รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาปฏิสัมพันธ์ระหว่างสิ่งมีชีวิตที่ไม่มีกระดูกสันหลัง (Invertebrate) และไวรัส (Virus) โดยใช้แบบจำลอง Mosquito-Parvovirus

โดย

ทรงศักดิ์ ฤกษ์หริ่ง

ตุลาคม 2548

สัญญาเลขที่ _____

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ภาควิชาเทคโนโลยีชีวภาพ และหน่วยวิจัยเพื่อความเป็นเลิศเทคโนโลยีชีวภาพกุ้ง คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล

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งานวิจัยอันเกี่ยวเนื่องกับไวรัสก่อโรคในกุ้งพบว่ากุ้งสามารถกระตุ้นทำให้เกิดการทนทานต่อโรคได้และมีการตอบสนองแบบจำเพาะมากและกลไกการตอบสนองยังไม่เป็นที่เข้าใจ ปรากฏการณ์ดังกล่าวไม่สะดวกในการศึกษาในรายละเอียดหากใช้กุ้งในการศึกษาวิจัยเนื่องมาจากสาเหตุหลักสองประการคือกุ้งมีวงจรชีวิตที่ยาว (1-2 ปี) และขณะนี้ยังไม่มีการเพาะเลี้ยงเนื้อเยื่อต่อเนื่อง (Continuous Cell Line) ไว้สำหรับการทดลอง คณะผู้วิจัยได้ใช้แบบจำลองยุงและเดนโซ่ไวรัส (Mosquito-densovirus) ซึ่งเป็นไวรัสกลุ่มพาร์โว (Parvoviruses) ในการศึกษาปฏิสัมพันธ์ระหว่างสิ่งมีชีวิตที่ไม่มีกระดูกสันหลังและไวรัสก่อโรคเพื่อตรวจว่าปรากฏการณ์ที่คล้ายคลึงกันนี้สามารถเกิดขึ้นได้หรือไม่ในแมลงเพื่อสนับสนุนแบบจำลองที่ประดิษฐ์ขึ้น กลุ่มผู้วิจัยได้รายงานการศึกษาถึงความสัมพันธ์ระหว่างไวรัสกลุ่มพาร์โวที่ก่อโรคในกุ้งและแมลงโดยเปรียบเทียบลำดับสายดีเอ็นเอ และกรดอะมิโน สำหรับไวรัสทั้งในกุ้งและแมลงนั้นจัดอยู่ใน subfamily Densovirinae แบ่งเป็นไวรัสจากกุ้งคือ Hepatopancreatic parvovirus (HPV) ในกุ้ง *Penaeus monodon* (HPVmon), HPVchin ในกุ้ง *P. chinensis*, spawner-isolated mortality virus ในกุ้ง *P. monodon* (SMVmon), infectious hypodermal and hematopoietic necrosis virus ในกุ้ง P. vannamei และไวรัสจากแมลงคือ *Aedes aegypti* densovirus (*Aae*DNV), *Ae. albopictus* densovirus (*Aal*DNV), *Junonia coenia* densovirus (*Jc*DNV), *Galleria mellonella* densovirus (*Gm*DNV), *Diatraea saccharalis* densovirus (*Ds*DNV) และ *Periplaneta fuliginosa* densovirus (*Pf*DNV) จากการศึกษาพบว่าความยาวสายดีเอ็นเอของไวรัสอยู่ระหว่าง 4 ถึง 6 กิโลเบส และเมื่อเปรียบเทียบ ลำดับสายดีเอ็นเอพบว่าไม่มีความเหมือนอย่างมีนัยสำคัญโดยใช้โปรแกรม Clustal W ยกเว้นพบ 77% ของ ความเหมือนระหว่างไวรัส HPVmon และ HPVchin อย่างไรก็ตามจากการใช้ Phylogenetic tree analysis สามารถจัดกลุ่มของไวรัสที่ศึกษาได้เป็น 2 กลุ่ม (Clade) คือ Clade 1 ประกอบด้วย SMVmon, *Pf*DNV, *Ds*DNV, *Gm*DNV, *Jc*DNV และ *Bm*DNV, Clade 2 ประกอบด้วย HPVmon, HPVchin, IHHNV, *Aal*DNV และ *Aae*DNV จะเห็นได้ว่าไวรัสก่อโรคในกุ้งทั้งสี่ชนิดกระจายอยู่ทั้งสองกลุ่มโดยไม่แบ่งแยกไวรัสกุ้งและแมลงออกจากกันอย่างชัดเจน แสดงว่าจากผลการศึกษาทั้งโดยใช้ลำดับสายดีเอ็นเอและโปรตีนนั้นอาจเป็นไปได้ว่าไวรัสกลุ่มนี้มีความใกล้ชิดและสามารถถ่ายทอดไปมาระหว่างกันได้ คณะผู้วิจัยได้ทำการศึกษาต่อโดยใช้แบบจำลองยุงและ เดนโซไวรัส (Mosquito-Densovirus) ในการตรวจสอบปรากฏการณ์การทนต่อเชื้อไวรัสก่อโรคที่เกิดขึ้นโดยวิธี ทำให้ยุงติดเชื้อจากไวรัสก่อโรคชนิด Thai densonucleosis virus หรือ *ATh*DNV พบว่าภายใน 5 รุ่นของยุง ที่ทำให้ติดเชื้อต่อเนื่องนั้นมีอัตรารอดเพิ่มขึ้นจากเริ่มต้น 15% ± 4.12 SD เป็น 55% ± 6.36 SD และคณะผู้วิจัยทดสอบการติดเชื้อใน ยุงที่รอดตายโดยใช้วิธี Polymerase Chain Reaction (PCR), Histology, In-Situ Hybridization และ Transmission Electron Microscope (TEM) พบมีการติดเชื้อในยุงกลุ่มดังกล่าวในปริมาณค่อนข้างสูง มีการติดเชื้อแบบถาวร และมีการเจริญเป็นปกติเมื่อเทียบกับกลุ่มชุดควบคุม คณะผู้วิจัยยังได้เปรียบเทียบลำดับสายนิวคลีโอไทด์ของ ไวรัสในยุงรุ่นที่ 5 กับไวรัสเริ่มต้น พบปริมาณของไวรัสที่ไม่สมบูรณ์ (Defective Interfering Particle, DIP) เพิ่มขึ้นมากในยุงรุ่นที่ 5 โดยพบ DIP 64% ± 5.0 SD และพบ DIP ในไวรัสเริ่มต้นเพียง 33% ± 6.7 SD จากการทดลองทำให้ทราบว่าปริมาณ DIP ที่สูงมีความสัมพันธ์กับอัตรารอดที่เพิ่มขึ้นซึ่งสนับสนุนกับรายงานการวิจัย ในการเพาะเลี้ยงเนื้อเยื่อยุงชนิด C6/36 ที่มีการติดเชื้อเดนโซไวรัสแบบถาวร ดังนั้นจึงมีความน่าจะเป็นที่จะพบ อัตราการรอดที่เพิ่มขึ้นในโฮสต์ที่มีปริมาณ DIP ที่สูงและมีความเป็นไปได้ว่าในสิ่งมีชีวิตกลุ่มแมลงสามารถพบการติดเชื้อแบบถาวรได้และมีการตอบสนองแบบจำเพาะอันจะนำไปสู่การลดความรุนแรงของการเกิดโรคซึ่งเป็นอันตรายต่อโฮสต์และไวรัสก่อโรคได้

Project Code :
(รหัสโครงการ)

Project Title : STUDY ON INVERTEBRATE HOST-VIRAL INTERACTION USING A MOSQUITO-PARVOVIRUS MODEL

ชื่อโครงการ : การศึกษาปฏิสัมพันธ์ระหว่างสิ่งมีชีวิตที่ไม่มีกระดูกสันหลัง (Invertebrate) และไวรัส (Virus) โดยใช้แบบจำลอง Mosquito-Parvovirus

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Project Period : 1 year
ระยะเวลาโครงการ : 1 ปี

ABSTACT

Research on cultivated shrimp suggests that they have the capability to tolerate viral p*ATh*ogens in a highly specific manner by mechanisms currently unknown. The phenomenon is difficult to study in detail because they have a generation time of 1-2 years and lack continuous cell lines. Therefore, we developed a mosquito-densovirus model to examine whether similar phenomena occur in insects. To support the invertebrate host-viral interaction model, we reported on the relationship between shrimp parvoviruses and known insect parvoviruses. Included in the computer analysis were the DNA and putative amino acid sequences of representative insect and shrimp parvoviruses (subfamily Densovirinae). These were the hepatopancreatic parvovirus (HPV) of *Penaeus monodon* (HPVmon) and *P. chinensis* (HPVchin), the spawner-isolated mortality virus from *P. monodon* (SMVmon) and the infectious hypodermal and hematopoietic necrosis virus (IHHNV) from P. vannamei. Insect viruses included were *Aedes aegypti* densovirus (*Aae*DNV), *Ae. albopictus* densovirus (*Aal*DNV), *Junonia coenia* densovirus (*Jc*DNV), *Galleria mellonella* densovirus (*Gm*DNV), Bombyx mori densovirus 5 (*Bm*DNV), *Diatraea saccharalis* densovirus (*Ds*DNV) and *Periplaneta fuliginosa* densovirus (*Pf*DNV). Virion size for all these viruses ranged between 18 and 30 nm in diameter and ssDNA genome length was between 4 and 6 kb. Using a Clustal W program with the sequence fra*Gm*ents available, no significant DNA homology was found except for 77% DNA identity between HPVmon and HPVchin. However, phylogenetic trees constructed by comparing DNA genome sequences for putative viral polypeptides, capsid proteins and nonstructural proteins placed the parvoviruses into two Clades: Clade 1 with SMVmon, *Pf*DNV, *Ds*DNV, *Gm*DNV, *Jc*DNV, and *Bm*DNV; and Clade 2 with HPVmon, HPVchin, IHHNV, *Aal*DNV and *Aae*DNV. The four shrimp parvoviruses fell into two different clades that grouped with different insect parvoviruses. Results based on DNA and proteins sequences suggested that viral transfers may occur between two distantly related arthropod groups. Using the mosquito-densovirus challenge model with a stock densovirus (*ATh*DNV) and 5 generations of mosquitoes, we found a progressive survival increase from 15% ± 4.12 SD to 55% ± 6.36 SD. Prevalence of *ATh*DNV infection in surviving mosquito larvae (confirmed by PCR, histology, in situ hybridization and transmission electron microscopy) was relatively high (e.g., 35% in F2) but they grew normally to establish each succeeding generation. After 5 generations, comparison of putative amino acid sequences from genome fra*Gm*ents revealed a significantly higher ($p = 0.003$) maximum estimated prevalence of defective targets in the survivor virus population (64% ± 5.0 SD) than in the stored viral population (33% ± 6.7SD). The results paralleled those reported for serially passaged C6/36 mosquito cell cultures infected with densoviruses. There, reduced infection rates are ascribed to the production of defective interfering particles (DIP). Thus, it is possible that the presence of prior *ATh*DNV infections with a high level of DIP contributed to improved survival in our challenged F4 mosquito population. If so, it suggests that persistent viral infections in *arthropods* may serve in a specific, adaptive manner to reduce the incidence and severity of disease.

Keywords: MOSQUITO, SHRIMP, DENSONUCLEOSIS VIRUS, PARVOVIRUSES, TOLERANCE, ADAPTATION

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เนื้อหางานวิจัย

INTRODUCTION

Global field experience with cultivated shrimp has shown that catastrophic introductions of exotic viral p*ATho*gens are followed within a few years by the appearance of grossly normal carriers of the exotic virus in natural, native shrimp populations (Flegel, 2001). The virus is transmissible and can be lethal to naïve shrimp. Results from using fry of mixed captured spawners to stock cultivation pon*Ds* suggests that tolerance depen*Ds* on factors related to individual production batches of shrimp (i.e., it is adaptive in nature) (Flegel, 2001). This adaptive pattern cannot be explained easily by current knowledge or theories regarding shrimp or crustacean defense. The natural phenomenon has been repeated experimentally by selection of survivors from successive viral challenges for the 2 shrimp p*ATho*gens infectious hypodermal and hematopoietic necrosis virus (IHHNV) and Taura syndrome virus (TSV) (Moss, 2002, Moss *et al.*, In press). The tolerance appears to be specific to each p*ATho*gen and not cross-protective (Moss, 2002, Moss *et al.*, In press). Specificity is such that even small changes in the viral genome of TSV, for example, have been shown to greatly alter virulence (Erickson *et al.*, 2002) or to overcome tolerance developed through breeding selection (Erickson *et al.*, In press).

Detailed study of host-viral interaction in shrimp is hindered by long shrimp generation times (1-2 years) and the lack of continuous shrimp cell lines. By contrast, many insects like mosquitoes have short generation times and are accompanied by well-characterized continuous cell lines. They are in the same phylum (Arthropoda) as shrimp, and interact with viral p*ATho*gens in a similar manner. For example, both lack an inflammatory response to viruses, both generally exhibit persistent infections and both lack antibodies. Insects have also been shown to develop specific resistance or tolerance to viral p*ATho*gens (Watanabe, 1986). For example use of baculoviruses for biological control of insect pests has been limited by such factors as a narrow effective *et al.*host range, speed of action, and development of resistance (Boucias *et al.*, 1980, Boucias & Nordin, 1977; Engelhard & Volkman, 1995; Evans, 1981, 1983; Magnoler, 1975; Stairs, 1965; Teakle *et al.*, 1985 & 1986; Whitlock, 1977). However, a problem with many published studies has been the tendency to measure "resistance" by survival rate and the general failure to determine whether survivors are infected or not.

To overcome the disadvantages with shrimp and to overcome the shortfalls in earlier work with insects, we describe here the use of an *Aedes aegytpi*, C6/36 cell line and mosquito densovirus (*ATh*DNV) model to examine the effect of challenging successive generations of an arthropod population with a viral p*ATh*ogen. As a prelude to this process, the relationship amongst the shrimp parvoviruses and known insect parvoviruses was examined by a comparison of known viral genomes and putative amino acid sequences of structural and non-structural proteins generated from them. It was hoped that this would provide some background information regarding the probable relevance of the mosquito/densonucleosis virus model to more general questions on host-viral interaction in shrimp and other *arthropods*.

LITERATURE REVIEW

1. Studying host-viral interactions

This thesis contains some knowledge and results of experiments dealing mainly with the question *"How do shrimp and other arthropods respond to viral pAThogens in such a manner that they are able to show tolerance to specific species and strains of species?* Aspects of this question have been reviewed Flegel (2001). This question arose from experience with viral infections in commercially cultured penaeid shrimp. Shrimp culture is a worldwide economic activity that is especially important for developed and developing countries in sub-tropical and tropical regions. However, the intensification of shrimp farming has been accompanied by problems from infectious diseases, especially those of viral origin. They have caused enormous economic losses as a result of either shrimp mortality or retarded growth. In cultured penaeid shrimp, about 20 shrimp viruses have been reported. Among them, white spot syndrome virus (WSSV) has been the most economically important in the past decade because it has caused high mortality (Zhang *et al.*, 2004). Other viruses that have caused major loss include yellow head virus (YHV), infectious hypodermal and hematopoietic necrosis virus (IHHNV) and Taura syndrome virus (TSV).

The epizootics caused by these viruses are characterized by lack of an inflammatory response in moribund shrimp and by a pattern of initial, widespread, catastrophic crop loss followed (within approximately two years) by sporadic crop losses but widespread occurrence of persistently infected pon*Ds* with significantly less mortality. Flegel (Flegel, 2001) suggested that the viruses carried by persistently infected shrimp remained lethal for naïve shrimp in cohabitation tests. This natural phenomenon of toleration to viral p*AThogens* over time has been repeated experimentally by selection of survivors from successive viral challenges for two shrimp p*AThogens*, IHHNV and TSV (Moss, 2002). The ability of shrimp to tolerate viral p*AThogens* in this manner appears to be specific to each p*AThogen* and not cross-protective (Moss, 2002). Specificity is such that even small changes in the viral genome of TSV, for example, have been shown to greatly alter virulence (Erickson *et al.*, 2002) or to overcome tolerance developed by breeding selection (Erickson *et al.*, in press).

Flegel and Pasharawipas (1998) proposed the viral accommodation theory as a testable hypothesis to explain these phenomena. Briefly, it proposed that crustaceans have developed a proactive viral binding and memory (A*BM*) system that is distinct from that involved in passive viral binding and infection (PVB), and that the function of this system is to actively accommodate viral p*ATh*ogens. It suggested that A*BM* results in specific memory, such that simultaneous or subsequent PVB (via different receptors) does not trigger cellular apoptosis (i.e. programmed cell de*ATh*). Since the A*BM* system is distinct from that for PVB, it may occur in the absence of infection. However, once initiated for a particular virus, A*BM* enables a susceptible host to tolerate its active infection without mortality. As a corollary, absence of A*BM* in a "naïve" susceptible host would result in de*ATh* by viral triggered apoptosis. This concept is supported by current information for both RNA and DNA viruses in shrimp farming systems in Asia and the Americas and by preliminary data on massive apoptosis in moribund shrimp challenged with lethal viruses (Khanobdee *et al.*, 2002; Sahtout *et al.*, 2001; Wongprasert *et al.*, 2003).

Active viral accommodation may have evolved in the crustaceans, insects and/or *arthropods* as a successful alternative to resistance. If this were so, new viruses or new viral strains exposed to a naïve, susceptible population would cause widespread mortality in the first (initial) interaction phase. As the prevalence of the virus increased in the environment, competent individuals in the shrimp population (early developmental stages?) would be exposed to the virus and A*BM* would occur leading to a second interaction phase characterized by the widespread occurrence of persistent infection accompanied by sporadic mortality. The second interaction phase would be characterized by long term positive selection processes for members of the shrimp population less affected negatively by the virus and for members of the viral population with less negative effect on the host. By contrast, there would be strong negative selection for members of the viral population with higher virulence. This process would lead to a final interaction phase characterized by mutual host/virus existence. Thus, active viral accommodation (AVA) may have evolutionary advantages in that the absence of host resistance pressure may slow the development of virulence and accelerate progression towar*Ds* mutual host/virus existence.

Due to the relatively few publications on the topic, some reference is made to the more extensive literature on insect response to viruses. Insects have been more deeply studied and may show some parallels to crustaceans since they are also *arthropods* (Flegel, 2001 & Flegel *et al.*, 2004). Detailed study of host-viral interaction in shrimp is hindered by the long shrimp generation time (1-2 years) and the lack of continuous shrimp cell lines that can be infected with viruses. By contrast, many insects such as mosquitoes have short generation times of a few weeks and are accompanied by well-characterized continuous cell lines. They are also in the same phylum (Arthropoda) as shrimp and interact with viral p*ATho*gens in a similar manner. For example, both lack an inflammatory response to viruses, both generally exhibit persistent infections and both lack antibodies. Insects have also been shown to be capable of specific resistance or tolerance to viral p*ATho*gens (Watanabe, 1986) that is dependent upon larval age, molting, metamorphosis, and rearing conditions such as temperatures, food quality, and contamination of agricultural chemicals. Other examples of specific resistance can be found against the Baculoviruses, double stranded DNA viruses restricted to arthropod hosts including shrimp (Lightner, 1996) and originally found to kill silk worms. Baculoviruses typically have a very narrow host range. Use of baculoviruses for biological control of insect pests has been limited by such factors as a narrow effective host range, speed of action, and development of resistance (Stairs, 1965; Boucias & Nordin, 1977; Whitlock, 1977; Boucias *et al.*, 1980; Evans, 1981 & 1983; Teakle *et al.*, 1985 & 1986; Engelhard & Volkman, 1995). However, a problem with many published studies has been that they tend to measure "resistance" by survival rate and fail to determine whether the survivors in such tests are infected or not. Infected survivors would be more appropriately labeled as "tolerant" than "resistant" to any particular virus.

To overcome the disadvantages with shrimp and to overcome the shortfalls in earlier work with insects, we describe here the use of an *Aedes aegypi* mosquito, *Ae. albopictus* C6/36 cell line and mosquito densovirus (*ATh*DNV) model to examine the effect of challenging successive generations of an arthropod population with a viral p*ATho*gen. The purpose of this thesis was to focus on using the mosquito (*Ae. aegypti*) and its viral p*ATho*gen Thai densonucleosis virus (*ATh*DNV) as a model to study the response of an arthropod host to its viral p*ATho*gen and specifically to examine what happens with both the virus and its host during infection. This interaction might serve as

a model for invertebrate host and viral interaction and might provide new insights for both basic and applied research.

2. *Aedes aegypti* mosquito and *Parvoviruses* is a model for studying host-viral interaction

2.1 *Aedes aegypti* mosquito

Aedes aegypti has many qualifications for study as a model insect. It was one of the earliest mosquitoes studied and has been cultivated and used in the laboratory for research on mosquito structure, bionomics, behavior and reactions (Christophers, 1960). In addition, *Ae. aegypti*, is the primary, worldwide arthropod vector for yellow fever and dengue viruses. As it is also one of the most tractable mosquito species for laboratory studies, it has been and remains one of the most intensively studied arthropod species (Severson *et al.*, 2004). It has a cosmotropical distribution between 30°N and 20°S (Christophers, 1960; Knight & Stone, 1977) and exhibits a distinct preference for human habitats, including artificial oviposition sites, e.g., tires, flower vases, water storage containers (Tabachnick, 1991). Dengue viruses are a threat to more than 2.5 billion people, with an annual disease incidence in the tens of millions and de*ATh*s reaching about 24,000 per year (WHO, 2002). Overall, mosquito-borne diseases have emerged or re-emerged as significant human he*ATh* problems due to a number of factors including lack of progress in vaccine development, emergence of drug resistance in p*ATho*gens, emergence of insecticide resistance in mosquitoes and a decline in socioeconomic conditions in many countries where the disease is endemic. All of this limits disease monitoring and mosquito control efforts (Gubler, 1998). With few exceptions, mosquito control remains the only viable strategy for preventing dengue and other mosquito-borne diseases. *Ae. aegypti* is considered the most tractable mosquito species for laboratory culture, and has been used for detailed laboratory investigations of mosquito biology including, morphology, physiology, genetics, and vector competence (Clements, 1992). Recently, it has been used in molecular evolution applications (Severson *et al.*, 2001).

2.1.1 *Aedes aegypti* provides broad utility for investigating mosquito-p*ATho*gen interactions

Severson *et al.* (2004) have provided very useful information on using *Ae. aegypti* mosquito as a model to study interaction between the mosquito and its p*ATho*gens.

For example, many investigators have used *Ae. aegypti* to study the genetic basis for dengue vector competence and found that oral infection of *Ae. aegypti* with dengue virus varies both within and among geographic strains (Gubler *et al.*, 1979; Tardieux *et al.*, 1990; Sumanochitrapon *et al.*, 1998; Bennett *et al.*, 2002). They also found that several environmental factors affect the extrinsic incubation factors (EIP) of arboviruses in mosquitoes. These include temperature, humidity and titer of the virus (Black IV *et al.*, 2002). This mosquito has also been used as a model to study the genetic basis of vector competence for malaria parasites (*Plasmodium gallinaceum*) (Kilama & Craig, 1969) and the human parasites, *Brigia malayi* and *Wulchereria bancrofti* (Macdonald, 1962a,b&1963). *Ae. aegypti* mosquito is not only used as a model for such studies but also to investigate comparative genomics. For instance, recent studies (Jaillon *et al.*, 2003; McCue *et al.*, 2002; Thomas *et al.*, 2003) clearly indicate that comparative genome sequence analyses will significantly enhance our understanding of fundamental evolutionary and genetic mechanisms that define genome organization. *Ae. aegypti*/*A. gambiae*/Drosophila genome comparisons (as well as other *arthropods*) will facilitate the discovery of conserved functional elements unique to *Culicidae* mosquitoes. This should provide important insights into mosquito chromosome evolution and allow for the identification of genes and gene function, either common to mosquitoes or perhaps unique to individual mosquito species that are specific to host-seeking and blood-feeding behaviors, as well as the innate immune response to p*ATho*gens encountered during blood-feeding.

2.1.2 *Aedes aegypti* is preferred for laboratory investigations

Aedes aegypti has been and will remain the preferred mosquito species for laboratory investigations of virtually all aspects of mosquito biology. Much of this relates directly to its superior tractability in the laboratory that facilitates basic research. The mosquito research community recognized early the broad utility of *Ae. aegypti* as one of the most tractable mosquito species for laboratory studies (Christophers, 1960).

2.1.3 Life history traits and suitability for experimentation

The evolution in *Ae. aegypti* of an egg quiescence period following oviposition is an extremely favorable basic biological attribute. That is, the life cycle for most mosquito species is continuous, wherein eggs are laid individually or in rafts, typically on the water surface and hatch soon thereafter. Such species, including *Anopheles* and *Culex*,

can be difficult to adapt to laboratory culture and even when successfully adapted require constant maintenance. This severely limits the number of laboratory colonies that can be maintained. In contrast, *Ae. aegypti* preferentially oviposit away from the water surface and their eggs show considerable tolerance to desiccation and can be stored on dried oviposition substrates (such as paper towels) for several months. The eggs can be induced to hatch by simply placing them in deoxygenated water where they rapidly develop to the adult stage within ca. 7 days. *Ae. aegypti* collected from the field also readily adapt to laboratory rearing conditions, including single-pair mating in small containers that greatly facilitates genetic studies. As such, individual laboratories are able to maintain a number of *Ae. aegypti* strains with a reasonable level of effort. While not comparable to the ease of maintaining hundreds or thousands of Drosophila cultures, individual laboratories can likely maintain more than 100 *Ae. aegypti* strains with a moderate level of effort.

2.1.4 Demand for genome sequence

Many laboratories that study mosquito biology include *Ae. aegypti* in their research program, either as their primary organism or as a critical complement to their target mosquito species. This is clearly evidenced by the PubMed citation data listed in Table 1. When compared with two other commonly studied mosquitoes, *A. gambiae* and *Culex pipiens*, research on *Ae. aegypti* has resulted in a greater number of total publications, and of publications in nearly every biological category. Availability of the complete genome sequence will clearly enhance interest in *Ae. aegypti* research within the mosquito research community and will undoubtedly attract interest from investigators outside medical entomology. A general assessment of the *A. gambiae* literature supports this scenario, and the availability of genome sequence data for both *A. gambiae* and *Ae. aegypti* will accelerate that interest for both species.

Table 1. Pubmed entries (as of 4/7/2004).

	Total	Physiology	Development	Immunity	Insecticide	Parasite	Pathogen	Genetics
Ae. aegypti	3228	1845	737	267	357	164	24	601
A. gambiae	1415	897	270	167	198	194	12	536
C. pipiens	1252	646	221	99	239	41	9	243

The availability of the complete and annotated *D. melanogaster* genome, the soon to be released, assembled whole genome sequence for *A. gambiae* and the whole genome

sequence for *Ae. aegypti* constitute important resources for gene discovery and comparisons in the two most important vector mosquito species. Moreover, a number of currently important scientific questions can be addressed more efficiently by comparative genomic approaches. For example, genetic studies in the laboratory have demonstrated that arbovirus susceptibility in *Ae. aegypti* is, in part, determined by multiple gene effects (Bosio *et al.*, 1998), and the general genome locations for quantitative trait loci (QTL) influencing both dengue virus midgut infection and midgut escape have been identified (Bosio *et al.*, 2000).

2.1.5 Genetic mapping and physical mapping

For genetic mapping, *Ae. aegypti* has been the subject of numerous genetic studies conducted in laboratories throughout the world. A relatively large number of morphological mutant stocks have been identified, and with isozymes, provided the tools for development of the first detailed genetic linkage map for any mosquito species (Munstermann & Craig, 1979). Moreover, *Ae. aegypti* has been used to study physical mapping. As with nearly all mosquito species, the *Ae. aegypti* genome is organized in three chromosomes. Of interest, no sex chromosome dimorphism is evident in *Ae. aegypti* or among other culicine mosquitoes. Instead, sex determination appears to be a function of a single autosomal gene locus (Gilchrist & Haldane, 1947; Anderson *et al.*, 2001). Physical mapping in *Ae. aegypti* is based on *in situ* hybridization to metaphase chromosomes, as its genome organization (likely due to the repetitive nature) is not conducive to producing useable polytene chromosome preparations. Both cosmid and BAC genomic libraries are available and have been used for developing a physical map using FISH technology (Brown *et al.*, 1995; Brown & Knu*D*son, 1997). In addition, the linkage and physical maps have been integrated by FISH mapping clones containing sequences for markers on the linkage map (Brown *et al.*, 2001). Finally, this mosquito has been used for gene discovery. Generation of large-scale EST data in *Ae. aegypti* is important for several reasons. First, they represent an opportunity to identify genes expressed collectively among various developmental stages and tissues including, for example, midgut, ovaries and salivary glan*Ds*, and thus are generally reflective of the entire transcriptome. Second, the examination of tissues from naïve, blood fed or p*AThog*en-infected mosquitoes will provide the opportunity to clone genes that are expressed in response to infection as well as those associated with blood-feeding. Comparison of genes from these sources with their homologues in *Drosophila* and *A.*

gambiae also may shed light on those evolutionary adaptations that have been necessary for obtaining, digesting and utilizing blood. Third, these libraries will represent a valuable immediate community resource, which will permit both subsequent full-length sequencing as desired and can be used for the production of sequence-verified, unique gene microarrays. Fourth, they will be important for training gene-finding software and subsequent annotation of the full genome. Finally, they will likely be useful in helping to determine whether *Drosophila* ORFs with no known homologues are in fact functional genes.

Ae. aegypti has been and will remain one of the most intensively studied mosquito species. Considerable genome information has been obtained for this mosquito, yet much remains unknown.

2.2 Parvoviruses

The virus family *Parvoviridae* comprises small animal viruses with linear single-strand DNA genomes (Lukashov & Gou*Ds*mit, 2001). Parvoviruses can be found in both invertebrate and vertebrate animals including humans (Siegl *et al.*, 1985; Kurstak & Tijssen, 1981). The genomes of parvoviruses are about 5 kb in length and contain two large open reading frames (ORFs). The first codes for two nonstructural proteins, NS-1 and NS-2, while the second encodes coat proteins VP-1 to VP2 or to VP3, which have substantial amino acid identity, being derived from overlapping reading frames.

The parvoviruses isolated from invertebrates, mainly insects, form a unique group of viruses. All are linked to fatal diseases, well described in *Galleria mellonella* lavae infected by the densonucleosis virus (Kurstak, 1972; Kurstak & Tijssen, 1977) . The common name of densovirus and densonucleosis virus (DNV) was first used by (Kawase & Kurstak, 1991). Densovirus posses a small amount of polyamines along with DNA and do not contain lipi*Ds*. Thus, they are resistant to organic solvents and have a high buoyant density of 1.4-1.45 g/ml in cesium chloride. The first densonucleosis virus was isolated in 1964 from lavae of the greater moth, *G. mellonella*. Subsequently, similar viruses were isolated in order Lepidoptera, Diptera, Orthoptera, Blattariae and Odonata. The host insect species infected with accepted and possible members of the group are distributed all over the world (Table 2).

Table 2. Densonucleosis viruses (DNV).

Host	Country of Isolation	Year of Isolation
Lepidoptera		
Aglais uricae	United Kingdom	1973
Agraulis vanillae	United Kingdom	1980
Bombyx mori	Japan	1973
	China	1982
Diatraea saccharalis	Guadeloupe	1977
Euxoa auxilliaris	United States	1970
Galleria mellonella	France	1964
Junonia coenia	United Kingdom	1973
Pieris rapae	China	1984
Sibine fusca	France	1977
Mythimna loreyi	Egypt	1995
Casphalia extranae	Egypt?	1986?
Diptera		
Aedes aegypti	Soviet Union	1972
Simulium vittatum	United States	1976
Culex pipiens	France	2000
Ae. aegypti & Ae. albopictus	Thailand	1999
Toxorhynchites amboinensis	-	1995
Haemagogus equinus cell line	-	1995
Odonata		
Lencorrhinia dubia	Sweden	1979
Orthrotera		
Periplaneta fuliginosa	Japan	1979
Decapoda		
Penaeus stylirostris	United States	1984
P. vannamei	United States	1984
P. monodon	United States	1993
	Thailand	1999
P. chinensis	Korea	1995
Hemiptera		
Myzus persicae	Netherlan*Ds*?	2003

2.2.1 Viral classification of the family *Parvoviridae*. The family *Parvoviridae* contains two subfamilies (Van Regenmortel *et al.*, 2000):

The *Parvovirinae*, including viruses from vertebrates,

The *Densovirinae*, including viruses from insects and other *arthropods*.

The subfamily *Parvovirinae* contains three genera: *Parvovirus*, comprising most parvoviruses from vertebrates; *Erythrovirus* comprising human B19 and V9 parvoviruses as well as parvoviruses from rhesus and pig-tailed macaques, and *Dependovirus*, which comprise adeno-associated viruses (AAV). Within *Densovirinae*, four genera are recognized. The current classification of parvoviruses is based primarily on their host range and their dependence on help from other viruses for replication. Accordingly, they are traditionally separated into three types: (i) autonomous virus of vertebrates, (ii) helper-dependent viruses of vertebrates, and (iii) autonomous viruses of insects and other *arthropods*.

Virions are unenveloped, 18-26 nm in diameter, and exhibit icosahedral symmetry. Virion relative molecular mass (Mr) is about 5.5-6.2 x 10^3 kDa. Virion buoyant density is 1.39-1.42 g/cm^3 in CsCl. The S_{20w} is 110-122S. Infectious particles are composed of about 80% protein and 20% DNA. Infectious particles with buoyant densities about 1.45 g/cm^3 may represent conformational or other variants, or precursors to the mature particles. Mature virions are stable in the presence of lipid solvents, or on exposure to pH 3-9, or for most species, on incubation at 56°C for at least 60 minutes. Viruses can be inactivated by treatment with formalin, β-propriolactone, hydroxylamine, or oxidizing agents. The genome is a linear molecule of ssDNA, 4-6 kb in size (Mr 1.5-20 x 10^6). The G+C content is 41-53%. Some members preferentially encapsidate ssDNA of negative polarity (i.e., complementary to viral mRNA species, such as *Mice minute virus*, MMV). After extraction, and depending on the amounts present, the complementary stran*Ds* may hybridize in vitro to form *Ds*DNA. Viruses generally have two to four virion protein species (VP1-4). Depending of the species, the Mr of VP1, VP2, VP3 and VP4 were 80-90 kDa, 64-85 kDa, 60-75 kDa and 49-52 kDa, respectively. The viral proteins represent alternative forms of the same gene product. Enzymes are lacking. Virions of viruses lack lipi*Ds* and none of the viral proteins is glycosidated (for more detail, please see van Regenmortel *et al.* (2000).

This review will be focused only on the subfamily *Densovirinae* that are viruses of insects and other *arthropods*. The molecular biology of the *Densovirinae* has been extensively reviewed by Bergoin and Tijssen (2000).

2.2.2 Taxonomic Structure of the Subfamily *Densovirinae*

Genus	*Densovirus*
Genus	*Iteravirus*
Genus	*Brevidensovirus*

Viruses assigned to the subfamily *Densovirinae* infect *arthropods*. The ssDNA genome of virions is either of positive or negative sense. Upon extraction, the complementary DNA stran*Ds* usually form *Ds*DNA. There are four structural proteins. Viruses multiply efficiently in most of the tissues of larvae, nymphs, and adult host species without the involvement of helper viruses. Cellular changes consist of hypertrophy of the nucleus with accumulation of virions therein to form dense, voluminous intranuclear masses. The known host range includes members of the *Dictyoptera*, *Diptera*, *Lepidoptera*, *Odonata* and *Orthoptera*. There is evidence that densovirus-like viruses also infect and multiply in crabs and shrimp. ssDNA viruses that infect shrimp include infectious hypodermal and hematopoietic necrosis virus (IHHNV) and hepatopancreatic parvovirus (HPV) (Flegel, 2001).

Genus *Densovirus*

The type species is *Junonia coenia* densovirus (*Jc*DNV). The ssDNA genome is about 6 kb in size. Populations of virions encapsidate equal amounts of positive and negative stran*Ds*. On one strand there are 3 ORFs which encode NS proteins using a single promoter (7 map units from the end). The four structural proteins are encoded on the complementary strand, using an mRNA promoter that is 9 map units from the end of that strand. *Jc*DNV has an inverted terminal repeat of 517 nts, the first 96 nts of which can fold to form a T-shaped structure of the type found in the ITR of AAV DNA. There are two species in this genus including *Jc*DNV (accession number S17265) and *Galleria mellonella* densovirus (*Gm*DNV, accession number L32896).

Genus *Iteravirus*

The one and only type species in this genus is *Bombyx mori* densovirus (*Bm*DNV) (accession numbers are M15123, M60583, M60584). The ssDNA genome is about 5 kb in size. Populations of virions encapsidate equal amounts of positive and negative stran*Ds*. ORFs for both the structural and NS proteins are located on the same strand. There is apparently one mRNA promoter upstream of each ORF. There is a small ORF on the complementary strand of unknown function. The DNA has an inverted terminal repeat of 225 nts, the first 175 nts are palindromic but do not form a T-shaped structure when folded.

Genus *Brevidensovirus*

The type species is *Ae. aegypti* densovirus (*Aae*DNV). The genome is about 4 kb in size. Populations of virions encapsidate positive and negative stran*Ds*, a majority of which are of negative polarity (85%). ORFs for the structural and NS proteins are on the same strand. There are mRNA promoters at map units 7 and 60. There is a small ORF of unknown function on the complementary strand. A palindromic sequence of 146 nts is found at the 3'-end of the genome and a different palindromic sequence of 164 nts at the 5'-end. Both terminal sequences can fold to form a T-shaped structure. There are two species in this genus that are *Aae*DNV and *Ae. albopictus* densovirus (*Aal*DNV). There are other tentative species both in the genus and in the subfamily. These are *Acheta domestica* densovirus (*Ad*DNV), *A. pseudoscutellaris* densovirus (*Ap*DNV), *Agraulis vanillae* densovirus (*Av*DNV), *Casphalia extranea* densovirus (*Ce*DNV), *Diatraea saccharalis* densovirus (*Ds*DNV),
Euxoa auxiliaris densovirus (*Ea*DNV), *Leucorrhinia dubia* densovirus (*Ld*DNV), *Lymantria dubia* densovirus (*Ld*DNV), *Periplanata fuliginosa* densovirus (*Pf*DNV), *Pieris rapae* densovirus (*Pr*DNV), *Pseudaletia includens* densovirus (*Pi*DNV), *Sibine fusca* densovirus (*Sf*DNV), *Simulium vittatum* densovirus (*Sv*DNV), hepatopancreatic parvo-like virus of shrimp (HPPLV), parvo-like virus of crab (PCV84) and IHHNV of shrimp. Phylogenetic relationships among the various members of the family *Parvoviridae* are shown in Figure 1 (Van Regenmortel *et al.*, 2000).

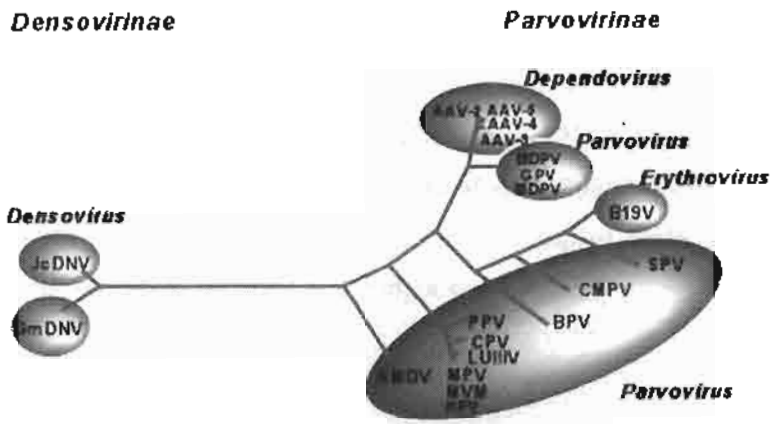
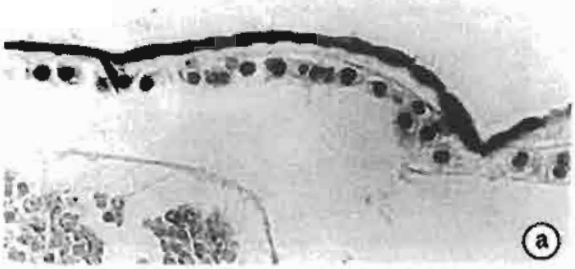


Figure 1. The phylogenetic relationships among the various members of the family *Parvoviridae*, determined by comparison of a conserved region of about 220 amino acid residues from the Rep and NS1 proteins, equivalent to the sequence between residues 254 and 562 of the NS1 protein of Mice minute virus. The sequences were aligned using the Clustal algorithim of the DNAStar program, and the most parsimonious phylogenetic relationships were determined using the Branch and Bound algorithim of the program PAUP (version 3.1.1). Thirty minimal phylogenies were derived, which all had the same overall topology, but which differed in arrangements of closely related sequences within some clades. The phylogeny shown is not rooted, and the branch lengths are proportional to the number of differences between sequences. (Contributed by Dr. Colin Parrish.)

2.2.3 Histop*ATho*logy

Histop*ATho*logical studies upon the virus in the subfamily *Densovirinae* have also been reported by several investigators (Shike *et al.*, 2000)(Figure 2A); (Barreau *et al.*, 1996;)(Figure 2B); (Lightner, 1996); (Flegel *et al.*, 1999)(Figure 2C). Histological sections of *Cp*DNV-infected *Culex pipiens* showed hypertopied nuclei in several tissues including hypodermal tissue, fat body and midgut epithelium.



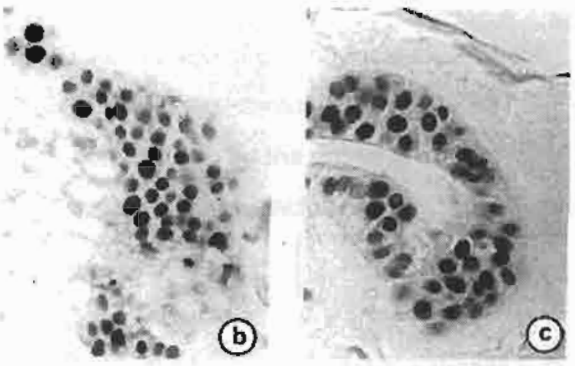
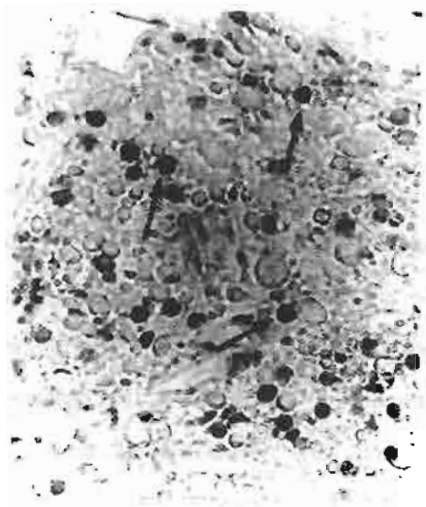


Figure 2A

Histological sections of *Cp*DNV-infected *Culex pipiens* larvae. Hypertrophied nuclei stained intensively with Feulgen reagent are observed in (a) hypodermis, (b) fat body and (c) midgut epithelium (×600).



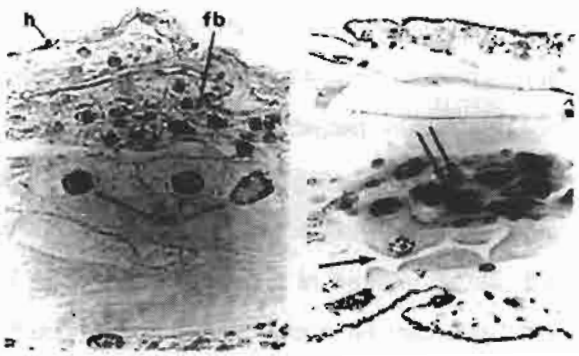
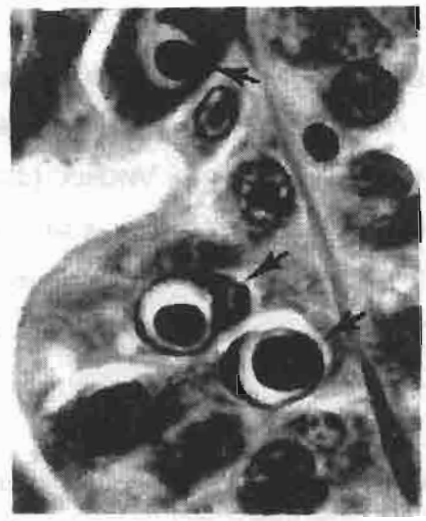
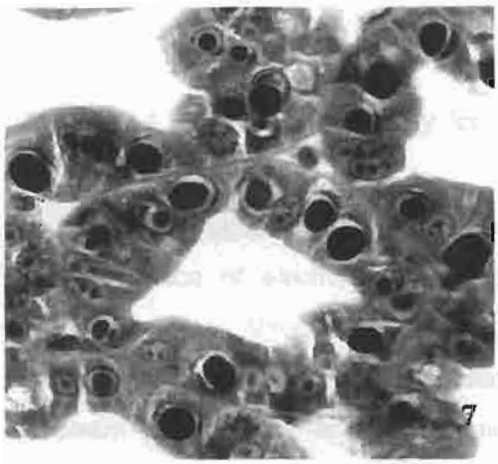


Figure 2B Light micrograph of *Ae. aegypti* lavae infected with *Aal*DNV showing parvo-type inclusion of hypodermal (*h*), fat body (f) and muscle (m).

Figure 2C. Typical histopathology for HPV of shrimp. HPV infected hepatopancreatic tissue showing normal nuclei together with hypertrophied nuclei containing inclusions typical for HPV (arrows).





The chromatin is marginated and the basophilic nucleolus is being compressed by the growing, more acidophilic viral inclusion.

Nuclei of infected tissue with densonucleosis viruses are enlarged and the cytoplasm is scarcely detected. The DNA content of individual *Bm*DNV-3 infected nuclei was examined by microspectrophotometry. The result revealed that DNA content in the case of infected nuclei was 13-16 times larger than that of noninfected nuclei. Tissue p*ATh*ology of *Aal*DNV-infected *Ae. aegypti* has been well investigated (Barreau *et al.*, 1996). There was no alteration in larval tissues at 2, 3 and 4 days postinfection. Anomalies appeared at Day 5 principally in cells of the fat body. In healthy larvae, the fat body had a spongy structure owing to the presence of numerous fat vacuoles. The nuclei were rounded or star-shaped, and the chromatin was set in regular granules. In infected fat body cells in the early stage of infection, the nuclear size increased significantly, chromatin was confined to the vicinity of the nuclear membrane and the internal structure was not discernible and generally lightly stained. Later, a voluminous dense homogeneous structure appeared in each affected nucleus and was strongly stained with Feulgen reaction. Finally, chromatin, nuclei, and cytoplasm were

indistinguishable (Figure 2B). The dense nuclei appeared in almost all of the larval tissues including muscular membranes, hypodermis, tracheal matrix, imaginal disks, and nervous tissue. However, malpighian tubules were infected in late development (Figure 2B). The tissue polytropism of *Aal*DNV was typical of that of most lepidopteran and orthopteran densonucleosis diseases (Kawase, 1985). *Aal*DNV was characterized by its nuclear tropism producing nuclear hypertrophy. The early damage in the fat body was also noted for other densonucleosis viruses such as *G. mellonella* DNV and *Aae*DNV. This *Aal*DNV infection showed no cytoplasmic inclusions, in contrast to *Aae*DNV, *Jc*DNV and *Ld*DNV (Kawase & Kurstak, 1991).

2.2.4 Electron microscopy for ultrastructural changes upon densonucleosis viral infection

The first ultrastructural change that occurs in silkworm infected with *Bm*DNV-1 is the appearance of electron-dense bodies in the nucleus, suggesting that there is virogenic stroma. Virogenic stromata iares not observed in the cytoplasm. The ultrastructural changes of midgut cells infected with *Bm*DNV-3 occur in both the cytoplasm and nucleus. In the nucleus at an early stage of infection, the chromatin disappears and many nucleoli appear. The nucleus hypertrophies greatly and is gradually filled with a network of electron-dense granules, which is probably the precursor of the virogenic stroma. During replication, two different patterns occur in infected nuclei. One replicates in linear array while the other disperses as patches that gradually condense and finally form large masses (Kawase & Kurstak, 1991).

In *Aae*DNV, the first changes are observed in the cytoplasm of DNV-infected cells. They consist of the formation of paracrytalline structures containing particles of 18-20 nm in diameter. Virogenic stroma and paracrytalline virion arrays are found in the nuclei of the virus-infected cells. A similar crystalline array of virus particles can be observed in the cytoplasm of fat body, muscle, and pericardial cells of *Periplaneta fuliginosa* infected with *Periplaneta* DNV. Thin sections of infected mosquito *C. pipiens* tissues contrasted with uranyl acetate and lead citrate and examined under the electron microscope revealed enlarged nuclei containing an electron-dense virogenic stroma filled with small paraspherical viral particles ~20 nm in diameter, occasionally arranged into paracristalline arrays (Figure 3a). The chromatin was greatly reduced and pushed to the

periphery of the nucleoplasm toward the nuclear inner membrane. These histop*ATho*logical and cytop*ATho*logical observations led to the conclusion that infected larvae exhibited salient features of an infection caused by a DNV (Jousset *et al.*, 2000).

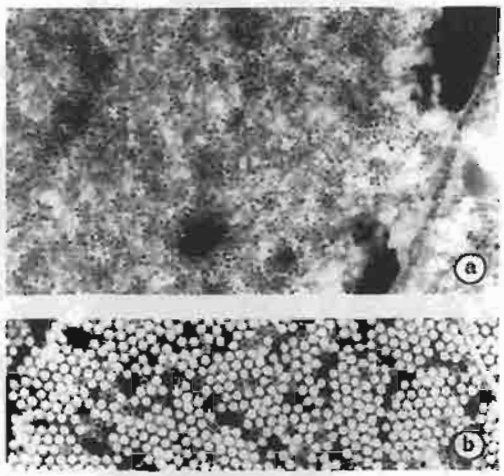


Figure 3 (a) Thin section of a hypertrophied nucleus from an adipose cell of a *C. pipiens* larva heavily infected with *Cp*DNV. Numerous viral particles are visible emerging from an electron dense virogenic stroma (×62500). (b) Purified suspension of *Cp*DNV virions negatively stained with 2% sodium phosphotungstate (×120000).

van Munster *et al.* (2003) reported the isolation, partial characterization and p*ATho*genic effects of a new virus infecting the green peach aphid, *Myzus persicae* (family *Aphididae*), named *Myzus persicae* densovirus (*Mp*DNV). The *Mp*DNV particles were always localized at the apical part of the cells, i.e., between the microvilli and the nucleus. Particles were either lining the inner membrane of vesicles, arranged randomly or organized as crystalline arrays inside vesicles (Figure 4A and B).

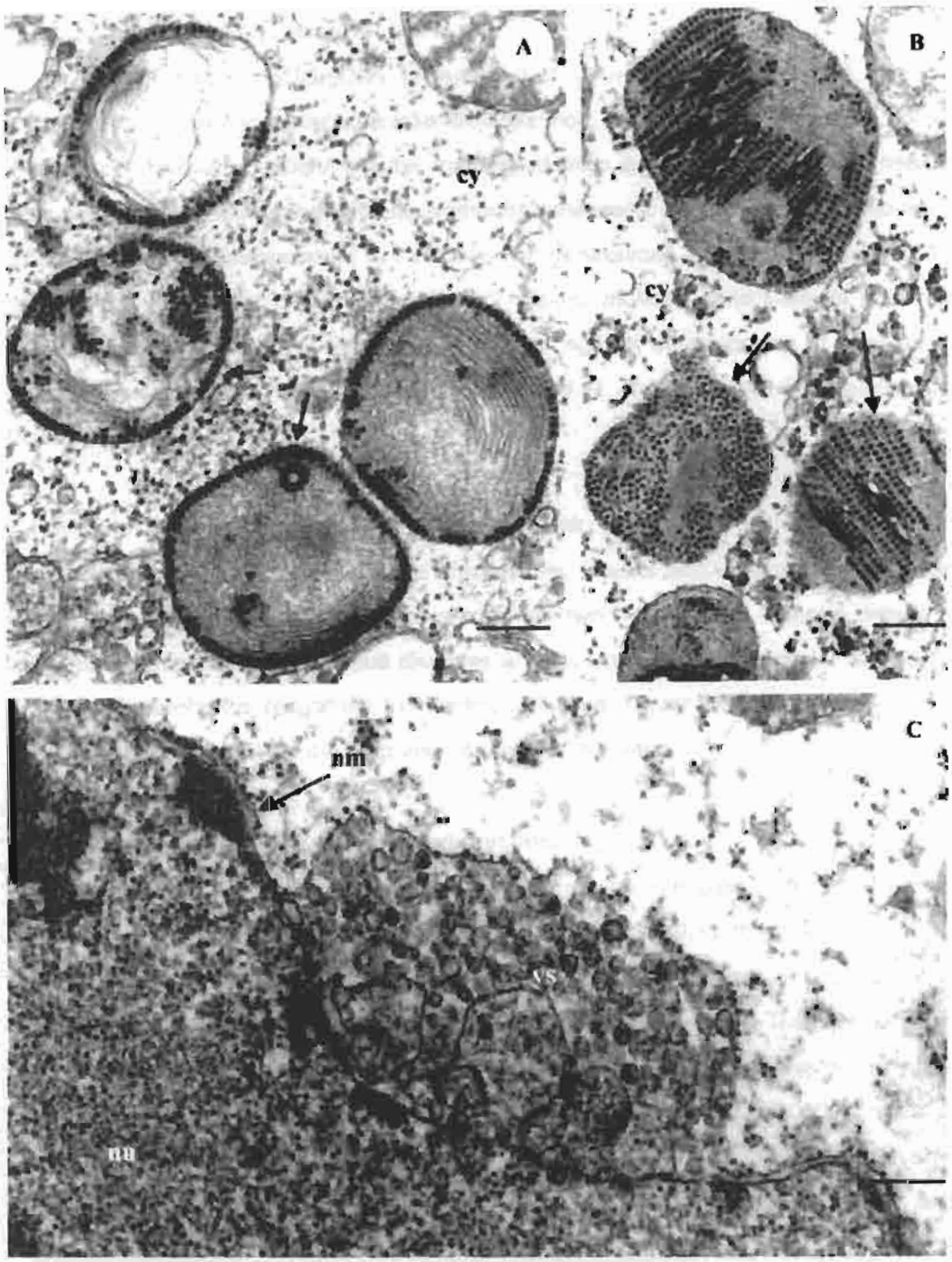


Figure 4. Electron micrographs of ultra thin transverse sections of third instar nymphs of *M. persicae* infected with *Mp*DNV. (A and B) Densovirus particles within vesicles in the cytoplasm (cy). (C) Vesicular structures (vs) between the outer and inner layers of the nucleus membrane (nm), nucleus (nu) (bar, 200 nm).

2.2.5 Densonucleosis viral host

The host range of *Gm*DNV is restricted to *G. mellonella*. In contrast, *Junonia coenia* DNV infects *Mamestra brassicae, Bombyx mori,* and *Lymantria dispar,* but not *G. mellonella*. However, *Gm*DNV can be transmitted from an infected larva to a healthy larva via the ovipositor of the parasite *Nemeritis canescens*. *Aae*DNV was infectious for different species of mosquitoes: *Ae. vexans, Ae. geniculatus, Ae. caspius dorsalis, Ae. cantans, Ae. albopictus, C. pipiens pipiens, C. pipiens molestus,* and *Culiseta annulata*. The infectivity of the DNV of *Periplaneta fuliginosa* against other species of *Periplaneta* has been investigated. Adults of *P. austraseae* and *P. americana* are less susceptible (Kawase & Kurstak, 1991).

3. Review of immunological details of invertebrates

The immune responses of invertebrate crustaceans, insects and *arthropods* have been studied in detail. The work will be reviewed under two headings: (i) General details of invertebrate responses to infectious diseases and (ii) Unusual and/or special viewpoints regarding invertebrates responses to infectious disease. Under the latter heading there will be special emphasis on the response to viral p*ATho*gens.

3.1 General details of invertebrate responses

Kavanagh and Reeves (2004) have reviewed the similarities of the vertebrate and insect innate immune responses to infection and identified the potential use of insects for *in vivo* evaluation of microbial p*ATho*genicity. Insects are one of the most successful and geographically widespread groups of animals on earth. They are found in almost every habitat and have succeeded in colonizing niches inaccessible to other animal life forms. Conservative estimates suggest that there are 750,000 species of insect but in reality this figure may be closer to 1,000,000. They are the most diverse and most abundant animal life form. From an evolutionary perspective, insects and vertebrates diverged approximately 500 million years ago. However, many aspects of their physiology remain similar. The innate immune system, unlike the adaptive immune system of insects and mammals (Klein, 1997; Arala-Chaves & Sequeira, 2000), shares a high degree of structural and functional homology. In particular, a number of features of the innate immune response are common to mammals and insects (Hoffman, 1995; Fallon & Sun, 2001) and analysis of insect responses to p*ATho*gens can provide an

indication of the vertebrate response to infection (Hoffmann, 1995; Kimbrell & Beutler, 2001). Since the innate immune response is the main line of defense in vertebrates against many microbial p*ATH*ogens, much effort has been focused on examining the mammalian and insect responses to microbial infection and a strong correlation between both systems has been demonstrated by Salzet (2001).

Research on the innate immune response of mammals has revealed similarities with the invertebrate immune system (Salzet, 2001). Insects have developed an acute response resembling that seen in humans, implicating similar effectors, receptors and regulators of gene expression. Mussels have developed intracellular phagocytosis resembling that seen in mammalian neutrophils, using cationic antibacterial peptides in phagolysosomes. Leeches, like amphibians, contain antibacterial peptides and immune stimulators that are derived from the processing of neuropeptide precursors. This pattern of similarities suggests that the vertebrate innate immune response resembles a patchwork of those responses seen in several invertebrate models. Cohn *et al.* (2001) have shown similarities in the innate immune response within mammals, insects and plants. They showed that signaling p*ATH*ways leading to activation of defense response genes in mammals, insects and plants share common components as shown in Figure 5.

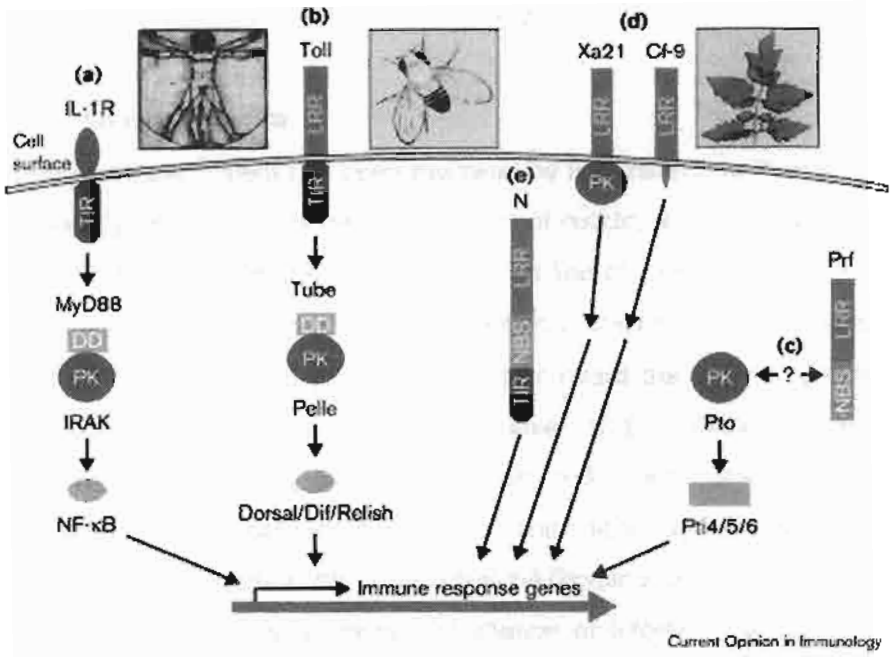


Figure 5. Signalling p*ATh*ways leading to activation of defense response genes in mammals, insects and plants share common components. For example, related PKs occur in the IL-1R, Toll and Pto–Prf defence p*ATh*ways in humans, *Drosophila* and tomato, respectively. Other common elements in defense p*ATh*ways include LRRs, D*Ds* and the TIR domain. Several R proteins in plants also contain putative NBSs. (a) In humans, IL-1 bin*Ds* IL-1R (which has a TIR); the adaptor MyD88 (which has a DD) then links this to IRAK (a PK); this releases NF-B from its inhibitor (I-B); NF-B then activates transcription of genes important in host defense. (b) A similar sequence occurs when Spätzle bin*Ds* Toll: eventually Dorsal is released from Cactus; in addition, subsequent Toll-mediated resistance to disease requires the transcription factors, Dif and Relish. (c) Tomato Pto-mediated signalling may involve complexes similar to those found in IL-1R- and Toll-mediated signalling: Pto (which requires Prf for its activity) is homologous to IRAK and Pelle. Pto interacts with Pti1, which is involved in the plant HR; Pto also interacts with Pti4/5/6, which are transcription factors that may be involved in regulation of PR genes. (d) Other plant proteins that may also signal in a similar fashion to IL-1R and Toll are tomato Cf-9 and rice Xa21: Cf-9 and Xa21 have extracytoplasmic domains

and ligand interaction may result in kinase activity; presumably, avr9 interaction with Cf-9 activates a CDPK that may be involved in host defence responses. (e) The tobacco N protein contains a TIR domain, an NBS and a LRR and is involved in resistance to TMV.

3.2 Insect immune system

The insect immune system has been reviewed by Kavanagh and Reeves (2004). They showed three types of insect barriers: (i) the insect cuticle, (ii) haemocytes and (iii) humoral factors. **(i) The cuticle** of the insects is the first line of defense against invading p*ATho*gens and serves a function analogous to the skin in mammals. It is a structurally and chemically complex barrier designed to prevent or retard the entry of p*ATho*gens into the haemoceol (the body cavity). The outer layer of the cuticle (epicuticle) is covered in a waxy layer containing lipi*Ds*, fatty aci*Ds* and sterols, which may display anti-microbial properties. The cuticle itself consists of chitin fibrils embedded in a protein matrix. The intact cuticle prevents entry of microbial p*ATho*gen,s but once it is ruptured by injury or degradation, there is an increased chance of infection (Teetor-Barsch & Roberts, 1983). Lesions may be plugged and subsequently repaired to restore the structural and functional integrity of the cuticle. Injury to the cuticle activates the humoral immune response, which lea*Ds* to the production of cecropins and attacins with anti-bacterial activity. Recent studies on antimicrobial peptides from *Drosophila* have shown that a variety of epithelial tissues in direct contact with the external environment can express the antifungal peptide drosomysin and the *imd* gene in *Drosophilla* plays a crucial role in the activation of this response to infection (Fallon & Sun, 2001). **(ii) Haemocytes** are found in the body fluid (haemolymph) that occupies the body cavity and serves a function analogous to blood in mammals. Haemolymph transports nutrients, waste products and signal molecules and it plays a role in crustacean but not insect respiration. In addition, haemolymph contains cells and anti-microbial peptides capable of immobilising and killing invading microorganisms. The volume of haemolymph varies between species and even within a species depending upon the developmental stage of the individual. The arthropod immune response to microorganisms has been shown to involve a change in the circulating haemocyte population and synthesis of new haemolymph proteins. The haemolymph is the main site of the immune response to microorganisms. The innate immune response consists of cellular and humoral mechanisms. Haemocytes function in a similar manner to phagocytes of mammals.

Blood cells of the crayfish, *Pacifitacus leniusculus*, have been shown by Johanson (1999) to release a cell-adhesive and opsonic peroxidase, peroxinectin. Cell-cell adhesion between blood cells and adhesion of blood cells to other cells, to the extracellular metrix or to infectious organisms occurs when blood cells move to and attach at site of inflammation or infection. Cell adhesion is essential in the cellular immune response of encapsulation and nodule formation. The majority of haemocytes circulate freely within the haemolymph but a significant number (up to 30% in some insect species) can be found associated with internal organs such as the fat body, trachea or digestive system. At least six types of haemocytes have been identified in lepidopterous (e.g., *G. mellonella*) although more or fewer types may exist in other species (Figure 6).

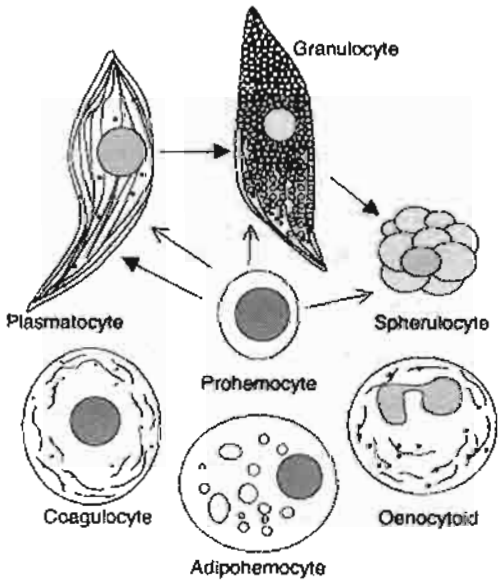


Figure 6. Haemocyte types involved in the cellular immune response. Linear maturation process in *Lepidoptera* (closed arrow) or differentiation of prohemocytes into different cell types (open arrows).

Haemocytes have been classified as prohemocyte, plasmatocytes, granulocytes (granular cells), coagulocytes, spherulocytes and oenocytoi*Ds*. Prohemocytes (6–13 µm in diameter) are small rounded cells with large nuclei that divide and may differentiate into other cell types (Figure 6). Plasmatocytes (40–50 µm) and granulocytes (45 µm) are the predominant phagocytic cells. Plasmatocytes contain lysosomal enzymes and are the most abundant cell type. Granulocytes possess a relatively small nucleus and granule-rich cytoplasm. Spherulocytes are oval or round cells (25 µm) with varying numbers of small spherical inclusions. Oenocytoi*Ds* are large, binucleate, non-phagocytic cells that may contain prophenoloxidase. Coagulocytes have also been termed hyaline hemocytes and are involved in the clotting process. Adipohemocytes are characterised by the presence of fat droplets. The plasmatocytes and granulocytes participate in phagocytosis, nodule formation and encapsulation, which are important elements of the insect's cellular defense against bacteria and unicellular fungi. Within *D. melanogaster*, plasmatocytes participate, to some extent, in the synthesis of anti-microbial peptides during the humoral response and assume the function of phagocytosis of micro-organisms while lamellocytes and crystal cells play respective roles in encapsulation and melanisation of larger intruders.

Crayfish is the best-studied invertebrate systems regarding cell adhesion in immunity (Johansson, 1999). The protein peroxinectin is most probably the best characterized invertebrate blood cell adhesion ligand. Other cell adhesion molecules in invertebrate blood have also been reported. These include hemagglutinin (Fujii *et al.*, 1992), dermatoponin (Superti-Furga *et al.*, 1993), hemocyanin (Kotani *et al.*, 1995) and the plasmocyte-spreading peptide (Clark *et al.*, 1997).

There are three main functions of invertebrate haemocytes. These are phagocytosis, nodulization and encapsulation as described by Kavanagh and Reeves (2004). For phagocytosis, the process of phagocytosis in insects and mammals appears to be very similar. In both cases there is the binding of opsonic ligan*Ds* to the surface of the particle that is then followed by recognition by specific receptors. An intracellular cascade results in the internalisation of the foreign body.

In the case of nodulisation, viable and degraded haemocytes, non-self materials and melanised debris may be attached to tissue or surrounded by haemocytes. Nodule

formation in insects is not fully characterised although it is known that it is lectin-mediated. For encapsulisation, large structures such as protozoa, nematodes and eggs and larvae of parasitic insects are encapsulated by being surrounded by layers of haemocytes that form a capsule of overlapping cell layers. In addition, circulating haemocytes of crustaceans and other invertebrates perform functions such as phagocytosis, encapsulation and lysis of foreign cells (Johansson *et al.*, 2000). **(iii) The humoral immune response** of insects consists of the processes of melanisation, haemolymph clotting and wound healing in response to injury. In addition, the humoral response involves the synthesis of a range of anti-microbial peptides and heat shock proteins. There are two clotting mechanisms characteristic of insects. The first involves polymerisation of clottable proteins and is catalysed by a calcium-dependent transglutaminase. The clottable proteins are lipophorins and vitellogenein-like proteins that contain a cysteine-rich region homologous to the "d" domain of the mammalian clottable proteins of the Von Willebrand's factor that is involved in the blood coagulation process. The second clotting mechanism is the haemocyte-derived clotting cascade, where clotable proteins are released into the haemolymph from cytoplasmic L-granules of haemocytes in response to activation by cell wall components of invading microbes.

Although insects do not produce antibodies, they are capable of generating a series of proteins that confer a degree of non-specific immunity to a range of microorganisms. Humoral factors involved in insect immunity to infection include lysozyme, lectins, the prophenoloxidase cascade, serine proteases and carbohydrases.

3.3 Adaptive-like, acquired and quasi immunity in invertebrates

Arala-Chaves and Sequeira (2000) have provided some interesting insights on the topic of adaptive immunity in invertebrates. They proposed that although invertebrates have a lower diversity of receptors for immunostimulants than vertebrates, this limited diversity does allow an immune response of different intensity, at least to some antigens. In addition, they proposed that an adaptive immune response can be detected in invertebrates after challenge with certain antigens but not with others. They proposed that a peculiar form of adaptive immune response, quantitatively and qualitatively different from that of vertebrates, probably exists in invertebrates as shown in Table 3.

Table 3. Comparison of postulated secondary adaptive immune response in vertebrates and invertebrates.

Cellular events	Vertebrates	Invertebrates
Proliferation	+++[a]	+
Morphological activation	+++	++
Memory cells	+++	-(?)
Molecular expression		
Igs	+++	-
TCRs	+++	-
MHC	+++	-
Adhesion IgSF molecules	+++	+++
Cytokines	+++	++(?)[b]

[a]Intensity of observation

[b]Evidences obtained by fluorescent staining but never been cloned so far

Adaptive immunity has been assumed to be absent from invertebrates because they lack the immunoglobulin (Ig), T cell receptor (TCR) and Major histocompatibility complex (MHC). Since adhesion Ig super family (SF) molecules that are known in mammals to be involved in adaptive immune response are present in invertebrates, it can be postulated that they may also be responsible for invertebrate adaptive immunity (Arala-Chaves & Sequeira, 2000). However, because invertebrate IgSF molecules are not phylogenetically homologous to those of vertebrates, the existence of an anticipatory immunity has not been accepted in invertebrates. It has also been postulated that the antigen receptors in invertebrates have a low range of diversity leading to similar responses to disparate immunostimulants. The investigators have observed that the haemocyte proliferation rate (HPR) of *Penaeus japonicus* was increased by a similar extent after stimulation with different mitogens, although at a lower magnitude than after fungal infections. Besides, *Drosophila* responses discriminate between fungus and bacteria. Furthermore, upon comparison of the HPR after a single and a second challenge with fungal antigens, they observed that after a second challenge there was

an increased HPR that correlated with cell activation. This increase was, however, much smaller than that observed in lymphocyte proliferation between a vertebrate primary and secondary immune response. This observation is suggestive of a peculiar form of adaptive immunity in invertebrates that can constitute, nevertheless, the basic tool for vaccination strategies.

In a related topic, evidence of specific tolerance to viral paghogens has been reported for the black tiger shrimp *P. monodon* in Thailand (Flegel, 1997). Briefly, the shrimp appeared to rapidly develop tolerance to the viruses WSSV and YHV within the lifetime of a single batch of shrimp, but the exact mechanism was unknown. Later, the "*viral accommodation theory*" was proposed to explain this phenomenon (Flegel & Pasharawipas, 1998). At the same time, a group of investigators from Japan reported the occurrence of a Quasi-immune response to WSSV in *Penaeus japonicus* (Venegas *et al.*, 2000; Namikoshi, 2004). This phenomenon was observed in shrimp that had survived a WSSV outbreak in a farm in Hiroshima, Japan.

A similar phenomenon has been reported suggesting that insects can also be immunized (Arala-Chaves & Sequeira, 2000; Little & Kraaijeveld, 2004). The latest example of an "acquired" response in an invertebrate has been briefly reviewed by Little and Kraaijeveld (2004) who demonstrated that prior infection with a parasite in the beetle *Tenebrio molitor* increased its chance of survival upon subsequent challenge with the same parasite. This indicated that past experience with a p*ATH*ogen could provide individual invertebrates or their descendants with enhanced immunity by a process they called "immunological priming" that resembled "acquired immune response" in vertebrates. So far, invertebrate immunological priming is known only from phenomenological studies of whole organisms and its mechanistic basis is not known. Linking functional to phenomenological studies will be exciting indeed, and should foster a new era in the evolutionary ecology and epidemiology of immunity and disease.

The idea of acquired immunity in invertebrates has long been refuted because they lack immunoglobulins. However, this do*Gm*a might now fall since studies such as those by Moret and Siva-Jothy (in press) show that at least some invertebrates have functional equivalents to the acquired response of vertebrates. The terms of "specific" and "general" immunity have been used by some groups of investigators (Little *et al.*,

2003; Huang & Song, 1999; Moret & Schmid-Hempel, 2001). However, the use of the word "specific" does not necessarily imply acquired immunity, as it does in vertebrate immunology (Little *et al.*, 2003). Evidence for memory in invertebrate immunity has been investigated by Kurtz & Franz (2003) using a copepod model system. The results indicated that an invertebrate defense system might be capable of specific memory.

Most investigations have been focused on the response of invertebrates to bacteria, fungi and parasites and little work has been done on the response to viral p*ATho*gens. Basic knowledge in this area is much needed and may lead to new strategies for disease prevention and control.

MATERIALS AND METHODS

1. DNA sequences used for the comparison of penaeid shrimp and insect parvoviruses

The shrimp DNA and putative protein sequences used for alignment and comparison were SMVmon (GenBank AF499102), HPVmon (GenBank AF456476), HPVchin (GenBank AY008257) and IHHNV (GenBank AF218266). Insect viral sequences from GenBank were *Aal*DNV (X74945), *Aae*DNV (M37899), *Bm*DNV (AB042597), *Ds*DNV (NC001899), *Gm*DNV (L32896), *Jc*DNV (S47266) and *Pf*DNV (NC000936). A key to the numbers and sources is shown in Table 4.

2. *Computer analysis*

BioEdit software (version 5.0.6) was used to manipulate the retrieved sequences (Hall, 1999). The alignment of sequences was performed by using clustal W software (Thompson *et al.*, 1994). For full-length genomes as well as noncoding regions, nucleotide sequences were aligned. For coding regions, the alignment was performed for amino acid sequences. Phylogenetic analysis was performed using several methods. For all methods, positions containing an alignment gap were excluded from pairwise sequence comparisons. Bootstrap re-sampling was performed for each analysis (100 replications). Nucleotide distances were analyzed by using the neighbor-joining algorithm (http://bioweb. pasteur.fr/ seqanal/ phylogeny/ phylip-uk.html) as implemented in the package (NEIGHBOR), based on the Kimura two-parameter distance estimation method or the proportion of differences (p distance). The program TreeView 3.2 was also used to view the phylograms (Page, 1996).

The phylogenetic tree for DNA was constructed based on 696 bp of aligned DNA sequences which encode an unknown protein and share similarity with capsid protein of Brevidensovirus. We used this 696 bp as the basis for the comparisons with all the other parvovirus sequences because it was the only sequence available from HPVmon. Proteins that were analyzed by using program from NCBI (Tatusova and Madden, 1999) and the most similar sequences were used for the phylogenetic analysis. For the analysis of protein similarities, the program version 2.0 (http://www. ch. embnet. org/

software/ LALIGN_form.html) was used to calculate global alignment of sequences (Myers & Miller, 1988).

3. Cell line

Cell line of C6/36 clone of *Aedes albopictus* was used (Igarashi, 1978). The densovirus-free line was grown in minimal essential medium (MEM, GIBCO™ Invitrogen), supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% nonessential amino acid, penicillin (100 units/ml), and streptomycin (0.1mg/ml) at pH7.0. Cells were propagated at 28°C and seeded at 4-day intervals in 25 cm^2 plastic flasks at 10^5 C6/36 cells per flask (Barreau *et al.*, 1996 & 1994). The live C6/36 cells were counted using 0.4% Trypan Blue and hemacytometer under light microscope every times before propagation. Morphology of healthy C6/36 was shown in Figure 7A.

Table 4. Some characteristics of insect and penaeid shrimp parvoviruses.

Abbreviation	Full name	Animal source	Common name	Sequence source	GenBank number
SMVmon	Spawner-isolated mortality virus	*P. monodon*	Black tiger shrimp	GenBank	AF499102
HPVmon	Hepatopancreatic parvovirus	*P. monodon*	Black tiger shrimp	GenBank	AF456476
HPVchin	Hepatopancreatic parvovirus	*P. chinensis*	Chinese white shrimp	GenBank	AY008257
IHHNV	Infectious hypodermal and hematopoeitic necrosis virus	*P. vannamei*	American white shrimp	GenBank	AF218266
*Aal*DNV	*Aedes albopictus* densovirus	*Aedes albopictus*	Mosquito	GenBank	X74945
*Aae*DNV	*Aedes aegypti* densovirus	*Aedes aegypti*	Mosquito	GenBank	M37899
*Bm*DNV	*Bombyx mori* densovirus	*Bombyx mori*	Silkworm	GenBank	AB042597
*Ds*DNV	*Diatraea saccharalis* densovirus	*Diatraea saccharalis*	Sugarcane borer	GenBank	NC001899
*Gm*DNV	*Galleria mellonella* densovirus	*Galleria mellonella*	Greater wax moth	GenBank	L32896
*Jc*DNV	*Junonia coenia* densovirus	*Junonia coenia*	Buckeye	GenBank	S47266
*Pf*DNV	*Periplaneta fuliginosa* densovirus	*Periplaneta fuliginosa*	Smokybrown cockroach	GenBank	NC000936

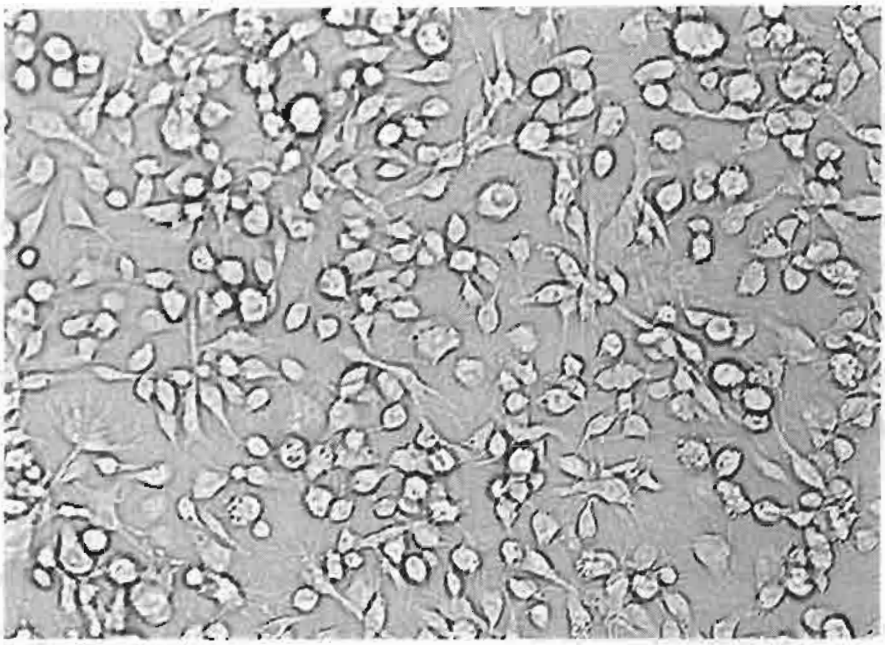


Figure 7. Healthy cell line of C6/36 clone of *Ae. albopictus*.

4. Virus and viral stock preparation

The Thai strain of densonucleosis virus used in this study was detected in a colony of *Ae. albopictus* and *Ae. aegypti* in 1999 by Dr. Pattamaporn Kittayapong from Center for Vectors and Vector-Borne Diseases (CVVD), Faculty of Science, Mahidol University, Thailand (Kittayapong *et al.*, 1999). It was the sixth mosquito densovirus described and was designated *ATh*DNV. Infected larvae from an *Ae. aegypti* colony were used as the source of the virus. About fifty moribund infected mosquito larvae were homogenized with 1.5 ml of GIBCO™ Minimum Essential Medium (MEM) culture medium (MEM; 1% non-essential amino aci*Ds*, antibiotics containing 100 units/ml penicillin and 0.1 g/ml streptomycin) at pH 7.2 without adding heated inactivated fetal bovine serum (FBS). The homogenized viral solution was filtered through 0.2 µm filter membrane and combined with cell culture medium to a final volume of 2 ml. A 3-day-old C6/36 clone free from densovirus infection and with approximately 80% confluence in a 25 cm^2 flask was washed 2 times with phosphate buffered saline (PBS) pH 7.2 before inoculation with 2 ml of the viral suspension for 2 hours at 30°C. The medium was then discharged and washed once with PBS before addition of 5 ml MEM containing 10% heat inactivated FBS and incubation for 3 days at 30°C. The cells were seeded at 4 day intervals with 10^5 cells per 25 cm^2 flask. The 2^{nd} passage of infected C6/36 cells was scraped from the flasks and aliquoted in 0.5 ml lots for storage in micro centrifuge tubes at -80°C.

5. Mosquitoes

An initiating batch of mosquito eggs (several thousand) was provided by the Ministry of Public Health, Thailand from a domestic colony of *Ae. aegypti* that had been maintained in captivity for more than 10 years. The eggs were hatched and reared to adults in cages in a mosquito insectary at 27°C and relative humidity 65-70%. Infected and uninfected mosquitoes were kept in separate rooms of the insectary to avoid contamination. Mosquito larvae were reared in 2 liter water bowls and fed on mice feeding powder while adults were fed on a 10% sugar solution. For egg maturation, females were fed on Wistar rats of 250-300 grams body weight provided by the National Laboratory Animal Center, Mahidol University, Thailand.

6. Challenge of mosquito larvae

First instar mosquito larvae were challenged with *ATh*DNV following a protocol slightly modified from that described by Barreau *et al.* (1996&1994). Four hours after hatching, 500 1st instar larvae were washed in sterile distilled water 2 times to remove feeding medium and then maintained in 5 ml of sterile distilled water in a beaker. Two tubes each containing 0.5 ml of viral stock at -80°C were thawed and added to the beaker with the larvae. These were kept at 30°C. Control larvae were treated in the same manner except that they were mock challenged with an equal volume of harvested C6/36 cells that were densovirus free. After 48 hours, the larvae had already molted to 2nd instar and were transferred to 2 liter water bowls where they fed daily with mouse feeding powder until all had emerged to the adult stage. Dead larvae, pupae and adults were collected daily and stored individually at –80°C.

7. Serial challenge protocol

The scheme for serial *ATh*DNV challenge of successive generations of *Ae. aegypti* is shown in Figure 8. In this model, aliquots of the same densovirus stock solution were used for all challenge tests. The starting population of F0 mosquitoes was screened for absence of *ATh*DNV by PCR, was maintained in parallel throughout the tests and remained *ATh*DNV negative by PCR. One subset of several hundred males and females from this population was used to produce eggs for an F1 challenge test. The larvae hatching from these eggs were placed in bowls in batches of 100 for viral challenge and mock challenge. Survivors were reared to adulthood to produce the next generation of larvae for subsequent challenge. This process was repeated serially for subsequent generations. For the F1 viral challenge, 5 replicates were used while 2 were used for the mock challenge. For later challenges, the number of viral challenge replicates was dependent on the number of survivors from the preceding generation while the mock challenge was held at 2 replicates. Dead, moribund and surviving larvae were counted. Dead and moribund larvae were pooled and stored at –80°C for PCR testing, while surviving adults were pooled for production of the next generation. Because the number of survivors (75) from the replicated F1 challenge was too low to allow for detailed analysis and reproduction for the next generation of larvae, a second pooled batch of 500 F1 larvae was challenged 2 weeks later and the surviving adults (approximately 75) were pooled with those from the first batch. After egg-laying (i.e., eggs for F2), 94 F1 adults were screened for *ATh*DNV by PCR and 15 were used for histology and FISH.

The F2 generation was used for more detailed analysis of *ATh*DNV infection status. For this purpose, a total of 800 F2 eggs was divided into 3 groups: 500 to reproduce the following generation of larvae for challenge tests; 200 to test for the presence of *ATh*DNV by PCR analysis at 3^{rd}-4^{th} instars; 100 to examine for the presence of *ATh*DNV by semi-thin sections and by TEM in 1^{st} instars within 2 hr after hatching (to avoid infections by horizontal transmission). For PCR analysis, it was necessary to rear to the 3^{rd}-4^{th} instar larvae stage in order to have sufficient DNA for the assays.

8. DNA extraction

To begin experiments the starting mosquito population was screened twice for devoiding from *ATh*DNV by selecting 150 larvae from a batch of approximately 3,000 so that a negative test would indicate with 95% probability the absence of *ATh*DNV at the level of 2% prevalence (Cameron, 2002). The larvae were pooled and homogenized with a glass rod in 5ml lysis buffer TF1 (50 mM Tris-HCl pH 9.0, 100 mM EDTA, 50 mM NaCl, 2% *SDS*, 1 mg/ml Proteinase K; National Center for Genetic Engineering and Biotechnology, Bangkok 10400 Thailand, Vol.1 No.1 March 2001). DNA was extracted using the phenol:chloroform technique of Sambrook *et al.* (1989) with commercial phenol saturated with TrisHCl. DNA in the final upper phase was precipitated with 2 volumes of cooled absolute ethanol and then washed with 70% ethanol before air-drying and resuspension in 100 µl TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0) containing 1 µl of 10 mg/ml RNase solution. The DNA was stored at -20°C and relative amount was measured using a spectrophotometer at wavelength 260 nm. After initial population screening, all tests were done with individual mosquitoes ground using a glass rod in lysis buffer TF1 at the ratio of 500 µl lysis buffer for 1 mosquito specimen.

9. *ATh*DNV screening by PCR

Screening of mosquito stocks and cell lines for *ATh*DNV was carried out by polymerase chain reaction (PCR) assay using specific primers from the densovirus open reading frame 3 (O'Neill *et al.*, 1995) newly designed by the Center for Vector and Vector Borne Diseases (CVVD)(unpublished data), Faculty of Science, Mahidol Univeristy, Bangkok

Thailand. One hundred and fifty micro liters of DNA extract was used for the PCR reaction. DNA extract from an *ATh*DNV infected *Ae. albopictus* C6/36 cell culture was used as the positive control while sterile double distilled water and DNA extract from *Ae. albopictus* C6/36 cells free of densovirus infection were used as negative control templates. Total DNA (50-150 ng in 3 µl) used as the template for PCR in a total of 20 µl containing 2 µl of 10x buffer (Promega, Madison, WI), 2 µl of 25 mM $MgCl_2$, 0.5 µl of dNTPs (10mM each), 0.5 µl of each primer (20mM each), and 1 unit of *Taq* DNA polymerase (Promega). The mixture was subjected to amplification in a PCR Thermal Cycler (Gene Amp PCR system 6900, Perkin Elmer, Foster City, CA, USA). For PCR, the initial denaturation (prePCR) was at 95°C for 5 min, followed by 35 cycles of denaturating at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, followed by final extension (postPCR) at 72°C for 7 min. PCR products were analyzed by standard 1.5% agarose gel electrophoresis together with a 1,500 bp and 100 bp ladder DNA marker (Promega). Samples that yielded products of the expected size (350bp, ORF3) were scored as positive.

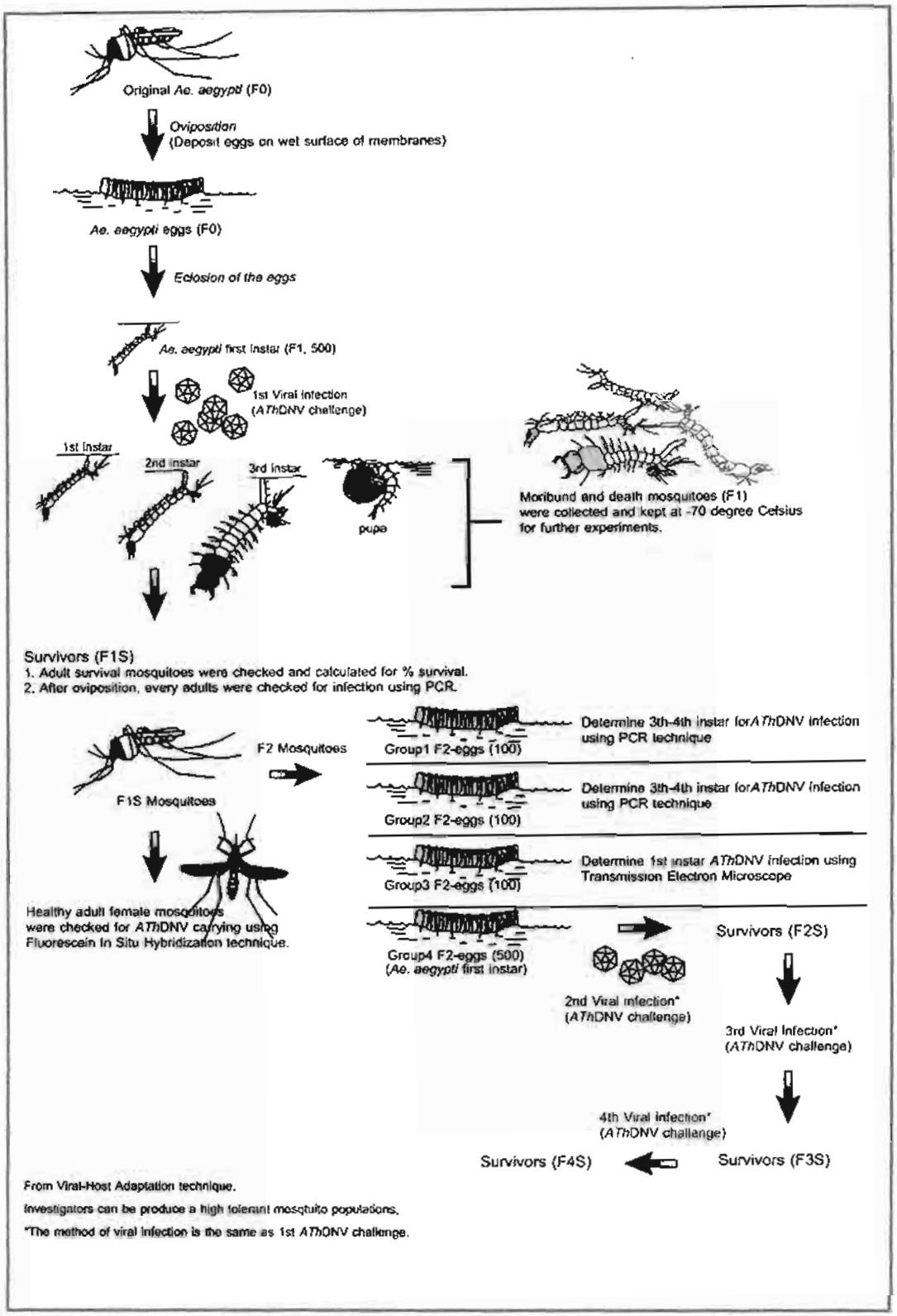


Figure 8. Diagram of the overall experimental protocol.

sections were cut in a Reichert ultramicrotome, mounted on formvar-coated copper grid, and examined in the transmission electron microscope (Hitachi H-7500) after staining in aqueous uranyl acetate and lead citrate. Semi-thin plastic sections for light microscopy (LM) were stained with 1% toluidine blue.

12. Cloning and sequencing of DNA

DNA fra*Gm*ents purified from agarose gels were ligated to pDrive cloning vector (QIAGEN) following the QIAGEN manual protocol as shown in Figure 9. The ligated product was transformed into *Escherichia coli* JM109. Transformed cells were spread together with 50μl of 20mg/ml X-gal in dimethylformamide solution on Luria Bertani (LB) agar (LB broth, Difco, Detroit, MI, USA, plus 1.5% agar) containing 50μg/ml of ampicillin. White colonies were selected as positive transformants. Recombinant plasmi*Ds* were then purified, DNA concentrations measured at OD_{260} and 150 ng used to check insertion of PCR fra*Gm*ents. All isolates showing inserts were sequenced from both stran*Ds* using and ABI PRISM Dye Terminator Cycle Ready Reaction Kit (Perkin Elmer) with and ABI PRISM 377 automated DNA sequencer by Macrogen Company Limitted (Chongro-Ku, South Korea).

10. *ATh*DNV detection by fluorescene *in situ* hybridization (FISH)

A fluorescein-labeled DNA probe was prepared by PCR using the procedure described above with a plasmid template containing an *ATh*DNV fra*Gm*ent from open reading frame 3 (O'Neill *et al.*, 1995) to amplify a 350 bp DNA fra*Gm*ent. Labelling was carried out using fluorescein-12-dUTP (Roche Molecular Biochemicals, Germany) according to instructions in the product manual (version 3, Sept.1999). The labeling mixture included sterile redistilled water, 10x PCR buffer without $MgCl_2$, 4mM $MgCl_2$, 200μM dNTP (PCR Fluorescein Labelling Mix), 1μM primers, 1U Taq DNA polymerase (Promega) and template of an extracted viral DNA 150 ng. The fluorescein-labeled probe was purified using a QIAquick PCR purification kit (QIAGEN, Germany) and stored in 100 μl of elution buffer at -20°C until used.

Mosquito samples were fixed in Davison's AFA for 2-4 h before being dehydrated and embedded in paraffin using standard histological metho*Ds* (Lightner, 1996). Sections (4 μm thick) were cut and mounted on Superfrost Plus Slides (Fisher Scientific, PA, USA). Prior to hybridization, slides with sections were incubated on their sides at 65°C for 45 min, deparaffined with xylene and then rehydrated through an ethanol series to water. The *in situ* hybridization protocol followed that described by Lightner (1996). The sections were then counterstained by immersing the slides in 40 ml of a freshly prepared propidium iodide solution (1 μg/ml in PBS, pH 7.4) for 15 min at room temperature. The sections were washed three times with de-ionized water 5 min and 1 drop of anti-fade solution (Verstashield, H-1000) was added to the sections before covering with a coverglass and viewing by fluorescence microscopy. Green fluorescence at 520±20 nm indicated a positive hybridization reaction while red fluorescence from propidium iodide at >620 nm indicated normal nuclei.

11. Normal histology and electron microscopy

Preparation of adult mosquitoes for normal histology was done according to Lightner (1996). Larval specimens for transmission electron microscopy (TEM) were fixed for 2 h in 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH7.8 and then washed twice in 0.1M cacodylate buffer, pH7.8 at 4°C. The samples were post-fixed for 2-3 h in 1% osmium tetroxide in 0.1M cacodylate buffer at pH 7.8. The samples were then dehydrated in a graded series of ethanol and embedded in an Epon-Aradite mixture. Silver to pale gold

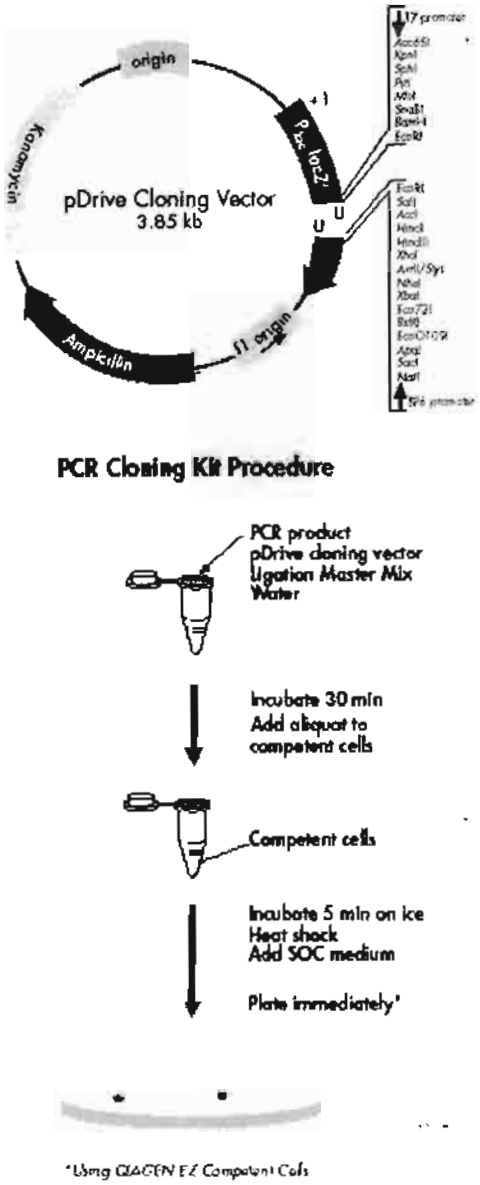


Figure 9. The pDrive cloning vector and procedure for cloning of PCR products (QIAGEN)(http://www1.qiagen.com/Products/Cloning/PCRCloningSystem.

RESULTS

1. Characteristics of insect and penaeid shrimp parvoviruses

From a literature review (Afanasiev *et al.*, 1991; Bando *et al.*, 1987a,b, 1990; Bonami *et al.*, 1990; Boublik *et al.*, 1994; Dumas *et al.*, 1992; Jousset *et al.*, 2000; Lightner & Redman, 1985; Lightner *et al.*, 1983, 1994; Owens *et al.*, 1991; Sukhumsirichart *et al.*, 1999), virion size for insect and penaeid shrimp viruses ranged between 18 and 30 nm diameter and all had genomes of ssDNA that ranged between 4 and 6 kb (Table 5). These characteristics would place them in the Family Parvoviridae. The smallest virion size (18 nm) and shortest length genome (4009 bp) belonged to the densovirus from *Aedes aegypti* (*Aae*DNV) and the longest (about 6 kb) to the densovirus from *Culex pipiens* (*Cp*DNV). The fact that these viruses are in the same family and all occur in *arthropods* opened the possibility that they might be ancestrally related in some way.

2. Sequences comparisons of insect and penaeid shrimp parvoviruses

Using programs to compare all of the tested nucleic acid sequences, the highest homology (77% DNA identity) was found between HPVmon and HPVchin. However, using the global alignment method from LALIGN program to detect homology of putative proteins from the two showed only 15.3% similarity. No other significant DNA homology was found. Results using the Clustal W program were the same. However, when Clustal W was used to compare isolated pairs of DNA sequences, some homology was found between IHHNV and *Aae*DNV or *Aal*DNV. A phylogenetic tree based on genomic DNA of shrimp and insect parvoviruses (Figure 10) gave two main clades (clusters) that included: Clade 1 with SMVmon, *Pf*DNV, *Ds*DNV, *Gm*DNV, *Jc*DNV, and *Bm*DNV; and Clade 2 with HPVmon, HPVchin, IHHNV, *Aal*DNV and *Aae*DNV.

Table 5. Some characteristics of insect and penaeid shrimp parvoviruses.

Virus name	Probable classification in the Family Parvoviridae	Their known natural and experimentally infected hosts	Virion size	Length of genome	References
IHHNV=Infectious Hypodermal and Hematopoietic Necrosis Virus (*Pst*DNV)	Densovirus	1. Litopenaeus: *Penaeus vannamei* *P. stylirostris* *P. setiferus* *P. schmitti* *P. occidentalis* 2. Penaeus *P. monodon* *P. esculentus* *P. semisulcatus* 3. Fenneropenaeus *P. chinensis* 4. Marsupenaeus *P. japonicus* 5. Farfantepenaeus *P. aztecus* *P. duorarum* *P. californiensis*	22 nm	4075 bp (IHHNV) 3873 bp (*Pst*DNV)	(Bonami *et al.*, 1990, Lightner *et al.*, 1983) (Shike *et al.*, 2000)
HPV= Hepatopancreatic Parvovirus	Parvovirus	1. Litopenaeus: *Penaeus vannamei* *P. stylirostris* *P. schmitti* 2. Penaeus *Penaeus monodon* *P. esculentus* *P. semisulcatus* 3. Fenneropaeus *Penaeus chinensis* *P. merguiensis* *P. indicus* *P. penicillatus* 4. 4. Marsupenaeus Penaeus japonicus	22-24 nm	4-4.3kb 5.8 kb	(Bonami *et al.*, 1995, Lightner & Redman, 1985) (Sukhumsirichart *et al.*, 1999)
SMV=Spawner-Isolated Mortality Virus	Parvovirus	Penaeus *Penaeus monodon*	20 nm	ND	(Fraser & Owens, 1996)
*Aae*DNV=*Aedes aegypti* Densonucleosis Virus	Densovirus	Mosquitoe: *Aedes aegypti*	18-26 nm	4009 bp	(Afanasiev *et al.*, 1991)

Table 5. (continued) Some characteristics of insect and penaeid shrimp parvoviruses.

Virus name	Probable classification in the Family Parvoviridae	Their known natural and experimentally infected hosts	Virion size	Length of genome	References
*Aal*DNV=*Aedes albopictus* Parvovirus	Contravirus	1. Mosquitoe: *Aedes aegypti* 2. *Aedes albopictus* C6/36 cell line	22 nm	4176 bp	(Boublik *et al.*, 1994)
*Bm*DNV=*Bombyx mori* Densovirus	Densovirus	Silkworm: *Bombyx mori*	ND	5084 bp	(Bando *et al.*, 1990, 1987b)
*Ds*DNV=*Diatraea saccharalis* Densovirus	Densovirus	Sugarcane borer (insects or their larvae)	ND	5941 bp	Boublik *et al.*, (unpublished data)
*Gm*DNV=*Galleria mellonella* densovirus	Densovirus	Lepidoptera: Pyralidae *Galleria mellonella* larvae	ND	6039 bp	Simpson *et al.*, 1998 (unpublished data)
*Jc*DNV=*Junonia coenia* Densovirus	Densovirus	Butterfly: *Junonia coenia*	ND	5908 bp	(Afanasiev *et al.*, 1991, Dumas *et al.*, 1992)
LPV=Lymphoidal Parvo-like Virus	Parvo-like virus	1. Penaeus *Penaeus monodon* *P. esculentus* 2. Fenneropenaeus *P. merguiensis*	25-30 nm	ND	(Owens *et al.*, 1991)
*Pf*DNV=*Periplaneta fuliginosa* densovirus (CSSV)	Densovirus	*Smoky-brown cockroach* *Periplaneta fuliginosa*	22	5454 bp	(Hu *et al.*, 1994)
*Cp*DNV=*Culex pipiens* Densovirus	Densovirus	Mosquito: *Culex pipiens*	ND	6 kb	(Jousset *et al.*, 2000)

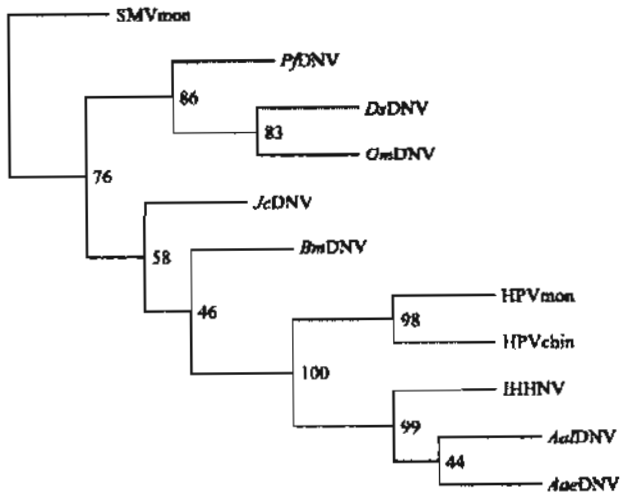


Figure 10. The neighbor-joining phylogenetic tree generated from a heuristic search of aligned insect and shrimp parvoviruses DNA sequences. The phylogenetic tree was constructed based on 696 bp of aligned DNA sequences. Bootstrap values are indicated as number at each branch (100 replications). For the sources of DNA sequences data are shown in Table 4.

A phylogenetic tree based on genomic DNA of shrimp and insect parvoviruses (Figure 10) gave two main clades (clusters) that included: Clade 1 with SMVmon, *Pf*DNV, *Ds*DNV, *Gm*DNV, *Jc*DNV, and *Bm*DNV; and Clade 2 with HPVmon, HPVchin, IHHNV, *Aal*DNV and *Aae*DNV. Comparisons were also made using amino acid sequences for nonstructural protein (NS1), structural protein, capsid/coat proteins (VP) and putative proteins of unknown function that showed similarity using global alignment from LALIGN program. There was some homology between the shrimp viral proteins and known capsid proteins of insect viruses (Figure 11A). Other proteins that showed homology were capsid protein of HPVchin and unknown protein of HPVmon (15.3%) and proteins of insect parvoviruses (Figure 12A). It was interesting that the capsid protein of IHHNV showed very low similarity to capsid proteins of *Aae*DNV and *Aal*DNV, even though its NS1 showed similarity to their NS1 (i.e. IHHNV and *Aae*DNV, 18.2%; IHHNV and *Aal*DNV, 20.4%). The results were used to construct a phylogenetic tree (Figure 11B).

(A)

HPVmon Unk	HPVmon Unk								
HPVchin Capsid	15.3	HPVchin Capsid							
IHHNV Capsid	0.4	0.2	IHHNV Capsid						
*Aae*DNV VP1	0.5	0.3	0.1	*Aae*DNV VP1					
*Aal*DNV VP	0.7	0.2	2.2	76.6	*Aal*DNV VP				
*Je*DNV orf1	5.4	0.5	0.1	1.1	0.6	*Je*DNV orf1			
*Gm*DNV VP1	0.1	0.7	0.2	1.0	0.6	87.5	*Gm*DNV VP1		
*Bm*DNV Capsid	0.3	0.2	0.3	0.1	0.2	15.2	15.6	*Bm*DNV Capsid	
*Pj*DNV orf5	2.5	0.5	0.2	2.0	0.2	8.6	9.7	4.8	*Pj*DNV orf5
*Ds*DNV VP1	0.1	0.5	0.0	1.0	0.2	83.1	78.7	14.9	8.6

(B)

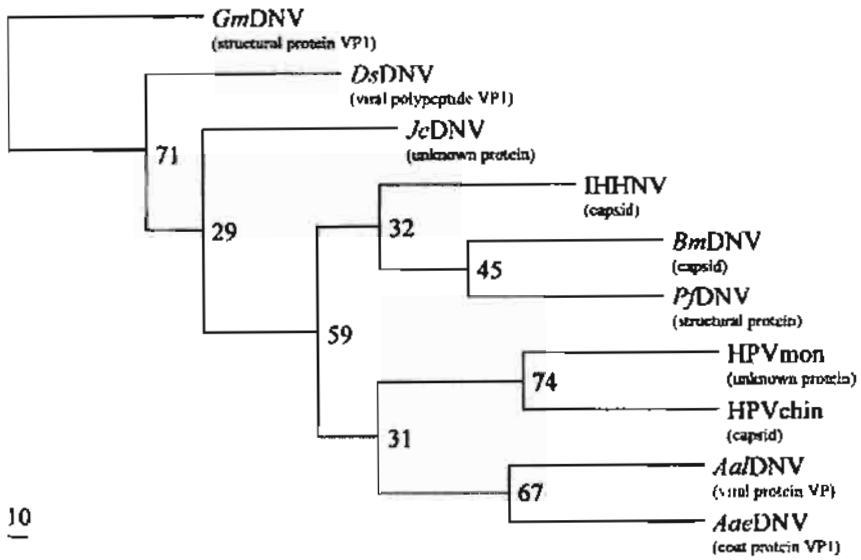


Figure 11. Comparisons based on putative protein sequences of capsid and structural proteins using a global alignment method (LALIGN). (A) Overall parvoviral protein similarities. (B) The neighbor-joining phylogenetic tree generated from a heuristic search of aligned insect and shrimp parvoviruses deduced amino acid sequences. The scale shown in the left-hand corner of the figure indicates the proportion of distance differences. Bootstrap values are indicated as number at each branch (100 replications).

(A)

HPVmon Unk	HPVmon Unk							
IHHNV NS1	1.6	IHHNV NS1						
*Aae*DNV NS	1.3	18.2	*Aae*DNV NS					
*Aal*DNV NS1	1.5	20.4	73.8	*Aal*DNV NS1				
*Jc*DNV Orf2	0.3	13.7	11.7	12.1	*Jc*DNV orf2			
*Bm*DNV NS orf1	0.5	0.1	0.2	0.4	17.7	*Bm*DNV NS orf1		
*Pj*DNV NS	1.1	0.2	0.4	11.2	28.0	18.0	*Pj*DNV NS	
*Ds*DNV NS1	0.3	14.3	29.7	11.5	92.8	17.8	29.7	*Ds*DNV NS1

(B)

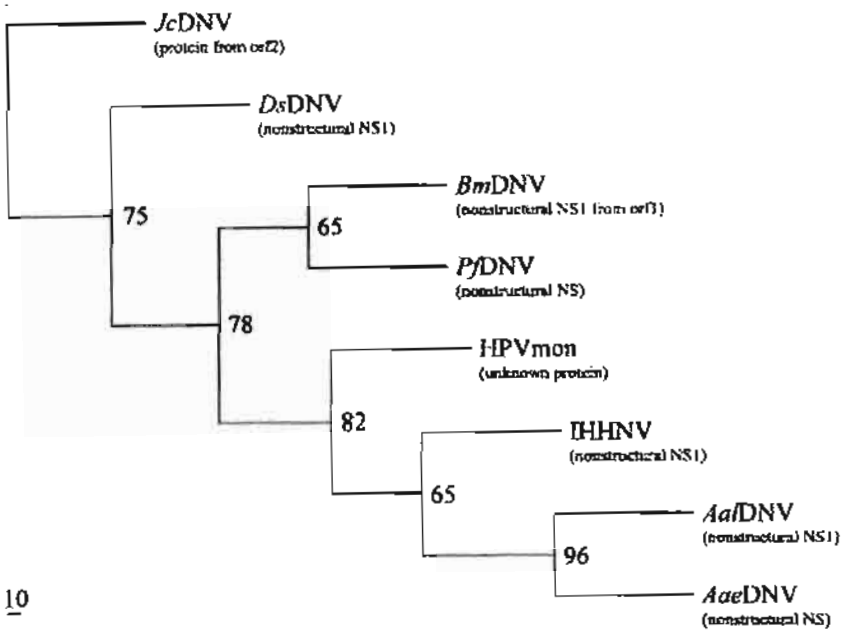


Figure 12. Comparisons based on putative protein sequences of insect virus nonstructural protein (NS) and most similar proteins using a global alignment method. (A) Overall parvoviral protein similarities. (B) The neighbor-joining phylogenetic tree generated from a heuristic search of aligned insect and shrimp parvoviruses deduced amino acid sequences. The phylogenetic tree was constructed based on deduced amino acid sequences of structural proteins. The scale shown in the left-hand corner of the figure indicates the proportion of distance differences. Bootstrap values are indicated as number at each branch (100 replications).

NS1 of IHHNV showed some similarity to the protein translated from ORF2 of *Jc*DNV (13.7%), to NS1 from *Ds*DNV (14.3%) and to NS1 or putative proteins of other viruses (Figure 12A). The results of the comparison were used to generate another phylogenetic tree (Figure 12B). A putative protein of HPVmon showed high homology to the capsid protein of HPVchin but both showed very low homology to the capsid protein of IHHNV. The phylograms based on capsid or related protein sequences (Figure 11B) and NS1 or related sequences (Figure 12B) were somewhat different.

3. Host-viral adaptation

From total 500 larvae of the offspring (F1) generated from naïve mosquito (F0) population, only 15% (75/500) ± 4.12 SD survived challenge with *ATh*DNV. By contrast, survival in the mock challenged control group was 95.5% (191/200) ± 0.71 SD. Challenges with succeeding generations gave increasing percent survival (Table 6), reaching 54.5% (109/200) ± 6.36 SD by the 4th generation while the control population survivals were always over 90%. Although survival increased with each generation, the numbers of produced eggs seemed to decrease. When the 4th generation tests had been completed, the virulence of the freezer virus was confirmed again by challenging a pool of 500 1st instar larvae derived from the maintained parental (F0) mosquito population with the stored *ATh*DNV. Survival was 18% and was thus comparable to survival for the original F1 challenge (*i.e.*, 15% ± 4.12 SD) indicating that infectivity of the freezer virus was unchanged after storage at -80°C for 6 months.

Table 6. Percent survival of each generation of *Ae. aegypti* infected with *ATh*DNV using the host-viral adaptation test. F1 means an offspring the naïve mosquito population.

Mosquito details	Mosquito challenged With *ATh*DNV		Mock challenged mosquitoes	
	Survivors from replicates of 100	% Survival ± SD	Survivors (adults)	% Survival ± SD
1st generation (F1) (naïve population)	75/500 (18, 15, 10, 20, 12)	15.0 ± 4.12	191/200 (96, 95)	95.5 ± 0.71
2nd generation (F2) (offspring of F1 survivors)	74/300 (20, 25, 29)	24.67 ± 4.51	182/200 (92,90)	91.0 ± 1.41
3rd generation (F3) (offspring of F2 survivors)	119/300 (47, 40, 32)	39.67 ± 7.51	181/200 (87,94)	90.5 ± 4.95
4th generation (F4) (offspring of F3 survivors)	109/200 (56, 59)	54.5 ± 6.36	187/200 (97,90)	93.5 ± 4.95

4. *ATh*DNV infection status of challenge survivors

From the challenge protocol, surviving larvae from the F1 challenge were reared to adulthood to generate the next generation for each subsequent test. Because of low number of only 75 F1 survivors, we repeated the same experiment of the first *ATh*DNV challenge and used a portion of F1 survivors for screening of the *ATh*DNV infection using PCR and FISH analyses. After egg-laying, a portion of the F1 adult survivors (94) were screened for *ATh*DNV by PCR and a smaller sub-set of females (15) were used for histology and FISH. Of 109 F1 surviving, adult females collected after laying eggs to generate F2, 94 were sampled by PCR assay and 13 (14%) were positive for *ATh*DNV. Of the remaining females 15 used for histology and FISH, 2 (13.3%) showed evidence of *ATh*DNV infection, giving an overall prevalence of 15/109 = 14%. Two batches of 100 F2 generation eggs hatched and sampled in sets of 50 and 42 larvae yielded 23 (46%) and 9 (21%) positive larvae, respectively. Altogether, there was a total of 32 (35%) positive in 92 taken as a sub-sample of 200 (Group 1 & Group 2 of 100 each, Figure 8) larvae. Using epidemiological computer software (Cameron, 2002), the estimated prevalence could have been as high as 41% in the 200 eggs (Figure 13).

Normal histology using H&E staining (Figure 14) revealed many enlarged nuclei containing typical, eosinophilic to basophilic parvovirus inclusions in adult F1 mosquitoes that arose from challenged larvae. They exibited grossly normal appearance and behavior. Despite the relatively large number of infected cells, there was no visible host inflammatory response and tissues did not appear to be disrupted or necrotic. FISH analysis confirmed the presence of *ATh*DNV nucleic acid in enlarged nuclei from these specimens (Figure 15). FISH and H&E staining for *ATh*DNV in adult *Ae. aegypti* mosquito were used to confirm that surviving adult female mosquitoes were infected with *ATh*DNV. Some eggs had been laid but some also still remained in the egg sac (Figure 16). From the hybridization test we found infected nuclei with green fluorescence (520±20 nm) in cells around the egg tissue/membrane but no egg nuclei were seen in the sections. Semi-thin sections of 1st instar F2 larvae (Figure 17A,17B) confirmed lack of tissue disruption, suggesting that normal functions were maintained despite viral infection. TEM revealed large numbers of 22-24 nm virions free in the nucleoplasm and collected into paracrystalline arrays (Figure 18,19). It also showed lack of tissue disruption. Of 6 blocks prepared from 100 specimens, only 1 showed evidence of viral particles. Application of epidemiological sampling software (Cameron, 2002)

indicated that estimated prevalence could be as high as 57% infection in the sampled population of 100.

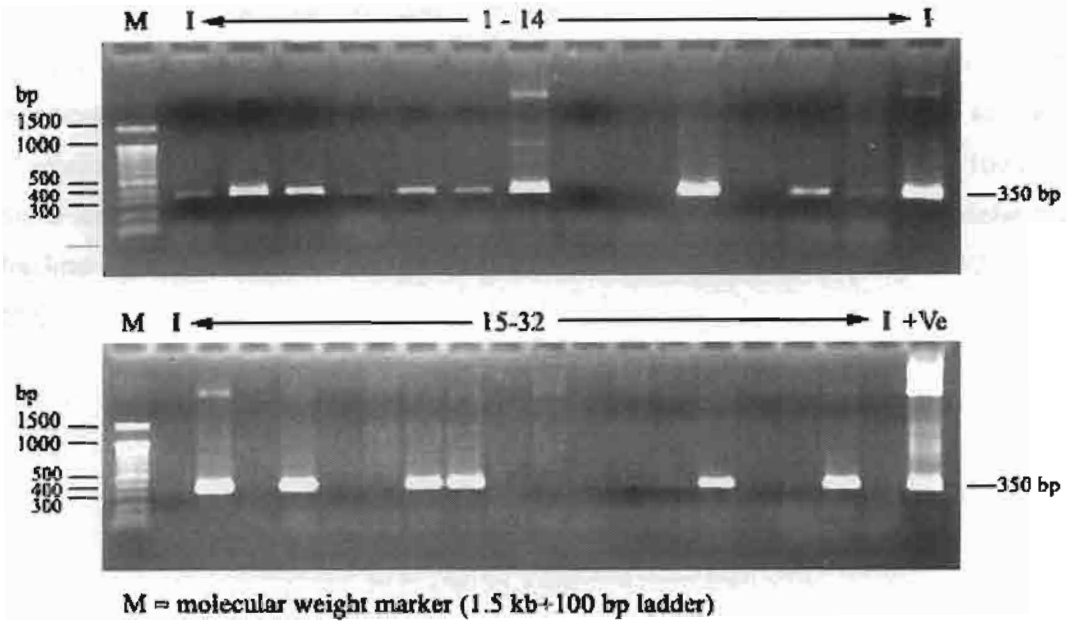


Figure 13. Screening of *ATh*DNV infection status of challenge survivors using PCR method with samples 1-32 showing 19/32 (59%) positive. Mosquito samples 33-94 (62 samples) were also checked by PCR and 13 (21%) out of 62 samples were positive (data not shown).

5. Genomic analysis of *ATh*DNV in stock and surviving mosquitoes

Fifty clones of inserted fra*Gm*ents were sequenced and used for analysis. Each clone was considered to have originated from a different target gene fra*Gm*ent and was translated into a putative amino acid sequence based on alignment to two amplicons from the NS1/NS2 overlap region and one amplicon from the NS1 region by comparison to the full sequence of *Ae. albopictus* densonucleosis virus (*Aal*DNV) genome (GenBank accession number NC_004285) as shown in Figures 20-22. Alignments with 2 portions of NS1 and 1 portion of NS2, revealed that the number of defective sequences in the freezer stock clones was 1/27 (4%) while the number in the F4 survivor clones was 7/23 (30%). In all cases, the defective sequences showed frame shifts resulting from base insertions or deletions. Although the alignments in the NS1/NS2 overlap region in Figures. 20 and 22 were done for NS1 and NS2, respectively, transcription products