histogenesis through the metamorphoses. Through the step of pharate adult stage for five days, the adult moth emerges by pupal-adult ecdysis. The adult is unable to take any food and all organs and tissues are in the final state of differentiation. Krishnaswami *et al.* (1973) reported the female of multivoltine variety of silkworm may lay an average number of 400 eggs while average number of egg for the univoltine and bivoltine variety was 300 and 600 eggs in one night, respectively. Ito and Kobayashi (1978) described two types of silkworm eggs, diapause and non-diapause. Silkworm races indigenous to temperate regions usually lay diapause eggs, while those of tropical regions lay mostly non-diapause eggs.

2. Nucleopolyhedrovirus (NPV)

2.1 Classification of NPV

Bilimoria (1986) described NPV as an entomopathogenic virus, belongs to the family Baculoviridae which was characterized by an enveloped, rod shaped virion (approximately 50 x 250 nm) containing a circular double-stranded DNA genome ranging from 50-100 million daltons. As described by Bilimoria (1986) the genus nucleopolyhedrovirus were previously divided into three subgenera based on their morphological properties. Subgenus A consists of the 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. Summers (1975) reported that the polyhedra of NPVs range from 0.5-15 μm in diameter and have a variety of shape: dodecahedra, tetrahedral, cubes and angular forms of irregular polygonal shape, and their sizes and shapes are considered to be characteristic of the virus isolate and the insect host in which it is growing. The single-nucleocapsid NPV (SNPV) (type species: *B. mori* SNPV) in which only one nucleocapsid is found per envelope and the multiple nucleocapsid NPVs (MNPVs) (type species: *Autographa californica* MNPV) in which several nucleocapsids (1-17) are packaged per envelope. Subgenus B consists of the granulosis viruses (GVs) (type

species: *Trichoplusia ni* GV). In this group, virions containing single nucleocapsid are packaged one per IB, 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 the nonoccluded viruses (NOVs) (type species: *Oryctes rhinoceros* NOV). van Regenmortel *et al*. (2000) recently reported that Baculoviridae comprises only two genera, NPVs and GVs and the terms nuclear polyhedrosis virus and granulosis virus have been replaced by nucleopolyhedrovirus and granulovirus, respectively.

2.2 Infectivity and pathology of NPV

Baculoviruses have been found in over 600 species of arthropod hosts. The majority of baculovirus host are within the order Lepidoptera (243 viruses). They also have 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). The most common mode of entry of baculoviruses into insect hosts is by ingestion of virus during feeding of larvae. Although the pupal and adult stages of some insect species may be infected, these insects are considered to have acquired the virus as larvae. Therefore, the larval stage is the most susceptible target for control with baculovirus pesticides (Granados and Williams, 1986).

Infection mostly takes place through feeding of polyhedra-contaminated mulberry leaf, rarely through wounding. The report of United Nation, (1990) indicated that factors influencing the outbreak of this disease are high temperature and humidity, the sudden fluctuations of these two parameters, poor ventilation in the rearing room, 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.

Gross pathological changes in NPV infected hosts had been described by Bilimoria, (1986). Prominent pathological changes usually occur late in infection. Within the family Baculoviridae, external changes are not of significant diagnostic value. Diseased larvae may exhibit characteristic behavior such as gathering in a characteristic fashion at the top of trees or hanging in an inverted position. Nucleopolyhedrovirus infection in the lepidopterous insects

frequently generalized. The integument often changes color and becomes flaccid and fragile. Large numbers of PIBs are released upon rupture of the body wall.

Keddie *et al.* (1989) described that nucleopolyhedrovirus produce two virion phenotypes during the infection cycle. Firstly, the polyhedra-derived virion (PDV), is occluded within the polyhedral bodies which are adapted for survival in the environment and instrumental in transmission of infection from insect to insect through the oral route. Secondly, the budded virion (BV) is responsible for cell to cell spreading within the insect after the infection is established by PDV in the midgut. The polyhedra-derived virion phenotype has also been called "occluded virus" (OV) and the alternate names for the BV phenotype include "nonoccluded virus" (NOV) and "extra cellular virus" (ECV or EV) (Blissard and Rohrmann, 1990).

Infection process of NPV in insect host had been reviewed by Keddie *et al.* (1989) as the followings: the PDV was responsible for natural infection as the insect larvae ingest occlusion bodies along with food. The alkaline environment in the insect midgut dissolves the polyhedra and releases the virions. The liberated PDVs infect midgut epithelial cells by receptor-mediated membrane fusion. These infected cells produce the budded virions, which are also referred to as extra cellular virus and are needed for lateral propagation of infection within the larvae via haemolymph and tracheae. Budded virions (BVs) are predominantly produced during an early phase of infection and acquire their envelopes as they bud through the plasma membrane. They are highly infectious *in vitro* and are responsible for the systemic spread of the virus in the infected insect. The budded virions infect many tissues in the larvae including fat bodies, ovaries and most endoepithelial cells. The budded virions enter the cell through receptor-mediated endocytosis. Following penetration of the plasma membrane, the nucleocapsids move towards the cell nucleus. At the nucleus, the viral nucleocapsids are uncoated and the DNAs get released. The nucleus becomes enlarged and a distinct electron dense granular structure, called virogenic stroma is formed. This structure gets associated with nuclear matrix and forms the site of nucleocapsids assembly. Viral transcription and replication take place in this region. Following virus replication by about 12 hours post-infection (hpi), the progeny BVs are produced and released into the extracellular compartment. Soon after, mature PDVs (surrounded by an envelope) get packaged into occlusion body. The infected larvae continue to feed till large

amounts of occlusion bodies accumulate. Eventually they stop feeding and undergo several physiological changes. The cuticle melanises, musculature becomes flaccid and the larva liquefies. Larval disintegration results in the release of polyhedra into the environment and the subsequent spread of virus infection. In the field, polyhedra protect the occluded virions from the environment and are required for horizontal transmission.

3. *Bombyx mori* NPV (BmNPV)

Grassery disease is known under a variety of names in different countries: named "Jaundice" in United State of America, "Grasserie" in France, "Giallume" in Italy, "Gelbsucht" in Germany, "Fettsucht" in Austria (Krassilstschik, 1896), "Nobyo" in Japan (Tanada and Kaya, 1993) and " Rok tur", " Rok taubeum", "Rok tauluang", or "Rok kati" in Thailand (Kaewwises, 2000).

3.1 Morphology of BmNPV

Matthews (1982) reported that the causal agent of grassery disease is the BmNPV which belongs to the subgroup A of the family Baculoviridae. The particle of BmNPV is rod shaped of a singled nucleocapsid (protein/ DNA core) surrounded by an envelope. The genome of BmNPV was double-stranded, covalently closed circular DNA with the varies molecular size such as 125 kbp (Maeda and Majima, 1990) or 128 kbp (Gomi *et al.*, 1999) or 130 kbp (Okano *et al*., 1999). DNA isolated from BmNPV could be observed in electron microscope preparations to have size with an upper range of approximately 60 μm (Faulkner and Henderson, 1972; Kok *et al.*, 1972).

Two types of BmNPV particles, thick rod-shaped of 80 × 330 nm and slender rod-shaped with the size of 45 × 340 nm (Khosaka *et al*., 1971). Ito and Kobayashi (1978) reported BmNPV particles were about 85 × 330 nm in size while Watanabe (2002) found that they were 45 × 330 nm. In Thailand, Attathom and Sinchaisri (1987) found the slender rod-shaped of BmNPV particles with the size of 45 × 340 nm. The inclusion body protein of BmNPV which surrounds the enveloped virions is called polyhedron (Summers, 1975).

After infesting the body of silkworm, the viral DNA replicated in the nucleus of the host cell, and formed polyhedra inclusion bodies (PIBs) within the nucleus. The PIBs, visible under the 400× microscope, were 2-7 μm in diameter with the shape of a tetragonal with rather round angles. However, the globular and hexagonal PIBs were unfrequently observed (Attathom and Sinchaisri, 1987). Hong *et al.* (2000) found the polyhedra morphology of BmNPV was irregular with a diameter of about 2-3 μm.

3.2 Symptomatology and pathology of BmNPV

Behavioral symptoms of NPV infection were described as early as 1909, when diseased *Lymantria monacha* L. larvae were reported to migrate to the top branches of pine trees, hence its initial description as "tree top disease" (Wahl, 1909). Infected larvae usually move away from the food plant or climb to an elevated location where they typically hang by the abdominal prolegs shortly before dying (Smirnoff, 1965). Pathology of BmNPV found in Thailand was described by Attathom and Sinchaisri (1987) as in the early stage of infection and no symptom was observed. With the progress of the disease, infected larva lost appetite and was characterized by a faint molting of fat body beneath the cuticle of the swollen abdominal segments. The larva appeared creamy white to yellowish in color and shrunken. After death the cadaver turned black and the integument became fragile, rupture upon touch, releasing viscous liquid containing polyhedra of the virus. 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 polyhedral body. Tracheal matrix and hypodermis tissue were heavily infected. Moderate infection was found in nuclei of silk glands and epithelial sheath of testes. No polyhedra were found in midgut cells, muscle cells and malpighian tubules of silkworm. However, the BmNPV was found in the follicular epithelium of the silk moth (Johassen *et al*., 1986).

Attathom and Sinchaisri (1987) observed several ultrastructural changes occurred in NPV infected cells of *B.mor*i. The hypertrophied nucleus undergoes chromatin margination 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 ın the nucleus or in line along periphery of the nucleus. Associated with the BmNPV development were the curved and vesicular membrane profiles appearing randomly in the infected nuclei. By the end of the infection cycle, the body of silkworm larva is liquefied and transformed into millions of new occlusion bodies that spread into the surrounding environment.

4. Transmission of NPV in insects

In field population of insects, transmission of pathogens probably consists of a combination of horizontal and vertical transmission. In horizontal transfer, pathogens are transmitted among individual hosts within a generation and between generations as environmental contamination, while vertical transmission occurs from parents to offspring (Fine, 1984). Ridhards *et al.* (1998) and Kukan (1999) reviewed that dispersal of baculoviruses in lepidopteran insects by adults may occur following vertical transmission to their progeny either inside the eggs (transovarial) or on the surface of the eggs (transovum).

4.1 Horizontal transmission of NPV

The discovery of NPV of *Gilpinia hercyniae* (Hartig) in bird dropping had given rise to the suggestion that birds might be important in virus dispersal. Advantage was taken of the continuing spread of sawfly and virus to sample larvae in an area with sawfly and virus and in adjacent areas with sawfly only: bird droppings were also collected in the latter. In previously virus-free area, NPV was identified in some larvae but not in bird droppings. It is therefore suggested that sawfly adults might be major agents of dispersal (Buse, 1977). Ali *et al.* (1987) fed the uniform and mixed-age of *H. zea* populations on caged soybean with *H. zea* NPV (HzNPV). Larval collections revealed viral disease outbreaks occurred in all treatments following release of infected larvae (primary infected larvae). Transmission of NPV in uniform-aged populations was related to the density of primary infected larvae released in the population. In mix-aged populations, horizontal transmission in the oldest larvae in the population was equal to that inuniform-aged populations, providing that primary infected larvae in the mix-aged population were all the age of the oldest noninfected cohorts. As the mix-aged population, transmission

increased and was generally higher than that in the uniform-aged population. Transmission was also higher when primary infected larvae were medium sized at death than when small or large at death.

4.2 Vertical transmission of NPV

Ham and Young (1974) fed adult *H. zea* with NPV (10^6-10^8 PIBs per insect). They found progeny infection range from 0-7.1% in controls and from 0.4-94.3% with virus treatment. Abul-Nasr *et al.* (1979) fed three concentrations of NPV (1.2×10^6, 1.2×10^7 and 1.2×10^8 PIBs/ml) to third and fifth instar *Spodertera littoralis*, the mortality in the progeny of the infected group was higher (38-48 %) than that of the control. Treatment of *Psuedoplusia includens* larvae at fourth, fifth and sixth instars inoculated with 4×10^3 -1.2×10^6 PIBs per mm^2 diet and mating of survivors gave levels of infection in progeny of between 0.5 and 8 % (Young and Yearian, 1982). Smits and Vlak (1988) observed that adults developing from *S. exigua* larvae infected as fifth instars with 1×10^5, 2×10^5 or 1×10^6 PIBs/ml transmitted virus to their progeny about 10-28%. Fuxa and Richter (1991) gave third to fifth instar *S. frugiperda* with NPV 5×10^4-3.5×10^7 PIBs per larva. They found vertical transmission of NPV to both pupae (control < 0.05% and virus treated 0.8-2%) and adults (control 0.2-0.5% and virus treated 8-10%). Fuxa and Richter (1992), reported the vertical transmission of NPV from five to seven generations in *S. frugiperda*. Goulson and Cory (1995) fed fourth and fifth instar larvae of *Mamestra brassicae* with NPV 10^3, 10^4, and 10^5 PIBs per larva. The results indicated that incidence of infection in progeny from adults surviving infection was 0.55%. Therefore, the vertical transmission may occur at low levels. However, Fuxa *et al.* (2002) reported the transovarial transmission of *T. ni* NPV (TnSNPV), *T. ni* Cypovirus (TnCPV), *A. californica* NPV (AcMNPV) in *T. ni*. population. Furthermore, Khurad *et al.* (2004) fed fifth instar larvae of *B. mori* with four different dosages of BmNPV (830, 1,300, 1,800 and 2,000 PIBs/larva). They found that a dosage of about 2,000 PIBs per larva suitable for obtaining infected adults and confirmed that the viral infection is vertically transmitted from the parent to progeny. Recently, Zhou *et al.* (2005) studied vertical transmission dynamics of wild-type *Helicoverpa armigera* single-nucleocapsid nucleopolyhedrovirus (HaSNPV-WT) and a genetically modified variant (HaSNPV-AalT) and found that the

recombinant virus was transmitted at lower rates in *H. armigera* populations than the wild-type virus.

5. Diagnosis and detection of NPV

Field detection and primary diagnosis of NPV generally rely on specific symptoms expressed by host insects. Infected larvae showed non-molting, milky white or yellow color body, incessant crawling and clipping of the caudal horn resulting in exudation of turbid haemolymph (Regional Sericulture Training Center, 1987).

Kaewwises *et al.* (2004) reported the disease symptom of grassery disease was usually clearly visualized at the final stage of larval growth. This late expression of the disease symptom leads to the difficulty in controlling spread of the disease in time. Nowadays, sericulturists try to breed multivoltine and bivoltine silkworm varieties resistant to BmNPV. However, the presumable most effective solution for the control of grassery disease is to detect viral infection as early as possible in order to stop spread of the disease in rearing house. Therefore technique that permits the detection of the grassery disease at early stage of infection is strongly needed.

Several conventional diagnostic methods have been described for detection of NPVs infection. Young *et al*. (1975) detected the infection of NPV in *H. zea* larvae by the agarose double diffusion technique using antiserum to alkali-solubilized polyhedra. In their field study, virus infection was detected by this method as early as three days after viral treatment. Langridge *et al.* (1981) detected the structural proteins of *A. californica* NPV (AcMNPV) and *H. zea* NPV (HzNPV) by indirect enzyme-linked immunosorbent assay (ELISA). The immunoassay detected less than one nanogram (ng) of AcMNPV protein and the polyhedral proteins of HzSNPV and AcMNPV were found to be immunologically identical. Ward *et al.* (1987) compared a dot-blot hybridisation assay to light microscopy and radioimmunoassay (RIA) for detection of NPV. Results showed the assay was more sensitive than the differential staining, phase-contrast microscopy or indirect solid-phase RIA with as few as 20 occlusion bodies (150 picograms (pg) DNA) being detected. Keating *et al.* (1989) used DNA hybridization assay to detect viral DNA in extracts from gypsy moth, *L. dispar* infected larvae.

The hybridization results were closely correlated with mortality observed in reared larvae. Furthermore, DNA dot-blot hybridization assay had been used by Ebling *et al.* (2000) to detect thresholds of NPV in whitemarked tussock moth, (*Orgyia leucostigma*) larvae. This detection system was proved to be reliable, efficient and simple method for early detection of NPV infection in large numbers of larvae. Vanapruk *et al.* (1992) compared ELISA and light microscopic observation for the detection of BmNPV by peroral infection into the third instar larvae and found that using ELISA, the virus can be detected within one day after inoculation, whereas, by light microscope the virus can be detected after three days post-inoculation. Using ELISA for viral detection, Shamim *et al*. (1994) employed murine monoclonal antibodies (MCAs) produced against *Borrelina bombycis* nuclear polyhedra. All five MCAs, they produced reacted with nuclear polyhedra of *B. bombycis* and to a variable extent with other strains of nucleopolyhedrovirus, namely *Amsaeta olbistriga*, *H. armigera* and *S. litura* NPVs. In sandwich ELISA employing purified rabbit polyclonal antibodies, virus in insect haemolymph could be detected as early as 96 hr after infection. These antibodies could be useful for detection of nuclear polyhedra infection at early stages, i.e., second to third instar of larvae. Nataraju *et al*. (1994) reported the use of colloidal textile dye-based dipstick immunoassay for BmNPV detection. The purified anti-BmNPV IgG was used to capture the antigen on nitrocellulose membrane attached to a dipstick, and the antigen was detected with colloidal textile dye labeled anti-BmNPV IgG. The sensitized dye was observed to be stable for three months period at 4° C.

6. PCR-based technique for viral detection

6.1 PCR-based method

Recently, PCR-based methods have proved to be more powerful techniques for disease detection and have been widely used for rapid detection of NPV in insect. Polymerase chain reaction is a highly sensitive technique which amplifies target DNA sequences of the causative agent of the disease. The 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 separated. 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 two 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).

6.2 Polyhedrin gene (*polh* gene)

Nucleopolyhedroviruses (NPVs) infect insects and produce proteinaceous inclusion bodies containing occluded virions (OVs) in the nuclei of infected cells. The inclusion bodies so called "polyhedra" composed of a single protein, polyhedrin or Polh protein which is encoded by viral gene so called *polh* gene. Polyhedrin protein is a phosphorylated molecule of 28.8 kDa (Gomi *et al*., 1999) which 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 *polh* 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 *polh* promoter (Iatrou *et al.*, 1985). Polyhedrin protein is highly expressed during the late phase of infection and is crystallized in the nuclei of the cells. Genes encoding *polh* of many NPVs have been cloned and sequenced. Amino acid sequences of *polh* are highly conserved among many NPVs (Zanotto *et al*., 1993).

6.3 PCR technique for NPV DNA detection

Burand *et al*. (1992) employed PCR for the detection of baculovirus DNA sequence from viral occlusion bodies (OB) contaminating the surface of gypsy moth eggs. The level of sensitivity of the technique was as low as five viral genome copies and DNA from one OB equivalent. Charpentier *et al.* (2003) used three specific DNA probes for detection of *L. dispar* NPV (LdNPV) genome. One of these probes was obtained by PCR. Using a combination of PCR and hybridization, LdNPV contamination of egg masses were detected. It was demonstrated that as little as ten copies of the LdNPV genome could be detected.

6.4 PCR technique for NPV *polh* gene detection

Polymerase chain reaction (PCR) technique was developed for detection of the NPV *polh* gene. A culture of *Mamestra brassicae* insects (MbLC) were used for the detection of a latent baculovirus (MbNPV). PCR amplification of *polh* gene sequences demonstrated that the latent virus was present throughout the life cycle of the insect; eggs, larvae, pupae and adults (Hughes *et al.,* 1993). Faktor and Raviv (1996) used PCR-based method for the detection of *polh* gene of *S. littoralis* NPV. This method enabled the detection of low levels of viral DNA directly from viral occlusion bodies or from total larval DNA. Woo (2001) compared the amino acid sequences of the *polh* gene in twenty-six NPVs. The viruses detected by this technique were the NPVs of *A. californica*, *B. mori*, *Hyphantria cunea*, *S. exigua*, *S. litura* and *L. dispar*.
The PCR amplified product size was 430 bp. Woo (2001) also suggested that this technique would be useful in monitoring the distribution of NPVs and release of the wild type and recombinant NPVs. Kaewwises *et al.* (2004) developed PCR-based method for early detection of grassery disease of silkworm in Thailand, based on the presence of *polh* gene of BmNPV infected silkworm, *B. mori*. Polymerase chain reaction technique could detect *polh* gene in all stages of silkworm. The method was sensitive enough to detect *polh* gene in only one infected individual egg. In larval stage, *polh* gene could be detected in all instars and using only one individual infected larva. In pupal and adult stages, *polh* gene was detected in both infected male and female. The whole process of BmNPV genomic extraction and detection could be done within five hours.

MATERIALS AND METHODS

1. Silkworm rearing

Colony of silkworm, *Bombyx mori* used in this study was initially obtained as eggs from Queen Sirikit Sericulture Center, Kanchanaburi and Nakhon Ratchasima provinces, Thailand. Nang noi variety, the commercial Thai mulberry silkworm, is a polyvoltine type. The larvae were fed with fresh mulberry leaves three times a day (09.00 am, 01.00 pm and 04.00 pm) and reared in clean rearing room using disinfected rearing appliances. The newly molted fifth instar silkworm larvae were divided into two groups. Larvae of the first group were continuously colonized by feeding with fresh mulberry leaves and rearing in clean rearing room. They were maintained as healthy stock colony for the entire experiments. Larvae of the second group were served as host for virus propagation.

2. Virus propagation

The *Bombyx mori* nucleopolyhedrovirus was propagated in Insect Pathology Laboratory, Department of Entomology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom province. The fifth instar silkworm larvae used as host for virus propagation were starved for six hours before inoculation with BmNPV. Per os inoculation was carried out by feeding the larvae with fresh mulberry leaves smeared with the suspension of BmNPV at the concentration of 10^8 PIBs/ml. Four to five days after inoculation, the larvae showed typical symptoms of grassery disease and died within 4-6 days. Heavily infected and dead larvae were collected and put in amber glass bottle contained distilled water and kept at room temperature. The silkworm cadavers were allowed to putrefy and release the virus inclusion bodies or polyhedra which precipitated at the bottom of the container leaving most of the remains in supernatant.

3. Polyhedral clarification

Polyhedral inclusion bodies (PIBs) of the virus were harvested from infected larvae and partially purified using the method described by Attathom (1978). In brief, BmNPV infected larvae were homogenized in distilled water and the homogenate was filtered through four layers of cheesecloth. The PIBs in the filtrate were pelleted by centrifugation at 5,000 rpm for 10 min at 4° C (JA-14 rotor, Beckman J-21C centrifuge) and the pellet was resuspended and washed with distilled water by centrifugation at 5,000 rpm for 10 min at 4° C. The precipitate contained PIBs were washed several times until the supernatant became colorless. The partially purified PIBs suspension was kept at -20° C for further study.

4. Evaluation of NPV transmission in silkworms

4.1 Samples preparation

4.1.1 Infected parent silkworms

Five hundreds of healthy and newly molted fifth instar *B. mori* larvae were starved for six hours. Four hundreds of them were used to initiate the infected parent silkworms by per os inoculation as follow: pieces of one cm^2 of mulberry leaves were coated with 10 μl suspension of BmNPV, 10^8 PIBs/ml and placed in plastic cups of 6 cm in diameter and 4 cm in height. After drying at room temperature, silkworm larvae were placed individually on BmNPV contaminated leaves and were allowed to completely consume the leaves. The other one hundred larvae were fed with pieces of mulberry leaves dipped in distilled water and were used as a group for the control unit. The inoculated and the control fifth instar larvae were further reared on fresh mulberry leaves until pupation and adult emergence. A few fifth instar larvae, pupae and adult moths developing from the healthy and infected silkworm samples were randomly collected for BmNPV observation by TEM and PCR amplification.

4.1.2 Eggs from infected adult moths

After viral inoculation, some of the silkworms in BmNPV-infected group died in the cocoon and some survived the viral infection and emerged into adult moths. The survivor adult moths were allowed to mate and lay eggs. Silkworms in control group grew normally to the adult stage. The eggs were randomly collected from each group and washed with distilled water twice, 10 min each before preparation for TEM observation and PCR amplification.

4.1.3 The F1 offspring of the infected silkmoths

The newly hatched larvae, F1 offspring of the silkmoths surviving infection were further reared on fresh mulberry leaves. Mortality due to transmission of BmNPV infection was recorded in every succeeding larval. Samples of the F1 offspring at each instar (first to fifth instar) were randomly collected. The collections were made on day 1 after each molt. The same procedure was also made with the control group. All samples were further prepared for TEM observation and PCR amplification.

4.2 Observation of BmNPV infection by Transmission electron microscope (TEM)

4.2.1 Viral observation in parent silkworms

At 4 days post-inoculation (p.i.), a few late fifth instar larvae from the control and BmNPV-infected groups were randomly collected for cytopathological observation. The rest were allowed to grow and reached their pupal and adult stages. Samples of the pupae and adult moths were also randomly collected.

Tissues preparations were as follows: healthy and infected silkworm (fifth instar larvae, pupae and adult moths) were dissected and pieces of fat body tissues, testes and ovaries were collected. Fixation was made in 2.5% glutaraldehyde (GA) for 3 hours, then rinsed with phosphate buffer, pH 7.0, 3 times (15 min each), post-fixed with 1% osmium tetraoxide in the same buffer for 2 hours in ice. The tissues were then rinsed with phosphate buffer for 10 min,

dehydrated through a standard acetone series, at 50%, 70%, 80%, 90%, 95% (10 min, each) and 100% for twice (10 min, each). The dehydrated samples were infiltrated with 100% acetone and embedding medium 1:1 for 30 min. The embedding medium composed of 5.2 ml of epon 812, 2.8 ml of dodecenyl succinic anhydride (DDSA), 2.0 ml of nadic methyl anhydride (NMA) and 0.15 ml of tridimethyl amino methyl phenol (DMP-30). The tissues were further infiltrated with 100% acetone and embedding medium 1:3 for 30 min and with the embedding medium for 60 min, twice. Finally, the tissues were embedded in embedding medium in gelatin capsule. The capsules were placed in oven regulated at 70° C for 36 hours for polymerization. Specimens were sectioned at 550 A^{o} with a diamond knife using ultra microtome (Leica ultracut uct), stained with 5% uranyl acetate for 60 min and lead citrate for 10 min. BmNPV infection was examined in each type of the tissues using TEM (JEOL-1230, JEOL, Ltd), operated at 100 kV.

4.2.2 Viral observation in eggs

Eggs from the control and infected silkmoths were randomly collected and washed with distilled water twice, 10 min each. Tissues preparation, ultrathin sectioning and TEM observation were made following the procedures described in 4.2.1

4.2.3 Viral observation in larval progeny

The first to fifth instar larvae, progeny of the control and of the silkmoths surviving BmNPV-infected were dissected and fat body tissues were collected. All samples were prepared, sectioned and observed for viral infection using TEM following the method described in 4.2.1

4.3 Detection of BmNPV infection using PCR- based method

4.3.1 Extraction of BmNPV DNA from silkworm samples

BmNPV DNA was extracted from the control and inoculated parent silkworms (fifth instar), pupae, adult moths, eggs and larval progeny from first to fifth instars.

The modified alkaline lysis method of Sambrook and Russell (2001) was used for DNA extraction. At least 50 μl of homogenate tissue samples were required for PCR detection. Therefore, at least 10 eggs and ten of the first and second instar larvae were used to extract the required DNA. But for pupa, adult moth, third to fifth instar larvae, only one individual provided sufficient homoginated tissue for DNA extraction.

In the extraction process, eggs were placed in a microtube and 50 μl of sterile distilled water were added, eggs were ground with a mini pestle. Sample of larva, pupa and moth was placed on mortar, steriled water was added at 1:1 ratio of body weight and ground. The 50 μl of homogenate was transferred to a new microtube; 200 μl of solution II (0.2 N NaOH, 1%SDS,) was added, the mixture was mixed thoroughly and 200 μl of cold solution III (3 M potassium acetate, 5 M glacial acetic acid) was added. The solution was vortexed and added with 200 μl chloroform: isoamyl alcohol (24:1). A mixture was centrifuged at 12,000 rpm for 5 min and the upper phase was transferred to a new microtube. DNA precipitation was made by adding 400 μl of isopropanol, mixed well and centrifuged at 12,000 rpm for 5 min at room temperature. Supernatant was discarded and DNA pellet was rinsed with 200 μl of 70% ethanol. DNA was allowed to vacuum dry for 20 mins and was suspended in 50 μl of filtered distill water.

4.3.2 PCR amplification of BmNPV *polh* gene

A set of specific primers was designed from the clone nucleotide sequence within Thai BmNPV *polh* gene (accession number AY779044). The oligonucleotide sequences of forward primer began from position 221-240 of *polh* ORF and reverse primers began from 616-644 of *polh* ORF. These primers amplified a 424 bp PCR product. The oligonucleotide sequences of forward and reverse primers were:

Forward primer: 5' AATTCGCAGTGAAACCCG 3'

Reverse primer: 5' AGAGTCTGTGCCGATGT 3'

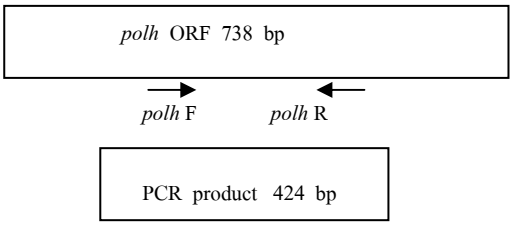

Figure 1 Diagram of the *polh* gene. Location of the primers used for PCR amplification is indicated. The predicted amplification product is 424 bp (Kaewwises *et al*., 2004).

The PCR amplification was performed in Thermal cycler PTC-100 (MJ Research, Inc.). The PCR mixture contained of 1 μl of total DNA (~50 ng), 0.2 μl of *Taq* DNA polymerase, 2.5 μl of 10x reaction buffer, 1 μl of 50 mM $MgCl_2$, 1 μl of 2.5 mM dNTP and 1 μl of 5 ρM/μl of each forward and reverse primers in a final volume of 25μl. The thermal profile for PCR was as follows; initiation at 94° C for 5 mins, and 35 repeated cycles of 94° C for 1 min, 56° C for 1 min, 72° C for 1 min, followed by a final extension at 72° C for 5 min (Kaewwises *et al*., 2004). The PCR products were separated on 2% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV illuminator.

RESULTS AND DISCUSSION

1. Observation of grassery disease in the silkworm

In the early stage of infection, the infected larva showed no obvious symptoms of grassery disease which caused by *Bombyx mori* nucleopolyhedrovirus (BmNPV). In general, the body of healthy silkworm larva is slender, long and white in color and shows no conspicuous segmentation (Figure 2A). The integument which composed of epidermal cells and cuticular layers is firm and leathery. Nearly 3-5 days after inoculation, the infected larva showed symptoms of disease infection. The larva was sluggish, no response to external stimuli.
Feeding activity decreased and eventually stopped feeding. As the disease progress, the typical symptoms of grassery disease were observed. A faint mottling of the fat body beneath the cuticle of the swollen abdominal segments become prominent which turned the infected larva to be robust and shorter than the healthy larva (Figure 2B). The larval body appeared creamy white to yellowish in color. Occasionally, some dark spots appeared randomly on the cuticle which at this stage was very thin and fragile (Figure 2C). Just prior to death, the larva showed characteristic symptoms of NPV infection in which the heavily infected larva hung by its prolegs head downward (Figure 2D) and died with the cuticle ruptured releasing plenty of NPV polyhedra in creamy gray-white liquified material.

The symptoms of grassery disease as demonstrated in the fifth instar larvae inoculated with BmNPV were similar to what described by Attathom and Sinchaisri (1987). They reported that no symptoms were observed in the early stage of BmNPV infection in silkworm, *B. mori*, but with the progress of the disease, infected larvae were characterized by a faint mottling of the fat body beneath the cuticle of the swollen abdominal segments. The symptoms were intensified as the larvae reach the fifth stage. After death the cadavers turned black and the integuments became very fragile, ruptured upon touch, releasing viscous liquid containing polyhedra of the virus. Young larvae were more susceptible to viral disease than the old ones, however, the disease outbreak often occurred and obviously seen during the fifth larval instar. A large number of silkworms were unable to perform the cocooning and died. As described by United Nation (1990), the rupture of the fragile skin liberates the liquified 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). Based on the symptoms observed, it was confirmed that the tested silkworms were infected by BmNPV, the causative agent of grassery disease.

Figure 2 Symptoms of grassery disease of silkworm, *Bombyx mori* caused by *Bombyx mori* nucleopolyhedrovirus (BmNPV)

A. Healthy fifth instar larva

B. Healthy (top) and BmNPV-infected (bottom) silkworms

C. Diseased larva with swollen abdominal segments and yellowish body

D. Dead larva hang head downward from the rearing container by its prolegs

2. Bombyx mori nucleopolyhedrovirus (BmNPV) infection in parent silkworms

Using transmission electron microscope (TEM), viral infection was observed in BmNPV-inoculated silkworm samples. Cells and tissues observed which included fat body tissue from the fifth instar larvae, pupae and adult moths and ovary and testes tissues from the adult moths were infected. In contrast, there were no BmNPV infection in fat body cells, testes and ovary tissues of the healthy or untreated samples of *B. mori*. Benz (1963) reported that in BmNPV-infected lepidopterous larvae, the polyhedra had been observed in nuclei of fat body, haemocytes, hypodermis, tracheoblasts, the intestinal epithelium, oenocytes, imaginal discs, silk glands, malpighian tubules, the brain, ganglionic cells, muscle cells and pericardial cells. Attathom and Sinchaisri (1987) described ultrastructure and pathological effect of strain of NPV of the local variety of Thai silkworm, *Bombyx mori*. They found that the evidence of NPV infection was the hypertrophied nuclei which almost filled the cells. The infected nuclei contained numerous polyhedra. The fat body, tracheal matrix and hypodermis were found to be the major tissues heavily infected by BmNPV. Moderate infection was found in the nuclei of silk glands. BmNPV infection in the fat body of fifth instar larvae of *B. mori* was also reported by Khurad *et al.* (2004). In addition, the infection was also found in the follicular epithelium of the silkmoths (Johanssen *et al*., 1986).

2.1 BmNPV observation in fat body tissues of infected parent silkworms

Cells of insect fat body are generally diffusely distributed within the body cavity and their arrangement varies considerably. They may be disposed in sheets, lobules or ribbons, typically most abundant in the abdomen but sometimes extending into the thorax and head. The amount and nature of food reserves within the fat body varies with the nutritional state, the molting cycle and also with the developmental stage of the insect (Smith, 1968).

Throughout the larval stage of silkworm, lipid particles were the most conspicuous food reserve in the fat body cells. This study revealed that there were many large and small lipid particles surrounding the nuclei in fat cells of healthy fifth instar larvae. In addition, the chromatin and nucleolus were obviously seen within the nuclei of healthy cells (Figure 3A).

However, in fat cells infected with BmNPV, several ultrastructures had been observed for example the membranous profiles and spheres that developed within the BmNPV-infected nucleus (Figure 3B). The nucleus hypertrophied and showed margination of cell chromatin and nucleolus disappearance. The nuclear membrane lost its structural integrity, establishing continuity with the cytoplasm (Figure 3B). Both single and multiple-enveloped virions were found occluded in the polyhedra (Figure 3C). This indicated that the nucleopolyhedrovirus of *B. mori* is the multiple-embedded type of NPV. The polyhedra located randomly in the nucleus or in line along periphery of the nucleus.

The same things were also observed in silkworm pupa. Many lipid droplets were found to surround the nucleus of the healthy fat cell of the pupa (Figure 4A). In the BmNPV-infected fat cells of the pupa, numerous polyhedra were found to locate randomly in the nuclei. The enlargement of the cell nucleus showed margination of chromatin and the nucleolus disappearance. The electron micrographs indicated that majority of the polyhedra were hexagonal and octagonal in shape (Figure 4B). Many embedded virions were in single, however, the multiple-embedded virions were also observed (Figure 4C).

In the healthy silkmoths, numerous lipid droplets were found in cytoplasm surrounding the nucleus of fat cells (Figure 5A). Electron micrographs showed considerably difference in lipid particle sizes, some of them were small but some of them were large (Figure 5A). In similar to the infected larvae, nucleus of the fat cells of BmNPV-infected adult moth showed margination of cell chromatin and nucleolus disappearance. In late phase of infection, the nuclei enlarged almost filled the cells. Many polyhedra were observed in the hypertrophied nuclei. They distributed randomly in the nucleus. At this stage nuclear membrane of the infected nuclei disintegrated, hence, nuclear mass and polyhedra of the virus were released into the cytoplasm. The electron micrographs showed polyhedra of different shaped which included pentagonal, hexagonal, octagonal and spherical shape (Figure 5B). Single-embedded virions were observed the most, however, the multiple-embedded virions were also observed within the mature polyhedron (Figure 5C).

Results of this observation revealed that BmNPV infected fat body tissue of the Thai variety of silkworm, which was in agreement with the reports of Attathom and Sinchaisri (1987) and Khurad *et al* (2004) which described the tissues that produce the heaviest of BmNPV infections were the fat body, tracheal matrix and hypodermis.

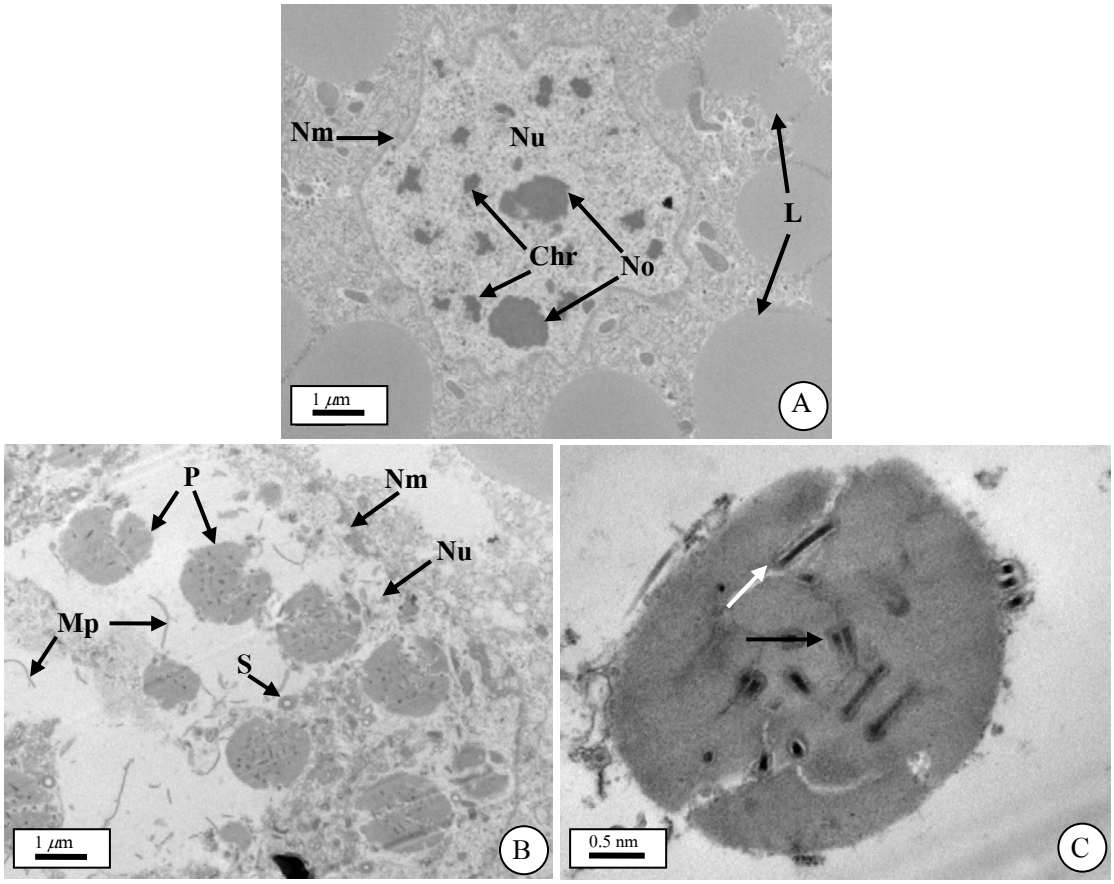

Figure 3 Electron micrographs of fat body tissues of healthy and BmNPV-infected fifth instar larva of silkworm, *Bombyx mori*

A. Nucleus of healthy fat cell surrounded with numerous lipid particles.

B. Nucleus of fat cell filled with polyhedra of BmNPV. Membranous profiles and spheres were observed. The nucleus hypertrophied and nuclear membrane disintegrated.

C. BmNPV polyhedron with single-embedded virions (white arrow) and multiple-embedded virions (black arrow).

Chr = Chromatin L = Lipid particle Mp = Membranous profiles

Nm = Nuclear membrane No = Nucleolus Nu = Nucleus

P = Polyhedra S = Spheres

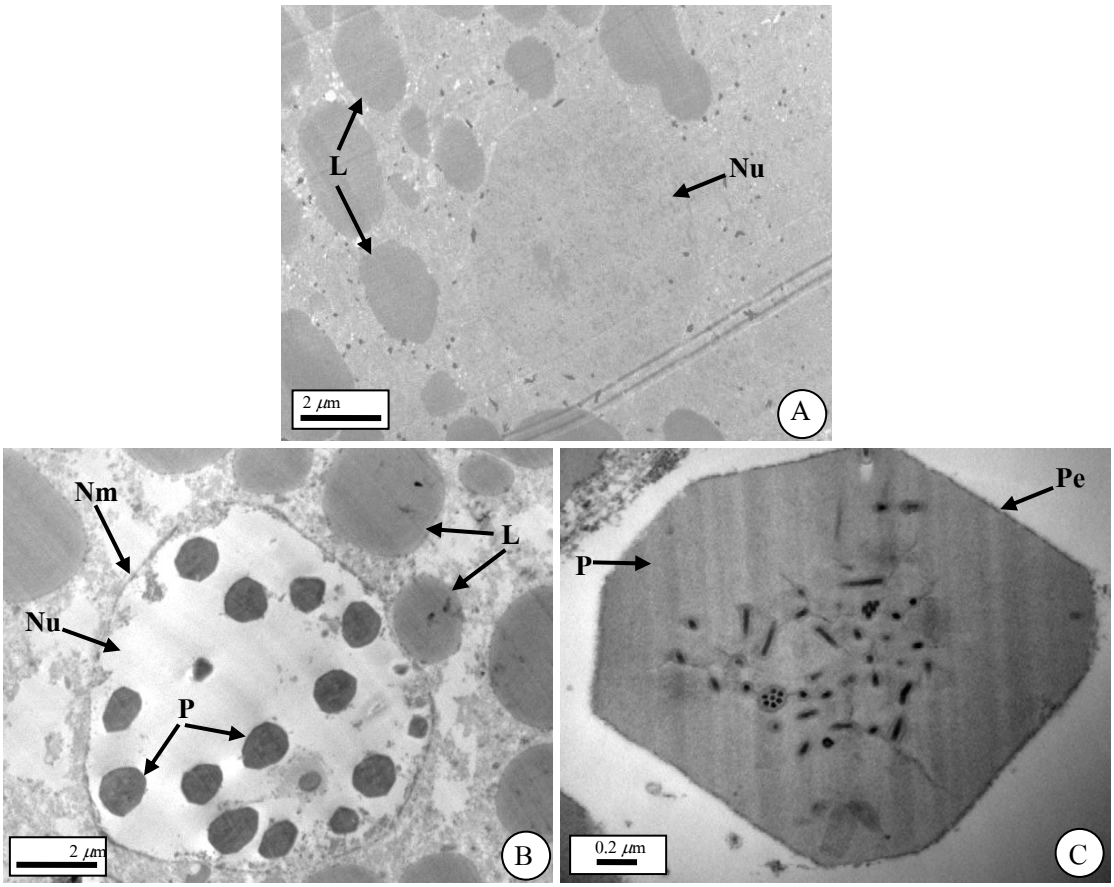

Figure 4 Electron micrographs of fat body tissues of healthy and BmNPV-infected pupa of silkworm, *Bombyx mori*

A. Healthy fat cell demonstrated uninfected nucleus surrounded with numerous lipid particles.

B. Nucleus of infected fat cell filled with polyhedra of BmNPV.

C. Mature BmNPV polyhedron with polyhedral envelope demonstrated several single-embedded and multiple-embedded virions within the polyhedron.

L = Lipid particle Nm = Nuclear membrane Nu = Nucleus

P = Polyhedra Pe = Polyhedral envelope

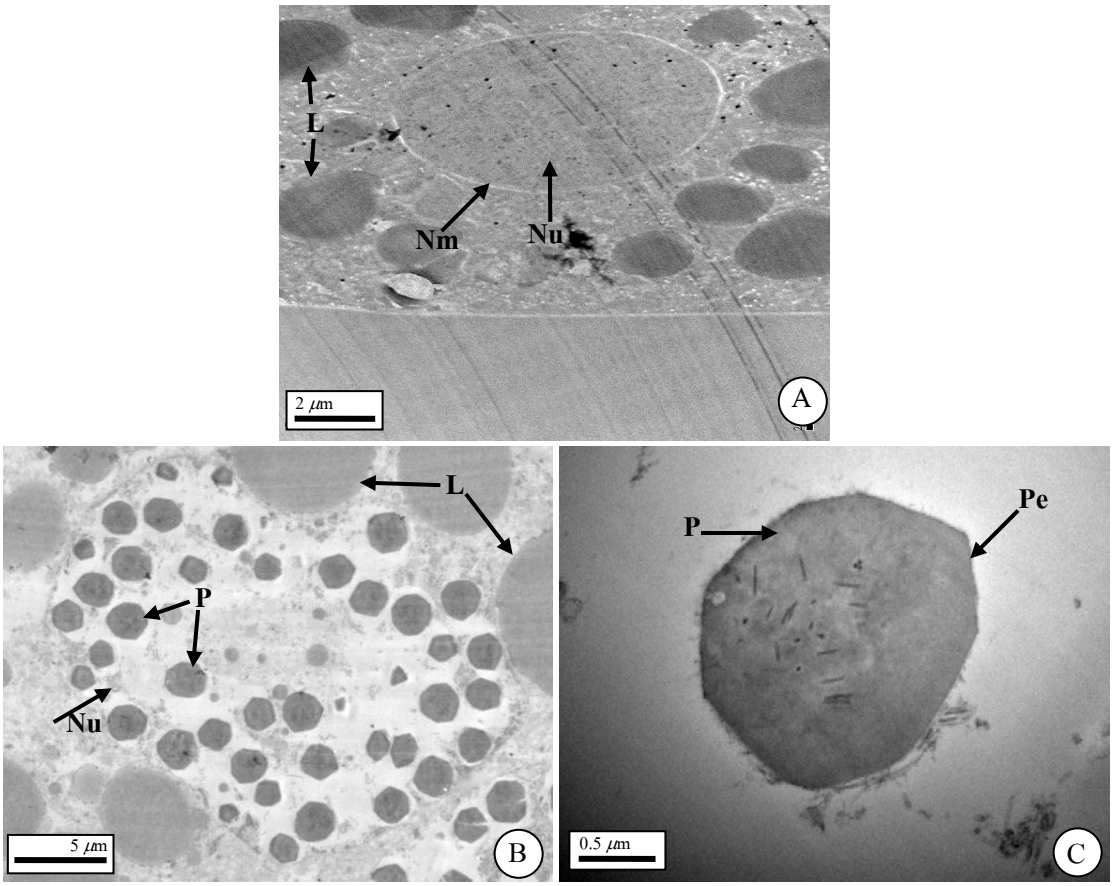

Figure 5 Electron micrographs of fat body tissues of healthy and BmNPV-infected adult moth of silkworm, *Bombyx mori*

A. Healthy fat cell demonstrated uninfected nucleus surrounded with lipid particles.

B. Nucleus of infected fat cell filled with polyhedra of BmNPV.

C. Mature BmNPV polyhedron with polyhedral envelope demonstrated several single-embedded and multiple-embedded virions within the polyhedron.

Nu = Nucleus Pe = Polyhedral envelope Nm = Nuclear membrane

L = Lipid particle P = Polyhedra

2.2 BmNPV observation in testes and ovaries of infected adult moths

Transmission electron microscopic observations of gonadal rudiments of healthy adult moth of silkworm*, B. mori* were compared with that of the BmNPV-infected adult moth. In general, the spermatozoa develop in testes and are stored in the seminal vesicles until mating occurs. In testes of the healthy adult moth, the spermatozoa lay in the testes lumen and no BmNPV infection was observed (Figure 6A). In contrast, ultrathin sections of the testes of BmNPV-infected adult moth revealed that nucleus of the external epithelial lining of testicular follicle was infected with BmNPV. Nuclear membrane ruptured establishing continuity with the cytoplasm (Figure 6B). Some of the polyhedra found in the nucleus contained no virions (Figure 6C). This is probably because the polyhedra were still in immature stage at the time of sample preparation. Abnormal ultrastructure for examples, many short electron-dense bands, frequently curved so called membranous profiles and spherical band so called spheres were randomly observed in the nuclei of the testes of the *B. mori* infected with BmNPV (Figure 6C).

BmNPV observation in ovary cells of the infected adult moth samples by TEM demonstrated that the nuclei of the ovary cells contained polyhedra of BmNPV as compared with the healthy samples. In BmNPV-infected adult moth, few polyhedra of the BmNPV were formed in the nuclei of the ovary cells (Figure 7A). The polyhedra located randomly in the nucleus or in line along periphery of the nucleus. The hypertrophy of nucleus, margination of cell chromatin and the nucleolus disappearance were observed. The virions of single-embedded type and multiple-embedded type were found within the polyhedra (Figure 7B).

Benz (1963) studied the initiation of NPV infection cycles in the susceptible larval gonadal tissues of *B.mori*. He found that both testes and ovaries were infected at various degree at the later stages of infection. Likewise, Khurad *et al.* (2004) found the infection of BmNPV in fat body tissue and gonadal tissues (testes and ovary) of BmNPV-infected silkworm. They found that the lightly and moderately infected larvae exhibited a few infected nurse cell nuclei of the young oocyte follicles in the ovarian rudiments. In male larvae the nuclei of the external epithelial lining of testicular follicle and spermatocytes inside the cyst were also containing polyhedra. They also observed that young oocytes leading to maturation and spermatocytes

leading to reduction division harboring virions in them and in survivors. The infection persisted in the eggs and spermatozoa during pupal and adult stages. Their observation were similar to our study in which BmNPV-infection was found in the gonadal tissues of the BmNPV-inoculated silkmoth.

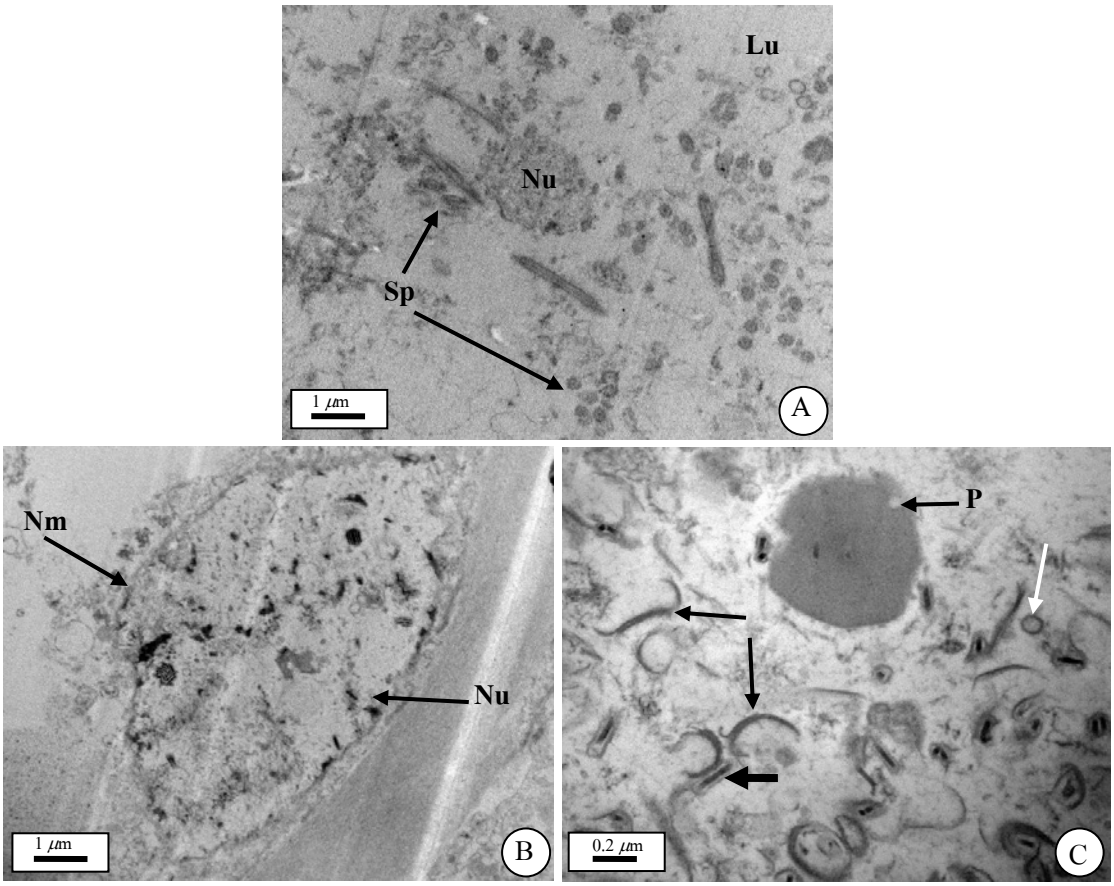

Figure 6 Electron micrographs of testes cell of healthy and BmNPV-infected adult moth of silkworm, *Bombyx mori*

A. Testes of the healthy adult moth revealed spermatozoa lay in the testes lumen.

B. Nucleus of testes of BmNPV-inoculated adult moth, the nuclear membrane lost its structural integrity (arrow).

C. BmNPV-infected nucleus of the testes cell showed immature polyhedron, virions (thick arrow), several curved membranous profiles (black arrows) and spheres (white arrows).

Lu = Lumen Nm = Nuclear membrane Nu = Nucleus

P = Polyhedra Sp = Spermatozoa

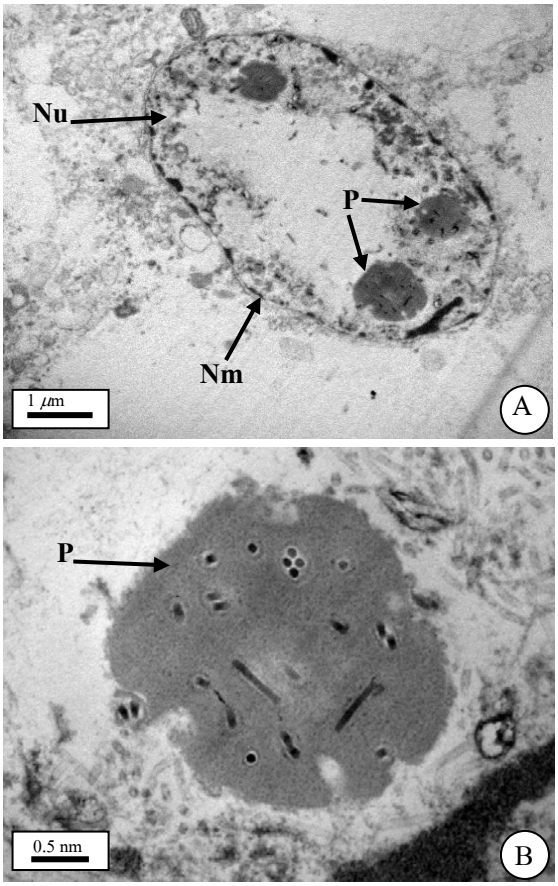

Figure 7 Electron micrographs of ovary cell of BmNPV-infected adult moth of silkworm, *Bombyx mori*

A. Nucleus of BmNPV-infected ovary cell revealed the developing polyhedra at the periphery of the nucleus.

B. Polyhedron of BmNPV with single-embedded and multiple-embedded virions.

Nm = Nuclear membrane Nu = Nucleus P = Polyhedra

3. *Bombyx mori* nucleopolyhedrovirus (BmNPV) observation in eggs and F1 offspring by TEM

The fifth instar larvae of silkworm, *Bombyx mori* were inoculated with the polyhedral inclusion bodies (PIBs) of BmNPV at the concentration 10^8 PIBs/ml. The survived larvae were allowed to pupate, emerge as adult moths and mate. Five pairs of silkmoth were randomly selected for observation and data collection. Numbers of egg laying, egg hatching and egg hatching percentage were recorded and summarized in Table 1. Within the selected five pairs of healthy silkmoths, number of egg laying on the first day ranged from 491-593, number of egg hatching on the first day ranged from 477-581 and egg hatching percentage ranged from 97.15-98.57. The total numbers of egg laying were 2,743, of egg hatching were 2,688 and percent of egg hatching was 97.99 (Table 1).

Within the selected five pairs of BmNPV-inoculated silkmoths, the number of egg laying on the first day ranged from 180-322, number of egg hatching on the first day ranged from 114-213 and egg hatching percentage ranged from 41.61-67.50. The total numbers of egg laying were 1,312, of egg hatching were 778 and percent of egg hatching was 59.30 (Table 1).

Results from this experiment indicated that number of egg laying, number of egg hatching and egg hatching percentage of the BmNPV-infected silkmoths were much lower than those of the healthy silkmoths. This may be due to the fact that BmNPV was able to infect ovary of silkmoth, hence, had an effect on egg production and viability. These results agreed with previous report of Khurad *et al*. (2004) who found that BmNPV-infected silkmoths showed low fecundity which may be attributed to improper vitellogenesis in the infected oocytes.

Numbers of dead larva and larval mortality percentage of the F1 offspring of healthy and BmNPV-infected silkmoths were recorded and summarized in Table 2. Number of dead larva in F1 offspring of the five pairs of healthy silkmoth ranged from 2-5 and the total percent mortality ranged from 0.42-0.91. The average percent mortalities of the first to fifth instar larvae of the F1 offspring of healthy silkmoths were 0.37, 0.19, 0.11, 0.00 and 0.00, respectively (Table 2).

In the F1 offspring of the five pairs of BmNPV-infected silkmoths, number of dead larva ranged from 44-52 and the total percent mortality ranged from 23.94-38.60. The average percent mortalities of the first to fifth instar larvae of the F1 offspring of BmNPV-infected silkmoths were 11.31, 6.81, 5.21, 5.82 and 5.12, respectively (Table 2).

Results from this study revealed the differences in number of dead larva and larval mortality percentage of the F1 offspring of healthy and BmNPV-infected silkmoths. The number of dead larva and larval mortality percentage of the F1 offspring of BmNPV-infected silkmoths were more than those of the F1 offspring of healthy silkmoths.

Transmission of NPV from parent to insect progeny was previously studied by many researchers. Ham and Young (1974) found the progeny infection of *Heliothis zea* NPV (HzNPV) ranged from 0.4-94.3% (10^6-10^8 PIBs/insect), Abul-Nasr (1979) observed that 38-48% mortality in progeny of infected *Spodoptera littoralis* (1.2 x 10^6-10^8 PIBs/ml), Smits and Vlak (1988) reported NPV transmission to *S. exigua* about 10-28% (1-2 x 10^5 -1 x 10^6 PIBs/ml) and Khurad *et al.* (2004) found 100% death of larval progeny of BmNPV-infected mating silkmoths (2,000 OBs/larva) occurred at first instar larvae of silkworm, *B. mori*.

In this study, even though the silkworms were inoculated with high concentration of BmNPV (10^8 PIBs/ml), percent mortality of all larval instars of the F1 offspring were quite low. Only 11.31% mortality was observed in the first instar larval progeny of the inoculated silkmoths as compared to 38-48% mortality in progeny of infected *S. littoralis*, 10-28% mortality in progeny of infected *S. exigua* and 100% mortality of the first instar larval progeny of the infected *B. mori* reported by Abul-Nasr (1979), Smits and Vlak (1988) and Khurad *et al.* (2004), respectively. These contradictory results can be explained by several reasons. The Thai BmNPV may be not as virulent as the strain of BmNPV used by Khurad *et al.* (2004). Vertical transmission may be occurred at low level in Thai BmNPV strain. Moreover, the data were randomly collected from only five pairs of silkmoth. It is possible that BmNPV infection in these silkmoths occurred only in the major tissues usually infected by this virus and had not yet infected the reproductive tissue of the silkmoth.

Table 1 Number of egg laying, egg hatching and egg hatching percentage of the healthy and BmNPV-inoculated silkmoths, *B. mori*.

Experiment	Pair of silkmoth	No. of egg laying	No. of egg hatching	% egg hatching
Healthy	First	539	530	98.33
	Second	559	551	98.57
	Third	561	549	97.86
	Fourth	593	581	97.98
	Fifth	491	477	97.15
	Total	2743	2688	97.99
BmNPV-inoculated (10^8 PIBs/ml)	First	180	114	63.33
	Second	250	155	62.00
	Third	240	162	67.50
	Fourth	322	134	41.61
	Fifth	320	213	66.56
	Total	1312	778	59.30

Table 2 Number of survived larva and larval mortality percentage of the F1 offspring of the healthy and BmNPV-inoculated silkmoths, *B. mori*.

Experiment	Pair of silkmoth	No. of egg hatching	No. of survived larva (instar)					No. of dead larva	Total mortality (%)
			First	Second	Third	Fourth	Fifth		
Healthy	First	530	529	529	527	527	527	3	0.57
	Second	551	549	548	548	548	548	3	0.54
	Third	549	546	544	544	544	544	5	0.91
	Fourth	581	579	577	576	576	576	5	0.86
	Fifth	477	475	475	475	475	475	2	0.42
	Total number	2688	2678	2673	2670	2670	2670	18	0.67
	Total mortality (%)		0.37	0.19	0.11	0	0		
BmNPV-inoculated (10^8PIBs/ml)	First	114	101	94	90	77	70	44	38.60
	Second	155	143	128	120	113	103	52	33.55
	Third	162	150	133	125	119	115	47	29.01
	Fourth	134	109	105	96	91	87	47	35.07
	Fifth	213	187	174	170	166	162	51	23.94
	Total number	778	690	634	601	566	537	241	30.98
	Total mortality (%)		11.31	6.81	5.21	5.82	5.12		

Transmission electron microscopic observation of the egg laid by healthy and BmNPV-inoculated silkmoths are shown in Figure 8. In the egg laid by healthy adult moth (Figure 8A), yolk granules and nuclei of the oocytes (egg cells) were surrounded by lipid particles. Clusters of yolk granule and lipid particles occupied nearly all parts of the oocytes. Most of the yolk granules were randomly located in the inner parts of the oocytes. In the egg laid by BmNPV-inoculated adult moth, the yolk granules and lipid particles scattered randomly in the oocytes. No BmNPV-infection was observed in the egg nuclei (Figure 8B). Takesue *et al.* (1976) described that yolk granules gradually aggregated into cluster from the periphery toward the inside of the egg during the period of blastoderm formation.

The results revealed no ultrastructural differences in the eggs laid by healthy silkmoths as compared to those laid by BmNPV-inoculated silkmoths. This may be because there was no BmNPV transmission from the infected silkmoth to their eggs or BmNPV could be transmitted from silkmoth to their eggs but it was in the early stage of infection, no virus particles and polyhedra were formed inside the eggs. As the result, viral infection could not be detected by TEM.

Fat cells of the first to fifth instar larvae, the F1 offspring of healthy and BmNPV-inoculated silkmoths were shown in Figures 9-13. In the first instar larva, fat cell of the healthy sample demonstrated nucleus with normal appearance (Figure 9A), fat cell of the BmNPV-inoculated samples showed nucleus of normal appearance and no BmNPV was observed in the nucleus (Figure 9B). In healthy F1 offspring larvae (second to fifth instars), the fat cells showed normal nuclei with complete nuclear membranes and surrounded with many lipid droplets (Figures 10A-13A). In the second to fifth instar larvae derived from BmNPV-inoculated silkmoths, the fat cells also showed no BmNPV-infection (Figure 10B-13B).

Results from this experiment demonstrated that even though the infection of BmNPV could be detected in the testes and ovary of BmNPV-inoculated silkmoths, there were no BmNPV in the eggs laid by the inoculated silkmoths and in all larval stages of their F1 offspring. It should, therefore be concluded that these eggs and all of the F1 offspring larvae were not

infected with BmNPV. In this study, the silkworms were inoculated with BmNPV when they were in the early fifth instar and were allowed to develop into adult for mating and laying eggs. It was found that most of the infected larvae died in the late fifth instar or the early pupal stage. The eggs laid by surviving adults and all larval stages of the F1 offspring which were employed in this experiment were probably the resistant ones or might not be infected by the BmNPV. On the other hand, this virus probably could be transmitted from the silkmoth via the eggs into their F1 offspring larvae but it was in the early stage of infection or it was a latent infection, resulting in no viral particles or polyhedra were formed in the observed samples. BmNPV infection was, therefore could not be observed by TEM.

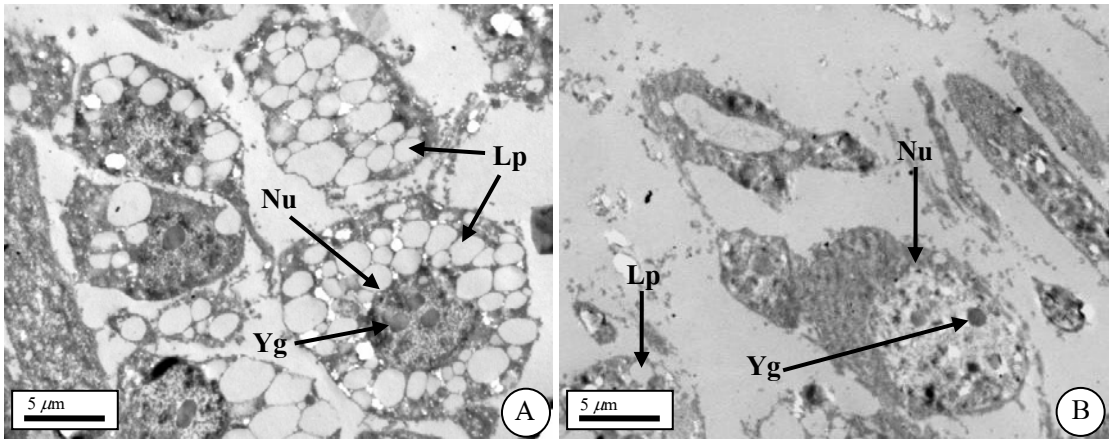

Figure 8 Electron micrographs of the eggs laid by healthy and BmNPV-inoculated silkmoths, *Bombyx mori*.

A. Healthy oocytes showed normal nuclei surrounded by lipid particles and yolk granules.

B. Oocytes of the egg laid by BmNPV-inoculated silkmoth, demonstrated yolk granules and lipid particles scattered randomly in egg cells.

Lp = Lipid particle Nu = Nucleus Yg = Yolk granule

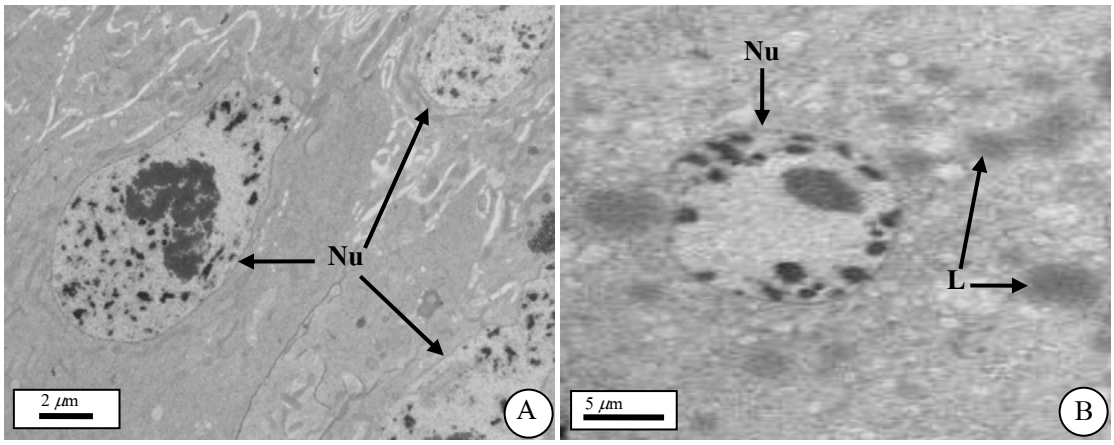

Figure 9 Electron micrographs of fat cells of the first instar larval offspring of silkworm, *Bombyx mori.*

A. Fat cells of the healthy larva, demonstrated nuclei with normal appearance.

B. Nucleus of the fat cell of the first instar larva, offspring of the BmNPV-inoculated silkmoth. No infection of BmNPV was observed.

Lp = Lipid particle Nu = Nucleus

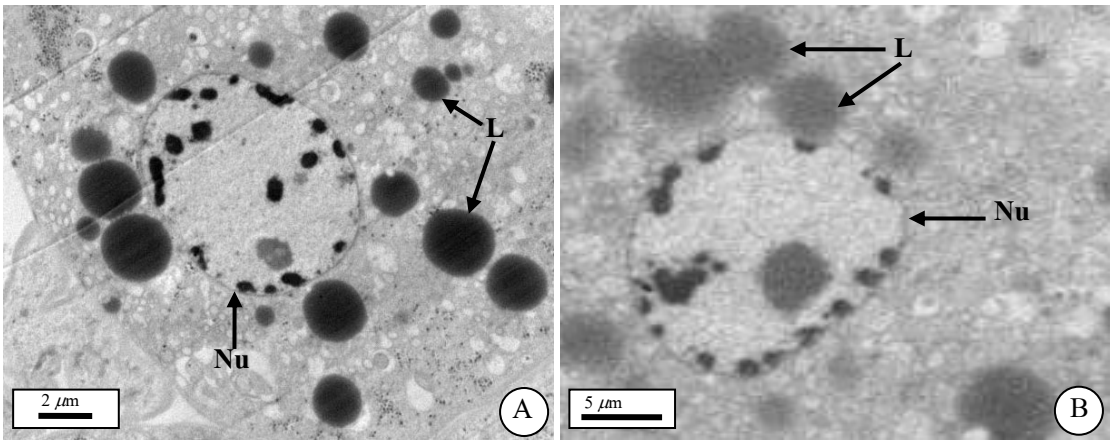

Figure 10 Electron micrographs of fat cells of the second instar larval offspring of silkworm, *Bombyx mori*.

A. Nucleus of fat cell of the healthy second instar larva surrounded with many lipid particles.

B. Nucleus of fat cell of the second instar larva, offspring of BmNPV-inoculated silkmoth. No BmNPV-infection was observed.

L = Lipid particle Nu = Nucleus

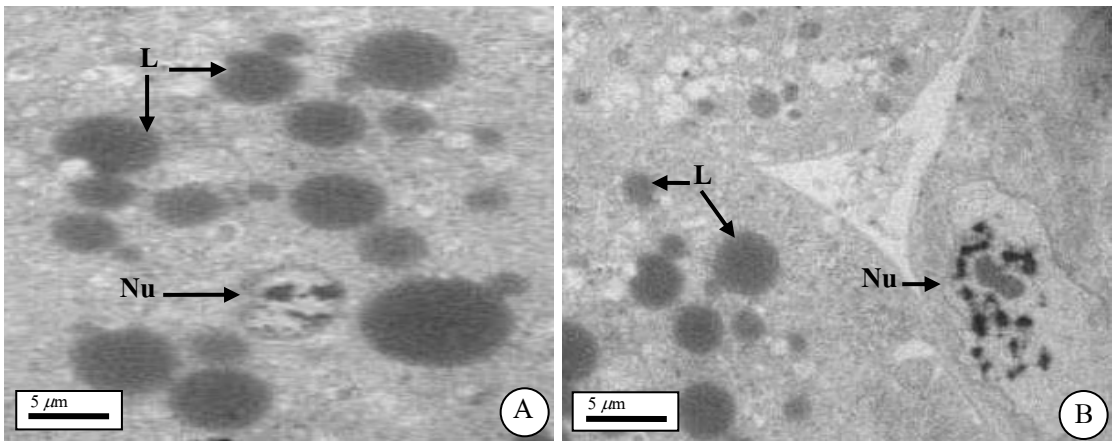

Figure 11 Electron micrographs of fat cells of the third instar larval offspring of silkworm, *Bombyx mori*.

A. Nucleus of fat cell of healthy larva surrounded with lipid particles.

B. Nucleus of fat cell of the third instar larva of BmNPV-inoculated silkmoth. No BmNPV-infection was observed. L = Lipid particle Nu = Nucleus

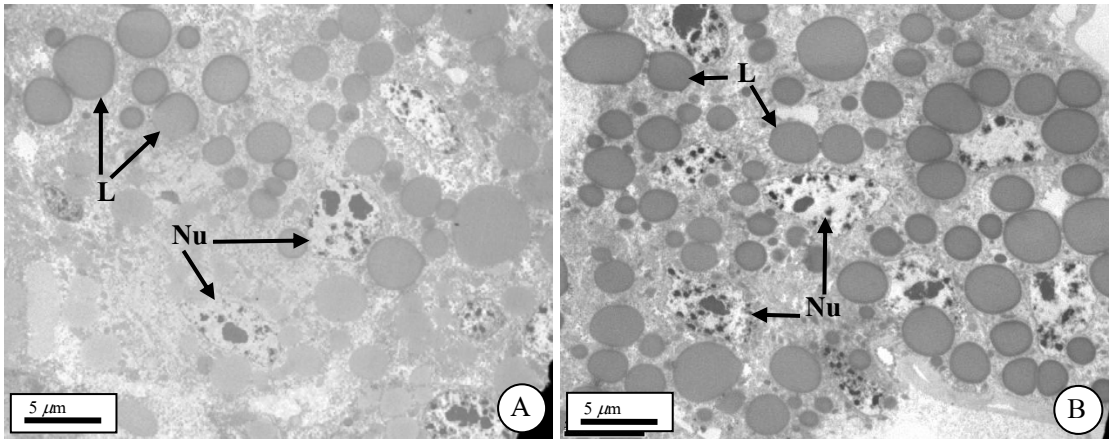

Figure 12 Electron micrographs of fat cells of the fourth instar larval offspring of silkworm, *Bombyx mori.*

A. Nuclei of fat cells of the healthy fourth instar larva surrounded by several lipid particles.

B. Nuclei of fat cells of the fourth instar larva of BmNPV-inoculated silkmoth. No BmNPV infection was observed. L = Lipid particle Nu = Nucleus

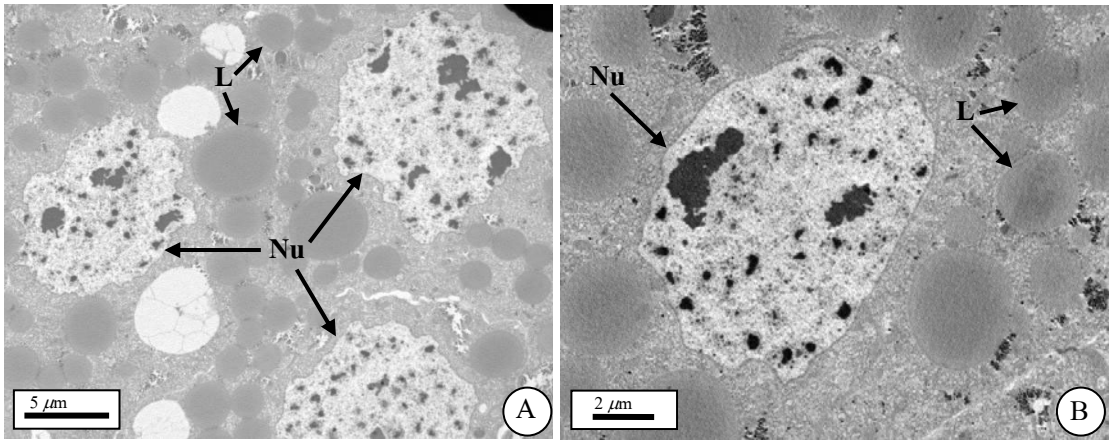

Figure 13 Electron micrographs of fat cells of the fifth instar larval offspring of silkworm, *Bombyx mori*.

A. Nuclei of fat cells of the healthy larva surrounded by the lipid particles.

B. Nucleus of fat cell of the fifth instar larva of BmNPV-inoculated silkmoth. No BmNPV infection was observed. L = Lipid particle Nu = Nucleus

4. PCR amplification

DNAs were isolated from healthy and BmNPV-inoculated parent silkworms and all larval stages of the progeny. PCR amplifications were conducted using complementary primers to BmNPV *polh* region. When the specific primers were used to amplify fragment of BmNPV *polh*, the expected PCR amplified products of 424 bp should be observed in the gels.

4.1 Detection of BmNPV in fifth instar larvae of silkworm, *B. mori*

PCR products of DNAs extracted from the fifth instar larvae of silkworm, *B. mori* were illustrated in Figure 14A. Upon PCR amplification, DNAs extracted from some of the inoculated fifth instar larvae yielded amplified products of 424 bp (lane A2 and A3) and no PCR products were observed when using DNA extracted from healthy larvae (lane N). The obtained PCR products were 424 bp as expected and were similar to those obtained from DNA extracted from BmNPV used as positive control (lane P). The results revealed that after inoculation, some of the fifth instar larvae were infected with BmNPV. PCR amplification using primers specific to BmNPV *polh* gene could detect the virus in the infected silkworm. Clear bands of PCR amplified *polh* gene of 424 bp were visualized on the gel and no nonspecific sequences were observed.

This result was similar to the study of Ikuno *et al*. (2004) and Kaewwises *et al.* (2004) who reported that PCR with complementary primers to the *polh* region could be used to detect BmNPV in haemolymph of all larval instars. Previous reports revealed methods for the detection of BmNPV in all larval instars including indirect ELISA (Vanapruk *et al.*, 1992) and DNA probe (Attathom *et al.*, 1994). However, these two methods were complicated and used rather expensive chemicals. In addition, in some other techniques such as sandwich ELISA, at least 5-6 second instar larvae were required as sample for the detection (Shamim *et al.*, 1994).

4.2 Detection of BmNPV in pupae of silkworm, *B. mori*

PCR products of DNAs extracted from the pupae of silkworm, *B. mori* were illustrated in Figure 14B. PCR amplified *polh* region of 424 bp was observed from some of the pupae (lane B2 and B3), while no PCR products were obtained from the DNA extracted from healthy pupae (lane N). DNAs isolated from the BmNPV-infected pupae yielded 424 bp of *polh* gene which were similar to those obtained from DNA extracted from BmNPV (lane P).
The results indicated that BmNPV inoculated into the fifth instar larvae of silkworm underwent pathogenesis in the larvae and passed into the pupae of silkworm.

There were few reports on BmNPV detection in pupal stage. In practical, grassery disease detection in silkworm at pupal stage is useless because if this disease is detected when silkworms reach pupal stage, it is too late to manage silkworms rearing and seldomly obtain high cocoon production. The only anticipated benefit is that one can eliminate infected pupae and keep, perhaps clean silkworm colony for the next generation. Previously, Vanapruks (1992) reported that the indirect ELISA could be used to detect BmNPV in the pupal stage after inoculation the fourth instar larvae with the virus at 10^6 PIB/ml. In addition, Attathom *et al*. (1994) illustrated the use of digoxiginin labeled probes to detect BmNPV DNA in infected pupae and Kaewwises *et al.* (2004) and Khurad *et al.* (2004) used PCR-based method for the detection of BmNPV in pupal stage of silkworm.

4.3 Detection of BmNPV in adult moths of silkworm, *B. mori*

PCR products of DNAs extracted from male and female silkmoths of silkworm, *B. mori* were shown in Figure 14 C and D, respectively. PCR amplification revealed that DNAs isolated from some BmNPV-infected males (lane C2 and C3) and females (lane D1 and D2) silkmoths yieled amplified *polh* region of 424 bp. PCR products of the same size were also obtained when using DNA extracted from BmNPV (lane P). No amplified products were observed when using the DNAs extracted from healthy silkmoths (lane N). The results showed that BmNPV inoculated into the fifth instar larvae caused disease in the larvae and the virus passed into the pupae and adult moths.

Results from this study agree with the reports by Kaewwises *et al.* (2004) and Khurad *et al.* (2004) who detected the BmNPV-infection in the adult moths of silkworm, *B. mori* by PCR-based method. Moreover, Attathom *et al.* (1994) was able to detect BmNPV in the adult moths using dot blot hybridization. However, Vanapruk *et al.* (1992) demonstrated that ELISA could detect BmNPV in all stages of silkworm except the adult moths which were previously inoculated with the virus at their fourth instar larva. This may be due to the level of the virus in the sample was too low to be detected by ELISA.

4.4 Detection of BmNPV in eggs of silkworm, *B. mori*

PCR products of DNAs extracted from some eggs laid by BmNPV-infected silkmoths, *B. mori* were illustrated in Figure 15A. The PCR products of the expected size 424 bp were obtained upon amplification (lane A1 and A3), which were similar to those obtained from DNA extracted from BmNPV (lane P). DNA extracted from eggs laid by healthy silkmoths gave no PCR products (lane N). The results indicated that BmNPV inoculated into the fifth instar larvae developed infection in the larvae and could persist through the egg stage.

Results of this experiment were similar to Ikuno *et al*. (2004) who reported the detection of BmNPV in infected eggs of silkworm using PCR with polyhedrin gene specific primers. Kaewwises *et al.* (2004) used PCR-based method for the detection of BmNPV in eggs laid by BmNPV-infected adult moths of silkworm, *B. mori* and the results revealed that genomic BmNPV DNA could be detected when using the egg sample as low as one egg. However, increasing the number of eggs for DNA extraction provided higher concentration of DNA template resulting in high intensity of DNA band visualized on the gel. The existing of BmNPV in the eggs could suggest that BmNPV may be transmitted vertically from parents to their offspring.

4.5 Detection of BmNPV in F1 offspring of the inoculated silkworm, *B. mori*

When the specific primers were used to amplify fragment of BmNPV *polh* gene in the F1 offspring (first to fifth instar larvae) of the inoculated parent silkworms, *B. mori*, the PCR

amplified products of expected size (424 bp) were observed in all of the observed samples as illustrated in Figure 15 (Figure 15B lane B1-B3, first instar larvae; Figure 15C lane C1-C3, second instar larvae; Figure 15D lane D1-D3, third instar larvae; Figure 15E lane E1-E3, fourth instar larvae and Figure 15F lane F1-F3, fifth instar larvae). PCR products of the Thai BmNPV *polh* gene from the F1 offspring samples had the same size as the DNA extracted from BmNPV (lane P). There were no amplified products when using DNA extracted from the healthy larvae (lane N) and no nonspecific sequences were observed. There were differences in band clearness when using DNAs extracted from the F1 offspring samples as shown in Figure 15B-F. This may be due to the difference in the virus concentration in the F1 offspring samples which transmitted from the infected parent silkmoths.

The results of PCR amplification of the DNAs isolated from the BmNPV-infected parents and the F1 offspring of Thai variety of silkworm had yielded 424 bp fragments of BmNPV *polh* gene. This confirmed that viral infection was vertically transmitted from the silkworm parents to the progeny. The results were similar to Khurad *et al.* (2004) who detected the vertical transmission of BmNPV from parents to their progeny in silkworm, *B. mori* by using PCR-based method. Ridhards *et al.* (1999) and Kukan (1999) reviewed that dispersal of baculoviruses by adult may occur following vertical transmission to larvae either inside the egg (transovarial) or to its surface (transovum). However, they pointed out that the dominant source of virus among generations is probably through environmental contamination. In this study, vertical transmission of BmNPV in Thai variety of silkworm was determined on the basis of the presence of viral DNA (*polh* gene of BmNPV) in the inoculated parents and their F1 offspring. The PCR amplification results confirmed that some of the larvae which were infected by the virus could survive and the moths derived from such larvae could successfully transmit the virus vertically to their offspring. Using PCR-based method, BmNPV could be detected in eggs and all larval developmental stages. The same method had been used by Kaewwises *et al*. (2004) who indicated that BmNPV DNA could be detected in all larval instars and only one individual BmNPV-infected larva provided adequate viral DNA to be used as PCR template. In addition, Fuxa *et al.* (2002) confirmed transovarial transmission of TnSNPV, TnCPV and AcMNPV in *T. ni* population by PCR-based method.

According to the situation in Thailand, nevertheless the sericulturists take precautionary measures before and during silkworm rearing such as decontamination of rearing house and appliances and use of disinfectants to destroy the polyhedral inclusion bodies of BmNPV released by infected silkworms of the previous crop, some polyhedral inclusion bodies may escape inactivation and become a source of infection to the next generation.

Khurad *et al.* (2004) stated that if the adult survivors of the sublethally infected larvae are used in the sericulture industry for egg production, the unfavorable effect on cocoon production might be quite large due to vertical transmission of NPV. Hence, it is important to confirm the viral-free nature of the eggs before they are used for seed multiplication purposes or for large-scale distribution to farmers. PCR-based technique offers an effective, fast, reliable and practical method for the detection of BmNPV in all stages of silkworm. However, further studies focusing on sensitivity of the PCR assay will provide even more efficient method of detection in order to prevent missing samples from the viral detection which may lead to the outbreak of the grassery disease in silkworm population.

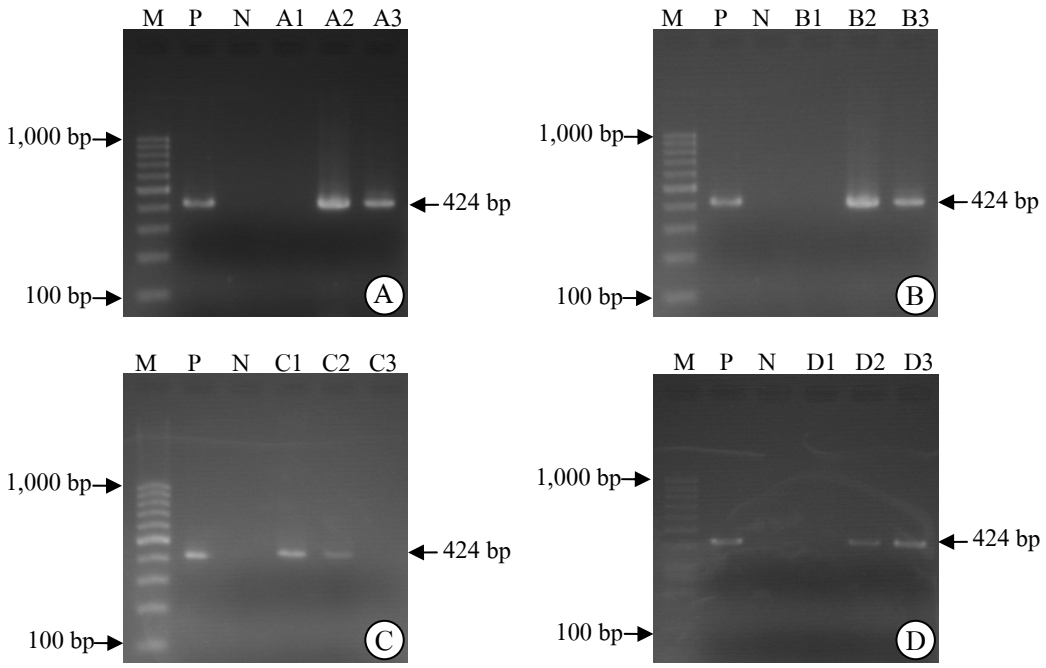

Figure 14 PCR products of DNAs extracted from the fifth instar larvae, pupae, male and female silkmoths of the silkworm, *Bombyx mori* inoculated with nucleopolyhedrovirus at their fifth larval instar.

A1-3 = Fifth instar larvae

B1-3 = Pupae

C1-3 = Male silkmoths

D1-3 = Female silkmoths

M = DNA marker (1,000 bp)

P = Positive control (BmNPV DNA)

N = Negative control (healthy silkworm DNA)

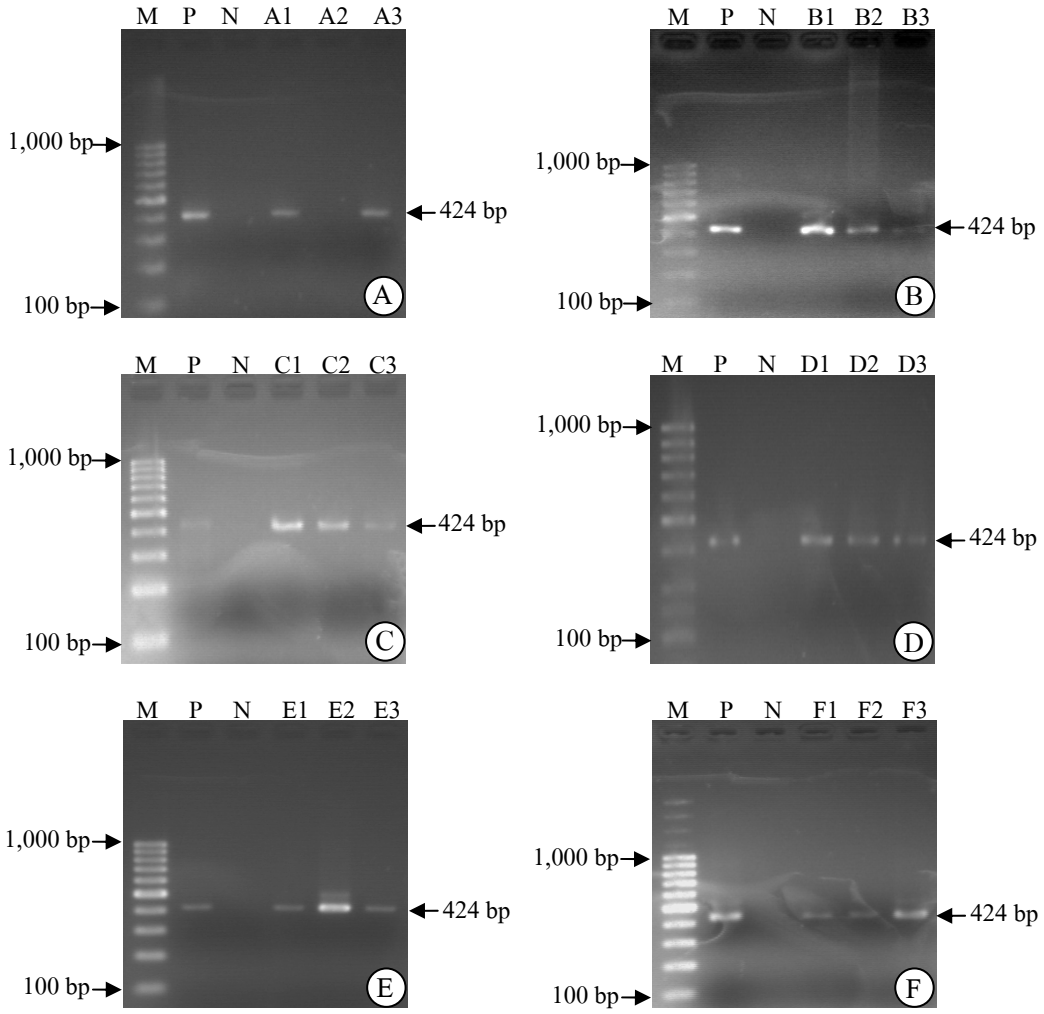

Figure 15 PCR products of DNAs extracted from eggs and F1 offspring (first to fifth instar larvae) of the silkworm, *Bombyx mori* inoculated with nucleopolyhedrovirus at their fifth larval instar.

A1-3 = Eggs laid by BmNPV-inoculated silkmoths

B1-3 = First instar larvae

C1-3 = Second instar larvae

D1-3 = Third instar larvae

E1-3 = Fourth instar larvae

F1-3 = Fifth instar larvae

M = DNA marker (1,000 bp)

P = Positive control (BmNPV DNA)

N = Negative control (healthy silkworm DNA)

CONCLUSION

Grassery disease caused by *Bombyx mori* nucleopolyhedrovirus (BmNPV) is considered one of the most destructive disease in Thai variety of silkworm, *B.mori*. In general, the infected larvae express the symptoms at the final stage of larval growth and die without cocoon production. To assess the current knowledge on vertical transmission of BmNPV will not only prevent the spread of the disease in silkworm sericulture but also help the farmers from wasting of time, money and energy to raise those infected young silkworms. This study aimed to verify vertical transmission of BmNPV from the parents to their F1 offspring in Thai variety of silkworm using transmission electron microscope (TEM) and PCR-based technique.

In healthy silkmoths, number of egg laying ranged from 491-593, number of egg hatching ranged from 477-581 and egg hatching percentage ranged from 97.15-98.57.
In BmNPV-infected silkmoths, number of egg laying ranged from 180-322, number of egg hatching ranged from 114-213 and egg hatching percentage ranged from 41.61-67.50. Number of dead larva in F1 offspring of the healthy silkmoths ranged from 2-5 and the total percent mortality ranged from 0.42-0.91. In the F1 offspring of the BmNPV-infected silkmoths, number of dead larva ranged from 44-52 and the total percent mortality ranged from 23.94-38.60.

Transmission electron microscopic observations demonstrated BmNPV infection in fat nuclei of BmNPV-inoculated parent silkworms (fifth instar larvae, pupae and adult moths).
The infection of BmNPV was also observed in testes and ovary of BmNPV-inoculated silkmoths. However, no BmNPV infection was found in the eggs laid by BmNPV-inoculated silkmoths and in fat nuclei of all larval instars of F1 offspring of the BmNPV-inoculated silkworm, *B. mori*.

Vertical transmission of BmNPV in Thai variety of silkworm was further verified using PCR-based method with complementary primers to *polh* region (BmNPV polyhedrin gene). Amplified products of expected size (424 bp) were obtained from DNAs extracted from some of the parent silkworm samples (fifth instar larvae, pupae and adult moths) and from all samples of the eggs and F1 offspring of BmNPV-inoculated silkmoths that employed in this experiment.

In conclusion, by TEM observation, BmNPV was observed in fat body tissues of fifth instar larva, pupa, adult moth and in testes and ovary of BmNPV-inoculated samples but no BmNPV was observed in the eggs and in fat body tissues of all larval instars of F1 offspring of BmNPV-inoculated silkmoths. This suggests that the eggs laid by surviving adults and all larval instars of the F1 offspring were probably the resistant one or may not be infected by BmNPV. On the other hand, this virus could be transmitted from the silkmoth via the eggs into the F1 offspring but it was in the early stage of infection or the virus caused latent infection, therefore no viral particles or polyhedra were formed in the infected cells. However, by PCR-based method, BmNPV was detected in some of the fifth instar larvae, pupae and adult moths of BmNPV-inoculated silkworms. The *polh* region PCR amplified products were also obtained from all of the observed eggs and larval instars of the F1 offspring.

This result suggests that PCR-based method was sensitive enough to detect the virus even if it was present in a small amount in the infected sample and moreover, the method was able to detect the virus at the DNA level. Polyhedrin gene (*polh*) is a highly conserved gene among members of nucleopolyhedrovirus and PCR primers used in this study were designed to be specific to the *polh* region. Results of PCR amplification of the DNAs isolated from the BmNPV-infected parents and the F1 offspring of Thai variety of silkworm had yielded 424 bp fragments of BmNPV *polh* gene. This confirmed that viral infection was vertically transmitted from the parent silkworms to their F1 offspring.

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