VIRULENCE OF *GALLERIA MELLONELLA* NUCLEAR POLYHEDROSIS VIRUS TO DIAMONDBACK MOTH, *PLUTELLA XYLOSTELLA* (L.) AFTER SERIAL PASSAGE IN *SPODOPTERA FRUGIPERDA* CELLS CULTIVATED *IN VITRO*

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หัวข้อวิทยานิพนธ์ ความรุนแรงในการทำให้เกิดโรคของ *Galleria mellonella* nuclear polyhedrosis virus ที่มีต่อหนอนใยผัก, *Plutella xylostella* (L.) ภายหลังผ่านการเพาะเลี้ยงอย่างต่อเนื่องในเซลล์ของ *Spodoptera frugiperda*
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บทคัดย่อ

การศึกษาครั้งนี้ได้ทำการเพาะเลี้ยง *Galleria mellonella* nuclear polyhedrosis virus (*Gm*NPV) อย่างต่อเนื่องในเซลล์ไลน์ *Spodoptera frugiperda* (Sf9) เป็นจำนวน 20 ครั้ง โดยในแต่ละลำดับขั้นของการเพาะเลี้ยง *Gm*NPV จะทำการศึกษาคุณลักษณะบางประการที่มีความสัมพันธ์กับการจำลองตัวของไวรัส ซึ่งประกอบด้วย เปอร์เซ็นต์การติดเชื้อ จำนวนผลึกไวรัส (polyhedra) ไตเตอร์ของไวรัสที่อยู่ภายนอกเซลล์ (extracellular virus titers) รวมทั้งทดสอบความรุนแรงในการทำให้เกิดโรคของ *Gm*NPV สายพันธุ์ต่างๆ ต่อหนอนใยผักวัยที่ 2

จากผลการทดลองที่ได้พบว่า การเพาะเลี้ยงไวรัสอย่างต่อเนื่องเป็นจำนวน 10 ครั้ง ส่งผลให้เปอร์เซ็นต์การติดเชื้อและจำนวนผลึกไวรัสลดลงอย่างมีนัยสำคัญทางสถิติ และมีการผลิตไวรัสที่อยู่ภายนอกเซลล์เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติในการเพาะเลี้ยงลำดับที่ 9 และ 10 จากการศึกษาภาพถ่ายอิเล็คตรอนพบความผิดปกติของรูปร่างผลึกไวรัสได้บ่อยขึ้นหลังการเพาะเลี้ยงลำดับที่ 10 และเมื่อทำการทดสอบเปรียบเทียบความรุนแรงในการทำให้เกิดโรคต่อหนอนใยผัก พบว่าไวรัสสายพันธุ์ดั้งเดิมมีความรุนแรงมากที่สุด (LC_{50} = 19,387.61 OBs/cm^2) เมื่อเปรียบเทียบกับสายพันธุ์ที่แยกได้จากพล๊าค (LC_{50} = 47,116.38 OBs/cm^2), สายพันธุ์ที่ได้จากการเพาะเลี้ยงลำดับที่ 1 (LC_{50} = 56,795.86 OBs/cm^2), สายพันธุ์ที่ได้จากการเพาะเลี้ยงลำดับที่ 5 (LC_{50} = 67,494.15 OBs/cm^2), และสายพันธุ์ที่ได้จากการเพาะเลี้ยงลำดับที่ 10 (LC_{50} = 68,899.53 OBs/cm^2) ($P<0.05$) เมื่อพิจารณาจากค่า LT_{50} พบว่าสายพันธุ์ที่นำมาทดสอบทั้งหมดให้ค่า LT_{50} ที่ไม่แตกต่างกัน ($P>0.05$) โดยไวรัสสายพันธุ์ดั้งเดิมสามารถฆ่าหนอนใยผักได้เร็วที่สุด (LT_{50} = 4.8 วัน) รองลงมาคือสายพันธุ์ที่ได้จากการเพาะเลี้ยงลำดับที่ 1 (LT_{50} = 5.1 วัน), สายพันธุ์ที่ได้จากการเพาะเลี้ยงลำดับที่ 10 (LT_{50} = 5.1 วัน), และสายพันธุ์ที่แยกได้จากพล๊าค (LT_{50} = 5.4 วัน), และสายพันธุ์ที่ได้จากการเพาะเลี้ยงลำดับที่ 5 (LT_{50} = 5.6 วัน) ตามลำดับ

Thesis title	Virulence of *Galleria mellonella* nuclear polyhedrosis virus to diamondback moth, *Plutella xylostella* (L.) after serial passage in *Spodoptera frugiperda* cells cultivated *in vitro*
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ABSTRACT

In this study, *Galleria mellonella* nuclear polyhedrosis virus (*Gm*NPV) was serially passaged 20 times in *Spodoptera frugiperda* cell line (Sf9). At each passage of virus production, some of the replication characteristics were analyzed including the percentage of infection, the production of polyhedra and extracellular virus titers. Besides, the comparative bioassay was done to determine the virulence of the various isolates of *Gm*NPV for 2nd stage larvae of *Plutella xylostella*.

After 10 passages in cell culture, significant reductions in infection percentage and the polyhedra production were observed. In the 9th and 10th passages, the ECV production was increased significantly. The electron microscopic observation also showed the abnormality of polyhedra morphology at high frequency after 10 serial passages. The comparative bioassay revealed that the virulence of wild isolate (LC_{50} = 19,387.61 OBs/cm^2) was highest when compared to the plaque-purified isolate (LC_{50} = 47,116.38 OBs/cm^2), the 1st passage isolate (LC_{50} = 56,795.86 OBs/cm^2), the 5th passage isolate (LC_{50} = 67,494.15 OBs/cm^2), and the 10th passage isolate (LC_{50} = 68,899.53 OBs/cm^2). The LT_{50} showed that all isolates killed larvae at similar rate ($P>0.05$) with the fastest incubation time in the wild isolate (4.8 days) followed by the 1st passage isolate (5.1 days), the 10th passage isolate (5.1 days), the plaque-purified isolate (5.4 days) and the 10th isolate (5.6 days), respectively.

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Warasirin Sornlake

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LIST OF ABBREVIATIONS

NPVs = nuclear polyhedrosis viruses

*Gm*NPV = *Galleria mellonella* nuclear polyhedrosis virus

*Ac*NPV = *Autographa californica* nuclear polyhedrosis virus

MP = many polyhedra

FP = few polyhesra

OBs = occlusion bodies

SNPV = singly enveloped virus

MNPV = multiply enveloped virus

PDV = polyhedra derived virus

OV = occluded virus

BV = budded virus

ECV = extra cellular virus

NOV = non-occluded virus

SDS = sodium dodesyl sulphate

NaCl = sodium chloride

TCID$_{50}$ = median tissue culture infected dose

LC$_{50}$ = median lethal concentration

LT$_{50}$ = median lethal time

CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the problems

The need to effectively control and manage crop pests is of global importance. To feed the world's growing population, food has to be produced on essentially the same acreage, which is now under cultivation. Not only the production of food must be increased on the arable land, control of insect pests is also necessary. Even though chemical pesticides are widely used but they have a number of disadvantages: some chemicals are carcinogenic, thus, unsafe for mankind; many insects become resistant to chemical pesticides; rising production costs and the cost of registration make development of new chemical pesticides and their production prohibitive [1, 2, 3]. Therefore, the control of insect pests by means other than chemical insecticides is rapidly becoming necessity. And also, there is a strong global movement away from non-specific chemical pesticides to the more species specific biopesticides.

The diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Yponomeutidae), is considered to be the most serious and widely distributed pest throughout the world [4] including Thailand. The efforts to control this pest solely through conventional insecticides has led to resistant development to several classes of insecticides even in *Bacillus thruringiensis* (*Bt*) [5, 6, 7]. The increasing cost of managing this pest with chemical insecticides has led to the development of technology for the production of biological pesticides that have more potential to control diamondback moth effectively.

The baculoviruses, especially nuclear polyhedrosis virus, have been repeatedly reported as an effective and safe pest control agents [8, 9, 10]. These viruses possess characteristics which are suitable for use in integrated pest management programs. They are exceedingly specific and often highly virulent to their hosts. The nuclear polyhedrosis viruses (NPVs) are restricted to arthropods, primarily insects. In addition to minimize ecological damage, their use in agroecosystems provides minimum disruption of beneficial arthropods [11].

Galleria mellonella nuclear polyhedrosis virus (*Gm*NPV), a wild type baculovirus, has been reported to be infective to diamondback moth and other lepidopterans [12, 13, 14, 15]. However, up to date, *Gm*NPV is not used extensively for diamondback moth control. The attempts to develop *Gm*NPV as bioinsecticide were carried out using cell culture *(in vitro)* technique.

Under the *in vitro* propagation of virus there were several advantages including controllable system, product purity, and low cost production. However, one particular problem associated with the virus propagation in cell culture is the propensity of virus to mutate into a form that produces few polyhedra [16, 17, 18, 19, 20, 21, 22, 23]. The occurrence of *few polyhedra* (*FP*) mutants during serial passage of virus in cell culture is impeded the development of virus production that would facilitate availability of virus for biological control.

In order to improve infectivity of baculoviruses, serial passage selection for more virulent variants had been reported by several workers both *in vivo* and *in vitro* technique [24, 25, 26, 27, 28, 29, 30, 31]. However, there was only one report for increased virulence following serial passage *in vitro* [29]. There is no report on increased virulence plaque purified isolate derived from serial passage of *Gm*NPV.

This study aimed to primarily determine some of the replication characteristics and virulence of the serial passage *Gm*NPV and plague purified isolate. During this study, *Gm*NPV was serial passaged in *Spodoptera frugiperda* cells cultivated in Grace's medium supplemented with 10% FBS for 20 passages. At each passage of virus production, the parameters analyzed include the production of polyhedra (OBs) and extracellular virus (ECV) titers. Finally, the virulence of polyhedra from the 1st passage isolate, 5th passage isolate, 10th passage isolate, plaque purified isolate and wild isolate of *Gm*NPV for second stage larvae of *Plutella xylostella* was determined by the *per oral* bioassay.

1.2 Objectives of the study

1.2.1 To determine some of the replication characteristics of *Galleria mellonella* nuclear polyhedrosis in *Spodoptera frugiperda* cell line (Sf9) after serial passages by analyzing the production of polyhedra (OBs) and the extra cellular virus (ECV) titers.

1.2.2 To determine the virulence polyhedra from the 1st, 5th, 10th passage isolates, plaque-purified isolate and wild isolate of *Gm*NPV for second stage larvae of *Plutella xylostella* by *per oral* bioassay.

1.3 Research planning

1.3.1 Preparation of *Spodoptera frugiperda* cell line.

1.3.2 Preparation of *Gm*NPV by routinely passaging in Sf9 cells for 20 passages and during this process the production of polyhedra and ECV titers were determined.

1.3.3 Preparation of polyhedra for comparative bioassay.

1.3.4 Collecting and mass-rearing of *Plutella xylostella* larvae for *per oral* bioassay.

1.3.5 Bioassay study

1.3.6 Data analysis

CHAPTER 2

LITERATURE REVIEW

2.1 Diamondback moth, *Plutella xylostella* (L.)

Diamondback moth, *Plutella xylostella* (L.), belongs to the family Yponomeutidae, order Lepidoptera. *Plutella xylostella* is also known by several common names such as diamondback moth, cabbage plutella, short hole worm, small cabbage moth, but the most common is diamondback moth.

The diamondback moth is a cosmopolitan species that probably originated in the Mediterranean region. It is found over much of North America, the southern portion of South America, Southern Africa, Europe, Southeast Asia, New Zealand, and parts of Australia [32].

Host plants of this pest include both cultivated and wild plants of the family Cruciferae, as well as several ornamentals, such as wallflower, candytuft, stocks, and alyssum. Cultivated crops that are attacked include broccoli, Brussels sprouts, cabbage, cauliflower, Chinese broccoli, Chinese cabbage, flowering white cabbage, head cabbage, mustard cabbage, and watercress. Weed hosts, such as mustard and radish, are important reservoir hosts for the species [33, 34].

Damage on plants caused by newly hatched caterpillars feed in the spongy plant tissue beneath the leaf surface forming shallow mines that appear as numerous white marks. The larvae are surface feeders in all subsequent stages. These larvae feed on the lower leaf surface 62-78% of time, chewing irregular patches in leaves [35]. All the leaf tissues are consumed except the veins. On some leaves, the larvae feed on all but the upper epidermis creating a "window" effect. The last stage larva is a voracious feeder, its cause more injury than the first three larval instar.

2.1.1 Life history

Life stages of the diamondback moth vary considerably depending on the environment under it develop. This insect pest prefers a warm environment for it development. The serious infestation usually occurs during dry season around February to April, when optimum climatic condition prevails, especially little to no rain, and food plants are readily available [35].

Egg stage: Eggs are laid either singly or in very small group on the upper or undersides of the leaves (Figure 2.1a). There are frequently deposited in the hollows along the vein, on the young stems or on partioles. The small (0.44 × 0.22 mm), flat, oval-shaped eggs are shiny yellow when first laid [36]. Just before hatching, the eggs darken and the young larva can be seen coiled beneath the eggshell [34]. Each female can lay up to 414 eggs with the mean of 288 eggs per female and incubation takes 3-8 days [37]. Under laboratory conditions, females produced an average of 233.5 eggs/female and the incubation period was 1.87 ± 0.34 days [38].

Larval stage: The caterpillar is pale green, widest in the middle of the body, and is about 12 mm long when fully grown (Figure 2.1b). Caterpillars are wriggle violently if disturbed and often drop off the leaf, remaining suspended from it by a thread. The total larval period varies from 14-28 days [37]. In Thailand, the larvae undergo 3 molting in 4 instars, mean duration of each instar was 2.42 ± 0.55, 1.43 ± 0.65, 1.89 ± 0.81 and 3.63 ± 0.91 days respectively [38].

Pupal stage: Pupation takes place inside a gauze-like silken cocoon about 9 mm long, which is stuck to the underside of a leaf. During the prepupal period, its body longitudinally shortened but was still active which last for 1 to 2 days [34]. Pupae are obtect shape. Young pupa was yellowish green, but 1 or 2 days later it turned brown and gradually change to dark brown (Figure 2.1c). Pupation is normally observed, on the lower surface of leaves close to the developing plant head [37]. Mean duration of prepupal and pupal stage were 1.11 ± 0.33 and 3.20 ± 0.62 days respectively [38].

Adult stage: The adult is small, grayish brown (Figure 2.1d). On the front pair of wing, the diamond shape is formed when the fore wings lies close to the sides of the body, and brighter in male. "Diamondback moth" is derived from this characteristic [34]. The wing expansion of the moth was measured to be 12.97 mm (male) and 13.0 mm (female). The adults are active at night and remain hidden during the day. Mating and oviposition took place at dusk of the first night of adult emergence. The oviposition occurred before midnight. During the rest of the night, the moths were not active. Mating lasted 1 to 2 hours and female mated only once. Most female laid eggs on the night of emergence [37]. The adult male lived for 16.17 ± 3.06 days whilst the female 9.50 ± 1.38 days. In the tropic breeding may continue, with as many as 15 generations in one year [38].

2.1.2 Development of insecticide resistance in diamondback moth

For controlling insect pests on cruciferous crops which has grown widely and continuously throughout the year, the growers relying primary on chemical insecticides. The improper use of insecticides form nursery to harvest stage had led to the resistance development of insect pests by the insecticidal selection pressure [39].

The diamondback moth, a major insect pest of cruciferous crops, is notorious for its ability to develop resistance to a wide range of insecticides since DDT resistance was first reported in Indonesia [40]. Currently, this insect pest shows resistance to all major groups of insecticides, including. chlorinated hydrocarbons, organophosphates, carbamates, synthetic pyrethroids, benzoylphenyl ureas [41, 42, 43, 44, 45, 46, 47, 48, 49] and *Bacillus thuringiensis* var. *kurstaki* [5, 6].

Previously, growers solved resistance problem by switching to new and more effective products, but this is no longer feasible. To avoid or retard the resistance development problem, it was essential to reduce the selection pressure of insecticide resistance. To reduce the selection pressure of an insecticide or a similar group of insecticide, the rotational use of different groups, which showed no cross-resistance was very important. In addition, other alternative control measures should be developed to support the resistant management in diamondback moth.

Figure 2.1 Various stages of diamondback moth, *Plutella xylostella* (L.):

a.) Egg stage

b.) Larval stage

c.) Pupal stage

d.) Adult stage

2.2 Nuclear polyhedrosis viruses

Nuclear polyhedrosis viruses (NPVs) are invertebrate pathogenic virus predominantly infect insect of the order Lepidoptera (moths and butterflies) [50]. NPVs have several advantages over conventional insecticides because they have narrow host range (sometimes limited to one or two species) and a good safety profile for humans and beneficial insects. Besides, these viruses possess the capability to persist in the environment, which can be utilized in the development of more ecologically base long-term control programs.

2.2.1 Classification of nuclear polyhedrosis viruses

Nucleopolyhedrosis viruses are members of the family Baculoviridae, genus *Nucleopolyhedrovirus*. Infection by NPV is characterized by the production of paracrystalline, proteinaceous occlusion bodies (OBs ; also called polyhedra) in the nuclei of NPV infected cells. The NPV OBs are 1-15 μm in diameter and compose of the closely related polyhedrin protein. NPV OBs or polyhedra, usually contain a large number of virions embedded within the matrix. Under the light microscope NPV OBs are readily observed (Figure 2.2) and under the electron microscope they seem to contain the enveloped bundles of bacilliform virus nucleocapsid (Figure 2.3). The number of nucleocapsids per envelope is a morphological feature that is used to further divide into singly (SNPV) or multiply (MNPV) enveloped viruses [50].

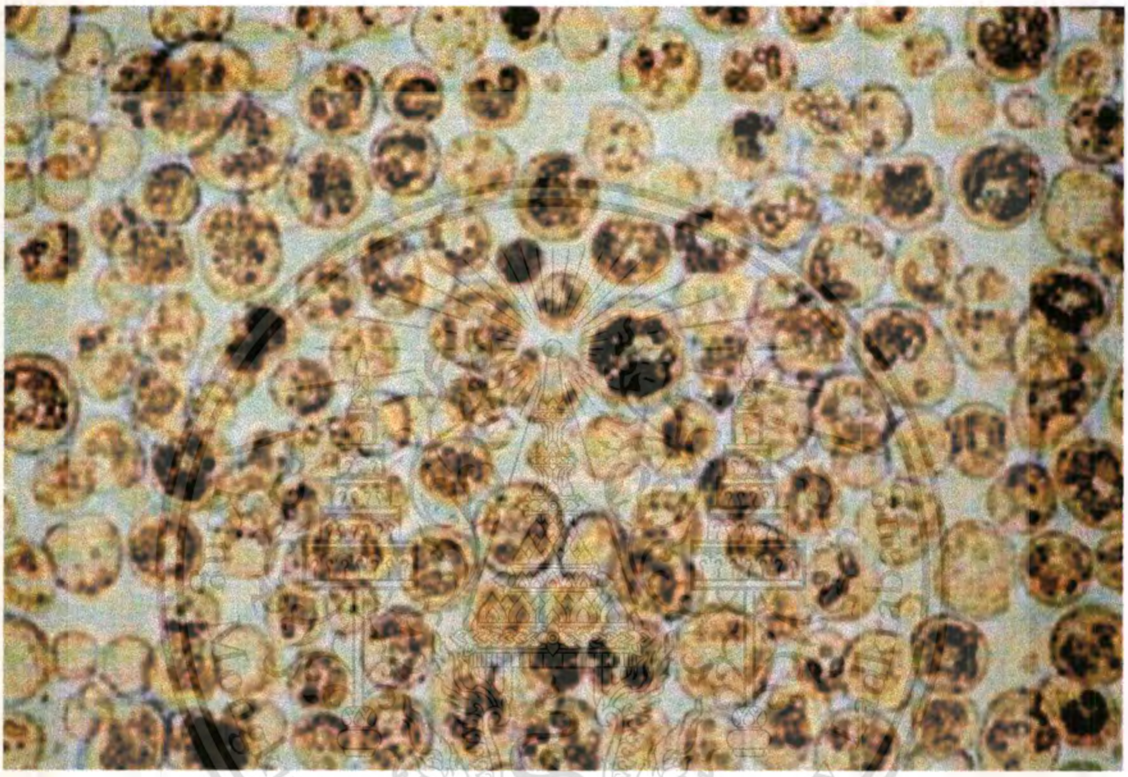

Figure 2.2 Light micrograph of *Spodoptera frugiperda* cell line (Sf9) infected with *Galleria mellonella* nuclear polyhedrosis virus (*Gm*NPV).

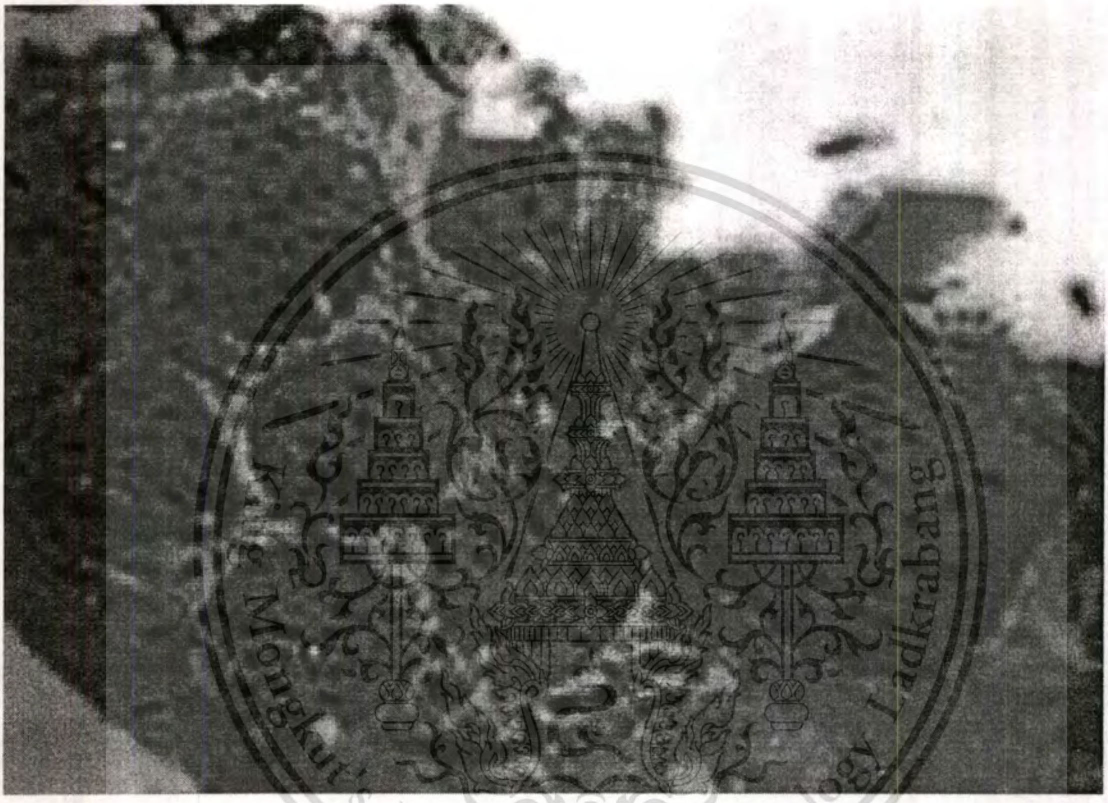

Figure 2.3 Electron micrograph of representative cross section through Sf9 cell infected with *Gm*NPV.

2.2.2 Virion structure

The NPV nucleocapsid is bacilliform in shape, usually 40-50 nm in diameter and 200-400 nm in length [51]. The length of the capsid can extend to accommodate larger DNA genomes such as those of recombinant virus carrying large inserts [52]. Nucleocapsids comprise a nucleoprotein core and a protein shell (the capsid). The core contains viral genome which is double-stranded, covalently closed, circular and 80-200 kbp in length [53]. The protein associated with the core structure include a predominant protaminelike protein know as p6.9 [54, 55].

NPVs are unusual among animal viruses in that the nucleocapsid of the virion lies within one of the two forms of viral envelope [56]; thus each species of NPV exists in distinctive virion phenotypes (Figure 2.4): 1.) the polyhedra derived virus (PDV), also called occluded virus (OV), the virus found in occlusion bodies, which makes natural horizontal transmission of the virus within an insect population possible; and 2.) the Budded virus (BV), also called extracellular virus (ECV), nonoccluded virus, (NOV), which is the form of the virus responsible for systemic spread of infection within the insect and for transmission of the virus in cell culture. Within the envelopes of both BV and PDV are rod-shaped nucleocapsids and are identical in biochemical and genetic composition [56, 57]. BV and PDV seem to differ only in the composition of their surrounding lipid envelopes and this is the reflection of their different routes of biosynthesis. In the BV , the envelope lipids are considered to be derived from their host cell plasma membrane. The envelope near one end has surface projection or peplomers that are thought to comprise the major virus-encoded glycoprotein, gp64. In the PDV, the origin of lipid envelope has not been clearly established. It has been suggested that this membrane is synthesized *de novo* [58].

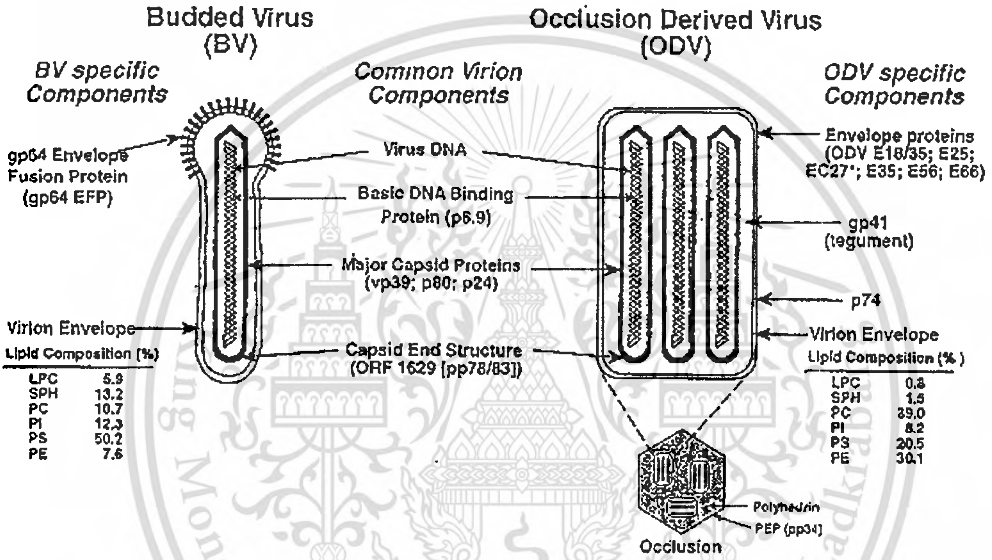

Figure 2.4 Baculovirus virions [56].

2.2.3 Infection process in the insect

In nature, NPV infection begins when an insect feeds on material contaminated with viral OBs. The OBs dissolved in the alkali condition of the mid gut (pH 9.5-11.5) and release polyhedra derived virions [59]. The virions enter the midgut cells by fusion with the membrane of microvilli [60]. Infection in the midgut cells results in BV release from the basement membrane side of the cell [61]. This BV can gain access to the hemocoel and is transported via the hemolymph to other tissues in the insect (insect has an open circulatory system). BV released from the midgut also infects the epithelial cells of tracheoles, which provide oxygen to the midgut, spreading the infection along the tracheal network [61].

During a typical NPV infection, the insect continues to feed during most of the infection process, which takes approximately five to seven days. The integument becomes swollen and changes in luster. Both BV and OBs are produced in most of the tissues infected during the secondary phase. Infection of a late instar larva probably involves approximately 10 generations of virus. Eventually, the insect becomes lethargic and stop feeding. The cuticle malanizes, a polyphenol oxidase-mediated process that results in a discoloration (browning) of the cuticle. The musculature disintegrates, and the larva becomes a cuticular sac of milky fluid containing OBs. Larval disintegration is sometimes described as melting or wilting. A substantial portion (e.g. ca.25%) of the dry weight of the liquefied carcass is polyhedra. The cuticle eventually ruptures and releases the OBs into the environment. Polyhedra are relatively stable in the environment, although they exhibit significant sensitivity to UV light. They are naturally dispersed by a variety of routes and may eventually be consumed by another permissive insect host, thereby reinitiating the infection cycle [62].

2.2.4 Infection process in cell culture

For replication in cell culture, the infection in cell culture can occur in three basic phases: early, late and very late.

Early phase

Infection in cell culture is mediated by the budded form of the virus entering by adsorptive endocytosis [56, 63]. Nucleocapsid migrate through the cytoplasm to the nucleus where they interact end-on with nuclear pores, thereby gaining entry to the nucleoplasm [60]. Viral RNA can be detected 30 minutes after inoculation indicating that arrival of viral DNA in the nucleus and initiation of early transcription are rapid processes [64]. Infected cells undergo significant changes during the first 6 hours of infection, a time period that constitutes the early phase of infection and precedes viral DNA replication. Cytoskeleton rearrangements occur, and the host chromatin disperses within the nucleus, which enlarges during this period. Some of these cytoplasmic and nuclear changes may be brought about by components of viral inoculum (i.e., protein in the infecting virions), while others are controlled by newly produced proteins from early viral gene [65].

Late phase

The early phase is followed by the late phase, a period of extensive viral DNA replication, late gene expression, and BV production. During this phase, a distinct electron-dense structure known as the virogenic stroma forms in the nucleus [66, 67]. Capsid sheaths appear to be assembled in pockets at the edge of virogenic stroma and are filled, while associated end-on with the stroma, with a nucleoprotein core to form nucleocapsids [52]. During the late phase, progeny nucleocapsids leave the nucleus, possibly by a variety of routes, and travel through the cytoplasm. Nucleocapsids usually bud individually from the cytoplasmic membrane although occasionally more than one nucleocapsid is observed in a BV [68].

Very late phase

The very late or occlusion-specific phase begins around 20 h pi. Electron microscopy reveals the elaboration of membrane envelope segments within the nucleus during this occlusion process [66]. Nucleocapcids become enveloped, either individually (SNPVs) or in groups (MNPVs) [52]. Envelopment of nucleocapsids appears to be an essential prelude to embodiment within the polyhedrin matrix of an occlusion body. During the occlusion phase, the occlusion bodies often form a ring around the inside of the nuclear membrane. Eventually, the nucleus becomes virtually filled with occlusion bodies [62].

2.2.5 Usage of NPVs as biological control agent

Since the increasing limitation on the use of chemical insecticides due to the widespread resistance of insect pests, environmental and health problems is well concerned, the usage of NPVs for insect pest control may be another alternative way to lessen the problems associated with the chemical insecticide usage. In contrast to chemical insecticides, NPVs have a narrow host range and safety profile for use as biological control agent. To date, several NPVs have been used for pest controlling in both agriculture and forest [69, 70].

For the forest insect pest management, *Orgyia pseudotsugata* nuclear polyhedrosis virus is used for the control of Douglas fir tussock moth (*Orgyia pseudotsugata*). The viral preparation registered by the EPA under the trade name "TM Biocontrol-1" in 1976. *Lymantria dispar* nuclear polyhedrosis virus is used for control gypsy moth (*Lymantria dispar*) under trade name "Gypchek" [71]. *Neodiprion sertifer* nuclear polyhedrosis virus is used for control European pine sawfly (*Neodiprion sertifer*), the viral preparation registered by the EPA under the trade name "Neochek-S" in 1983. In the same year, preparation of *Neodiprion lecontei* nucleopolyhedrosis virus was registered by Agriculture Canada under name "Lecontvirus", this virus is used for control redheaded pine sawfly (*Neodiprion lecontei*) but carried restricted label and can only be used under the supervision of the Forest service [70].

In agricultural crops; *Mamestra brassicae* nuclear polyhydrosis virus (Mamestrin™) is used for control of cabbage moth (*Mamestra brassicae*), American bollworm (*Heliothis armigera*), diamonndback moth (*Plutella xylostella*), potato tuber moth (*Phthorimaea operculla*) and grape berry moth (*Lobesia botrana*). *Spodoptera litoralis* nuclear polyhedrosis virus (Spodopterin™) is used for control *Spodoptera litoralis*. *Heliothis zea* single capsid nuclear polyhedrosis virus (Gemstar™ biological insecticide, Biotrol™, Elcar™) is used for controlling *Heliothis spp.* *Spodoptera exigua* nuclear polyhedrosis virus (Spod-X™) is used for control beet army worm (*Spodoptera exgua*). *Autographa californica* nuclear polyhedrosis virus (Gusano™) is used for control Alfalfa looper (*Autographa californica*) and miscellaneous lepidoptera [70, 72].

2.3 Cell culture method for propagation of NPVs

In order to be commercially viable, virus based insecticides must be produced on a large scale feasible and efficiently, under strictly controlled and reproducible conditions. The use of an *in vitro* system for virus production, using insect cell culture are currently recognized as being more suitable for large scale production of viral insecticides.

2.3.1 Advantages in using *in vitro* system

Large-scale production of virus in insect cell culture has many attractive features constitute clear advantages over *in vivo* production method [72].

- Reduced capital costs for cell propagation equipment compare to the elaborate facilities require for growing and maintaining whole insect colonies.
- Reduced labors cost.
- Reduced costs of various consumable supplies, especially when serum free media are used.
- Reduced costs for harvesting and downstream processing operations.
- Production of clean viral products, substantially free from microbial contamination (often occurring in insect larvae) and from unwanted impurities.
- Closer monitoring and control of nutritional and physiological environment leading to reliable quality control.
- Potential for plant automation and for scale-up based on the principals of chemical reaction engineering.

- Increased productivity of a cell production facility by applying emerging breakthoughts in genetic engineering and in bioreactor design.

2.3.2 General considerations for *in vitro* propagation of NPVs

The use of *in vitro* system for virus production is very similar to mass cultivation of vertebrate animal cells, but different in some details. The insect cell cultivation must be based on several theoretical and practical considerations. An adequate understanding of nutritional and physical requirements of the candidate cell line for growth and virus proliferation, coupled with information on the behavior of the cells in the culture condition [72].

Repeated passage of several NPVs *in vitro* with ECV as virus inoculum has been reported to generate spontaneous mutants, which produce fewer occlusion bodies in infected cells than the wild type virus [16, 17, 18, 19, 20, 21, 22, 23]. These mutants, termed *FP* (few polyhedra) mutants, easily detected because the distinctive plaque morphology. Previous study [20] showed that the *FP* plaques are large and the infected cells contained fewer polyhedra than the *MP* plaques. In addition, the *MP* plaques always produced *FP* plaques after serial passage in cell culture or by injection of larvae.

According to [73, 74, 75, 76, 77, 78] *FP* mutants have been reported to generate from the genetic modification in viral genome such as deletions and insertions in essential genes. Fraser *et al.* [73, 74] reported that *FP* mutants can be generated by serial passage *Gm*NPV for 3 passages in *Trichoplusia ni* (TN-368) cells. These mutants had acquired cell DNA sequences ranging from 0.8-2.8 kbp in a specific region between 35.0 and 35.7 m.u. of viral genome. The studies also showed that the *FP* mutants failed to synthesize a 25,000 MW polypeptide normally detected in cells infected with wild type virus. The deletions in viral 25KDa gene have also reported to be involved in the *FP* phenotype [76, 77]. Moreover, serial passage of undiluted virus stocks (i.e., high MOIs) results in the accumulation of defective interfering particles. The presence of significant proportions of these particles in a virus stocks is detected in restriction fragment profiles of DNA compare from virus. particles. The defective interfering particles lack approximately 43% of the genome from 1.7 to 45 map units (m.u.) on the *Ac*NPV genome so that restriction fragments from this region are present in submolar quantities. These particles are not infectious but interfere with wild type virus replication [79].

Electron microscopic studies revealed that *FP* mutants had an initial defect of envelopment of PDV during occlusion phase, and released 2-5 fold more infectious virus particles (p.f.u.) into the media of infected Sf9 cells, possibly by the defective envelopment of PDV nucleocapsids. The non-occluded PDV from an *FP* infection might contribute to the ECV titer by passing from the nuclei and budding from infected cells and acquired an ECV envelope [78, 80].

The major problem resulting from the passage effect is the loss of virulence of polyhedra for target insects since, the polyhedra are devoid of occluded virus. Bioassays of *MP* and *FP* polyhedra of *Gm*NPV demonstrated a 350-fold reduction in virulence for *FP* polyhedra [20]. In most cases, the generation of FP mutants would greatly impede the cell culture technique for NPV production.

2.4 Virulence of *Galleria mellonella* nuclear polyhedrosis virus

Galleria mellonella nuclear polyhedrosis virus (*Gm*NPV), normally infects the larvae of wax moth, but is also capable of causing disease in other insect hosts such as *Agrotis ipsilon, Heliothis virescens, Heliothis zea, Plathypena scabra, Pseudoplusia includens, Spodoptera litura, Spodoptera ornithogalli, Trichoplusia ni* and *Plutella xylostella* [14].

In Malaysia, *Gm*NPV was used in laboratory test against *Plutella xylostella* larvae. Although pathogenicity of *Gm*NPV to diamondback moth is only marginally virulent, but this virus shows faster speed of killed when compared with other viruses being used in the test [15].

Preliminary experiments conducted on *Gm*NPV produced in cell cultures adapted to serum-free medium demonstrated the virulence of this virus to *Plutella xylostella* in Taiwan [81].

CHAPTER 3

MATERIALS AND METHODS

3.1 Propagation of insect cells

Sf9 cell line (cloned from *Spodoptera frugiperda* IPLB-Sf21 cell line) used in the experiment was kindly provided from Professor Dr. Serge Belloncik, INRS-Institut Armand-Frappier, Quebec', Canada.

Sf9 cell line was routinely maintained in Grace's medium (GIBCO #350-1590AJ) supplemented with 10% FBS (GIBCO #230-6140), 0.0666 g/l lactalbumin hydrolysate (GIBCO #670-8080AG), 0.0666 g/l yeastolate (GIBCO #670-8190AG). A final of concentration of gentamycin (GIBCO #15710-015) 50 μg/ml was added into the medium. Monolayer culture was incubated at 28°C (temperature-controlled incubator: Shillab™: model 2020) in 25 cm^2 tissue culture flasks (Corning™ sterile disposable polystyrene: 25 cm^2 #25102, 75 cm^2 #25110).

For subculturing the cells, the old medium was discarded from the flask and replaced with 5 ml of pre-warmed fresh medium. Sf9 cells usually grew loosely attached, and were easily resuspended by gentle pipetting.

Complete cell detachment was ensured by observing cell monolayer with an inverted phase-contrast microscope (Diaphot; phase-contrast objectives: 4, 10, 20, and 40x; eyepieces: 10x, Nikon™: model Phase-contrast-2 ELWD 0.3). A passage or subculture was defined as the transfer of the confluent cells from one cultured flask to another, with dilution to obtain 10^6 cells in a final culture medium volume of 5 ml.

Cell counts were performed, using a hemacytometer (Bright-Line™ Neubauer 0.1 mm: Reichert-Jung Model 1475) and trypan blue exclusion method, by adding 0.1 ml trypan blue (Sigma # T6164) to 0.4 ml well-suspended Sf9 cells. Viable and uncolored cells were counted in the center square of both chambers of the hemacytometer, and the numbers of cells per milliliter were calculated using the following formula:

$$\textbf{Number of viable cells per ml} = [(A+B)/2] \times 10^4 \times 5/4$$

where A is equal to the total number of viable cells in the center square of the first chamber of the hemacytometer and B is equal to the total number of viable cells in the center square of the second chamber of the hemacytometer. The cells were subcultured every 4 days.

3.2 Serial passage and light microscopic studies

The strain of *Galleria mellonella* nucleopolyhedrosis virus used in the experiment was kindly provided from Professor Dr. Serge Belloncik, INRS-Institut Armand-Frappier, Quebec', Canada.

For the serial passage of *Gm*NPV, Sf9 cells were seeded in triplicate in 25 cm^2 plastic tissue culture flasks at a concentration of 2×10^5 cells/ml and the final volume of medium was 5 ml/flask. Cells were incubated at 28°C overnight to allow the cells to completely attach to the culture surface. In the following day, the cells were infected: the old tissue culture medium from the cell monolayers was aspirates, and replaced with 1 ml of virus inoculum. In 1^{st} passage virus inoculum was derived from the diluted hemolymph of *Galleria mellonella* larva infected with *Gm*NPV and in subsequent passages virus inoculum was obtained from infectious medium from previous passage. For the control, non-infectious medium (1ml/flask) was added instead of virus inoculum. The healthy and infected cells were routinely examined with an inverted phase-contrast microscope and Light micrographs of the infected cells were taken every 24 hours post infection.

3.2.1 Percentage of infection

After 7 days of post-infection, infected cells were detached from the flasks by gently pipetting with medium. The cell suspension was collected for enumeration of infected and normal cells (3 times) using hemacytometer and trypan blue exclusion method (as described before). The percentage of infection was calculated using the following formula:

$$\textbf{\% infected cells} = [\textbf{infected cells per ml / total cells per ml}] \times 100$$

3.2.2 Production of polyhedra

The remaining cell suspension from each flask, was collected for the purification of occlusion bodies, according to a modified protocol described by Miller and Dawes [82]. Triplicate counts of occlusion bodies were performed using hemacytometer. The method for purification of occlusion bodies were as follows:

1.) Harvested infected cells by centrifugation at 1000×g (HERMILE™: model Z383K) for five minutes at room temperature.

2.) Discarded supernatant and resuspended cells in 0.5% SDS (ten milliliters of 0.5% SDS per 2×10^8 cells/ml is a convenient volume).

3.) Centrifuged at 5000×g for five minutes and discarded supernatant.

4.) Resuspended the pellet in 1 ml of sterile 0.5M NaCl.

5.) Centrifuged at 5000×g for five minutes and discarded supernatant.

6.) Resuspended the pellet of occlusion bodies in sterile distilled water.

The number of occlusion bodies per ml was calculated using the following formula:

$$\textbf{Number of occlusion bodies per ml} = [(A+B)/2] \times 10^4$$

where A is equal to the total number of occlusion bodies (OBs) in the center square of the first chamber of the hemacytometer and B is equal to the total number of occlusion bodies (OBs) in the center square of the second chamber of the hemacytometer.

3.2.3 Production of extracellular virus titers

A virus titration is a quantitative determination of viral activity, i.e. the smallest amount of viral suspension which can be produce disease, lesion, or some recognizable effect in host. The smallest amount of virus which produces this reaction is termed as the infectious unit, and the titer is usually expressed as the reciprocal of the highest dilution of the virus which caused a specified reaction in 50% of the material inoculated with, or exposed to, that dilution of infectious material. Where cell cultures are used as indicator, titer are expressed as 50% tissue culture infective dose ($TCID_{50}$) [83].

In this study, the extracellular virus titers of *Gm*NPV were determined by the tissue culture infectious dose ($TCID_{50}$) as follows:

The cells were diluted to a concentration of 1×10^5 cells/ml with complete tissue culture medium and seeded with 100 μl aliquots of the cell suspension into each well of 96-well microtitre plate (Corning™: #25860) and incubated overnight. In the following day, a 10 μl aliquots of each virus dilution was added to each well of a 96-well microtitre plate (ten serial dilutions were prepared from infected culture media). One inoculum of 96-well microtitre plate, or 8 wells was used for each dilution of virus. Four replications were done for each virus dilution and incubated at 28°C for 7 days. Wells were observed at 7 days post infection for the presence of polyhedra and the $TCID_{50}$ values were calculated by Karber method [84].

All the data obtained from serial passage study were submitted to statistical analysis by using ANOVA model and LSD test (with the significant level 0f 0.05) to determine the significant differences in each data series.

3.2.4 Preparation of occlusion bodies

Occlusion bodies were enumerated with hemacytometer, directly from the purification of OBs as previously described, and their concentration was adjusted to 10^7 OBs/ml in sterile distilled water, and kept as a stock solution at -20°C.

3.3 Electron microscopic study

For ultrastructural analysis, the culture medium was removed from the cells by centrifugation. Cell pellets were fixed with 2.5% glutaraldehyde in cacodylate buffer pH 7.2-7.4 at 4°C for 1 hour. They were then washed with cacodylate buffer pH 7.2-7.4 at 4°C for 10 minutes two times, post-fixed with 1.3% osmium tetraoxide (usually buffered) at room temperature for 1 hour and washed with distilled water. During each step of procedure cells were suspended in the solution after pelleted by centrifugation (at 7000g for 5 minutes).

After the distilled water were removed from the cells, they were dehydrated in a graded series of acetone solutions (25%-50%-75%-95%-100%-100%) and embedded in resin, and cut with diamond knife. The ultra thin sections were stained with uranyl acetate and lead citrate. They were examined and photographed with an electron microscope (Philip™ 300). The thin sections were observed for the polyhedra morphology and the number of virions present.

3.4 Plaque purification of virus isolates

Sf9 cells were seeded in duplicate in 60 mm tissue culture dishes (Corning™ #: 25010) with 2×10^6 cells/plate. The final volume of tissue culture medium should be 4 ml per plate. Monolayer culture was incubated at 28°C for 30 minutes to allow the cells to attach.

Ten serial dilutions of virus derived from 1st, 10th and 15th passage were prepared. The tissue culture medium from the cell monolayers was aspirated and 0.5 ml of virus inoculum was added. For the control, 0.5 ml of tissue culture medium was added instead of virus. The cells were incubated at room temperature for 1 hour.

While the cells were incubating, 0.5% overlay was prepared and warmed to 40-42°C. The virus inoculum was removed from the cells and 4 ml of agarose overlay was added to each plate. The cells were left at room temperature for 15-20 minutes until the agarose had hardened. The cells were incubated at 28°C for 5 days.

After 5 days post-infection, plaques would appear as clear, circular area. The well-isolated plaques on a plate that has 10 plaques or less were selected and examined under the microscope to ensure that the virus is pure.

3.5 Bioassay study

Plutella xylostella larvae were originally obtained by collecting from Klong Rangsit vegetable cultivated area, Pathumthanee province, Thailand. The larvae used throughout the experiments were reared in the laboratory on Chinese kale leaves.

The virulence of *Gm*NPV to the second stage larvae of *Plutella xylostella* was examined by using leaf disk contamination technique [85]. The various concentration of occlusion bodies of *Gm*NPV (10^7, 10^6 and 10^5 OBs/ml) derived from various virus isolates including wild type (from infected larvae of *Galleria mellonella*), 1st passage, 5th passage 10th passage and plaque purified isolate were made in sterile distilled water.

A volume of 5 µl of each test concentration was applied to the surface of Chinese kale leaf disk (6 mm in diameter). Allowed the water to evaporate and placed second-instar larva on the treated leaf disk. For the control, of 5 µl of sterile distilled water was applied on the leaf disk instead of virus. At each test concentration, bioassay was replicated three times (twenty-five larvae per replication).

After 24 hours, larvae that had consumed the entire exposed leaf area were transferred individually into another container containing fresh leaves and fed until dead and pupated. Mortality recording were scored daily for all bioassays.

To confirm diagnosis of *Gm*NPV infection, the dead larvae were removed a thin smear was prepared, and stained by using Buffalo black application. The presence of infected OBs were microscopically observed at 100x under oil immersion.

The results of the bioassays including LC_{50} and LT_{50} were estimated by the Probit analysis [86]. Chi-square tests were used to assess the goodness of fit of the model to the data. Significant differences in potency between different virus preparations was determined by one-way ANOVA and LSD test with significant level of 0.05 (Spss® 10.0.1 for Win98NT) [87].

CHAPTER 4

RESULTS

4.1 Serial passage of *Galleria mellonella* nuclear polyhedrosis virus

In this study, *Gm*NPV was serially passaged through *Spodoptera frugiperda* cell line (Sf9) 20 times in monolayer culture. In order to determine some of the replication characteristics, the virus was passaged every week, and the percentage of infection, the polyhedra and extracellular virus titer production were documented as shown in Table 4.1.

4.1.1 Percentage of infection

As shown by the histogram in Figure 4.1, the percentage of infection obtained from 1st passage up to 20th passage varied from 71.3 to 8 %. From the 1st passage through the 10th passage, the percentage of infection seemed to decrease slightly with the highest infection percentage detected in the 2nd passage. After about 10 passages in cell culture the percentage of infection declined sharply. The statistical analysis revealed that after the 10th passages onward, the percentage of infection decreased significantly ($P<0.05$). In this experiment the reduction in percentage of infection was observed even higher or lower MOI were applied to cell culture.

4.1.2 Production of polyhedra

During the serial passage of *Gm*NPV in cell culture the stability of polyhedra production was investigated through the total number of polyhedra production per culture (5 ml). As shown by the chart in Figure 4.2, the polyhedra production seemed to decline slightly from the 1st passage up to 10th passage but after the 10th passage marked reduction in polyhedra production was observed. The data obtained from this experiment were subjected to statistical analysis and the results revealed that the polyhedra production was significantly decreased after the 10th passage ($P<0.05$). The reduction in yields indicated that serial passage of *Gm*NPV in cell culture by using ECV as virus inoculum resulted in fewer polyhedra production.

4.1.3 Production of extracellular virus titers

In this study, extracellular virus titer at each passage level was determined by end point dilution method as described above. As shown by the chart in Figure 4.2, from the 1st passage up to 8th passage the titer of ECV fluctuated from $10^{7.2}$ to $10^{9.67}$ TCID$_{50}$/ml, with the lowest titers detected in the 6th passage. In the 9th and 10th passages the titers of extracellular virus were observed to increase about 1,000 and 1,000,000 folds when compared with the 1st passage. The data were also subjected to statistical analysis and the results indicated that the ECV titers obtained from the 9th and 10th passages were significantly higher than other passages ($P<0.05$).

Table 4.1 Serial passage of *Gm*NPV in Sf9 cells.

Virus.passage NO.	Percentage of infection	Total No. of polyhedra per culture (5 ml)	$TCID_{50}$/ml
1	69.8^{a}	$10^{6.76e}$	$10^{8.20i}$
2	71.5^{a}	$10^{6.45ef}$	$10^{9.67ij}$
3	64.9^{a}	$10^{6.08g}$	$10^{7.50i}$
4	49.1^{ab}	$10^{6.08g}$	$10^{7.50i}$
5	62.4^{ab}	$10^{6.15f}$	$10^{8.25i}$
6	58.3^{ab}	$10^{6.06g}$	$10^{7.20i}$
7	57.8^{ab}	$10^{6.08g}$	$10^{8.10ij}$
8	40.8^{b}	$10^{6.50e}$	$10^{9.20j}$
9	42.2^{b}	$10^{6.08g}$	$10^{11.20j}$
10	47.7^{b}	$10^{6.11g}$	$10^{14.20l}$
11	21.3^{d}	$10^{4.08h}$	$10^{7.30i}$
12	11.3^{d}	$10^{5.11h}$	$10^{6.70i}$
13	8.5^{d}	$10^{5.11h}$	$10^{6.40i}$
14	9.3^{d}	$10^{4.78h}$	$10^{6.30k}$
15	13.0^{d}	$10^{4.78h}$	$10^{6.30k}$
16	9.0^{d}	$10^{4.95h}$	$10^{6.17k}$
17	10.9^{d}	$10^{4.41h}$	$10^{6.60k}$
18	8.0^{d}	$10^{4.60h}$	$10^{5.20k}$
19	11.3^{d}	$10^{4.90h}$	$10^{6.80i}$
20	9.6^{d}	$10^{4.40h}$	$10^{6.20k}$

Mean values of % infection and polyhedra production were calculated from 3 replications, and mean values of ECV titer production was calculated from 4 replications.

Means followed by the same letter were not significantly different (P>0.05).

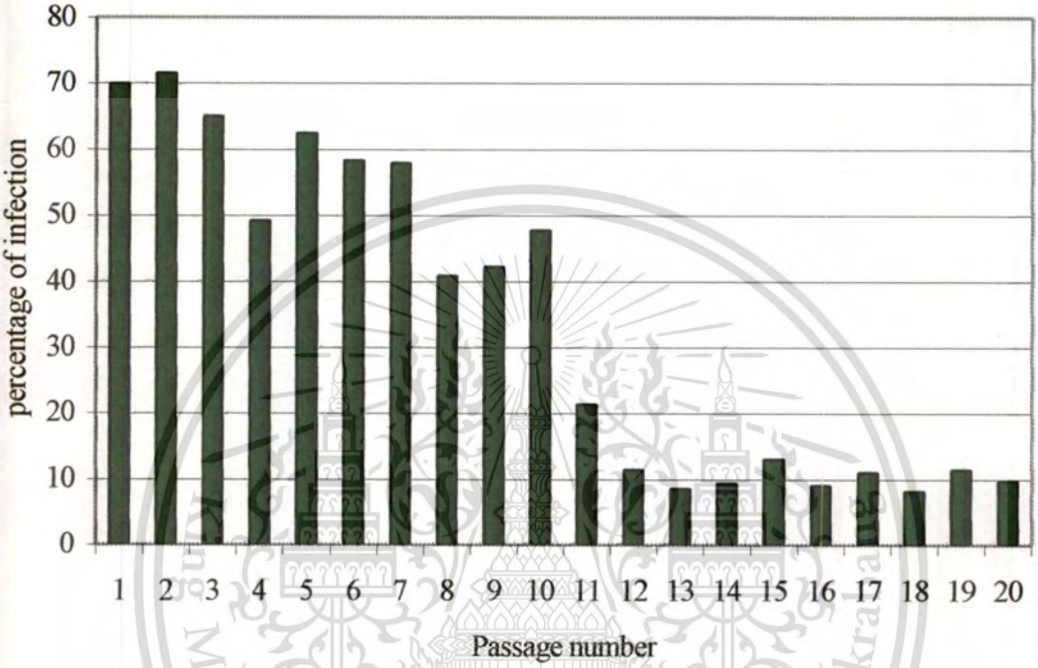

Figure 4.1 The percentage of *Gm*NPV infection at each passage level.

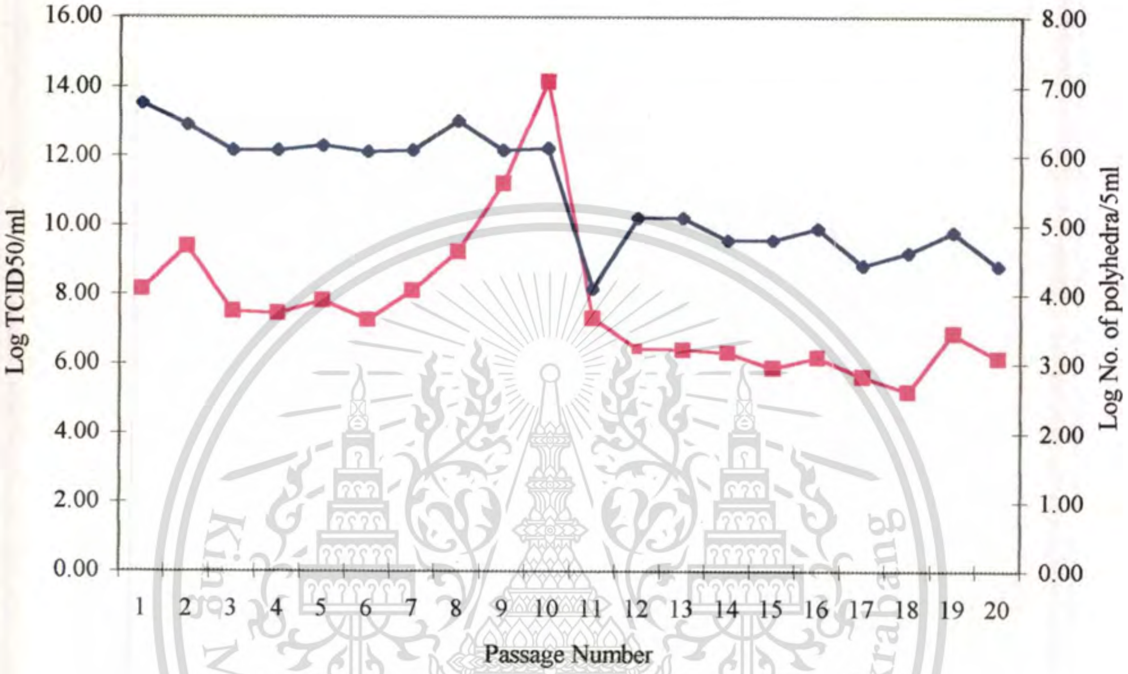

Figure 4.2 The ECV titers and total polyhedra productions during serial passage *in vitro*. The relationship between extracellular virus titer production and serial passage level was represented in pink color (■). The relationship between total polyhedra production and serial passage level was represented in blue color (◆).

4.2 Light microscopic study

At each passage level of *Gm*NPV production, the infected cells were examined routinely by phase-contrast light microscope to ensure the infection. The hypertrophy of nuclei and the presence of developing polyhedra were the criterion of the virus infection. As observed in present study, the infection of *Gm*NPV was generally observed within 24 hours post infection and the infected cells appeared to be filled with mature polyhedra by 48 hours post infection and mostly disrupted in the late time post infection.

The healthy non-infected Sf9 cells (untreated control) were illustrated in Figure 4.3 and those heavily infected with *Gm*NPV were illustrated in Figure 4.4. The light micrographs of the infected cells obtained from the 1st, 5th, 10th, 15th, and 20th passages at 48 hours post infection were shown in Figure 4.5-4.9. In the early passage (1st passage up to 10th passage) a large number of polyhedra were produced in infected nuclei and there was just a small difference in the level of polyhedra production. As shown by the light micrographs in Figure 4.5-4.7, the polyhedra formed in the infected cells in those passages were quite similar in size and shape.

In contrast, after the 10th passage marked reduction in polyhedra production in the infected nuclei was generally observed (Figures 4.8 and 4.9). Some of the infected cells contained no polyhedra but exhibited typical signs of NPV infection such as hypertrophy of nuclei. Instead of forming polyhedra many granular were appeared inside the cells. Moreover, the abnormal in size (arrow) of polyhedra can be seen easily after the 10th passage.

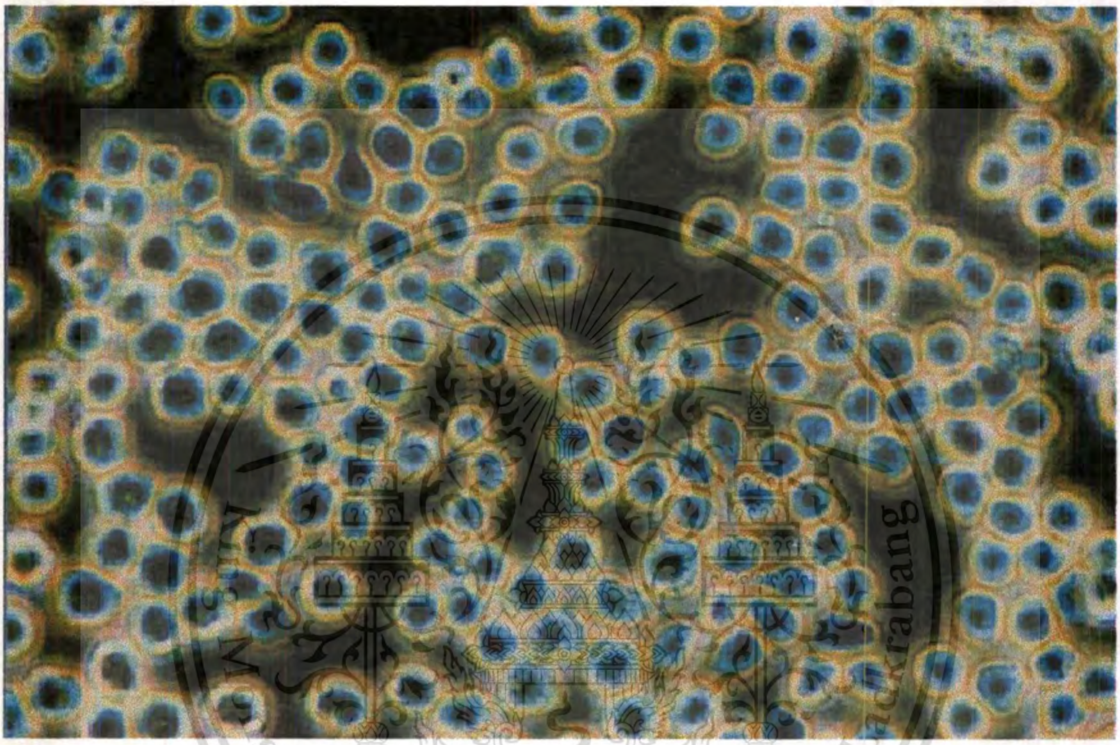

Figure 4.3 Phase-contrast micrograph of non-infected Sf9 cells (48 hour old).

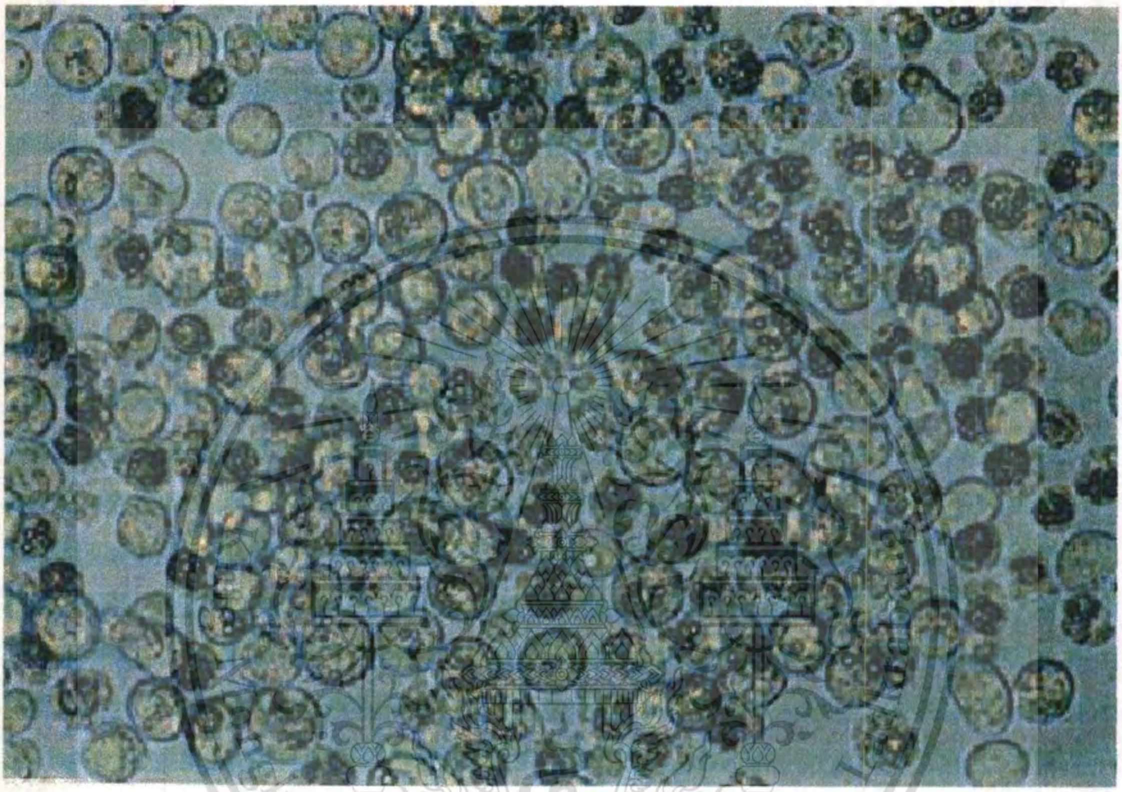

Figure 4.4 Phase-contrast micrograph of Sf9 infected with *Gm*NPV at 72 hours post infection.

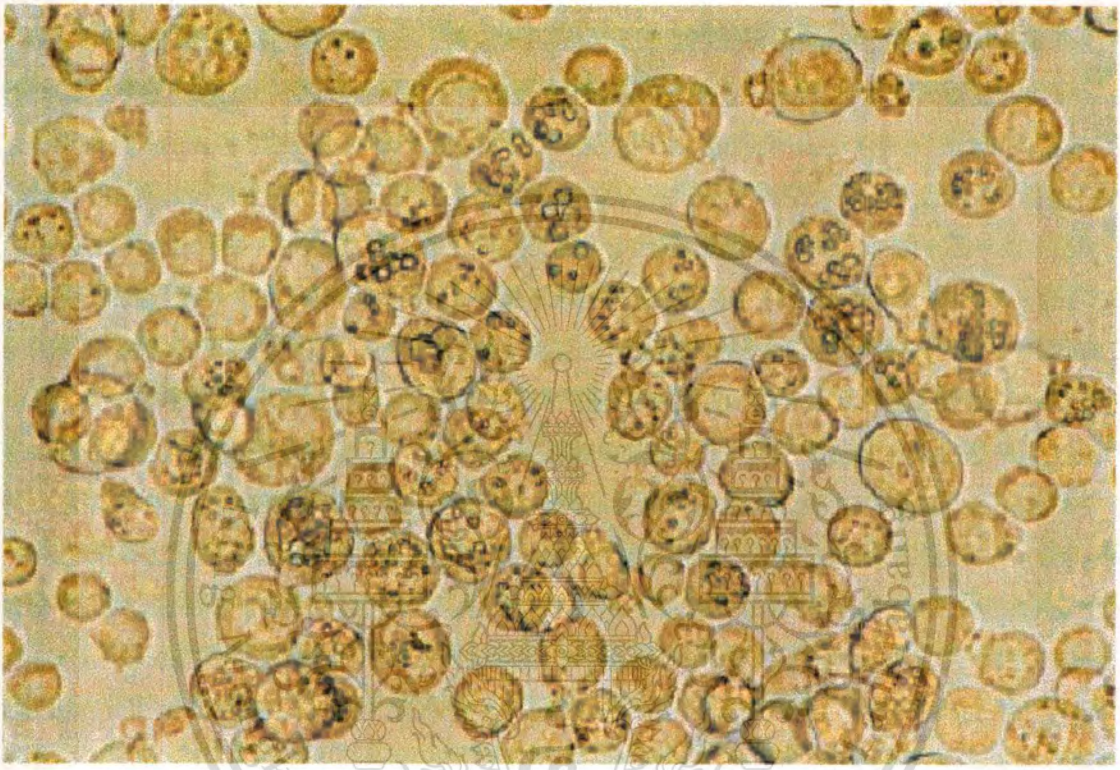

Figure 4.5 Phase-contrast micrograph of Sf9 cells infected with *Gm*NPV from the 1st passage at 48 hours post infection.

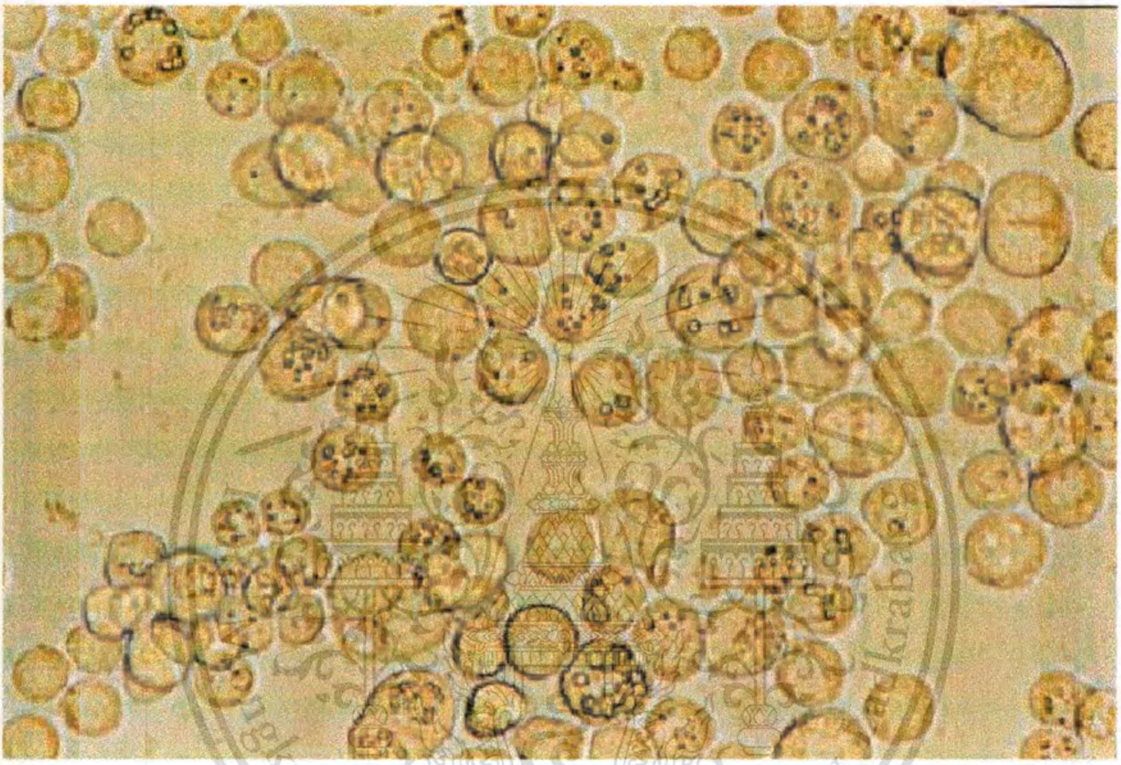

Figure 4.6 Phase-contrast micrograph of Sf9 cells infected with *Gm*NPV from the 5th passage at 48 hours post infection.

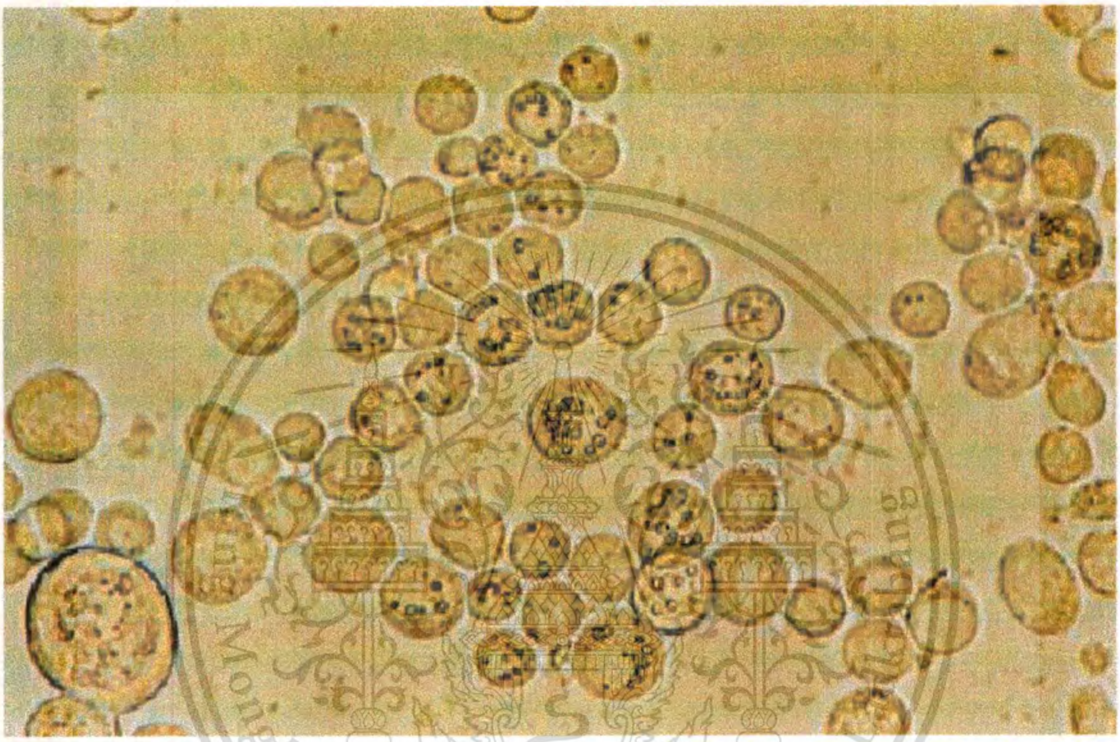

Figure 4.7 Phase-contrast micrograph of Sf9 cells infected with *Gm*NPV from the 10th passage at 48 hours post infection.

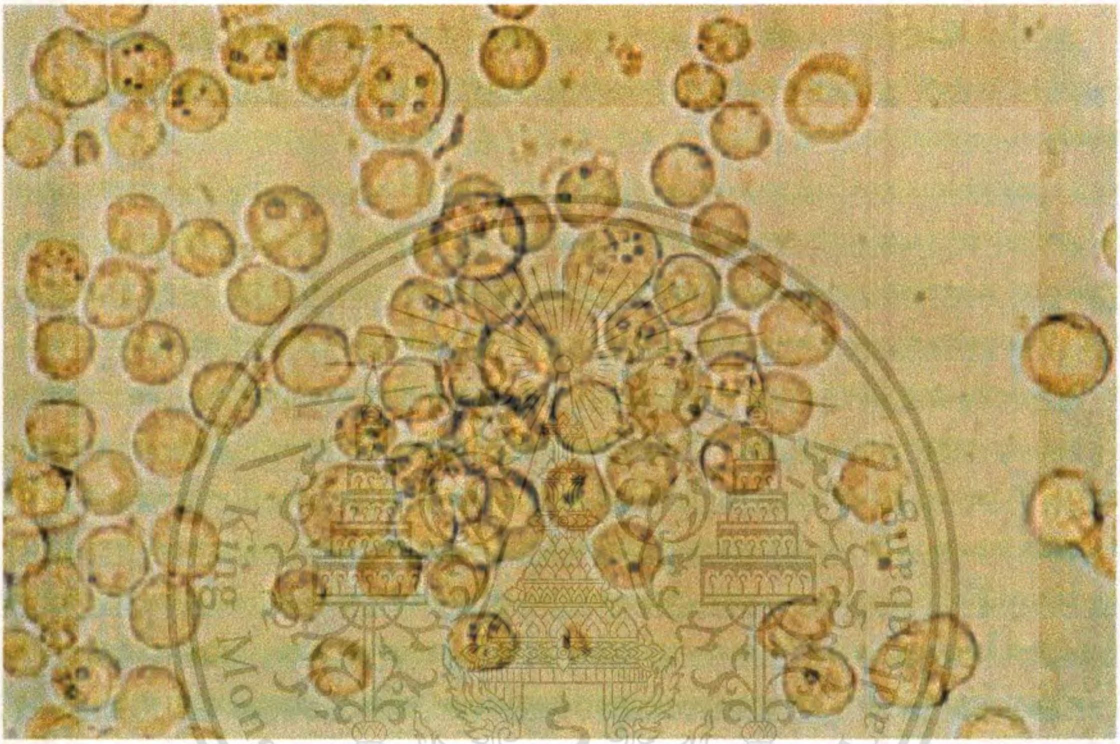

Figure 4.8 Phase-contrast micrograph of Sf9 cells infected with *Gm*NPV from the 15th passage at 48 hours post infection.

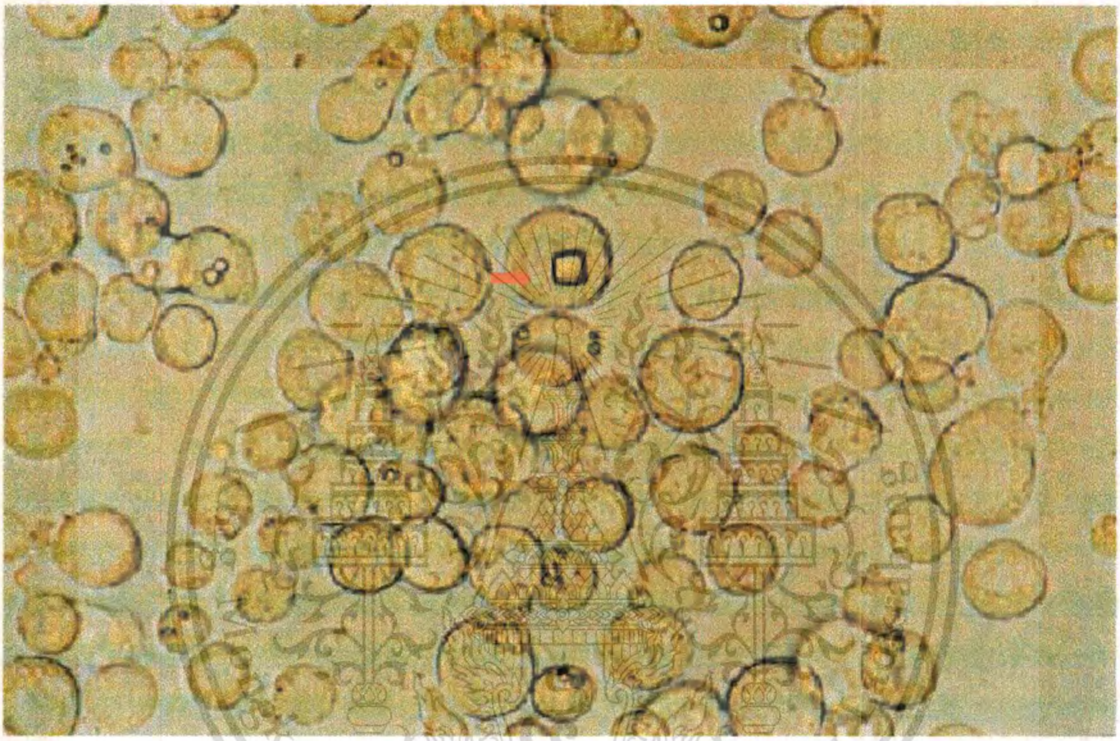

Figure 4.9 Phase-contrast micrograph of Sf9 cells infected with *Gm*NPV from the 20th passage at 48 hours post infection.

4.3 Electron microscopic study

The electron microscopic examination of the *Gm*NPV infected cells obtained from the 1st, 5th, 10th, 15th, and 20th passages are shown in Figures 4.10-4.14.

In the 1st passage, normal replication and polyhedra production were observed (Figure 4.10). Inside each polyhedron, many occluded viruses were presented within polyhedra and the polyhedra appeared in normal shape and size. Similarly, in the 5th and 10th passages the viral replication and polyhedra production were quite similar to the 1st passage (Figures 4.11 and 4.12). Although, it is not apparent in figure represented, the presence of polyhedra that contained few or no occluded virions can be seen but not predominant.

After about 10 passages of virus *in vitro*, it became apparent that the viral replication and polyhedra production were abnormal. As shown in Figure 4.13 and 4.14, the abnormal in polyhedra production was observed both in shape and size. Inside the polyhedra there was very few or no occluded virions.

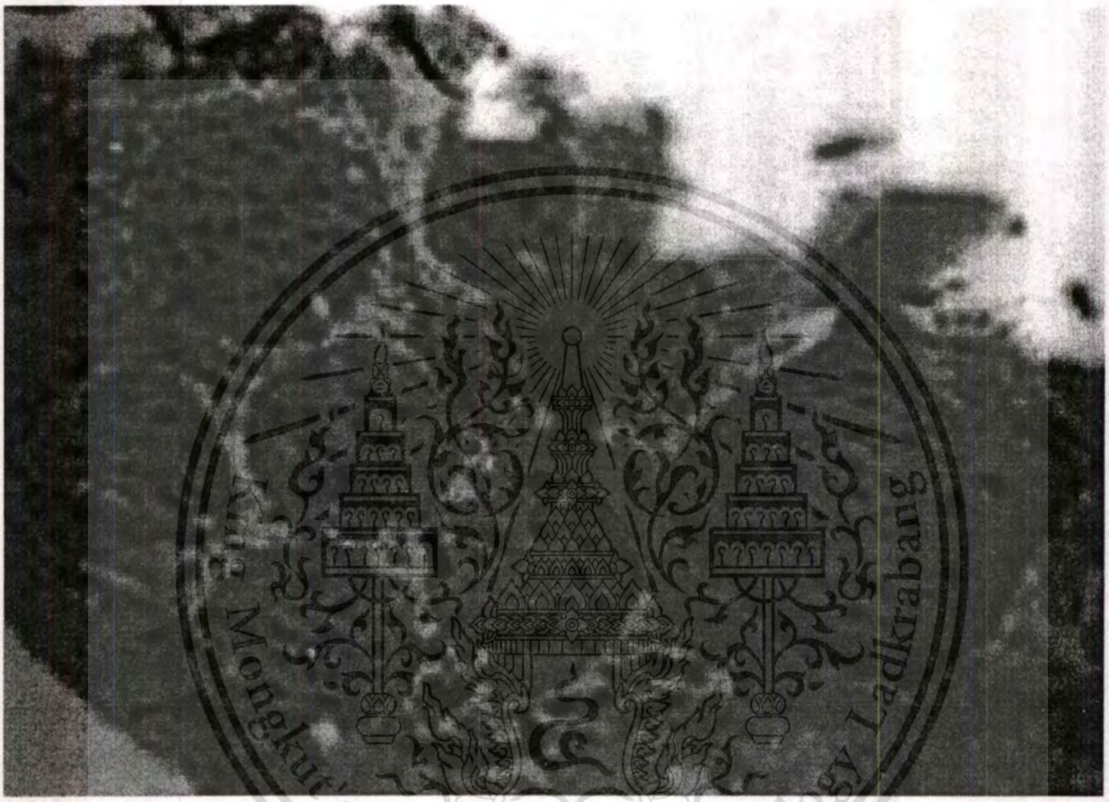

Figure 4.10 Electron micrograph of Sf9 cell infected with *Gm*NPV from the 1st passage.

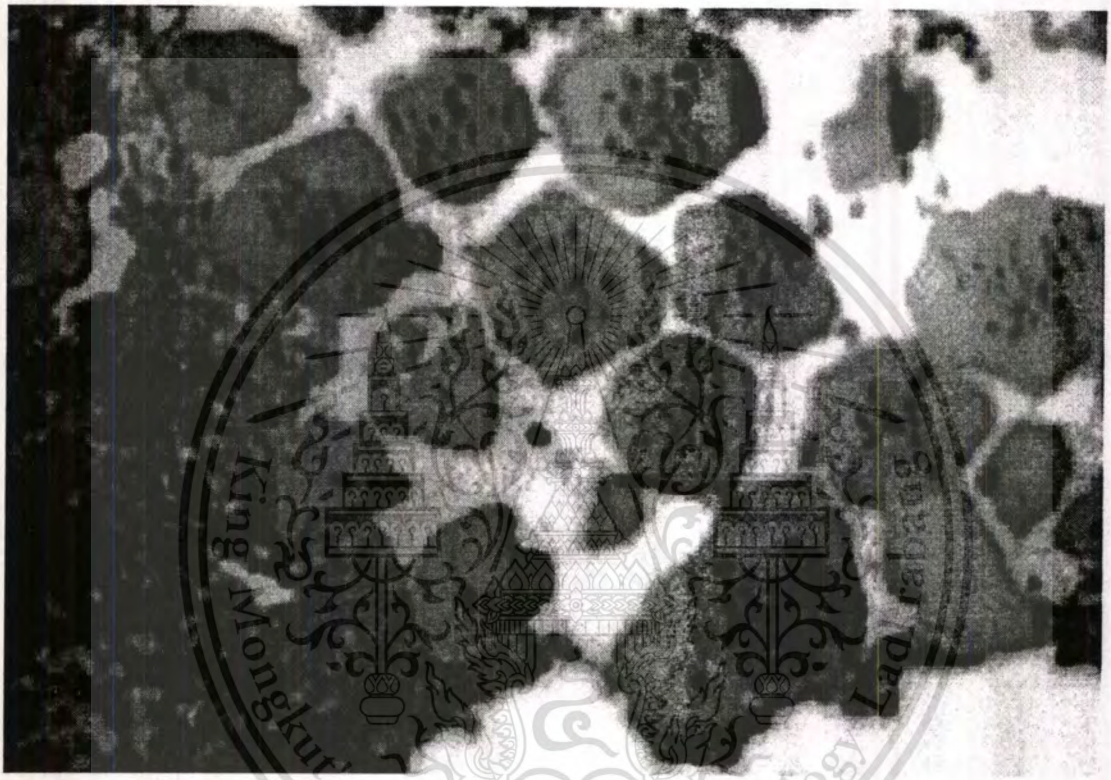

Figure 4.11 Electron micrograph of Sf9 cell infected with *Gm*NPV from the 5th passage.

Figure 4.12 Electron micrograph of Sf9 cell infected with *Gm*NPV from the 10th passage.

Figure 4.13 Electron micrograph of Sf9 cell infected with *Gm*NPV from the 15th passage.

Figure 4.14 Electron micrograph of Sf9 cell infected with *Gm*NPV from the 20th passage.

4.4 Plaque purification of virus isolates

The extracellular virus obtained from the 1st, 10th and 15th passages were plaque-purified 3 times in Sf9 cell line. In this experiment, five plaque-purified isolates were obtained (Table 4.2). In order to determine the production of polyhedra and titer of extracellular virus, each plaque-purified isolate was used as virus inoculum to infect Sf9 cells.

The results indicated that one plaque purified isolate designated as P-10 exhibited high productivity of polyhedra and extracellular virus titer. In another word, this plaque-purified isolate exhibited either high infectivity or productivity. Therefore, the polyhedra produced from P-10 were further used in bioassay studies to compare the infectivity to *Plutella xylostella*.

Table 4.2 The plaque purified isolates obtained from plaque purification.

Plaque isolates	Total No. of polyhedra per culture (5 ml)	$TCID_{50}$ /ml
P-1a	$10^{5.55}$	$10^{8.8}$
P-1b	$10^{5.72}$	$10^{8.67}$
P-10	$10^{6.38}$	$>10^{17}$
P-15a	$10^{5.99}$	$10^{16.63}$
P-15b	$10^{6.01}$	$10^{16.75}$

4.5 Bioassay study

The comparative bioassays of the 1st, 5th, 10th passage isolates, plaque-purified isolate and wild isolate of *Gm*NPV to 2nd stage larvae of diamondback moth were performed using leaf disk bioassay. Normally, the noticeable symptoms of infected larvae were observed after 2-3 days of post infection. When the infected larvae were not fed, they turned to pale green color. Mortality of infected larvae was observed within 2 days feeding was terminated. Dead larvae were dark brown in color (Figure 4.15) and easily disrupted when gently touched by camel brush.

The light microscopic examination of infected tissue from the cadavers that exhibited the symptoms of NPV infection revealed plenty of polyhedra (Figure 4.16). The infected nuclei were filled with a large number of polyhedra and the lysis of cells yielded free polyhedra which was responsible for dissemination in the environment. In case of metamorphosis, the incomplete pupation of some infected larvae was observed but these malformed pupae usually died before emerging to adults.

The concentration-mortality responses obtained from the experiment are shown in Table 4.3. The percentage of mortality was calculated from the total number of dead-infected larvae. The data were then submitted to Probit analysis [85]. The concentration-mortality response lines for 2nd stage larvae of *Plutella xylostella* are shown in Figure 4.17.

According to the calculated median lethal concentration values (LC_{50}s), the wild isolate of *Gm*NPV was most pathogenic to second-instar of *Plutella xylostella* with the LC_{50} value of 19,387.61 OBs/cm^2 (Table 4.4). It was found that the LC_{50} value of wild type *Gm*NPV was significantly higher than the 1st, 5th, 10th passage isolates and plaque purified isolate when treatment means were compared using ANOVA and LSD test ($P<0.05$). For the other 4 isolates, *Gm*NPV from 1st passage (LC_{50} = 56,795.86 OBs/cm^2), 5th passage (LC_{50} = 67,494.15 OBs/cm^2) 10th passage (LC_{50} = 68,899.53 OBs/cm^2) and plaque-purified isolate (LC_{50} = 47,116.38 OBs/cm^2) showed no significant different in virulence ($P>0.05$).

Figure 4.15 Photograph of *Plutella xylostella* larva infected with *Gm*NPV, discoloration of the cuticle can be observed.

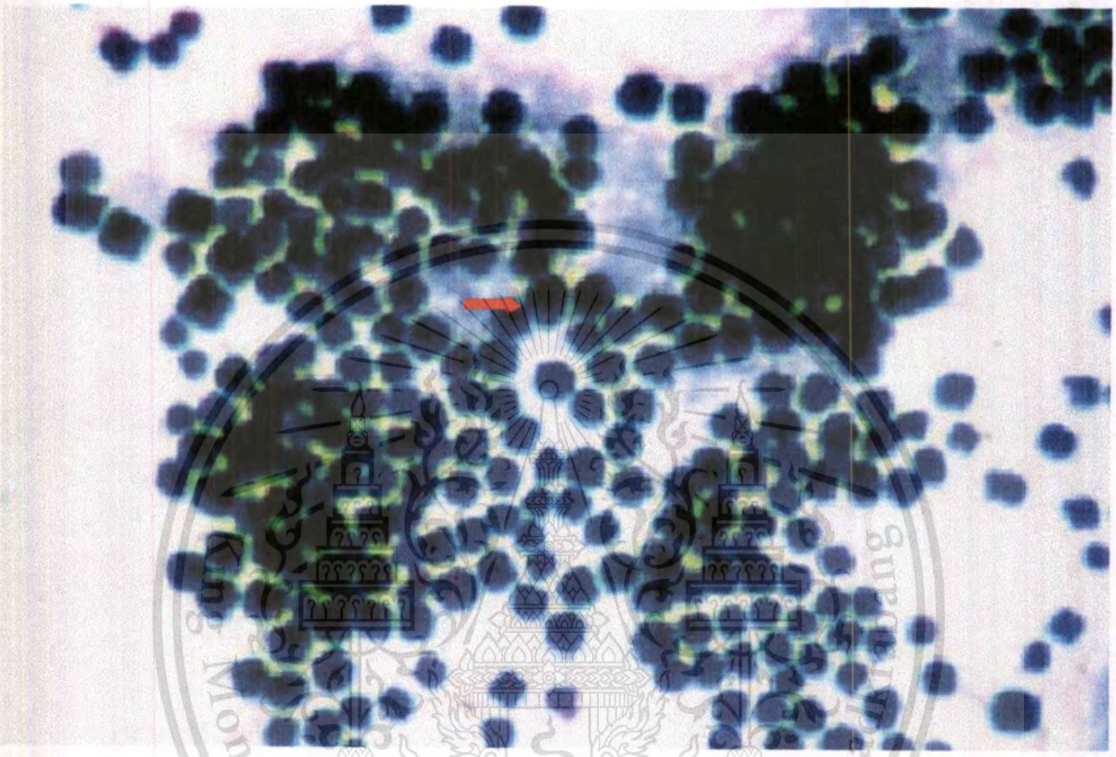

Figure 4.16 Light micrograph of Buffalo black stained smear from infected tissue of *Plutella xylostella* larva. The presence of polyhedra (arrow) had confirmed the infection in the insect.

Table 4.3 Concentration-mortality response of 2nd stage larvae of *Plutella xylostella* after fed leaf disk contaminated with 5 isolates of *Gm*NPV.

Virus isolate	Number of polyhedra/ml	Number of tested larvae	Number. of response	% Mortality
Wild type isolate	100000	75	3	4.00
	1000000	75	44	58.67
	10000000	75	64	85.33
1st passage isolate	100000	75	0	0.00
	1000000	75	25	33.33
	10000000	75	52	69.33
5th passage isolate	100000	75	2	2.67
	1000000	75	27	36.00
	10000000	75	46	61.33
10th passage isolate	100000	75	2	2.67
	1000000	75	19	25.33
	10000000	75	51	68.00
Plaque-purified isolate	100000	75	2	2.67
	1000000	75	29	38.67
	10000000	75	53	70.67

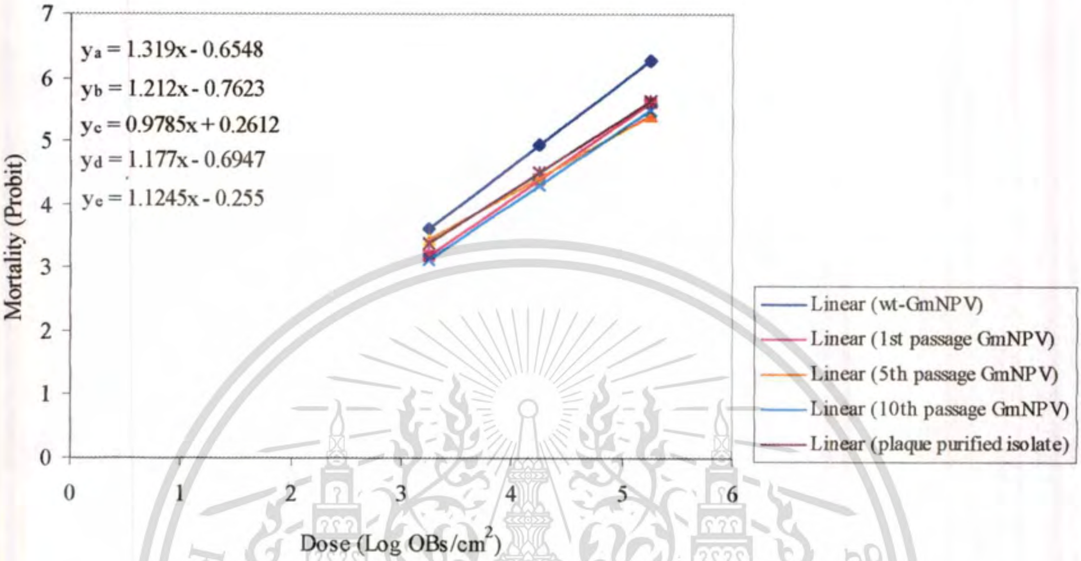

Figure 4.17 Concentration-mortality response regression lines for 2nd stage larvae of *Plutella xylostella* treated with 5 isolates of *Gm*NPV.

y_a = Regression line of the wild isolate

y_b = Regression line of the 1st passage isolate

y_c = Regression line of the 5th passage isolate

y_d = Regression line of the 10th passage isolate

y_e = Regression line of plaque-purified isolate

Table 4.4 Mean median lethal concentration values (LC_{50}s) of the 1st passage isolate, 5th passage isolate, 10th passage isolate, plaque-purified isolate and wild isolate of *Gm*NPV for 2nd stage larvae of *Plutella xylostella.*

Virus isolate	Slope	LC_{50}(OBs/cm^2)	95% Fiducial limits
Wild type isolate	1.3190	19,387.61^a	13,698.40-27,673.53
1st passage isolate	1.2120	56,795.86^b	38,950.40-87,917.96
5th passage isolate	0.9785	67,494.15^b	42,801.71-119,998.60
10th passage isolate	1.1770	68,899.53^b	46,284.26-110,653.70
Plaque-purified isolate	1.2145	47,116.38^b	31,789.84-73,968.02

Means followed by the same letter were not significantly different (P>0.05, one-way ANOVA; LSD test)

On the basis of time-mortality responses, the median lethal time values (LT_{50}s) of *Gm*NPV 5 isolates were calculated from the same concentration (10^7 OBs/ml). The time-mortality responses of *Gm*NPV 5 isolates at certain dosage are shown in Table 4.5. All data were also subjected to Probit analysis and time-mortality response lines for 2nd stage larva of *Plutella xylostella* are shown in Figure 4.17.

The values of LT_{50} were calculated and summarized in Table 4.6. For the most pathogenic virus, wild type *Gm*NPV showed highest speed of kill (LT_{50} = 4.8 days). For the other 4 isolates, *Gm*NPV from 1st passage, 5th passage, 10th passage and plaque purified isolate showed LT_{50} equal to 5.1 days; 5.6 days, 5.1 and 5.4 days, respectively. However, there was no significant difference among 5 *Gm*NPV isolates (P>0.05).

Table 4.5 Time-mortality response for 2nd stage larvae of *Plutella xylostella* after fed leaf disk contaminated with 5 isolates of *Gm*NPV.

Virus isolate	Days (post application)	Number of tested larvae	Number of response	Collected response	% Mortality
Wild type isolate	3	75	3	3	4.00
	4	75	11	14	18.67
	5	75	38	52	69.33
	6	75	10	62	82.67
	7	75	2	64	85.33
1st passage isolate	3	75	1	1	1.33
	4	75	24	25	33.33
	5	75	21	46	61.33
	6	75	5	51	68.00
	7	75	1	52	70.67
5th passage isolate	3	75	1	1	1.33
	4	75	15	16	21.33
	5	75	22	38	50.67
	6	75	6	44	58.67
	7	75	2	46	61.33
10th passage isolate	3	75	5	5	6.67
	4	75	21	26	34.67
	5	75	16	42	56.00
	6	75	6	48	64.00
	7	75	3	51	68.00
Plaque-purified Isolate	3	75	2	2	2.67
	4	75	11	13	17.33
	5	75	25	38	52.00
	6	75	11	49	65.33
	7	75	4	53	73.33

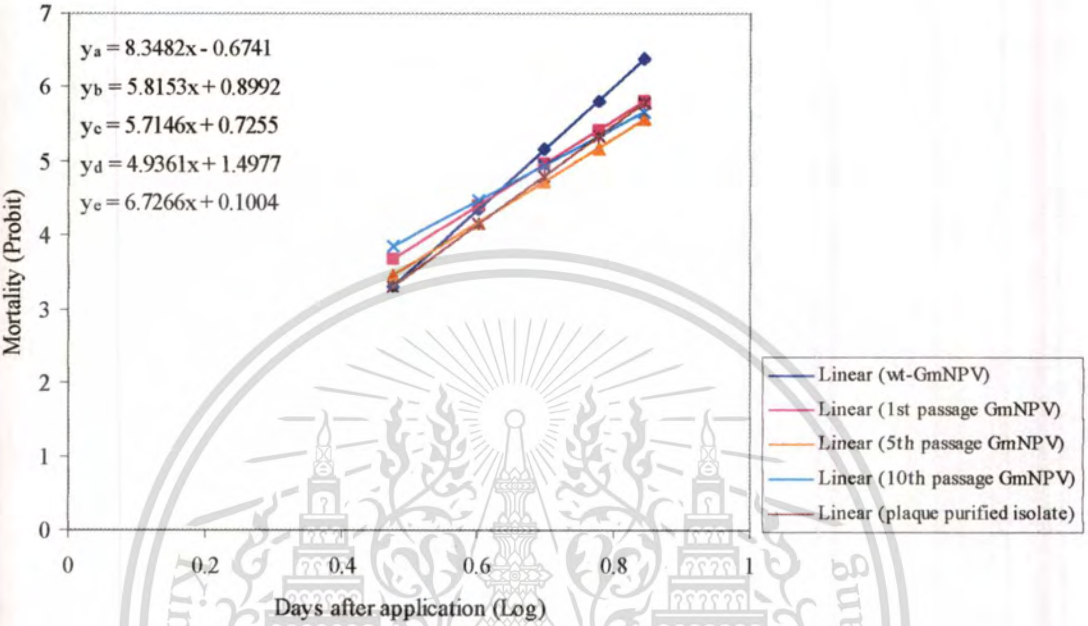

Figure 4.18 Time-mortality response regression lines for 2nd stage larvae of *Plutella xylostella* treated with 5 isolates of *Gm*NPV.

y_a = Regression line of the wild isolate

y_b = Regression line of the 1st passage isolate

y_c = Regression line of the 5th passage isolate

y_d = Regression line of the 10th passage isolate

y_e = Regression line of the plaque-purified isolate

Table 4.6 Mean median lethal time values (LT$_{50}$s) of the 1st passage isolate, 5th passage isolate, 10th passage isolate, plaque-purified isolate and wild isolate of *Gm*NPV for 2nd stage larvae of *Plutella xylostella.*

Virus isolate	Slope	LT$_{50}$ (days)	95% Fiducial limits
Wild type isolate	8.3507	4.8^{a}	4.4-5.2
1st passage isolate	5.8834	5.1^{a}	4.6-5.6
5th passage isolate	5.6741	5.6^{a}	5.1-6.4
10th passage isolate	4.9361	5.1^{a}	4.6-5.8
Plaque-purified isolate	6.8297	5.4^{a}	4.9-5.9

Means followed by the same letter were not significantly different ($P>0.05$, one-way ANOVA; LSD test)

CHAPTER 5

DISCUSSION

In this study, serial passage of *Gm*NPV has been investigated in various aspects of replication and potency to determine its stability and suitability for the use as biopesticide. Based upon the previous studies, serial passage of several NPVs in cell/virus system resulted in the generation of mutation at high frequency. Some genetic modifications resulted from the alteration within viral genome such as deletions and insertions of DNA in essential gene [73, 74, 75, 76, 77, 78]. However, the results obtained from serial passage study will be useful for the development of bioinsecticides in large-scale production in order to avoid problems associated with serial passage effect.

Serial passage of *Gm*NPV resulted in significant reductions in percentage of infection and polyhedra production, which were apparent after 10 passages in cell culture. Also, the titer of ECV production was increased significantly by the 9th and 10th passages. All of these observations were quite similar to the previous reports [16, 17, 18, 19, 20, 21, 22, 23]. Namely, the reduction in polyhedra production decreased while passage level was increased. These results indicated that the normal virus was degenerated into the defective forms, which produced low yield of polyhedra. The generation of these mutants during serial passage was known to be the serial passage effect when viruses were passaged several times *in vitro* by using ECV. Effects of serial passages of *Gm*NPV in Sf9 cells adapted to serum free-medium were also recently investigated. Despite the fact that stability of recombinant protein expression over 18 serial passages was noted, serial passages of the *Gm*NPV *in vitro* resulted as stated in our work, in abnormality of polyhedra morphology, and occlusion of virions as well as decrease of polyhedra production and cell infection rate in serum-free condition [88, 89]

Serial passage of *Gm*NPV in cell culture or larvae by using ECV as virus inoculum always produces spontaneous mutants, which produce fewer polyhedra than the wild type virus [20]. These mutants termed as FP (few polyhedra) mutants usually detected within three or four passages and became predominant in cell culture by the 6th passage. The electron microscopic observation indicated that most of the polyhedra produced by FP mutants were devoid of nucleocapsid, whereas some contained fewer nucleocapsids.

It was found in the present study that the steep reduction in percentage of infection involved with more than one class of virus. MacKinnon *et al.* [17] reported the presence of another class of mutants termed defective interfering particles, which have been correlated with the interfering of normal virus replication and the reduction in virulence of the virus. Kool *et al.* [79] suggested that the presence of defective interfering particles is perhaps associated with the formation of FP mutants in that the insertion sequences in FP mutants are located within the deletion sequences of defective interfering particles.

It is well known that serial passage in cell culture frequently leads to generate spontaneous mutations at high frequency. In this regard, changes in virulence of polyhedra for target insect seem to be the major problem and have been identified in several types of viruses. As described by Fraser and Hink [20] polyhedra obtained from *FP* infected larvae were 350 times less virulence than *MP* polyhedra. The reduction in virulence may be because *FP* polyhedra contained few or no virion inside.

Fraser and Hink [20] used electron microscopy to investigate the reason for the reduction in virulence of polyhedra derived from FP mutants of *Gm*NPV. They found that FP infections proceed with a minimum amount of de novo membrane synthesis in the nucleus. Hence, most progeny nucleocapsids are released from the nucleus into the cytoplasm instead being occluded within polyhedra. Primary membrane acquisition for the cell-released nonoccluded virus of the FP mutants occurs by budding from the cell membrane. This would explain the smaller yield of occlusion bodies produced and the decrease in virulence of polyhedra to larvae from virus at the higher passage level as observed in these experiments. In contrast, MP infection produces many nucleocapsids in the nucleus indicating that a high degree of de novo membrane synthesis takes place.

Since after the 10th passage, total number of polyhedra production was decreased significantly and there were several reports on the low infectivity of polyhedra obtained from the late passage level [16, 17, 18, 19, 20, 21, 22, 23]. Thus, only the polyhedra recovered from certain passage including 1st, 5th, 10th passages were used in bioassay study. The data obtained from comparative bioassay of *Gm*NPV five isolates (1st passage isolate, 5th passage isolate, 10th passage isolate, plaque-purified isolate and wild isolate) indicated that *Plutella xylostella* larvae are susceptible to *Gm*NPV infection in which the infected larvae has shown the symptoms of NPV infection. Based on the LC_{50} values, the wild isolate of *Gm*NPV showed highest in virulence to 2nd stage larvae of *Plutella xylostella* with the LC_{50} value of 19,387.61 OBs/cm^2 when compared with other four isolates. These differences were significantly different when statistical analyzed using ANOVA model ($P<0.05$). Among the three passaged isolates, the virulence of polyhedra derived from the 1st passage was found to be highest with the LC_{50} of 56,795.86 OBs/cm^2 while the 5th and 10th passaged isolates showed indifferent in LC_{50} values (67,494.15 OBs/cm^2 and 68,899.53 OBs/cm^2, respectively). However, among these passaged isolates the statistical analysis showed no significant differences in LC_{50} values. In this case it was probably due to the accumulation of *FP* mutants was not notable during 1st to 10th passages.

According to the plaque-purification study, there was one plaque-purified isolate designated as P-10 exhibited high productivity of ECV and polyhedra. Because of the notable increase in ECV production this plaque-purified isolate was suspected to enhance the rate of systemic transmission within the insect. Consequently, this plaque-purified isolate was submitted to bioassay study in order to test the hypothesis. The data obtained from bioassay indicated that the virulence of P-10 isolate was higher than those passaged isolates. Although, it was considered to be no significant differences among the plaque-purified isolate and the three passaged isolates. From this result, it may postulated that the plaque-purified isolate was undergone some genetic modifications that influences the level of virus production. However, the mechanism involved the generation of this plaque-purified isolate was not clearly understood.

The comparison of infectivity of different virus isolates for *Plutella xylostella* obtained from both current and previous studies were shown in Table 5.1. The concentration-mortality responses indicated that the plaque-purified isolate (*Px*NPVCL3) obtained from a new baculovirus isolate recovered from infected larvae of *Plutella xylostella* was most pathogenic to 1st stage larvae of *Plutella xylostella* (LC_{50} = 5.54 OBs/cm^2) followed by *Af*NPV (*Anagrapha falcifera* nuclear polyhedrosis virus) (LC_{50} = 9,224.48 OBs/cm^2) and *Ac*NPV (LC_{50} = 11,600.20 OBs/cm^2 [30]. Based on the infectivity bioassay performed by Abdul Kadir [15] he found that the pathogenicity of *Gm*NPV and *Ac*NPV were indifferent. As this result, it may conclude that *Gm*NPV have moderate virulence in controlling *Plutella xylostella* which should be ranked between *Af*NPV and *Ac*NPV. However, the developmental stage of the tested larvae was different but the comparison can be done. Despite the fact that the increasing age of tested larvae will affect the susceptibility of *Plutela xylostella* to NPVs, it is true that the concentration needed to kill 50 percent of 2nd stage larvae of *Plutella xylostella* should be higher.

Table 5.1 Infectivity of various virus isolates for *Plutella xylostella*.

Virus isolate	Slope	LD$_{50}$ (OBs/cm^2)	95% Fiducial limits
*Px*NPVCL3[a]	0.60	5.54	3.10-8.91
*Af*NPVCL1[a]	0.82	9,224.48	6,480.63-13,229.39
*Ac*NPV[a]	1.02	11,600.20	8,534.90-15,776.60
*Gm*NPV (wild isolate)[b]	1.32	19,387.61	13,698.40-27,673.53
1st passaged *Gm*NPV [b]	1.21	56,795.86	38,950.40-87,917.96
5th passaged *Gm*NPV [b]	0.98	67,494.15	42,801.71-119,998.60
10th passaged *Gm*NPV [b]	1.18	68,899.53	46,284.26-110,653.70
Plaque-purified isolate of *Gm*NPV [b]	1.12	47,116.38	31,789.84-73,968.02

[a] Infectivity bioassay was performed by using 1st stage larvae of *Plutella xylostella* [20].

[b] Infectivity bioassay was performed by using 2nd stage larvae of *Plutella xylostella* [current study].

Based on the LT$_{50}$ values, the average incubation period of *Gm*NPV infection in 2nd stage larvae of *Plutella xylostella* was 5.2 days after ingestion of polyhedra. It was found that all isolates of *Gm*NPV appeared to kill the larvae at similar rate. This result was confirmed by the statistical analysis, which resulted in the nonsignificant differences in these values. Abdul Kadir [15] found that LT$_{50}$ values of *Gm*NPV were dose-dependent from the lowest to the highest doses. However, in the present study it was found that in the higher doses the increasing number of polyhedra had little effect on LT$_{50}$ value.

Although several NPVs have been reported to be pathogenic to *Plutella xylostella* including *Ac*NPV, *Gm*NPV [12, 13, 14, 15, 30], *Af*NPV and *Px*NPVCL3 [31] up to now no one has used these viruses for controlling *Plutella xylostella* which is epidemic in Thailand. This current study is first report on the usage of *Gm*NPV and its plaque-purified isolate to control *Plutella xylostella* in Thailand. Further study, large-scale production of *Gm*NPV based biopesticide media should be developed to obtain easily prepared and low-cost formulation. In addition, long-term passage of *Gm*NPV by using ECV is not recommended because the infectivity and polyhedra production of this virus began to decline after 10 passages. Usage of alkaline solution or alkaline solution and insect gut fluid could diminish such problems but it still led to the reduction in polyhedra production when passage level was increased [90]. However, the reduction in polyhedra production was observed in lesser degree than that passage with ECV. Fraser and Hink [20] suggested that serial passage of *Gm*NPV in insect host by using PDV could also avoid serial passage effect.

Furthermore, in attempt to use *Gm*NPV as biocontrol agent several trials should be carried out including formulation, host range determination and field application study. In Thailand NPVs were accepted, two NPVs have already been used in integrated pest management programs for long time including NPV of *Spodoptera exigua* and *Heliothis armigera*. These viruses have been developed and produced by Department of Agriculture in Bangkok using laboratory-reared larvae. The viral preparations have been used successfully in controlling of *Spodoptera exigua* and *Heliothis armigera* in several agricultural crops by the distribution from the agricultural extension service.

CHAPTER 6

CONCLUSION

It was shown in present study that long termed serial passage of *Galleria mellonella* nuclear polyhedrosis virus (*Gm*NPV) in *Spodoptera frugiperda* cell line (Sf9) had led to the significant reductions in percentage of infection and the polyhedra production after 10 passages. Also, the significant increase in the ECV production was observed in the 9th passage and 10th passage. Besides, the cytopathology study of infected cells by electron microscope had revealed that the morphological abnormality of polyhedra was observed at high frequency after the 10 serial passages. Since the significant reduction in polyhedra production was observed after the 10th passage only the polyhedra derived from particular isolates of *Gm*NPV including the 1st passage islate, the 5th passage isolate, 10th passage, plaque-purified islate and wild isolate were subjected to comparative bioassay. The data obtained from bioassay study indicated that the wild isolate of *Gm*NPV was most pathogenic to 2nd stage larvae of *Plutella xylostella* (LC_{50} = 19,387.61 OBs/cm^2) when compared with the plaque-purified isolate (LC_{50} = 47,116.38 OBs/cm^2), the 1st passage isolate (LC_{50} = 56,795.86 OBs/cm^2), the 5th passage isolate (LC_{50} = 67,494.15 OBs/cm^2), and the 10th passage isolate (LC_{50} = 68,899.53 OBs/cm^2) ($P<0.05$). Based on the LT_{50} values *Gm*NPV in all isolates appeared to kill larvae at similar rate ($P>0.05$) with the average incubation time of 5.2 days after ingestion of the leaf disk contaminated with polyhedra. The fastest incubation time was observed in the wild isolate (4.8 days) followed by the 1st passage isolate (5.1 days), the 10th passage isolate (5.1 days), the plaque-purified isolate (5.4 days) and the 10th isolate (5.6 days).

The results obtained from present study suggested that long-term passage of *Gm*NPV by using ECV is not recommended because the infectivity and polyhedra production of this virus began to declined after 10 passages. In order to avoid these problems, which was known to be the result of passage effect usage of PDV derived from the passage of *Gm*NPV in homologous or heterologous insect host was recommended. For further study, large-scale production of *Gm*NPV based biopesticide the media should be developed to obtain easily prepared and low-cost formulation. Moreover, in attempt to develop *Gm*NPV as biocontrol agent several experiments should be carried out including formulation, host range determination and field application study.

REFERENCES

[1] Luck R.F., Van den Bosch R., and Garcia R. "Chemical insect control a troubled pest management strategy." *Biological Science* 1977 ; 8 : 601-611.

[2] Boraiko A.A. "The pesticide dilemma." *National Geographic* 1980 ; 157 : 145-183.

[3] Carter J.B. "Virus as pest control agents." *Biotechnology and Genetic Engineering Review* 1984 ; 1 : 375-405.

[4] Bonnemaison L. "Insect pests of crucifers and their control." *Ann. Rev. Entomol.* 1965 ; 10 : 233-256.

[5] Shelton A.M., Wyman J.A., Cushing N.L., Apfelbeck K., Dennehy T.J., Mahr S.E.R., and Eigenbrode S.D. "Insecticide resistance of diamondback moth, *Plutella xylostella* (lepidoptera : Plutellidae), in North America." *J. Econ. Entomol.* 1993 ; 86 : 11-19.

[6] Shelton A.M., Robertson J.L., Tang J.D., Perez C., Eigenbrode S.D., Preister H.K., Wilsey W.T., and Cooley R.J. "Resistance of diamondback moth (Lepidoptera : Plutellidae) to *Bacillus thuringiensis* subspecies in the field." *J. Econ. Entomol.* 1993 ; 86 : 697-705.

[7] Talekar N.T. and Shelton A.M. "Biology, ecology and management of the diamondback moth." *Ann. Rev. Entomol.* 1993 ; 38 : 275-301.

[8] Yearian W.C. and Young S.Y. " Control of insect pests of agricultural importance by viral insecticides," 387-423. *In* Kurstak E. (ed.). *Microbial and viral insecticides*. New York : Marcel Dekkar. 1982.

[9] Hubner J. "Use of baculoviruses in pest management programs," 181-202. *In* Granados R.R. and Federici B.A. (eds.). *The biology of baculoviruses vol. II.* Florida : CRD press. 1986.

[10] Payne C.C. "Insect pathogenic viruses aspect control agents," 183-200. *In* Franz J.M. (ed.). *Biological plant and health protection : Biological control of plants and of vectors of human and animal diseases. progress in zoology vol. 32.* Stuttgart : Gustav Fischer Verlag. 1986.

[11] Weiss S.A., Thomus W.K., Dunlop B.F., Georgis R., Vail P.V., and Hoffman D.F. "*In vitro* production of viral pesticides : Key elements," 57-63. *In* Monsour C.J., Reid S., and Teakle R.E. (eds.). Opportunities for Australian industry :

Proceeding of the 1st Brisbane symposium biopesticides. Australia : CSIRO. 1994.

[12] Vail P.V., Hunter D.K., and Jay D.L. "Nuclear polyhedrosis of the diamondback moth, *Plutella xylostella*." *J. Invertebr. Pothol.* 1972 ; 20: 216-217.

[13] Biever K.D. and Andrews P.L. "Susceptibility of lepidopterous larvae to *Plutella xylostella* nuclear polyhedrosis virus. *J. Invertebr. Pathol.* 1984 ; 44 : 117-119.

[14] Abdul Kadir H.B. and Payne C.C. "Relationship of *Plutella xylostella* nuclear polyhedrosis virus (NPV) to NPV of *Galleria mellonella*." *J. Invertebr. Pathol.* 1989 ; 53 : 113-116.

[15] Abdul Kadir H.B. "Potential of several baculoviruses for the control of diamondback moth and *Crocidolomia binotalis* on cabbages," 205-212. *In* Talekar N.S. (ed.). *Diamondback moth and other cruciferous pests* : Proceedings of the 1st international workshop. Asian Vegetable Research and Development Center, Tainan, Taiwan. 1992.

[16] Hink W.F., and Strauss E. "Replication and passage of alfalfa looper nuclear polyhedrosis virus plaque variants in cloned cell cultures and larval stages of four host species." *J. Invertebe. Pathol.* 1976 ; 27 : 49-55.

[17] MacKinnon E.A., Henderson J.F., Stoltz D.B., and Faulkner P. "Morphogenesis of nuclear polyhedrosis virus under conditions of prolonged passage *in vitro*." *J. Ultrastruct. Res.* 1974 ; 49 : 419-435.

[18] Potter K.N., Faulkner P., and MacKinnon E.A. "Strain selection during serial passage of *Trichoplusia ni* nuclear polyhedrosis virus." *J. Virol.* 1976 ; 18 : 1040-1050.

[19] Potter K.N., Jaques R.P., and Faulkner P. "Modification of *Trichoplusia ni* nuclear polyhedrosisvirus passaged *in vivo*." *Intervirology* 1978 ; 9 : 76-85.

[20] Fraser M.J. and Hink W.F. "The isolation and characterization of the *MP* and *FP* plaque variants of *Galleria mellonella* nuclear polyhedrosis virus." *Virology* 1982 ; 117 : 336-378.

[21] Slavicek J.M., Podgwaite J., Lanner-Herrera C. "Properties of *two Lymantria dispar* nuclear polyhedrosis virus isolates obtained from the microbial pesticide Gypchek." *J. Invertebr. Pathol.* 1992 ; 59 : 142-148.

[22] Slavicek J.M., Hayes-Plazelles N., and Kelly M.E. "Rapid formation of few polyhedra mutants of *Lymantria dispar* multinucleocapsid nuclear polyhedrosis virus during serial passage in cell culture." *Biol. Control.* 1995 ; 5 : 251-261.

[23] Chakraborty S., and Reid S. "Serial passage of a *Helicoverpa armigera* nucleopolyhedrovirus in *Helicoverpa zea* cell cultures." *J. Invertebr. Pathol.* 1999 ; 73 : 303-308.

[24] Shapiro M. and Ignoffo C.M. "Nucleopolyhedrosis of *Heliothis* : Activity of isolates from *Heliothis zea*." *J. Invertebr. Pathol.* 1970 ; 16 : 107-111.

[25] Maleki-Milani N. "Influence de passages repetes du virus de la polyhedrose nuleaire de *Autographa californica* chez *Spodoptera litoralis* (Lep; Noctuidae). *Entomophaga*. 1978 ; 23 : 217-224.

[26] Shapiro M., Martignoni M.E., Cunningham J.C., and Goodwin R.H. "Potential use of the saltmarsh caterpillar as a production host for nucleopolyhedrosis viruses." *J. Econ. Entomol.* 1982 ; 75 : 69-71.

[27] Shapiro M., Lynn D.E., and Dougherty E.M. "More virulent of biotypes isolated from wild-type virus." *United States Patent* 1992 ; 5 : 132, 220.

[28] Matignoni M.E. and Iwai P.J. "Propagation of multicapsid nuclear polyhedrosis virus of *Orgyia pseudotsugata* in larvae of *Trichoplusia ni*." *J. Invertebr. Pathol.* 1986 ; 47 : 32-41.

[29] Slavicek J.M., Mercer M.J., Kelly M.E., and Hayes-Plazolles N. "Isolation of a baculovirus variant that exhibits enhanced polyhedra production stability during serial passage in cell culture." *J. Invertebr. Pathol.* 1996 ; 67 : 153-160.

[30] Kolody-Hirsch D.M. and Van Beek N.A.M. "Selection of morphological variant of *Autographa californica* nuclear polyhedrosis virus with increased virulence following serial passage in *Plutella xylostella*." *J. Invertebr. Pathol.* 1997 ; 69 : 205-211.

[31] Kariuki C.W. and McIntosh A.H. 'Infectivity studies of a new baculovirus isolate for the control of the diamondback moth (Plutellidae: Lepidoptera)." *J. Econ. Entomol.* 1999 ; 92(5) : 1093-1098.

[32] Hardy J.E. "*Pluella maculipennis* Curt., its natural and biological control in England." *Bull. Entomol. Res.* 1938 ; 29 : 343-372.

[33] Harcourt D.G. "Biology of the diamondback moth, *Plutella maculipennis* (Curt.) (Lepidoptera: Plutellidae), in eastern Ontario." *Rpt. Quebec Soc. Prot. Plants.* 1955 ; 37 : 155-160.

[34] Harcourt D.G. "Biology of the diamondback moth, *Plutella maculipennis* (Curt.) (Lepidoptera: Plutellidae), in eastern Ontario. II. Life-histry, behavior, and host relatioships." *Can. Entomol.* 1957 ; 89 : 554-564.

[35] Rushtapakornchai W. and Vuttanatangum A. "Present status of insecticidal control of diamondback moth in Thailand," 307-312. *In* Griggs T.G. and Talakar N.S.(eds.). *Diamondback moth management.* AVRDC, Taiwan. 1986.

[36] Ho T.H. "The life-history and control of the diamondback moth in Malaya." *Bull. No. 118, Div. of Agric.* 1965 : 26.

[37] Hill D.S. and Walker J.M. *Pests and diseases of tropical crops : Field handbook.* Hong Kong : Longman group (FE). 1990.

[38] Pongprasert V. "Ecological investigation on the diamondback moth, *Plutella xylostella* L. (Lepidoptera; *Plutellidae*) and its natural enemies in Thailand." *M.S. thesis, Kasetsart Univ.*, Bangkok, Thailand. 1985.

[39] Miyata T.H., Kawai H., and Saito T. "Insecticide resistance in the diamondback moth, *Plutella xylostella* L. (Lepidoptera: Yponomeutidae)." *Appl. Entomol. Zool.* 1982 ; 17 : 539-542.

[40] Ankersmith G.W. "DDT-resistance in *Plutella maculipennis* (Curt.) (Lepidoptera) in Java." *Bull. Entomol. Res.* 1953 ; 44 : 421-425.

[41] Sun C.N., Chi H., and Feng H.T. "Diamondback moth resistance to diazinon and methomyl in Taiwan." *J. Econ. Entomol.* 1978 ; 71 : 551-554.

[42] Sudderuddin K.I. and Kok P.F. "Insecticide resistance in *Plutella xylostella* collected from the Cameron highlands of Malaysia." *Rev. Appl. Enomol. Ser. A.* 1979 ; 67(7) : 349.

[43] Cheng E.Y. "Insecticide resistance study in *Plutella xylostella* L. II. a survey (1980-81)." *J. Agri. Res. China* 1981 ; 30 : 277-284.

[44] Liu M.Y., Tzeng L.J., and Sun C.N. Insecticide resistance in the diamonback moth." *J. Econ. Entomol.* 1982 ; 75 : 153-155.

[45] Ohasawa S., Toyama Y., and Saito M. "Insecticides susceptibility of the diamondback moth in Fukushima prefecture." *Rev. Appl. Entomol. Ser. A.* 1985 ; 73(1) : 876.

[46] Telekar N.S., Yang H.C., Lee S.T., Chen B.S., and Sun L.Y. (eds.). *Annotated bibliography of diamondback moth.* Asian Vegetable Research and Development Center, Tainan, Taiwan. 1985.

[47] Chen J.S. and Sun C.N. "Resistance of diamondback moth (*Plutella xylostella*) (Lepidoptera : Plutellidae) to a combination of fenvalerate and piperonyl butoxide." *J. Econ. Entomol.* 1986 ; 79(1) : 22-30.

[48] Griggs T.G. and Talekar N.S. (eds.). *Diamondback moth management.* Asian Vegetable Research and Development Center, Tainan, Taiwan. 1986.

[49] Pering F.S., Yao M.C., Hung C.F., and Sun C.N. "Teflubenzuron resistance in diamondback moth." *J. Econ. Entomol.* 1988 ; 8 : 1277-1282.

[50] Murphy F.A., Fauquet C.M., Bishop D.H.L., Ghabrial S.A., Javis A.W., Martinelli G.P., Mayo M.A. and Summers M.D., "Virus taxonomy: Sixty report of the International committee on taxonomy of viruses." *Arch. Virol. Suppl.* 1995 ; 10.

[51] Harrap K. "The structure of nuclear polyhedrosis virus II. The virus particle." *Virology* 1972 ; 50 :124-135.

[52] Fraser M.J. "Ultrastructural observations of virion maturation in *Autographa californica* nuclear polyhedrosis virus infected *Spodoptera frugiperda* cell cultures." *J. Ultrastruc. Mol. Struct. Res.* 1986 ; 95 : 189-195.

[53] Burgess S. "Molecular weights of Lepidopteran baculovirus DNAs : derivation electron microscopy." *J. Gen. Virol.* 1977 ; 37 : 501-510.

[54] Tweeten K.A., Bulla L.A., and Consigli R.A. "Characterization of an extremely basic protein derived from granulosis virus nucleocapsids." *J. Virol.* 1980 ; 33 : 866-876.

[55] Wilson M.E., Mainprize T.H., Friesen P.D., and Miller L.K. "Location, transcription and sequence of a baculovirus gene encoding a small arginine-rich polypeptide." *J. Virol.* 1987 ; 61 : 661-666.

[56] Volkman L.E. "The 64-k envelope protein of budded *Autographa californica* nuclear polyhedrosis virus," 103-118. *In* Doerfler W. and Boehm P. (eds.). *Current topics in microbiology and immunology.* Berlin : Springer-Verlag. 1986.

[57] Cochran M.A., Brawn S.E., and Knudson D.L. "Organization and expression of the baculovirus genome," 108-216. *In* Granados R.R. and Federici B.A.(eds.). *The Biology of baculovirus vol. I.* Florida : CRD Press. 1986.

[58] Stoltz D., Pavan C., and Da Cunha A. "Nuclear polyhedrosis virus: a possible example of *de novo* intranuclear membrane morphogenesis." *J. Gen. Virol.* 1973 ; 19 :145-150.

[59] Harrap K. and Longworth J. "An evaluation of purification methods for baculoviruses." *J. Invertebr. Pathol.* 1974 ; 24 : 55-62.

[60] Granados R.R. and William K. "*In vivo* infection and replication of baculoviruses, 89-108. *In* Granados R.R. and Federici B.A. (eds.). *The Biology of baculovirus vol. I.* Florida : CRD Press. 1986.

[61] Keddie B.A., Aponte G.W., and Volkman L.E. "The pathway of infection of *Autographa californica* nuclear polyhedrosis virus importance for the 64K envelope glycoprotein." *J. Gen. Virol.* 1989 ; 66 : 1195-1200.

[62] O'Reilly D.R., Miller L.K., and Luckow L.A. *Baculovirus expression vector, A laboratory manual.* New York : W.H. Freeman and company. 1992.

[63] Volkman L.E. and Goldsmith P.A. "Mechanism of neutralization of budded *Autographa californica* nuclear polyhedrosis virus by a monoclonal antibody: inhibition of entry by adsorptive endocytosis." *Virology* 1985 ; 143 : 185-195.

[64] Chisholm G.E. and Henner D.J. "Multiple early transcripts and splicing of the *Autographa californica* nuclear polyhedrosis virus IE-1 gene." *J. Virol.* 1988 ; 62 : 3193-3200.

[65] Charlton C.A. and Volkman L.E. "Sequential rearrangement and nuclear polymerization of actin in baculovirus infected *Spodoptera frugiperda* cells." *J. Virol.* 1991 ; 65 : 1219-1227.

[66] Harrap K. "The structure of nuclear polyhedrosis virus III Virus assembly." *Virology* 1973 ; 50 : 133-139.

[67] Kelly D. "Baculovirus replication: electron microscopy of the sequence of infection of *Trichoplusia ni* nuclear polyhedrosis virus in *Spodoptera frugiperda* cells." J. *Gen. Virol.* 1981 ; 52 : 209-219.

[68] Raghow R. and Grace T. " Studies on a nuclear polyhedrosis virus in *Bombyx mori* cells *in vitro.*" *J. Ultrastructure Res.* 1974 ; 47 : 384-399.

[69] Burges H.D. *Microbial control of pests and plant diseases.* London : Academic press. 1981.

[70] Granados R.R. and Federici B.A. (eds.). *The biology of baculoviruses Vol. II.* Florida : CRC press. 1986.

[71] Reardon R., Podgwaite J.P., and Zerillo R.T. "Gypchek-the gypsy moth nucleopolydrosis virus product." *USDA Forest Service Publication FHTET.* 1996 : 96-16.

[72] Maramorosch K. and Sherman K.E. *Viral insecticides for biological control.* Florida : Academic press. 1985.

[73] Fraser M.J., Smith G.E., and Summers M.D. "Acquisition of host cell DNA sequences by baculoviruses: Relationship between host DNA insertion and *FP* mutants of *Autographa californica* and *Galleria mellonella* nuclear polyhedrosis viruses." *J. Virol.* 1983 ; 47 : 287-300.

[74] Fraser M.J., Brusca J.S., Smith G.E., and Summers M.D. "Transposon-mediated mutagenesis of a baculovirus." Virology 1985 ; 145 : 356-361.

[75] Kumar, S. and Miller L.K. "Effects of serial passage of *Autographa californica* nuclear polyhedrosis virus in cell culture." *Virus Res.* 1987 ; 7 : 335-350.

[76] Beames B. and Summers M.D. "Location and nucleotide sequence of the 25K protein missing from baculovirus few polyhedra (FP) mutants." *Virology* 1989; 168: 344-353.

[77] Beames B., and Summers M.D. "Sequence comparison of cellular and viral copies of host cell DNA insertions found in *Autographa californica* nuclear polyhedrosis virus.: *Virology* 1990; 174: 354-263.

[78] Harrison R.L. and Summers M.D. "Mutations in the *Autographa californica* multinucleocapsid nuclear polyhedrosis virus 25kDa protein gene result in reduced virion occlusion, altered intranuclear envelopment and enhance virus production." *J. Gen. Virol.* 1995 ; 76 : 1451-1459.

[79] Kool M., Vonken J.W., Vanlier F.L.J., Tramper J., and Vlak J.M. "Detection and analysis of *Autographa californica* nuclear polyhedrosis virus mutants with defective interfering properties." *Viroloy* 1991 ; 183 : 739-746.

[80] Miller L.K. "The genetics of baculoviruses, 217-238. *In* Granados R.R. and Federici B.A. (eds.). *The Biology of baculovirus vol. I.* Florida : CRD Press. 1986.

[81] Belloncik S. 19__. "Preliminary experiments on virulence of *Gm*NPV produced in cell cultures adapted to serum-free medium to *Plutella xylostella* in Taiwan". Unpublished data.

[82] Miller F.A. and Dawes K.P. "Restriction endonuclease analysis for the identification of the baculovirus pesticides." *Appl. Environ. Microbiol.* 1978 ; 35 : 411-421.

[83] Rovozzo G.C. and Burke C.N. *A manual of basic virological techniques.* New Jersey : Prentice-Hall Inc. 1973.

[84] Karber G. "Eeitrag zur kollektiven behandlung pharmakologischer reihenversuche. " *Arch. Exptl. Pharmakol.* 1931 ; 192 : 480-483.

[85] Evans H.F. "Quantitative assessment of the relationships between dosage and response of the nuclear polyhedrosis virus of *Mamestra brassicae.*" *J. Invertebr. Pathol.* 1980 ; 37 : 101-109.

[86] Finney D.L. *Statistical methods in biological assay.* 3rd ed. London : Charles Griffin and Co. 1978.

[87] SPSS Based 10.0.1 for Windows Application guide. SPSS Inc. 2000.

[88] Michaud J.F. and Belloncik S. "Stabilité de production de baculovirus dans un système cellulaire sans sérum de veau foetal". *68e Congrès de l'Acfas* 15-19 mai 2000 Université de Montréal. 2000.

[89] Michaud J.F. "Réplication de baculovirus sauvage et recombinant dans un systè me cellulaire en absence de sérum de veau faetal". *MSc thesis INRS-Institut Armand-Frappier*, Quebec', Canada. 2001.

[90] Lynn D.E. "Enhanced infectivity of occluded virions of the gypsy moth nuclear polyhedrosis virus for cell cultures". *J. Invertebr. Pathol.* 1994 ; 63 : 268-274.

APPENDIX I

I.1 Preparation of Grace's medium supplemented with 10% FBS

Reagents and solutions	Quantity
Grace's basal insect cell culture medium	428 ml
Yeastolate (1X)	10 ml
Latalbumin hydrolysate (1X)	10 ml
Gentamycin solution	2 ml
Fetal bovine serum	50 ml

Procedures for preparation of Grace's medium suppremented with 10% FBS are as follows:

1.) Preheat the fetal bovine serum in water bath at 56°C for 1 hour (for toxin inactivation).

2.) Dispense 2 ml of gentamycin solution in Grace's basal tissue culture medium.

3.) Filter (1X) yeastolate and (1X) lactalbumin hydrolysate 10 ml each through 0.2 μm membrane filter.

4.) Filter heat-inactivated serum 50 ml through 0.2 μm membrane filter.

5.) Add those filtered ingredients (3. and 4.) in Grace's basal tissue culture medium (2.).

6.) Mix into a homogenous mixture.

7.) Adjust the pH of the complete tissue culture medium to 6.18-6.20.

8.) Check the contamination in complete tissue culture medium if necessary.

I.2 Cryopreservation of cultured insect cells

I.2.1 Freezing insect cells

1.) Prepare desired quantity of cells by harvesting in the mid-logarithumic.

2.) Determine the viable cells count, and calculate the required volume of cryopreservation medium to yield a cell density of $0.5\text{-}1.0\times10^7$ cells/ml.

3.) Prepare that volume of freeze medium consisting of 7.5% DMSO in 50% fresh medium and 50% conditioned medium. Hold this preparation at 4°C.

4.) Centrifuge cells from culture medium at 100g for 6min. Resuspend pellet in the determine volume of 4°C cryopreservation medium.

5.) Incubate cell suspension at 4°C until well chilled.

6.) Dispense aliquots of this suspension to cryovials.

7.) Freeze the cryovials gradually in 20°C freezer for 2 hours followed by -70°C freezer (these cryovials can kept 3 months long) or further freeze in liquid nitrogen for long-term preservation (Frozen cells are stable indefinitely in liquid nitrogen storage).

I.2.2 Recovery of viable cells from frozen culture

1.) Recover cultures from frozen storage by rapidly thawing a vial of cells in a water bath at 28 ± 0.5 °C followed by rapid aseptic transferring of the entire contents of the vial into a complete cell culture medium.

2.) Maintain culture between 3×10^5 and 1×10^6 cells/ml for the first two subcultures after recovery; thereafter returning to the normal maintenance schedule.

I.3 Staining method for light microscopy

Among the many possible strains for light microscopy, two principal methods are commonly employed to aid diagnosis of viruses. Particularly those producing occlusion bodies. Buffalo Black and Giemsa's stain offer simplicity in use and rapid diagnosis without the necessity for complex fixation and mounting procedures. Both are virtually permanent stains, enabling slides to be stored in light-tight containers for many years without significant loss of detail.

Buffalo Black stains protein blue-black and thus is a positive stain that allows crystalline protein to be distinguished from a range of different backgrounds. Giemsa's stain is, for the majority of occluded viruses, a negative stain in that the Buffalo Black can remain unstained while the background stains in blues and reds. It is a particularly useful "all-round" stain that can aid diagnosis of some bacteria, fungal spores and particularly, Microsporidia as well as occluded insect viruses.

I.3.1 Buffalo Black staining

Buffalo black is also known as Buffalo Black 12B or Naphthalene Black 12B or Amido Schwartz or Acid Black 1. Procedures for Buffalo Black staining are as follows:

1.) Make a thin smear of dead insect covering the width of the slide.

2.) Air dry the preparation to be stained.

3.) Heat the Buffalo Black solution to 40-45 °C in a staining rack on hotplate.

4.) Immerse the slide in the Buffalo Black solution for 5 minutes.

5.) Wash the slide under the running tap water for 10 seconds.

6.) Dry the slide and examine under oil immersion for the presence of occlusion bodies.

<u>Preparation of the Buffalo black solution</u> (total volume 100 ml):

1.) Mix these following solutions together:

Buffalo black	0.1 g
Absolute methanol	50 ml
Distilled water	20 ml
Glacial acetic acid	30 ml

2.) Stir for 1-2 hour(s) until all solutions are well mixed.

I.3.2 Giemsa's staining

Giemsa's stain is a differential stain that clearly distinguishes nuclear and cytoplasmic cellular details and, aids in the diagnosis of site of replication of various virus groups. Procedures for Giemsa's staining are as follows:

1.) Make a thin smear of dead insect covering the width of the slide.

2.) Immerse slides with air dried smears for 2 minutes in Giemsa's fixative.

3.) Rinse slides under running tap water for 10 seconds.

4.) Stain for 45 minutes in 10% Giemsa stain in 0.02M phosphate buffer, pH 6.9.

5.) Rinse under running tap water for 10 seconds. If the slide appears to be very red (overstained) immerse in 0.02M buffer until the red color disappears.

6.) Air dry the slide and examine under oil immersion.

APPENDIX II

Probit analysis

Probit analysis is the log-normal transformation of the data to enable the sigmoid curve dosage-response curve to be linearized and and compared for LC_{50} and slope value. Analysis of normally uses a maximum likelihood procedure to estimate the LC_{50} value iteratively using the basic probit transformation initially and then a set of calculated probits from the transformed curve. Iterations continue until the values and their standard error stabilize. Fiducial limits (normally at the 95% level) and the degree of heterogeneity (based on chi-square estimation) are then determined.

Determination of LC_{50} value:

The steps in computing the estimation of the probit regression line are as follow:

1.) Column x, the logarithms (base 10) of concentrations which were selected.

2.) Column n, the number of tested insects corresponds to each concentration (column x) and column r, the number of tested insects respond to each concentration. These data were recorded during the experiment.

3.) Column p, the mortality percentage (empirical response) corresponds to each concentration.

4.) Each mortality percentage was converted to probit by using probit transformation table.

5.) The probits (Y axis) were plotted against log-concentrations (X axis) on the probit-log paper and a probit regression line was drawn freehand.

6.) Read the expected probit (Y) on the Y axis at each tested concentration.

7.) From each expected probit, the weighting coefficient (W) can be obtained from weighing coefficient table.

8.) The next two columns, nW and nWx are the result of multiplication of data in columns n, W and x.

9.) At each tested concentration, expected probit (Y) and mortality percentage were used to determine the working probit (y) by using working probit table.

10.) Each value of working probit was used to determine the nWy value and put in column nWy.

11.) Compute:

Σ nW = the sum of column nW

Σ nWx = the sum of column nWx

Σ nWy = the sum of column nWy

$S_1 = \Sigma nWx^2$ = the sum of the products of the elements in columns x and nWx.

$S_2 = nWxy$ = the sum of the products of corresponding in columns nWx and y.

$S_3 = \Sigma nWxy^2$ = the sum of the products of corresponding in columns y and nWy.

$S_{xx} = S_1 - (\Sigma nWx)^2/\Sigma nW$

$S_{xy} = S_2 - (\Sigma nWx)(\Sigma nWy)/\Sigma nW$

$S_{xy} = S_3 - (\Sigma nWy)^2/\Sigma nW$

12.) Compute:

$b = S_{xy}/S_{xx}$

$\overline{x} = \Sigma nWx/\Sigma nW$

$\overline{y} = \Sigma nWy/\Sigma nW$

The relationship between probit and concentration may be written:

$Y = \overline{y} + b(x - \overline{x})$..................................①

13.) In order to test the goodness of fit of the linear to the data, the χ^2-test is used. The χ^2-test goodness of fit are as follows:

$$\chi^2 = Syy - (Sxy)^2/Sxx$$

Criteria for conclusion:

- If χ^2 calculated $\leq \chi^2_{1-\infty}$, k-2 (k = number of selected concentrations), the line adequately represent the data.
- If χ^2 calculated $\geq \chi^2_{1-\infty}$, k-2 then the straight line does not adequately represent describe the relationship between stimulus and response.

14.) After the statistical prove of the fitted line, the estimation of LC_{50} is:

$m = \overline{x} + 1/b\,(5 - \overline{y})$

The compute variance of estimate LC_{50} is:

$S_m^2 = 1/b^2\{1/\Sigma nW + (m-\overline{x})^2/Sxx\}$

The 95% confidence interval estimate of LC50 is the interval of m ± 1.96 S_m.

Determination of LT_{50} value:

Relationships between dosage and mortality response are measure in terms of absolute and by reference to the time taken to reach a given response. The later, usually concerned with the LT_{50} value, is a useful measure of the rate of expression of response and can be used to compare assays where LD_{50} may be similar. Analysis of the results as a plot of probit value against time can be used to visually estimate the LT_{50} value in the same way as the LC_{50} value.

APPENDIX III

III.1 Statistical analysis of percentage of infection during serial passage *Gm*NPV in Sf9 cells for 20 passages.

III.1.1 Descriptives

Passage	N	Mean	S.D.	S.E.	95% C.I. for Mean Lower Bound	95% C.I. for Mean Upper Bound	Min	Max
1	4	69.75	5.93	2.97	60.31	79.19	65.50	78.30
2	4	71.50	6.41	3.21	61.30	81.70	66.70	80.80
3	4	64.95	3.78	1.89	58.94	70.96	60.00	68.80
4	4	49.10	3.74	1.87	43.15	55.05	43.80	52.60
5	4	62.43	5.97	2.99	52.93	71.92	55.60	70.00
6	4	58.25	9.07	4.53	43.82	72.68	49.00	67.00
7	4	57.80	7.04	3.52	46.59	69.01	50.00	67.00
8	4	40.75	6.34	3.17	30.65	50.85	33.00	48.00
9	4	42.00	6.06	3.03	32.37	51.64	33.00	46.00
10	4	47.68	4.39	2.20	40.69	54.67	43.50	53.40
11	4	21.25	4.27	2.14	14.45	28.05	16.00	26.00
12	4	11.25	2.50	1.25	7.27	15.23	8.00	14.00
13	4	8.50	1.73	0.87	5.74	11.26	7.00	10.00
14	4	9.35	1.73	0.87	6.60	12.11	7.40	11.60
15	4	12.95	2.99	1.49	8.20	17.70	9.40	15.80
16	4	9.03	1.74	0.87	6.25	11.80	6.80	13.70
17	4	10.88	2.24	1.12	7.32	14.431	8.80	13.70
18	4	8.03	1.10	0.55	6.27	9.78	6.80	9.10
19	4	11.25	2.50	1.25	7.27	15.23	8.00	14.00
20	4	9.58	0.99	0.50	7.99	11.16	8.80	11.00
Total	80	33.81	24.42	2.73	28.38	39.25	6.80	80.80

III.1.2 ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	45828.13	19	2412.01	113.73	.00
Within Groups	1272.48	60	21.21		
Total	47100.61	79			

III.2 Statistical analysis of polyhedra production during serial passage *Gm*NPV in Sf9 cells for 20 passages.

III.2.1 Descriptives

Passage	N	Mean	S.D.	S.E.	95% C.I. for Mean Lower Bound	95% C.I. for Mean Upper Bound	Min	Max
1	3	6.80	0.30	0.17	6.06	7.52	6.50	7.10
2	3	6.53	0.25	0.15	5.91	7.16	6.30	6.80
3	3	6.07	0.25	0.15	5.44	6.69	5.80	6.30
4	3	6.07	0.25	0.15	5.44	6.69	5.80	6.30
5	3	6.20	0.10	0.06	5.95	6.45	6.10	6.3
6	3	6.10	0.27	0.15	5.44	6.76	5.80	6.30
7	3	6.07	0.25	0.15	5.44	6.69	5.80	6.30
8	3	6.47	0.15	0.09	6.09	6.85	6.30	6.60
9	3	6.07	0.25	0.15	5.44	6.69	5.80	6.30
10	3	6.07	0.25	0.15	5.44	6.69	5.80	6.30
11	3	4.10	0.10	0.06	3.85	4.35	4.00	4.2
12	3	5.07	0.25	4.44	4.44	5.69	4.80	5.30
13	3	5.10	0.10	0.06	4.85	5.35	5.00	5.20
14	3	4.80	0.20	0.12	4.30	5.30	4.60	5.00
15	3	4.80	0.20	0.12	4.30	5.30	4.60	5.00
16	3	4.93	0.15	0.09	4.55	5.31	4.80	5.10
17	3	4.43	0.25	0.15	3.81	5.06	4.20	4.70
18	3	4.60	0.10	0.06	4.35	4.85	4.50	4.70
19	3	4.87	0.21	0.12	4.35	5.38	4.70	5.10
20	3	4.40	0.10	0.06	4.15	4.65	4.30	4.50
Total	60	5.48	0.84	0.11	5.26	5.69	4.00	7.10

III.2.2 ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	39.91	19	2.10	47.38	0.00
Within Groups	1.77	40	0.04		
Total	41.68	59			

III.3 Statistical analysis of ECV production during serial passage *Gm*NPV in Sf9 cells for 20 passages.

III.3.1 Desriptives

Passage	N	Mean	S.D.	S.E.	95% C.I. for Mean		Min	Max
					Lower Bound	Upper Bound		
1	4	8.15	0.24	0.12	7.77	8.53	7.80	8.30
2	4	9.38	0.60	0.30	8.43	10.32	8.50	9.75
3	4	7.52	0.28	0.14	7.08	7.96	7.20	7.88
4	4	7.45	0.24	0.12	7.07	7.83	7.20	7.70
5	4	7.83	0.26	0.13	7.41	8.24	7.60	8.20
6	4	7.26	0.39	0.20	6.64	7.88	6.90	7.75
7	4	7.10	0.29	0.15	7.36	8.57	7.80	8.50
8	4	9.22	0.21	0.11	8.88	9.56	9.00	9.50
9	4	11.00	0.81	0.41	9.71	12.29	10.00	11.80
10	4	14.15	0.31	0.16	13.66	14.65	13.80	14.50
11	4	7.32	0.22	0.11	6.97	7.66	7.13	7.63
12	4	6.44	0.49	0.24	5.67	7.21	5.75	6.88
13	4	6.41	0.38	019	5.81	7.01	6.00	6.88
14	4	6.32	0.30	0.15	5.85	6.79	5.88	6.50
15	4	5.88	0.62	0.31	4.89	6.87	5.38	6.75
16	4	6.18	0.12	0.06	5.99	6.36	6.00	6.25
17	4	5.63	0.10	0.05	5.47	5.79	5.50	5.75
18	4	5.19	0.07	0.04	5.08	5.30	5.13	5.25
19	4	6.88	0.14	0.07	6.65	7.11	6.75	7.00
20	4	6.15	0.17	0.08	5.88	6.42	5.90	6.25
Total	80	7.62	2.07	0.23	7.16	8.08	5.13	14.50

III.3.2 ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	331.16	19	17.43	132.57	0.00
Within Groups	7.89	60	0.13		
Total	339.05	79			

III.4 Statistical analysis of LC$_{50}$ values of *Gm*NPV 5 isolates obtained from bioassay study.

III.4.1 Descriptives

Isolate	N	Mean	S.D.	S.E.	95% C.I. for Mean Lower Bound	95% C.I. for Mean Upper Bound	Min	Max
1	3	19,388	4,394	2,632	8,462	31,286	14,748	23,620
2	3	56,796	20,261	11,698	9,606	110,266	40,393	80,845
3	3	67,494	23,151	13,366	16,844	131,865	49,416	95,163
4	3	68,900	16,521	9,539	29,661	111,743	56,363	88,769
5	3	47,116	11,460	6,616	16,363	73,298	34,544	57,182
Total	15	55,816	24,893	6,427	40,154	67,725	14,748	95,163

(1 = wild type isolate of *Gm*NPV; 2 = 1st passage isolate of *Gm*NPV; 3 = 5st passage isolate of *Gm*NPV; 4 = 10st passage isolate of *Gm*NPV; 5 = plaque-purified isolate).

III.4.2 ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5,931,456,697.64	4	1,482,864,174.41	5.405	0.014
Within Groups	2,743,708,444.04	10	274,370,844.40		
Total	867,516,141.68	14			

III.5 Statistical analysis of LT$_{50}$ values of *Gm*NPV 5 isolates obtained from bioassay study.

III.5.1 Descriptives

Isolate	N	Mean	S.D.	S.E.	95% C.I. for Mean		Min	Max
					Lower Bound	Upper Bound		
1	3	4.80	0.16	0.94	4.30	5.11	4.60	4.89
2	3	5.10	0.04	0.25	4.64	4.86	4.72	4.80
3	3	5.60	0.16	0.92	4.92	5.71	5.22	5.50
4	3	5.10	0.42	0.24	4.00	6.06	4.62	5.45
5	3	5.40	0.17	0.99	4.81	5.66	5.12	5.43
Total	15	5.20	0.32	0.83	4.83	5.18	4.60	5.50

(1 = wild type isolate of *Gm*NPV; 2 = 1st passage isolate of *Gm*NPV; 3 = 5st passage isolate of *Gm*NPV; 4 = 10st passage isolate of *Gm*NPV; 5 = plaque-purified isolate).

III.5.2 ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.64	4	0.16	2.68	0.094
Within Groups	0.60	10	0.06		
Total	1.24	14			

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