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).

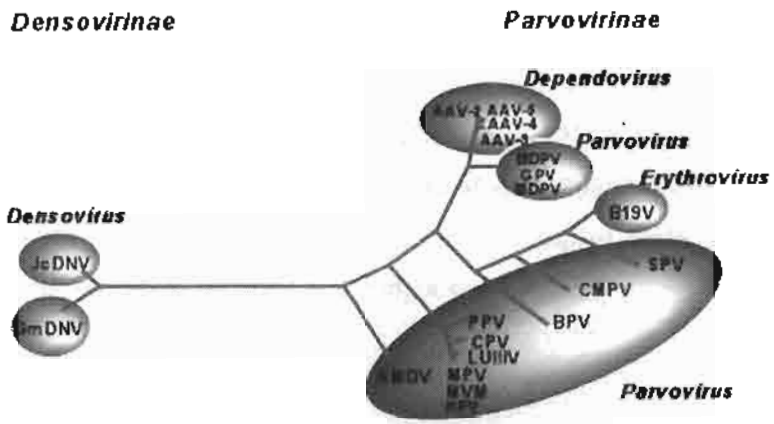
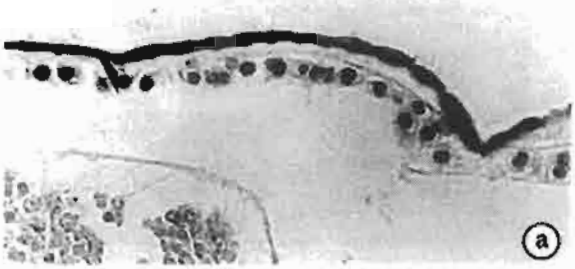


Figure 1. The phylogenetic relationships among the various members of the family *Parvoviridae*, determined by comparison of a conserved region of about 220 amino acid residues from the Rep and NS1 proteins, equivalent to the sequence between residues 254 and 562 of the NS1 protein of Mice minute virus. 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.



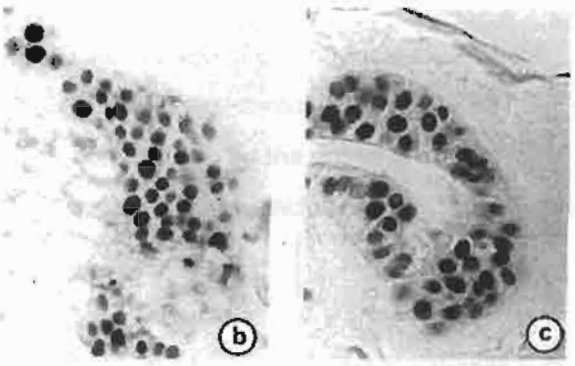
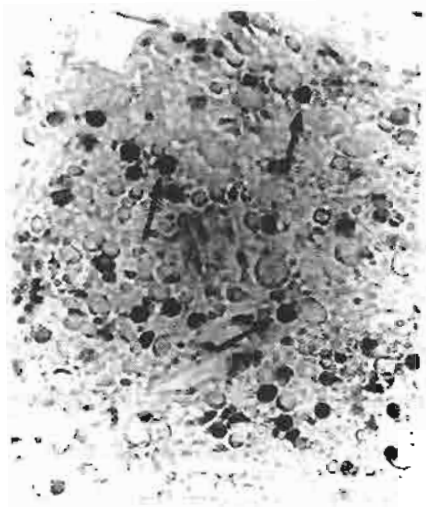


Figure 2A

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).



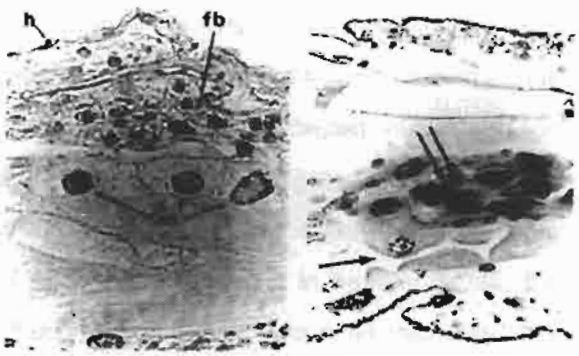
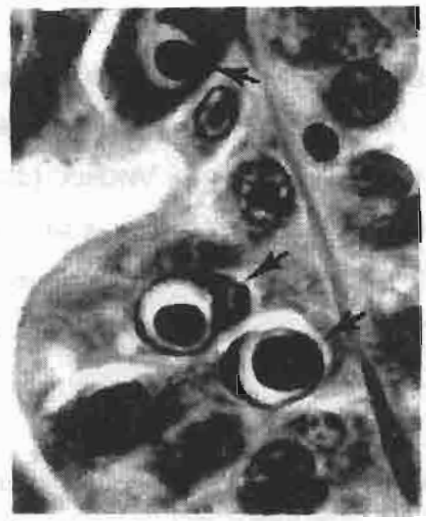
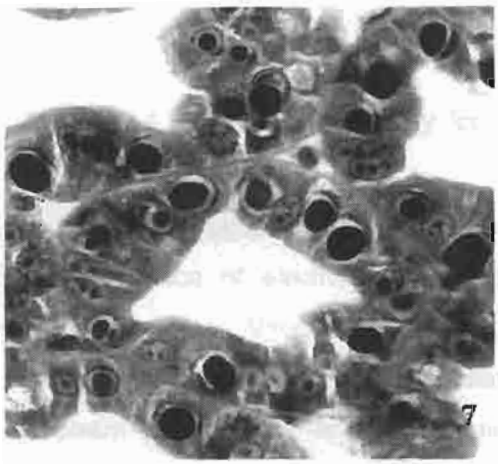


Figure 2B Light micrograph of *Ae. aegypti* lavae infected with *Aal*DNV showing parvo-type inclusion of hypodermal (*h*), fat body (f) and muscle (m).

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).

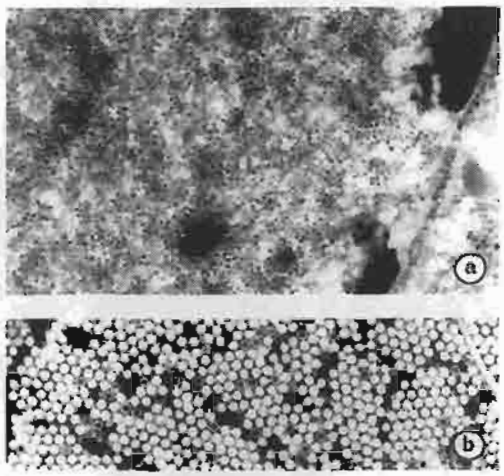


Figure 3 (a) Thin section of a hypertrophied nucleus from an adipose cell of a *C. pipiens* larva heavily infected with *Cp*DNV. Numerous viral particles are visible emerging from an electron dense virogenic stroma (×62500). (b) Purified suspension of *Cp*DNV virions negatively stained with 2% sodium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

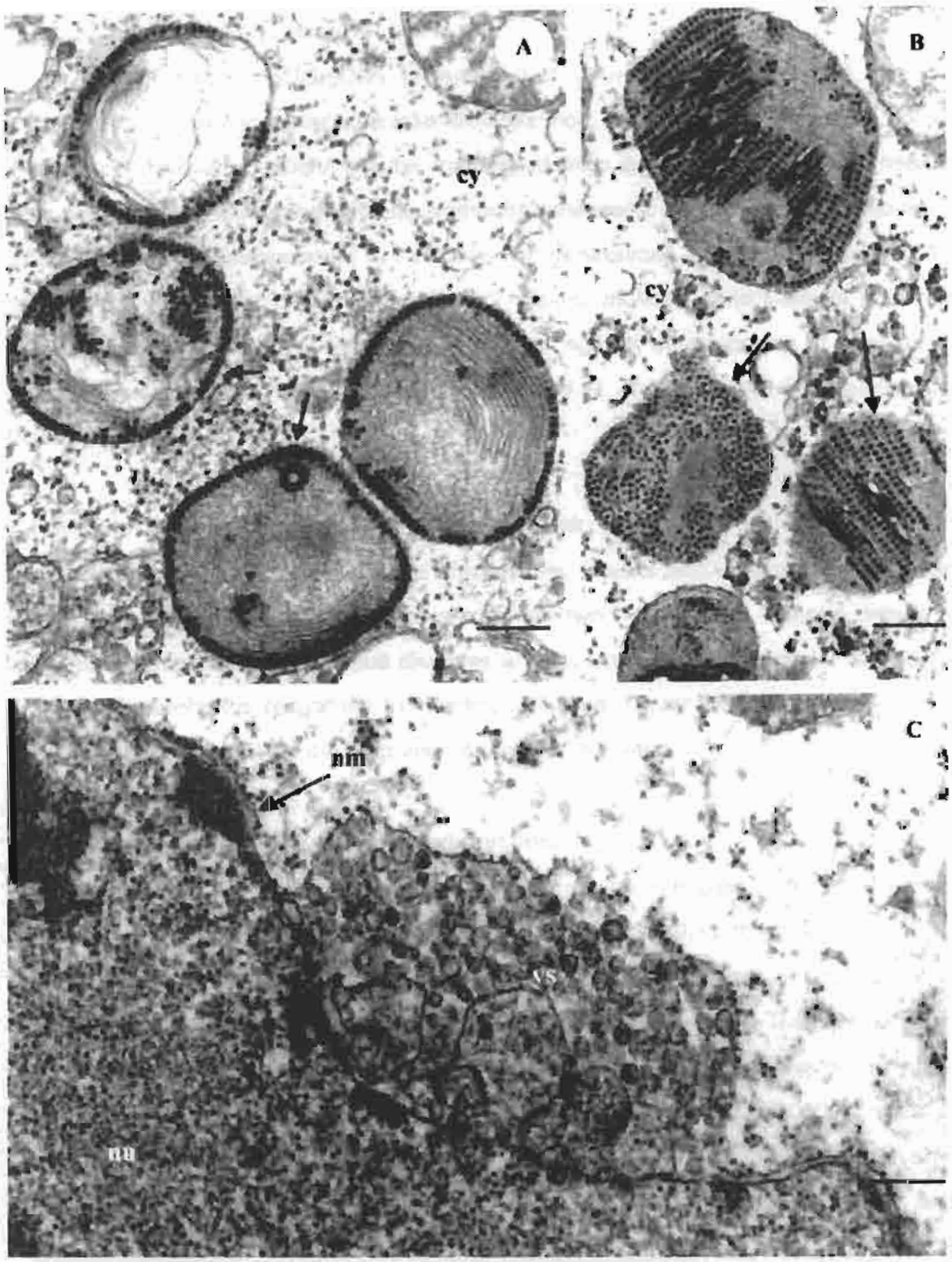


Figure 4. Electron micrographs of ultra thin transverse sections of third instar nymphs of *M. persicae* infected with *Mp*DNV. (A and B) Densovirus particles within vesicles in the cytoplasm (cy). (C) Vesicular structures (vs) between the outer and inner layers of the nucleus membrane (nm), nucleus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

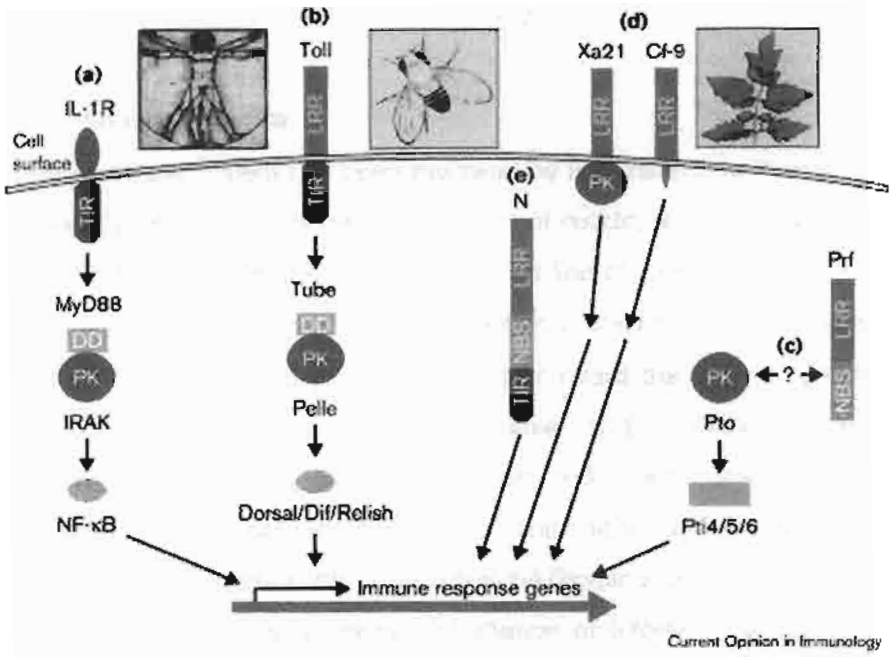


Figure 5. Signalling p*ATh*ways leading to activation of defense response genes in mammals, insects and plants share common components. For example, related PKs occur in the IL-1R, Toll and Pto–Prf defence p*ATh*ways in humans, *Drosophila* and tomato, respectively. Other common elements in defense p*ATh*ways 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).

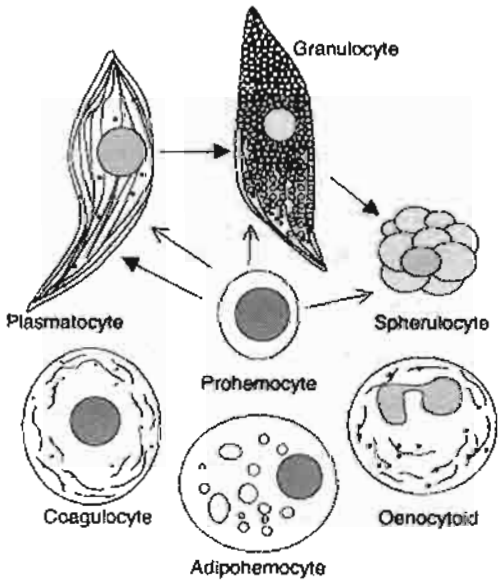


Figure 6. Haemocyte types involved in the cellular immune response. Linear maturation process in *Lepidoptera* (closed arrow) or differentiation of prohemocytes into different cell types (open arrows).

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

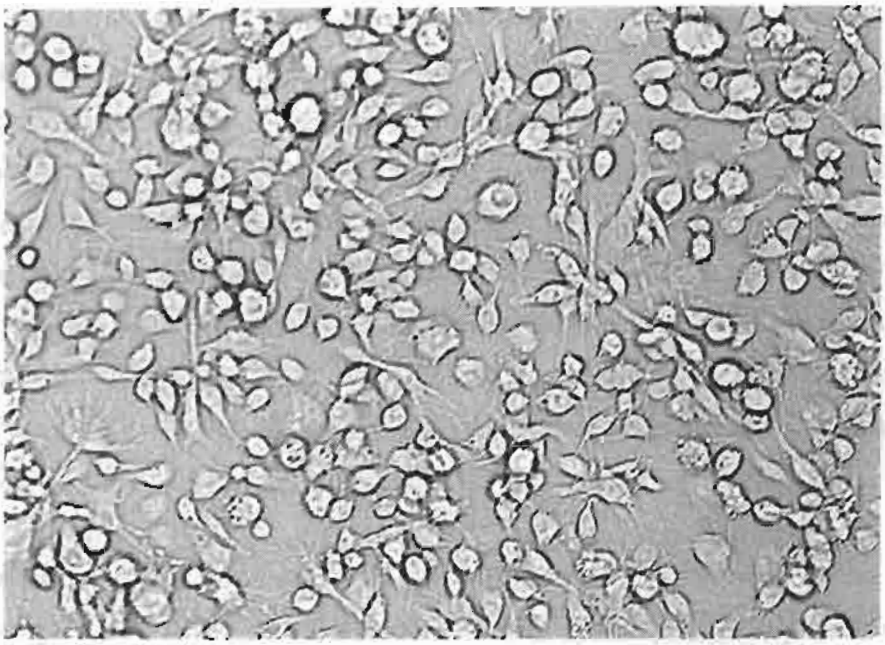


Figure 7. Healthy cell line of C6/36 clone of *Ae. albopictus*.

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.

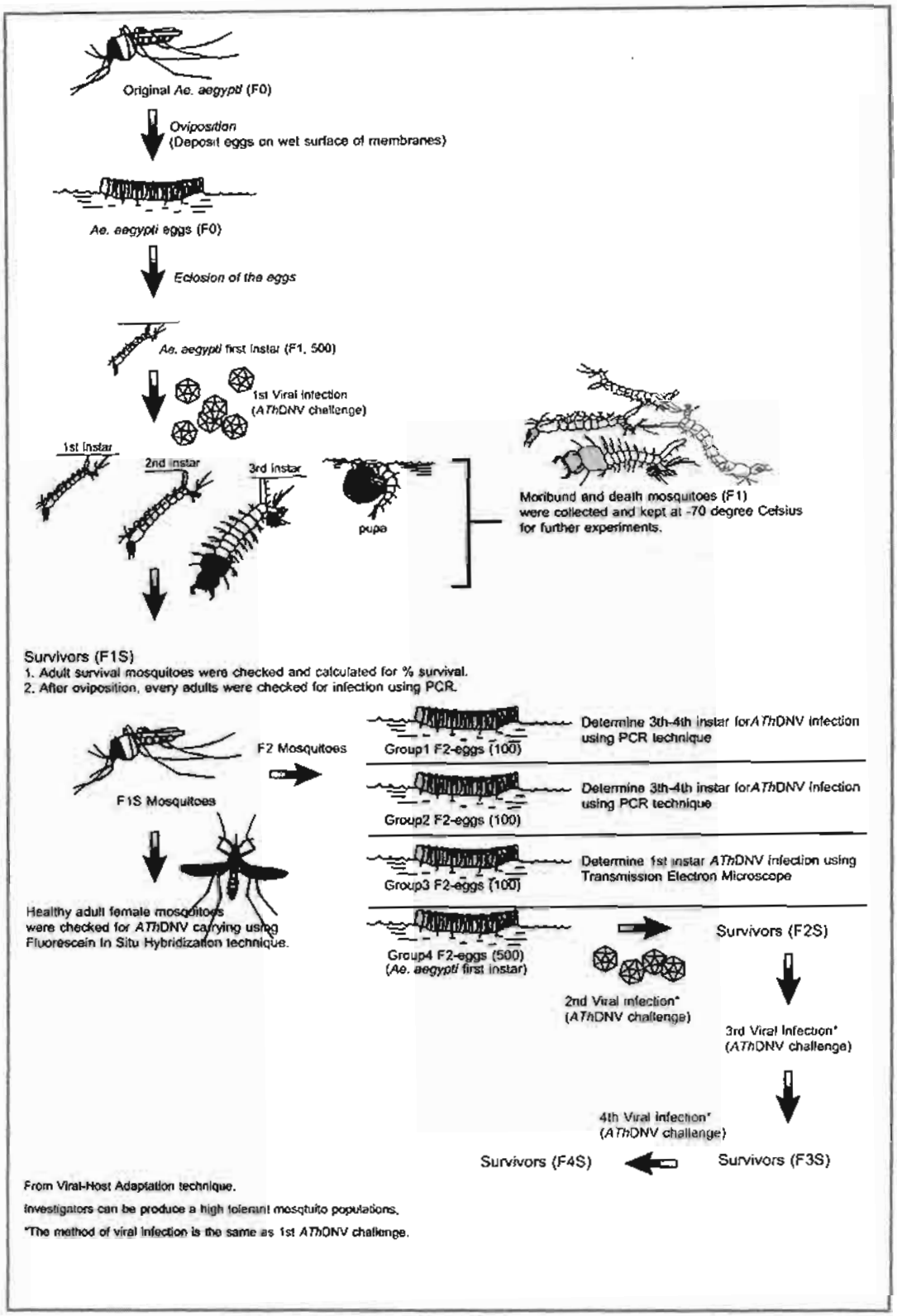


Figure 8. Diagram of the overall experimental protocol.

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

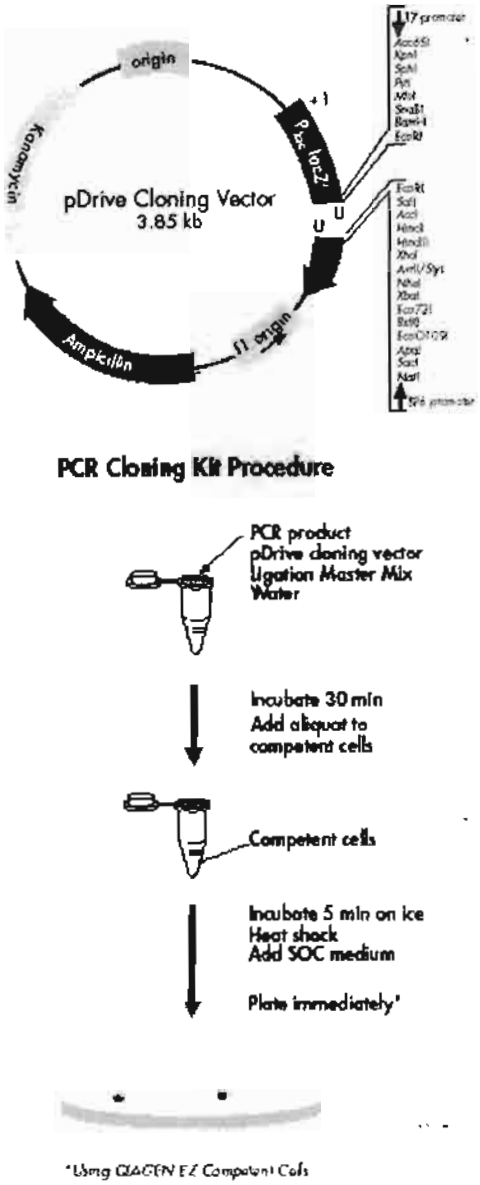


Figure 9. The pDrive cloning vector and procedure for cloning of PCR products (QIAGEN)(http://www1.qiagen.com/Products/Cloning/PCRCloningSystem.

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)

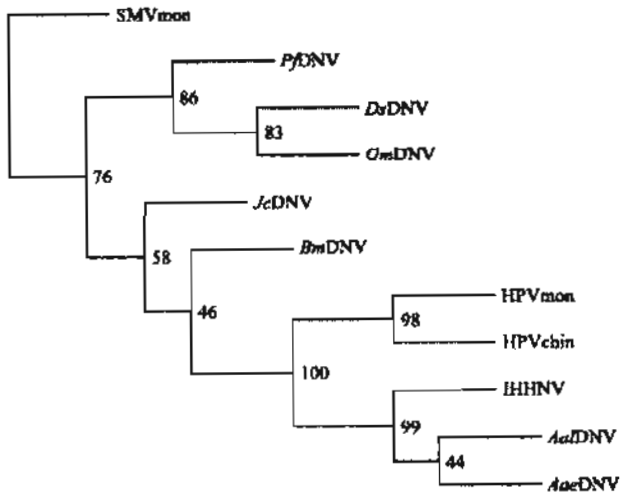


Figure 10. The neighbor-joining phylogenetic tree generated from a heuristic search of aligned insect and shrimp parvoviruses DNA sequences. The phylogenetic tree was constructed based on 696 bp of aligned DNA sequences. Bootstrap values are indicated as number at each branch (100 replications). For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

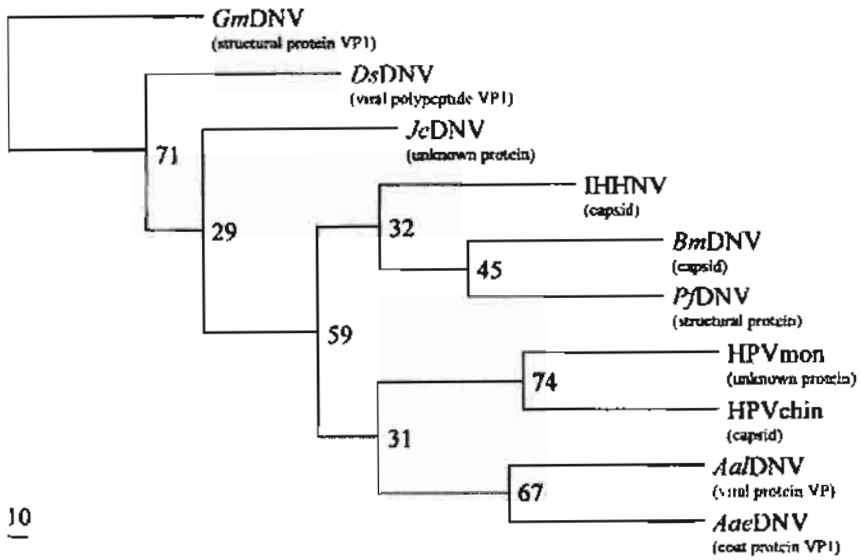


Figure 11. Comparisons based on putative protein sequences of capsid and structural proteins using a global alignment method (LALIGN). (A) Overall parvoviral protein similarities. (B) The neighbor-joining phylogenetic tree generated from a heuristic search of aligned insect and shrimp parvoviruses deduced 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)

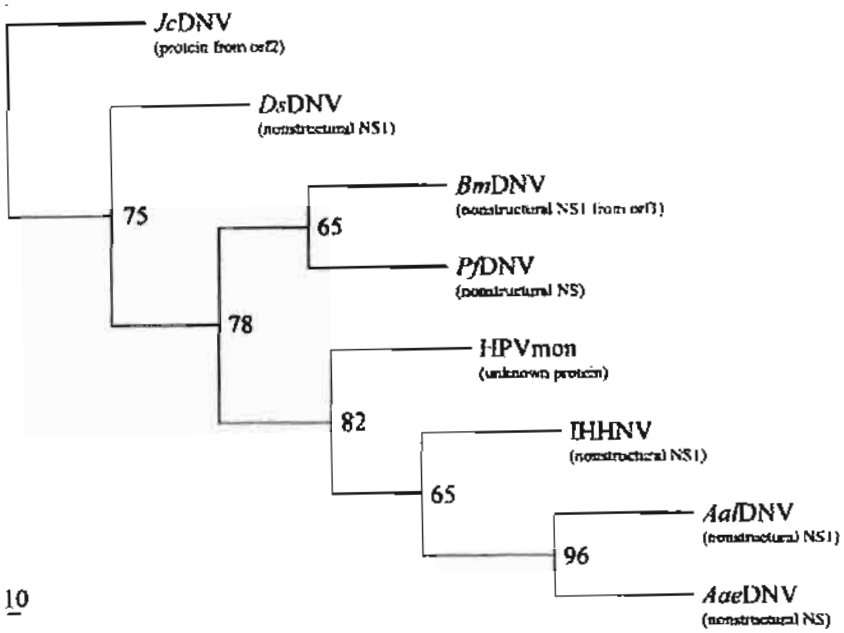


Figure 12. Comparisons based on putative protein sequences of insect virus nonstructural protein (NS) and most similar proteins using a global alignment method. (A) Overall parvoviral protein similarities. (B) The neighbor-joining phylogenetic tree generated from a heuristic search of aligned insect 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.

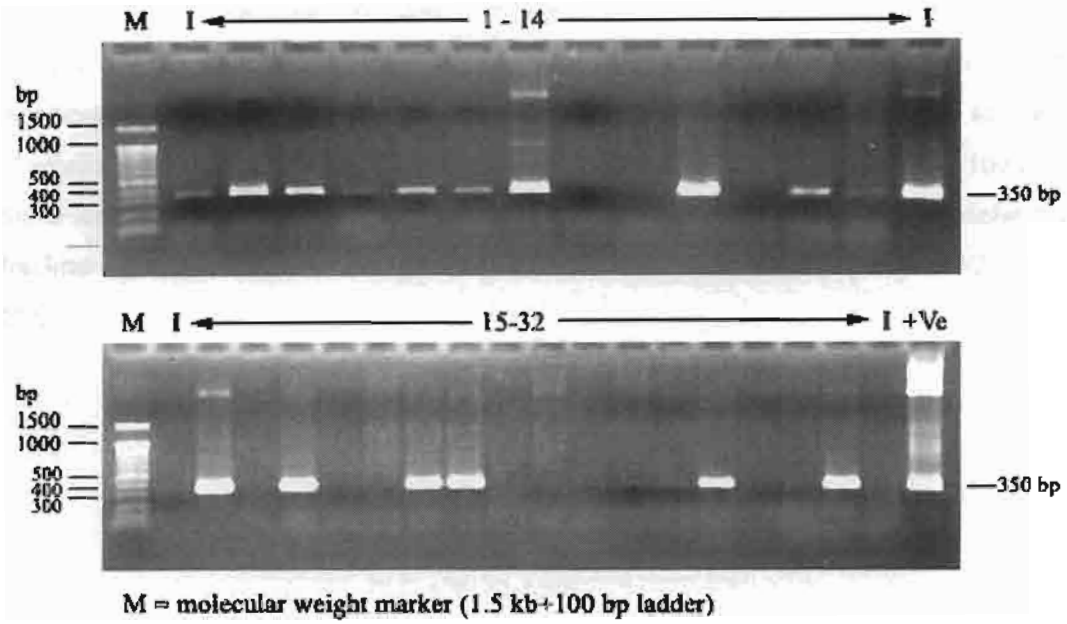


Figure 13. Screening of *ATh*DNV infection status of challenge survivors using PCR method with samples 1-32 showing 19/32 (59%) positive. Mosquito samples 33-94 (62 samples) were also checked by PCR and 13 (21%) out of 62 samples were positive (data not shown).

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

would be defective for the reading of both NS1 and NS2 proteins because the single base deletions and insertions occurred after the start codons for both proteins. Calculating from the percentage of putative defective sequences in each of the 3 sampled regions, the mean prevalence of defective sequences in the freezer group was 3.3% ± 5.8 SD while that in the F4 group was 29.7% ± 10.0 SD. The difference was significant ($p = 0.017$). Using an alternative method with epidemiological software (Cameron, 2002) and considering target viral populations well in excess of 100,000, there was a significant difference ($p = 0.003$) in the estimated prevalence of defective fra*Gm*ents between the freezer group (33% ± 6.7 SD) and the F4 group (64% ± 5.0 SD).

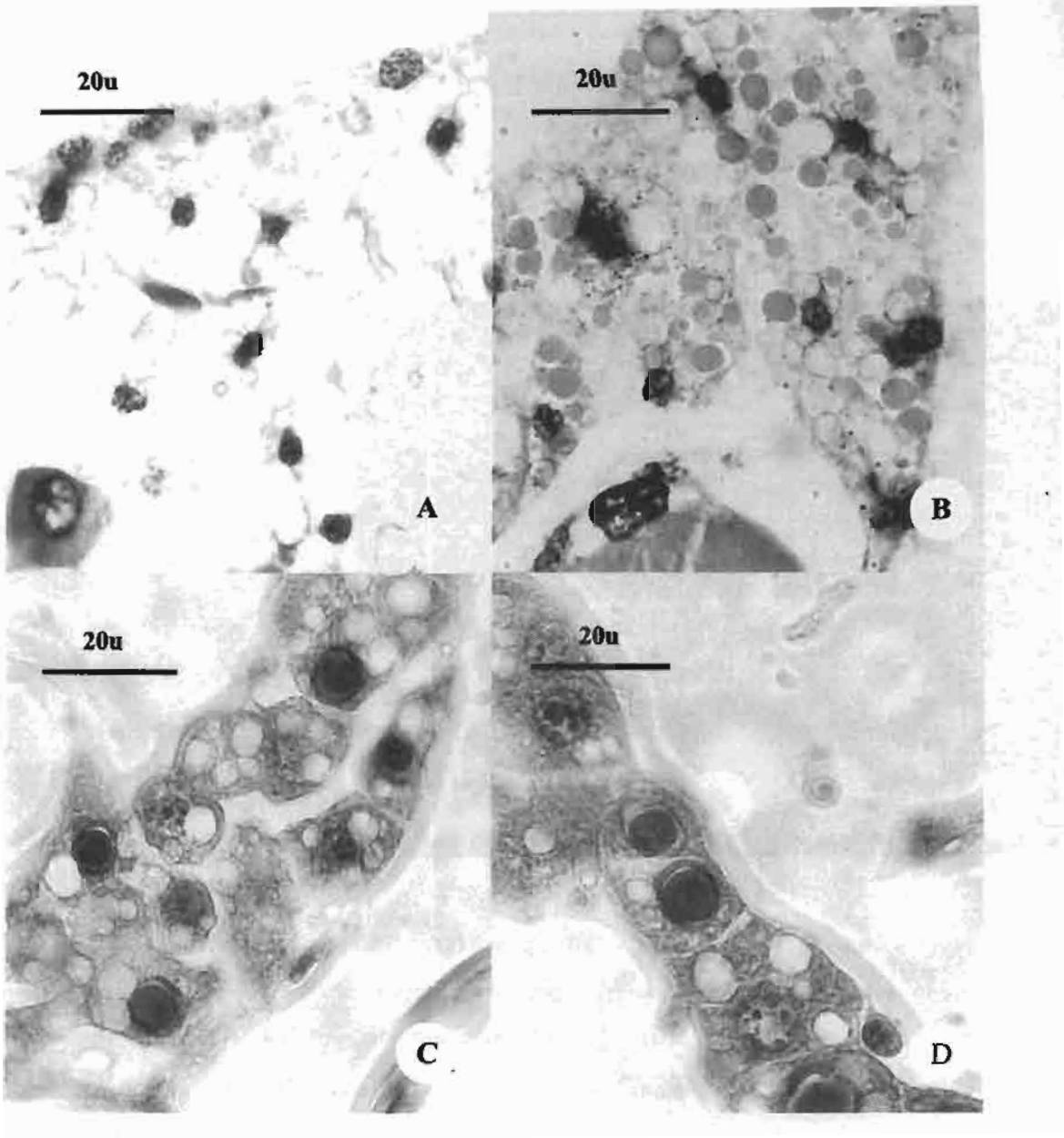


Figure 14. Hematoxylin and Eosin (H&E) staining with sections of normal (A&B) *Ae. aegypti* adults and *ATh*DNV-infected (C&D). Parvovirus-type inclusions in fat body tissues were seen as large, basophilic intranuclear inclusions in nuclei with marginated chromatin (C&D). These nuclei contrast sharply with smaller normal nuclei (A&B).

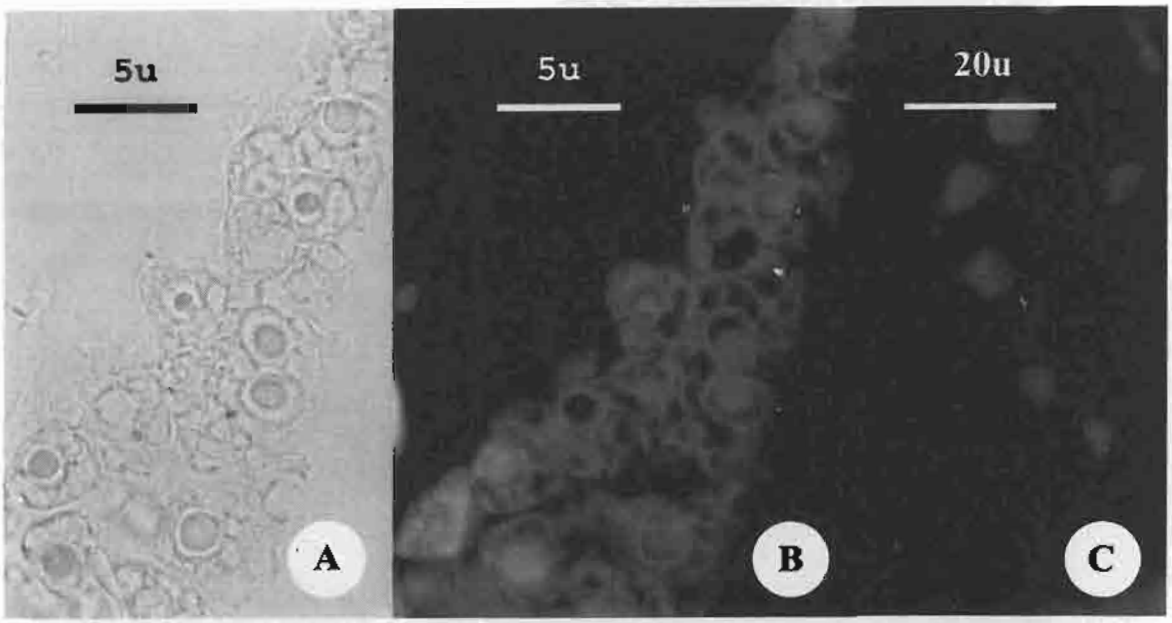


Figure 15. Fluorescene *in situ* hybridization for *ATh*DNV in adult *Ae. aegypti* mosquitoes. The bright field micrograph (A) shows enlarged nuclei that give green fluorescence positive for the presence of *ATh*DNV (B). Red fluorescence from propidium iodide at >620 nm indicated a normal nuclei reaction in uninfected mosquitoes (C).

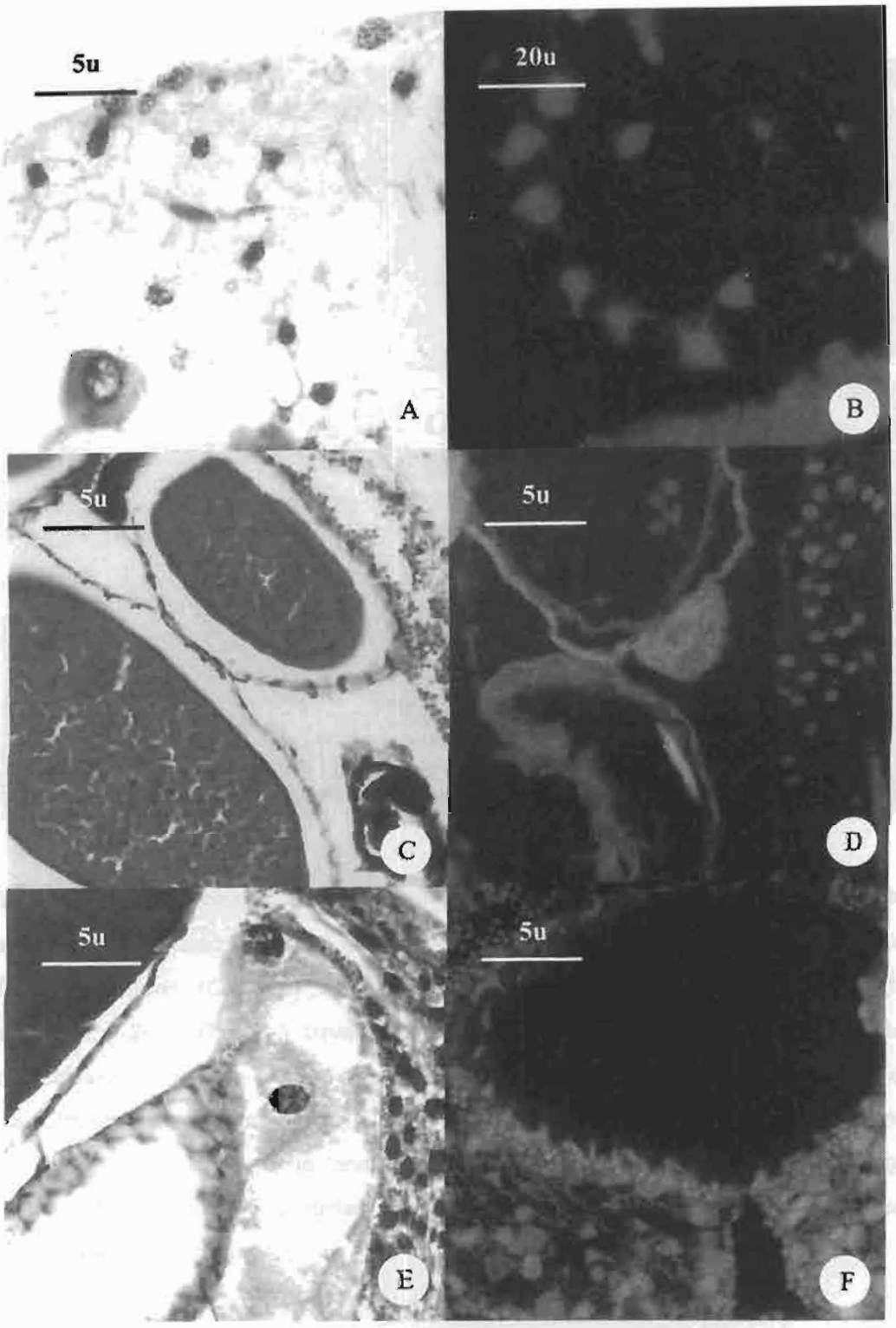


Figure 16. Fluorescene *in situ* hybridization (B,D&F) and H&E staining (A,C&E) for *ATh*DNV infected tissue and normal tissue in adult *Ae. aegypti* mosquitoes. Normal tissues were shown in A, B, C&D while *ATh*DNV infected tissues were shown in E&F.

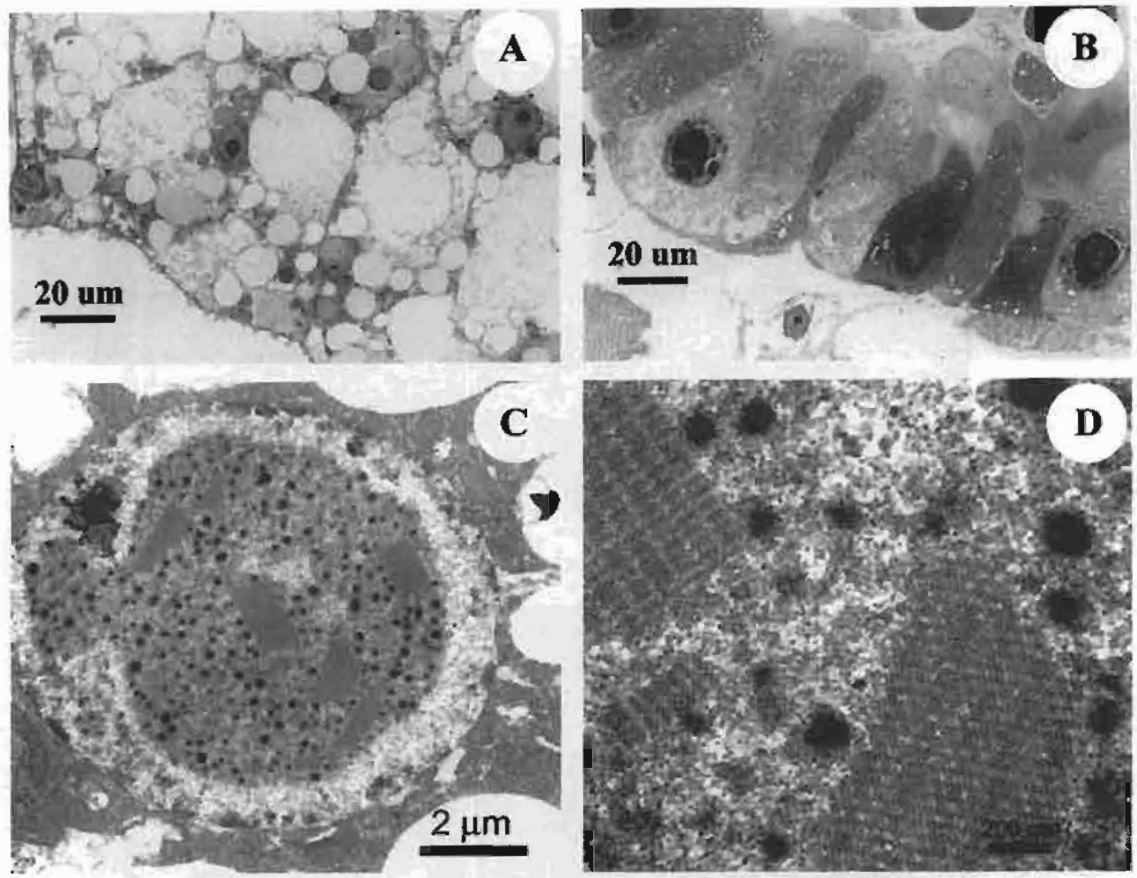


Figure 17. Light microscopy and ultastructure of normal and *ATh*DNV-infected mosquitoes. Light microscopy of semi-thin sections (A&B) and electron microscopy of ultra-thin sections (C&D) of Epon-Aradite embedded mosquito larva of the 3rd generation (F2) of Group 3 larvae. (A) Normal control mosquito larva showing no intranuclear inclusions in fat body tissue. (B) Enlarged nuclei in gut epithelial cells of mosquito larva. Low magnification (C) and high magnification (D) electron micrographs of fat body nuclei of mosquito larva showing intranuclear inclusions containing large numbers of 22-24 nm viral particles either free or in para-crystalline arrays. Note also the marginated chromatin in C.

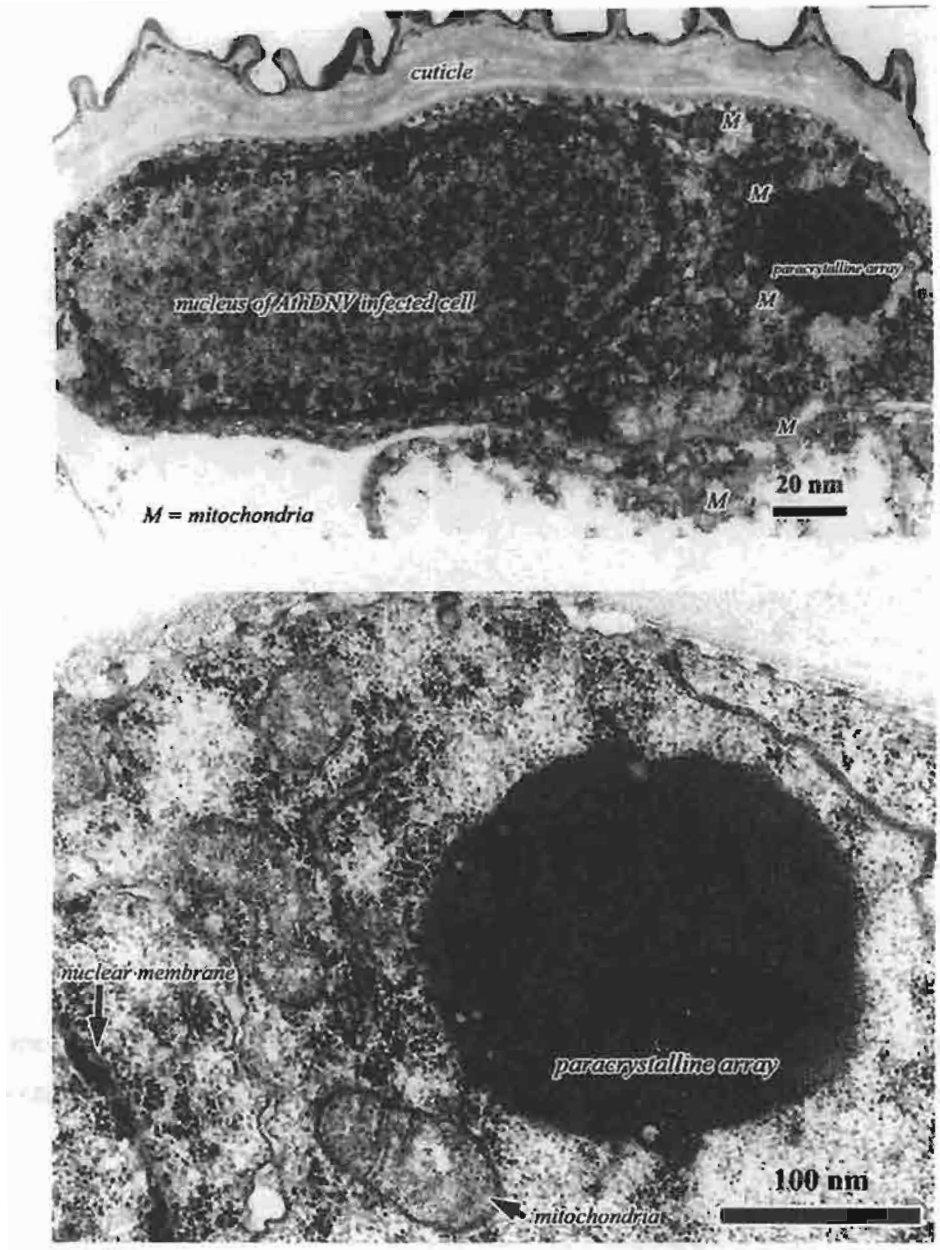


Figure 18. Electron micrographs at low (upper) and high (lower) magnification showing a subcuticular epithelial cell infected with *ATh*DNV. Note that the nucleus contains large numbers of free virions while the pracrystalline array is contained in the cell cytoplasm.

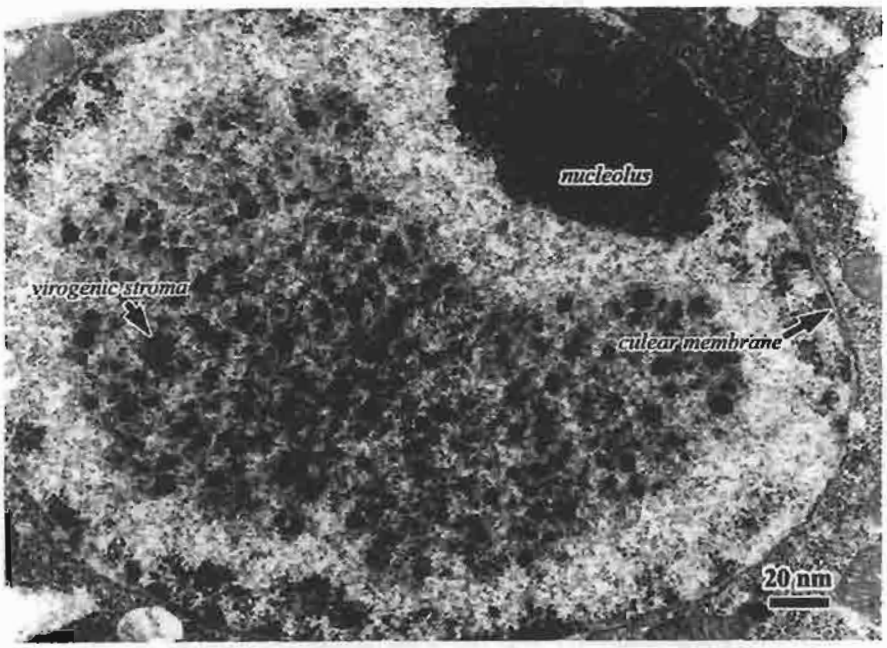


Figure 19. Electron micrograph of the nucleus of an *ATh*DNV-infected subcuticular epithelial cell showing large numbers of free virions. Note the marginaged chromatin.

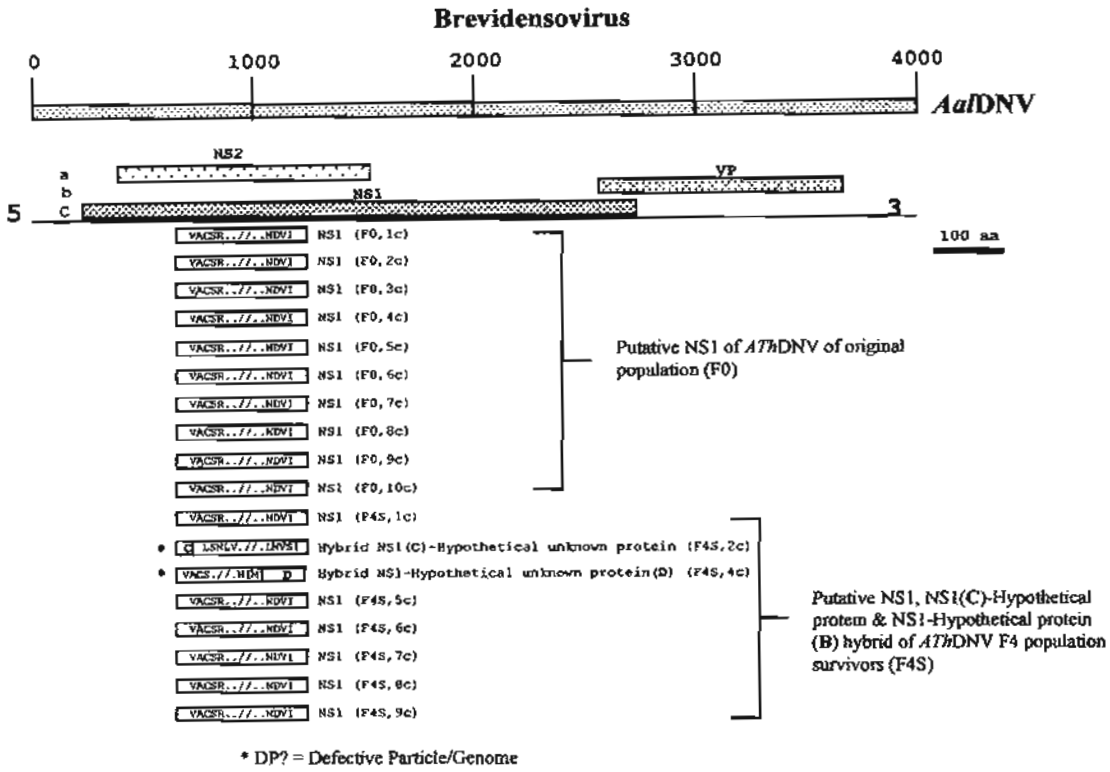


Figure 20. Descriptive model comparing region 1 of deduced NS1 protein sequences from the freezer virus and the F4 survivor virus populations. Putative defective sequences are marked with an asterisk.

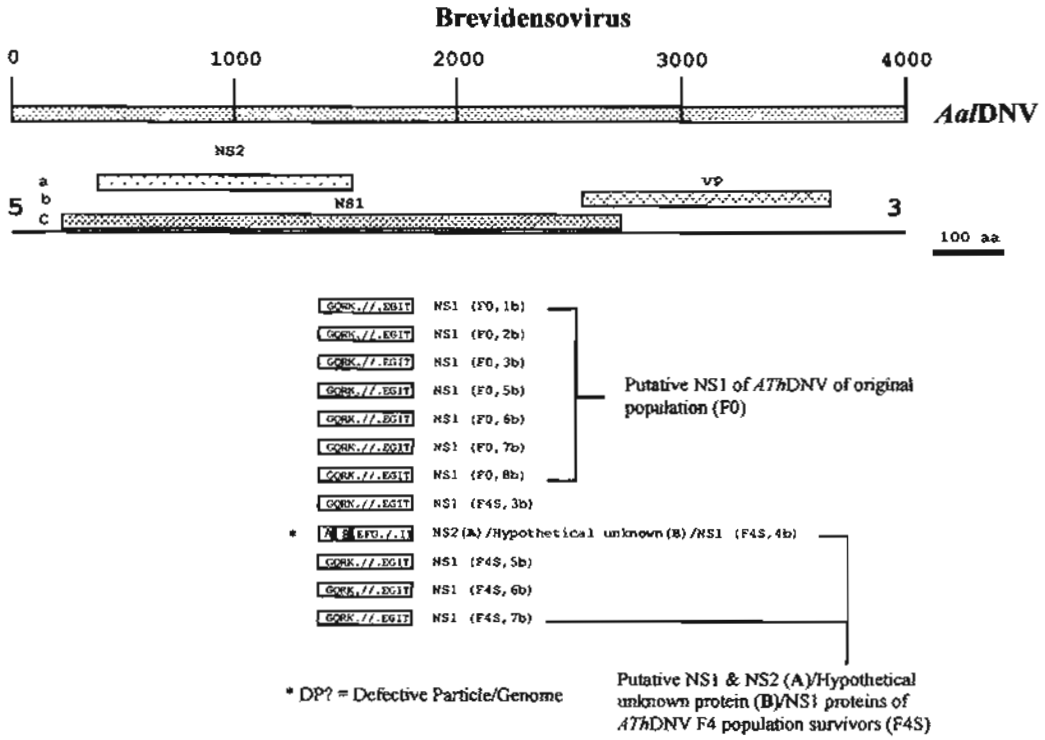


Figure 21. Descriptive model comparing region 2 of deduced NS1 protein sequences from the freezer virus and the F4 survivor virus populations. Putative defective sequences are marked with an asterisk.

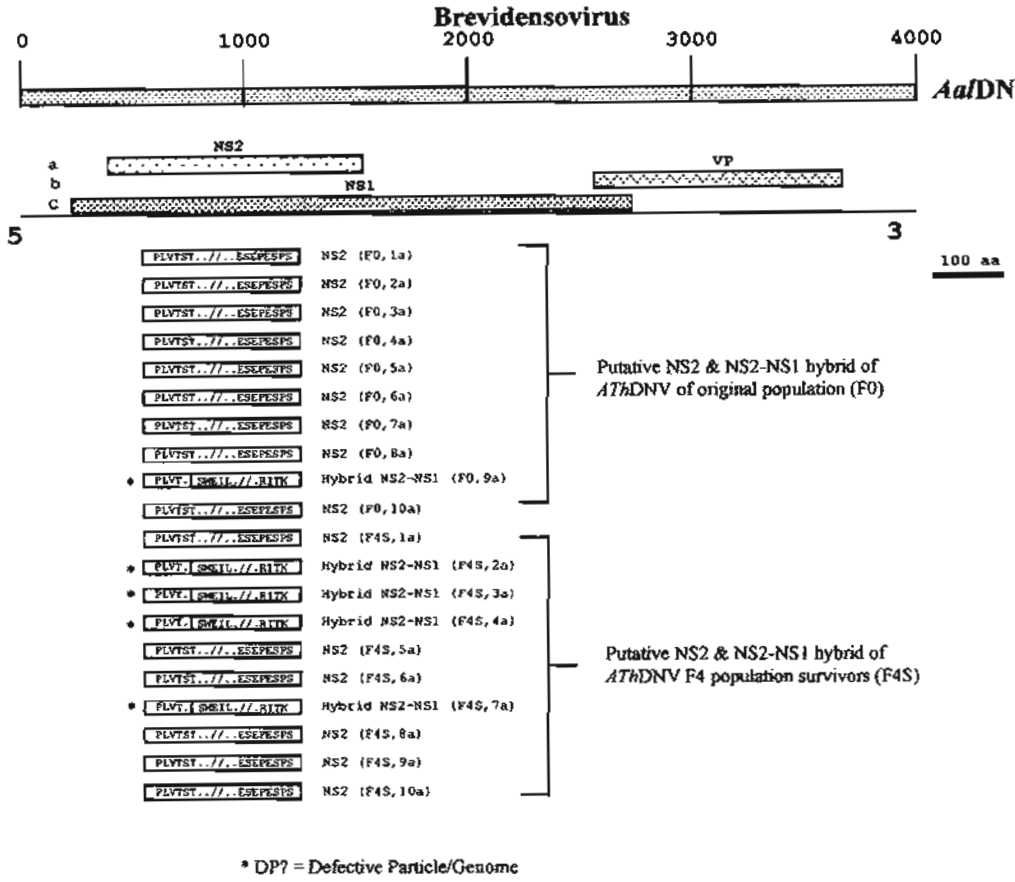


Figure 22. Descriptive model comparing deduced NS2 protein sequences from the freezer virus and the F4 survivor virus populations. Putative defective sequences are marked with an asterisk.

DISCUSSION

Part I.

Comparison of penaeid shrimp and insect parvoviruses suggests that viral transfers may occur between two distantly related arthropod groups

We have used an insect model for host-virus interaction because such studies in shrimp are 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. In this model, we used a parvovirus from the same virus family that contains the shrimp viruses IHHNV, spawner-isolated mortality virus (SMV) and HPV.

Of the parvoviruses in crustaceans, only those of shrimp have been studied in detail. These include hepatopancreatic parvovirus (HPV) in *Penaeus chinensis* (HPVchin) (Lightner & Redman, 1985) and *P. monodon* (HPVmon) (Sukhumsirichart *et al.*, 1999), SMV from *P. monodon* (SMVmon) (Fraser & Owens, 1996; Owens *et al.*, 1998; Owens & McElnea, 2000) and IHHNV (Lightner *et al.*, 1983; Bonami *et al.*, 1990; Shike *et al.*, 2000). These viruses have caused disease in cultivated shrimp in many countries all over the world. Shike *et al.* (2000) recently reported that IHHNV of shrimp and the mosquito densoviruses *Aae*DNV (Afanasiev *et al.* 1991,1994) and *Aal*DNV (Barreau *et al.*, 1996; Jousset *et al.*, 1993) shared similar overall genomic organization. Specifically, the left ORF of these viruses most likely encoded the major NS protein (NS1) since it contained conserved replication initiator motifs and NTP-binding and helicase domains similar to those in NS1 from all other parvoviruses and the putative NS1 of IHHNV shared 33-35 % amino acid (aa) sequence identity with NS1 of the mosquito densoviruses (Shike *et al.*, 2000). It was of interest to know the relationship amongst the IHHNV, SMV and HPV of shrimp parvovirus and also their relationship to known insect parvoviruses. To this end, recent sequence information from these three shrimp virus types was compared to that published for the insect parvoviruses. From the study on characteristics of insect and penaeid shrimp parvoviruses, we found that virion size both

from insect and shrimp ranged between 18-30 nm in diameter and all had genome of ssDNA that ranged between 4 and 6 kb.

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. The grouping of IHHNV with the mosquito brevidensoviruses *Aae*DNV and *Aal*DNV was in agreement with the study of Shike *et al.* (2000) who found the same relationship in a phylogenetic tree based on a 199-aa conserved region characteristic of the NTP-binding and helicase domains of the nonstructural protein (NS1) of IHHNV, of insect parvoviruses and vertebrate parvoviruses. However, the clustering of SMV with a different group of insect densoviruses and the separation of HPV into yet another group suggests that the shrimp parvoviruses studied are of diverse origin and that they are not closely related. Shike *et al.* (2000) reported that IHHNV was the first example of a densovirus with a host range outside the class Insecta that diverged from the Crustacea over 500 million years ago. Our analysis suggests that SMVmon and HPVmon/HPVchin may represent two other groups of distantly related densoviruses in the class Crustacea.

Comparisons were 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. The phylograms based on capsid or related protein sequences (Figure 11B) and NS1 or related sequences (Figure 12B) were somewhat different. The phylogenetic tree based on NS1 was similar to that described by Shike *et al.* (2000) with the closest relationship found between IHHNV and *Aae*DNV or *Aal*DNV. However, when capsid proteins were used, IHHNV fell closer to *Bm*DNV and *Pf*DNV than to *Aae*DNV or *Aal*DNV. The phylogram based on NS1 sequences corresponded most closely to the phylogram constructed based on DNA sequences and it suggested that NS1 might be more conserved than the capsid protein. Jousset *et al.* (2000) reported that there was a lack of sequence homology between *Cp*DNV and *Aal*DNV genomes, and that lack of antigenic cross-reactivity between capsid polypeptides indicated that the two mosquito viruses *Aae*DNV and *Aal*DNV were also phylogenetically distant. They also reported that *Cp*DNV appeared to be related to *Junonia coenia* densovirus (*Jc*DNV) both serologically and

genomically. Due to lack of appropriate sequence information we were unable to include *Cp*DNV in our analysis, but based on the work of Jousset *et al.* (2000), it might eventually cluster with *Jc*DNV, distant from *Aal*DNV. In contrast to their work, however, our results indicated that *Aae*DNV and *Aal*DNV were closely related. Based on DNA sequence comparisons, we were surprised to find that the shrimp parvoviruses did not form one cluster and the insect parvoviruses another. The fact that the three shrimp viruses fell into two different parvovirus clades, including two different insect virus groups suggests that there may have been and may still be viral transfers between these distantly related *arthropods*. Indeed, Lo *et al.* (1996) found that a PCR probe for white spot syndrome virus of penaeid shrimp gave positive results with aquatic insect larvae and suggested that they were possible reservoir hosts. Since this work was not followed up with more detailed tests such as *in situ* DNA hybridization assays, it may have been that the insect larvae were simply mechanical carriers of the shrimp virus. On the other hand, cultivated penaeid shrimp larvae and some insect larvae are carnivorous and known by shrimp farmers to eat each other when sizes are appropriate and opportunity arises. It would be worthwhile looking in the shrimp environment to determine whether any aquatic insect larvae there can be infected with shrimp parvoviruses and act as reservoirs for them.

As a result of this phylogenetic analysis, we became concerned that HPV might be transmitted to shrimp by aquatic insect carriers. Thus, we carried out an unpublished survey of aquatic insects in Thai shrimp pon*Ds* using an HPV-specific PCR detection method. This revealed 4 PCR positive aquatic insect species, 3 of which were in the predatory insect family *Gerridae* (pond skaters, water striders), *Corixidae* (water boatman), *Notonectidae* (backswimmers) and one (mayfly larvae) in the Order *Ephemeroptera*. This has resulted in precautionary measures to prevent HPV transmission from these potential carriers. It is particularly important for mayfly larvae, since many shrimp farmers raise them intentionally to feed the live to shrimp larvae. In the meantime, further studies are underway to determine whether these insects are infected or mechanical carriers.

Part II.

Challenging successive mosquito generations with a densonucleosis virus yield persistent, innocuous infections and progressive improvements in survival

Using this relatively simple mosquito-virus model, we have been able to demonstrate increasing survival (i.e., from 15% ± 4.12 SD to 55% ± 6.36 SD) in successive generations challenged with a fixed viral stock of *ATh*DNV. This parallels the situation reported for shrimp (Flegel, 2001; Santamaria, 1999; Moss, 2002). Also similar to shrimp, we have shown that many challenge survivors are actually infected with *ATh*DNV although they show no gross signs of disease and are capable of normal maturation and reproduction. The high number of infected cells and the presence of paracrystalline arrays of viral particles in transmission electron micrographs of 1st instar larvae collected within 2 hours of hatching suggested that vertical transmission of the virus was possible by the transovum route. Vertical transmission has previously been reported for *Aal*DNV (Barreau *et al.*, 1997). However, the transmission of virus through the egg surface is termed transovum and that within the egg itself is designed tranovarial. Investigators did sections in the positive female but they showed no dense nucleus within the nucleus. The transovarial transmission was found in *Aae*DNV since surface sterilization of the ova did not prevent larval infection (Lebedinets & Kononko, 1989). We do not exclude the possible transmission at later stages by horizontal transfer amongst larvae and adults, although study of the possibility and rate was not included in our work. Horizontal viral transmission amongst larvae has been reported. Barreau *et al.* (1997 & 1996) described transmission via defecation or regurgitation while Vasconcelos (1996) described nucleopolyhedrovirus transmission with *Mamestra brassicae* larvae via both cannibalism and release of virus prior to the de*ATh*. Rosen described horizontal transmission of dengue-2 virus by *Ae. albopictus* males (Rosen, 1987). From the viewpoint of our study, the route of transmission to successive generations was not particularly relevant. It was sufficient to show that challenge survivors were infected in significant numbers and capable of transmitting that infection to future generations.

Our results indicated that the number of eggs produced with each succeeding generation of mosquitoes dropped. Although this could have been the result of

*ATh*DNV infection, it could also have resulted from inbreeding in the groups of survivor mosquitoes. Barreau *et al.* (1997) reported that fecundity of *Aal*DNV-infected females was not significantly different from *Aal*DNV-free females. By contrast, Kuznetsova & Buchatsky (1988) observed significant changes of eggs in *Ae. aegypti* females from *Aae*DNV infection. Buchatsky also showed that some of females did not suck blood, so that both fertility and number of eggs laid were reduced. Specifically designed tests would be needed to distinguish between these possibilities with our model system.

The feature that our survivors to *ATh*DNV challenge and survivors from other viral challenged *arthropods* generally carry the challenge virus for life is a highly significant difference from vertebrates. In our opinion, this difference should be emphasized by use of the term "tolerant" r*ATh*er than "resistant" to describe such survivors (Flegel, 2001). In addition, the full significance of these persistent viral infections should be determined.

The lack of host mosquito inflammatory response to the presence of *ATh*DNV also parallels the situation with viral infections in shrimp where even large numbers of infected cells generally solicit no mobilization of hemocytes (Flegel, 2001). This contrasts sharply with the shrimp response to bacterial and fungal p*ATh*ogens (Johansson *et al.*, 2000; Thornqvist & Soderhall, 1997) where host response usually lea*Ds* to p*ATh*ogen clearance and where long-term chronic infections are characterized by hemocytic aggregation leading to encapsulation and granuloma formation (Flegel *et al.*, 1992; Lightner, 1996). Also noteworthy was the lack of tissue disruption when *ATh*DNV-infected cells held positions in the epidermis and gut epithelium of infected mosquitoes. Since the cuticle over infected epidermal cells was normal, cellular function appeared to be unaffected by presence of the virus.

Tolerance to a densovirus infection can also occur in insect cell lines. For example, it has been shown that serial passage of C6/36 cells infected with the densovirus *Aal*DNV results in a gradual decline in number of infected cells from around 90% to a steady state of approximately 20% from the 10^{th} passage onwar*Ds* (Burivong, 2003). This effect has been attributed to defective interfering particles (DIP) that had previously been suggested to reduce viral production or to compete with infective particles for cell surface receptors (Frank, 2000; Kirkwood & Bangham, 1994; Rhode, 1978). It has been shown by electron microscopy that empty particles are mixed with purified *Aal*DNV

virions from infected C6/36 cells (Jousset *et al.*, 1993). Interference by defective particles has also been claimed as an additional viral interference factor in parvovirus replication in porcine parvovirus (PPV) (Choi *et al.*, 1987), parvovirus H-I (Rhode, 1978), and in parvovirus minute virus of mice (MVM) (Clement *et al.*, 2001). Infection with mixtures of various ratios of empty and full porcine parvovirus particles markedly inhibited viral production in a manner related to the concentration of empty particles added (Choi *et al.*, 1987).

The fact that the percentage of infected cells in persistently-infected cell cultures cannot be increased by super-challenge with the same virus corresponDs to a process called superinfection exclusion with a homologous virus. It has been reported in several viruses and is defined as the phenomenon where cells infected with one virus cannot be productively infected with the same or a closely-related virus at some later time. Cultured C6/36 cells persistently infected with Dengue virus type 1 were found to be resistant to superinfection with Dengue type 3 after 20 h of primary infection (Dittmar *et al.*, 1982). *Ae. albopictus* cell lines persistently infected with Sindbis virus excluded the replication of both homologous (variant strains) and heterologous alphaviruses but exclusion was restricted to alphaviruses (Kar*Pf et al.*, 1997). Bunyamwera virus persistently infected C6/36 cells were also resistant to superinfection with Bunyamwera virus and other bunyaviruses (Elliott & Wilkie, 1986). Most simply, superinfection exclusion may result from the production of DIP that compete for host cell receptors, but the phenomenon may also involve intracellular host factors such as interferon, interferon-like substances or other antiviral substances.

The results of our experiments with this mosquito-densovirus experimental model are consistent with previous works with shrimp and with mosquito cell lines. Populations surviving *ATh*DNV challenge were infected with *ATh*DNV and successive *ATh*DNV challenges lead to decreasing severity of disease caused by *ATh*DNV. Since the decrease in severity of disease was associated with high levels of defective *ATh*DNV genomes (DIP) in the surviving mosquito population, it may be proposed, by analogy to insect cell cultures, that DIP are instrumental in the process. It could be argued, in turn, that persistent viral infections act as a kind of "specific memory" that can serve to reduce the severity of disease. Such memory would satisfy a currently missing

mechanism for memory in the viral accommodation theory proposed by Flegel and Pashariwipas (1998).

Briefly, the viral accommodation theory proposed that crustaceans have an active viral binding and memory system (*ABM*) that is distinct from that involved in binding for infection and that the function of the system is to actively accommodate viral p*ATh*ogens and specifically block viral triggered apoptosis (programmed cell de*ATh*) (Flegel, 2001; Flegel & Pasharawipas, 1998). Since 1998, tests of the theory have shown that moribund shrimp for two major shrimp viruses do exhibit increasing levels of apoptosis as lethal viral infections progress (Khanobdee *et al.*, 2002; Wongprasert *et al.*, 2003). It has also been found that viral challenges often result in cryptic infections (Hsu *et al.*, 1999; Tsai *et al.*, 1999; Yoganandhan *et al.*, 2003). Invoking specific memory provided by persistent infections can greatly simplify the viral accommodation theory and improve its alignment with current research results. Up to date, it would propose that "crustaceans and other *arthropods* have an active system to accommodate viral p*ATh*ogens as persistent infections that function to specifically reduce the severity of disease (by the production of defective viral particles) and to dampen viral triggered apoptosis".

If persistent infection accompanied by high production of DIP is a general arthropod phenomenon, then is important to understand the mechanism of DIP formation. Early proposals suggested that they arose from production of randomly truncated viral genomes that retained an origin of replication and that they out-competed full genome sequences because of their small size (Frank, 2000; Kirkwood & Bangham, 1994). However, recent work with a baculovirus indicates that DIP production involves more complex, rapid generation of internal deletions that may require somewhat sophisticated cellular machinery (Pijlman *et al.*, 2001). Our results also revealed internal (albeit small) deletions in the viral genome. Given the small size of the densovirus genome and the fact that it generates only 3 or 4 proteins, the machinery involved in DIP formation must be of host cell origin. Altogether, a picture begins to emerge of a concerted process that may have arisen by evolutionary advantage in the arthropod line. Using the virus itself as the template for DIP would allow the host to immediately respond to any viral variation that might arise. By accommodating viruses in persistent infections without mortality, there would be positive selection of viral variants with the least negative effect

on the host and positive selection for host variants least affected by presence of the virus. There would be strong negative selection against increased virulence in the viral population and high disease in the host population. The process would lead to rapid mutual existence. This appears to occur in the relationship between infectious hypodermal and hematopoeitic necrosis virus (IHHNV) and the black tiger shrimp *Penaeus monodon* where no negative effects are generally seen (Flegel, 2001).

A second advantage of persistent infections may be a reduction in severity of disease from super infection with second virus. This has been reported for shrimp (Tang *et al.*, 2003) and for mosquito cells (Burivong, 2003) although the molecular basis for the process is unknown. In both cases, it is important to understand that the second infection is successful but does not lead to mortality. It is possible that presence of a resident virus helps to dampen viral triggered apoptosis that would normally lead to de*ATh*.

One predicted outcome of viral accommodation would be a slow rate of evolution in arthropod viral p*ATh*ogens when compared to vertebrate viral p*ATh*ogens where viruses are under strong selective pressure to evade host defenses. This appears to be the case. Holland (1996) has stated, "...the rates of evolution (mutation fixation) for the flaviviruses, the alphaviruses and other aboviruses are generally lower by an order of magnitude or more than are the rates for many other (non-arthropod-borne) viruses." Although Holland (1996) suggested that the lower rate of evolution might have arisen from the need for arboviruses to maintain efficient replication capacity in quite different selective environments (i.e., vertebrate and arthropod hosts), it could as easily be that viral accommodation in the arthropod host populations would tend to continually pull the p*ATh*ogen back to the most stably accommodated types.

The implications of these phenomena with respect to viral control of insect pests and vectors are not good. They suggest that widespread use of such p*ATh*ogens would quickly lead to persistently infected populations with increasing tolerance over time. On the other hand, understanding the nature of persistent infections and selecting well-tolerated viruses could open the way for new control strategies. For example, genetic modification of a widespread and well-tolerated virus to a lethal factor such as scorpion toxin, would leave the hosts with no means of defense. The disadvantage of this

approach would be the need to constantly re-apply the virus from new, artificially generated stocks. A better option might be to genetically modify a well accommodated, innocuous virus to contain lethal signals for a second agent of concern (e.g., modify an innocuous densovirus to interfere with Dengue replication). The only disadvantage to that process would be application of selective pressure on the Dengue population to overcome the newly applied interference. Obviously, it would be wise to carefully select the mode of interference to avoid this possibility.

BIBLIOGRAPHY

Afanasiev, B. N., Galyov, E. E., Buchatsky, L. P. & Kozlov, Y. V. (1991). Nucleotide sequence and genomic organization of *Aedes* densonucleosis virus. *Virol* **185**, 323-336.

Afanasiev, B. N., Kozlov, Y. V., Carlson, J. O. & Beaty, B. J. (1994). Densovirus of *Aedes aegypti* as an expression vector in mosquito cells. *Exp Parasitol* **79**, 322-339.

Anderson, J. R., Grimstad, P. R. & Severson, D. W. (2001). Chromosomal Evolution among Six Mosquito Species (Diptera: Culicidae) Based on Shared Restriction FraGment Length Polymorphisms. *Mol Phylogenet Evol* **20**, 316-321.

Arala-Chaves, M. & Sequeira, T. (2000). Is there any kind of adaptive immunity in invertebrates? *Aquaculture* **191**, 247-258.

Bando, H., Choi, H., Ito, Y. & Kawase, S. (1990). Terminal structure of a Densovirus implies a hairpin transfer replication which is similar to the model for AAV. *Virol* **179**, 57-63.

Bando, H., Kusuda, J., Gojobori, T., Maruyama, T. & Kawase, S. (1987a). Organization and nucleotide sequence of a densovirus genome imply a host-dependent evolution of the parvoviruses. *J Virol* **61**, 553-60.

Bando, H., Kusuda, J. & Kawase, S. (1987b). Molecular cloning and characterization of *Bombyx* densovirus genomic DNA. Brief report. *Arch Virol* **93**, 139-146.

Barreau, C., Jousset, F. X. & Bergoin, M. (1997). Venereal and vertical transmission of the *Aedes albopictus* parvovirus in *Aedes aegypti* mosquitoes. *Am J Trop Med Hyg* **57**, 126-131.

Barreau, C., Jousset, F.-X. & Bergoin, M. (1996). PATHogenicity of the *Aedes albopictus* parvovirus (AaPV), a denso-like virus, for *Aedes aegypti* mosquitoes. *J Invertebr PAThol* **68**, 299-309.

Barreau, C., Jousset, F. X. & Cornet, M. (1994). An efficient and easy method of infection of mosquito larvae from virus-contaminated cell cultures. *J Virol MethoDs* **49**, 153-156.

Bennett, K. E., Olson, K. E., Munoz, M. D. L., Fernandez-Salas, I., Farfan, J. A., Higgs, S., Black IV, W. C. & Beaty, B. J. (2002). Variation in vector competence for dengue 2 virus among 24 collections of *Aedes aegypti* from Mexico and the United States. *Am J Trop Med Hyg* **67**, 84-92.

Bergoin, M. & Tijssen, P. (2000). Molecular biology of Densovirinae. In *Parvoviruses. From molecular biology to pAThology and therapeutic uses. Contrib Microbiol*, pp. 12-32. Edited by F. S. Rommelaere: Basel, Karger.

Black IV, W. C., Bennett, K. E., Gorrochotegui-Escalante, N., Barillas-Mury, C. V., Fernandez-Salas, I., de Lourdes Munoz, M., Farfan, J. A., Olson, K. E. & Beaty, B. J. (2002). Flavivirus susceptibility in *Aedes aegypti*. *Arch Med Res* **33**.

Bonami, J. R., Mari, J., Poulos, B. T. & Lightner, D. V. (1995). Characterization of hepatopancreatic parvo-like virus, a second unusual parvovirus p*ATho*genic for penaeid shrimps. *J Gen Virol* **76**, 813-817.

Bonami, J. R., Trumper, B., Mari, J., Brehelin, M. & Lightner, D. V. (1990). Purification and characterization of the infectious hypodermal and haematopoietic necrosis virus of penaeid shrimps. *J Gen Virol* **71**, 2657-2664.

Bosio, C. F., Beaty, B. J. & Black IV, W. C. (1998). Quantitative genetics of vector competence for dengue-2 virus in *Aedes aegypti*. *Am J Trop Med Hyg* **59**, 965-970.

Bosio, C. F., Fulton, R. E., Salasek, M. L., Beaty, B. J. & Black, W. C., 4th (2000). Quantitative trait loci that control vector competence for dengue-2 virus in the mosquito *Aedes aegypti*. *Genetics* **156**, 687-698.

Boublik, Y., Jousset, F. X. & Bergoin, M. (1994). Complete nucleotide sequence and genomic organization of the *Aedes albopictus* parvovirus (AaPV) p*ATho*genic for *Aedes aegypti* larvae. *Virol* **200**, 752-63.

Boucias, D. G., Johnson, D. W. & Allen, G. E. (1980). Effects of host age, virus dosage, and temperature on the infectivity of a nucleopolyhedrosis virus against the velvetbean caterpillar, *Anticarsia gemmatalis*, larvae. *Environ Entomol* **9**, 59-61.

Boucias, D. G. & Nordin, G. L. (1977). Interinstar susceptibility of the fall webworm, *Hyphantria cunea,* to its nucleopolyhedrosis and granulosis viruses. *J Invertebr PAThol* **30**, 68-75.

Brown, S. E. & Knu*D*son, D. L. (1997). FISH landmarks for *Aedes aegypti* chromosomes. *Insect Molecular Biology* **6**, 197-202.

Brown, S. E., Menninger, J., Difillipantonio, M., Beaty, B. J., Ward, D. C. & Knu*D*son, D. L. (1995). Toward a physical map of *Aedes aegypti*. *Insect Mol Biol* **4**, 161-167.

Brown, S. E., Severson, D. W., Smith, L. A. & Knu*D*son, D. L. (2001). Integration of the *Aedes aegypti* mosquito genetic linkage and physical maps. *Genetics* **157**, 1299-1305.

Burivong, P. (2003). Densovirus: its kinetics of growth and interaction with infectivity of Dengue virus in mosquito cells. In *Medical Science*, pp. 79. Bangkok: Mahidol University.

Cameron, A. (2002). Survey toolbox for aquatic animal diseases; a practical manual and software package, pp. 375. Canberra: Australian Centre for International Agricultural Research.

Choi, C. S., Molitor, T. W. & Joo, H. S. (1987). Inhibition of porcine parvovirus replication by empty virus particles. *Arch Virol* **96**, 75-87.

Christophers, S. R. (1960). *Aedes aegypti* (L.) The Yellow Fever Mosquito: Its Life History, Bionomics and Structure, Cambridge University Press, Cambridge.

Clark, K. D., Pech, L. L. & Strand, M. R. (1997). Isolation and identification of a plasmatocyte-spreading peptide from the hemolymph of the lepidopteran insect *Pseudoplusia includens*. *J Biol Chem* **272**, 23440-23447.

Clement, N., Avalosse, B., El Bakkouri, K., Velu, T. & Brandenburger, A. (2001). Cloning and sequencing of defective particles derived from the autonomous parvovirus minute virus of mice for the construction of vectors with minimal cis-acting sequences. *J Virol* **75**, 1284-1293.

Clements, A. N. (1992). The Biology of Mosquitoes, Chapman & Hall, London.

Cohn, J., Sessa, G. & Martin, G. B. (2001). Innate immunity in plants. *Curr Opin Immunol* **13**, 55-62.

Dittmar, D., Castro, A. & Haines, H. (1982). Demonstration of interference between dengue virus types in cultured mosquito cells using monoclonal antibody probes. *J Gen Virol* **59**, 273-282.

Dumas, B., Jourdan, M., Pascaud, A. M. & Bergoin, M. (1992). Complete nucleotide sequence of the cloned infectious genome of *Junonia coenia* densovirus reveals an organization unique among parvoviruses. *Virol* **191**, 202-22.

Elliott, R. M. & Wilkie, M. L. (1986). Persistent infection of *Aedes albopictus* C6/36 cells by Bunyamwera virus. *Virol* **150**, 21-32.

Engelhard, E. K. & Volkman, L. E. (1995). Developmental resistance in fourth instar *Trichoplusia ni* orally inoculated with *Autographa californica* M nuclear polyhedrosis virus. *Virol* **209**, 384-389.

Erickson, H. S., Poulos, B. T., Bradley-Dunlop, D. & Lightner, D. V. (In press). Taura syndrome virus from Belize represents a unique TSV isolate: comparison of

antigenicity with MAB and molecular composition of the structural coding region with TSV isolates from other geographic regions. *Dis Aquat Org.*

Erickson, H. S., Zarain-Herzberg, M. & Lightner, D. V. (2002). Detection of Taura syndrome virus (TSV) strain differences using selected diagnostic metho*Ds*: diagnostic implications in penaeid shrimp. *Dis Aquat Org* **52**, 1-10.

Evans, H. F. (1981). Quantitative assessment of the relationship between dosage and response of the nuclear polyhedrosis virus of *Mamestra brassicae*. *J Invertebr PAThol* **37**, 101-109.

Evans, H. F. (1983). The influence of larval maturation on responses of *Mamestra brassicae* L. (Lepidoptera: Noctuidae) to nuclear polyhedrosis virus infection. *Arch Virol* **75**, 163-170.

Fallon, A. M. & Sun, D. (2001). Exploration of mosquito immunity using cells in culture. *Insect Biochem Mol Biol* **31**, 263-278.

Flegel, T. W. (1997). Special topic review: Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. *World J Microbiol Biotechnol.* **13**, 433-442.

Flegel, T. W. (2001). The shrimp response to viral p*ATh*ogens. In *The new wave. Proceedings of the special session on sustainable shrimp aquaculture, World Aquaculture 2001 , Orlando*, pp. 190-214. Edited by C. L. Browdy & D. E. Jory. Boca Raton: World Aquaculture Society.

Flegel, T. W., Fegan, D. F., Kongsom, S., Vuthikornudomkit, S., Sriurairatana, S., Boonyaratpalin, S., Chantanachookhin, C., Vickers, J. E. & Macdonald, O. D. (1992). Occurrence, diagnosis and treatment of shrimp diseases in Thailand. In *Diseases of Cultured Penaeid Shrimp in Asia and the United States*, pp. 57-112. Edited by W. Fulks & K. L. Main. Honolulu, HI: Oceanic Institute.

Flegel, T. W., Nielsen, L., Thamavit, V., Kongtim, S. & Pasharawipas, T. (2004). Presence of multiple viruses in non-diseased, cultivated shrimp at harvest. *Aquaculture* In press.

Flegel, T. W. & Pasharawipas, T. (1998). Active viral accommodation: a new concept for crustacean response to viral p*ATh*ogens. In *Advances in Shrimp Biotechnology*, pp. 245-250. Edited by T. W. Flegel. Bangkok: National Center for Genetic Engineering and Biotechnology.

Flegel, T. W., Thamavit, V., Pasharawipas, T. & Alday-Sanz, V. (1999). Statistical correlation between severity of hepatopancreatic parvovirus infection and

stunting of farmed black tiger shrimp (*Penaeus monodon*). *Aquaculture* **174**, 197-206.

Frank, S. A. (2000). Within-host spatial dynamics of viruses and defective interfering particles. *J Theor Biol* **206**, 279-290.

Fraser, C. A. & Owens, L. (1996). Spawner-isolated mortality virus from Australian *Penaeus monodon*. *Dis Aquat Org.* **27**, 141-148.

Fujii, N., Minetti, C. A. S. A., Nakhasi, H. L., Chen, S.-W., Barbehenn, E., Nunes, P. H. & Nga Yen, N. (1992). Isolation, cDNA cloning, and characterization of an 18-kDa hemagglutinin and amebocyte aggregation factor from *Limulus polyphemus*. *J Biol Chem* **267**, 22452-22459.

Gilchrist, B. M. & Haldane, J. B. S. (1947). Sex linkage and sex determination in a mosquito, *Culex molestus*. *Hereditas* **33**.

Gubler, D. J. (1998). Resurgent vector-borne diseases as a global health problem. *Emerg Infect Dis* **4**, 442-450.

Gubler, D. J., Nalim, S., Tan, R., Saipan, H. & Sulianti Saroso, J. (1979). Variation in susceptibility to oral infection with dengue viruses among geographic strains of *Aedes aegypti*. *Am J Trop Med Hyg* **28**, 1045-1052.

Hall, T. A. (1999). BioEdit: a user-friendly program for Windows 95/98/NT. *Nucl AciDs Symp Ser* **41**, 95-98.

Hoffmann, J. A. (1995). Innate immunity of insects. *Curr Opin Immunol* **7**, 4-10.

Holland, J. J. (1996). Evolving virus plagues. *Proc Nat Acad Sci USA* **93**, 545-546.

Hsu, H. C., Lo, C. F., Lin, S. C., Liu, K. F., Peng, S. E., Chang, Y. S., Chen, L. L., Liu, W. J. & Kou, G. H. (1999). Studies on effective PCR screening strategies for white spot syndrome virus (WSSV) detection in *Penaeus monodon* brooders. *Dis Aquat Org* **39**, 13-19.

Hu, Y., Zheng, J., Iizuka, T. & Bando, H. (1994). A densovirus newly isolated from the smoky-brown cockroach *Periplaneta fuliginosa*. *Arch Virol* **138**, 365-372.

Huang, C. C. & Song, Y. L. (1999). Maternal transmission of immunity to white spot syndrome associated virus (WSSV) in shrimp (*Penaeus monodon*). *Dev Comp Immunol* **23**, 545-552.

Igarashi, A. (1978). Isolation of a Singh's *Aedes albopictus* cell clone sensitive to dengue and Chikungunya viruses. *J Gen Virol* **40**, 531-544.

Jaillon, O., Dossat, C., Eckenberg, R., Eiglmeier, K., Segurens, B., Aury, J. M., Roth, C. W., Scarpelli, C., Brey, P. T., Weissenbach, J. & Wincker, P. (2003). Assessing

the *Drosophila melanogaster* and *Anopheles gambiae* genome annotations using genome-wide sequence comparisons. *Genome Res* **13**, 1595-1599.

Johansson, M. W. (1999). Cell adhesion molecules in invertebrate immunity. *Dev Comp Immunol* **23**, 303-315.

Johansson, M. W., Keyseer, P., Sritunyalucksana, K. & Soderhall, K. (2000). Crustacean haemocytes and haematopoiesis. *Aquaculture* **191**, 45-52.

Jousset, F. X., Baquerizo, E. & Bergoin, M. (2000). A new densovirus isolated from the mosquito *Culex pipiens* (Diptera: culicidae). *Virus Res* **67**, 11-16.

Jousset, F. X., Barreau, C., Boublik, Y. & Comet, M. (1993). A parvo-like virus persistently infecting a C6/36 clone of *Aedes albopictus* mosquito cell line and p*ATho*genic for *Aedes aegypti* larvae. *Virus Res* **29**, 99-114.

Kar*Pf*, A. R., Lenches, E., Strauss, E. G., Strauss, J. H. & Brown, D. T. (1997). Superinfection exclusion of alphaviruses in three mosquito cell lines persistently infected with Sindbis virus. *J Virol* **71**, 7119-7123.

Kavanagh, K. & Reeves, E. P. (2004). Exploiting the potential of insects for in vivo p*ATho*genicity testing of microbial p*ATho*gens. *FEMS Microbiol Rev* **28**, 101-112.

Kawase, S. (1985). P*ATho*logy associated with densoviruses. In *Viral insecticides for biological control*, pp. 197. Edited by K. Maramorosch & K. E. Sherman. London: Academic Press.

Kawase, S. & Kurstak, E. (1991). Parvoviridae of Invertebrate: Densonucleosis Viruses. In *Viruses of Invertebrates*, pp. 315. Edited by E. Kurstak. New York: Marcel Dekker, INC.

Khanobdee, K., Soowannayan, C., Flegel, T. W., Ubol, S. & Withyachumnarnkul, B. (2002). Evidence for apoptosis correlated with mortality in the giant black tiger shrimp *Penaeus monodon* infected with yellow head virus. *Dis Aquat Org* **48**, 79-90.

Kilama, W. L. & Craig, G. B., Jr (1969). Monofactorial inheritance of susceptibility to Plasmodium Gallinaceum in *Aedes aegypti*. *Annals Trop Med Parasitol* **63**, 419-432.

Kimbrell, D. A. & Beutler, B. (2001). The evolution and genetics of innate immunity. *Nat Rev Genet* **2**, 256-67.

Kirkwood, T. B. L. & Bangham, C. R. M. (1994). Cycles, chaos, and evolution in virus cultures: A model of defective interfering particles. *Proc Nat Acad Sci USA* **91**, 8685-8689.

Kittayapong, P., Baisley, K. J. & O'Neill, S. L. (1999). A mosquito densovirus infecting *Aedes aegypti* and *Aedes albopictus* from Thailand. *Am J Trop Med Hyg* **61**, 612-617.

Klein, J. (1997). Homology between immune responses in vertebrates and invertebrates: Does it exist? *Scandinavian J Immunol* **46**, 558-564.

Knight, K. L. & Stone, A. (1977). A Catalog of the Mosquitoes of the World, Geo.W.King Co, Baltimore, MD.

Kotani, E., Yamakawa, M., Iwamoto, S., Tashiro, M., Mori, H., Sumida, M., Matsubara, F., Taniai, K., Kadono-Okuda, K., Kato, Y. & Mori, H. (1995). Cloning and expression of the gene of hemocytin, an insect humoral lectin which is homologous with the mammalian von Willebrand factor. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression* **1260**, 245-258.

Kurstak, E. (1972). Small densonucleosis virus (DNV). *Adv Virus Res* **17**, 207.

Kurstak, E. & Tijssen, P. (1977). Densonuclosis virus (Parvoviridae). In *The Atlas of Insects and Plant Viruses*, pp. 67. Edited by K. Maramorosch. New York: Academic Press.

Kurstak, E. & Tijssen, P. (1981). Animal parvoviruses: Comparative aspects and diagnosis. In *Comparative Diagnosis of Viral Diseases*, 4 edn. Edited by E. Kurstak & C. Kurstakm. New York: Academic Press.

Kurtz, J. & Franz, K. (2003). Innate defence: Evidence for memory in invertebrate immunity. *Nature* **425**, 37-38.

Kuznetsova, M. A. & Buchatsky, L. P. (1988). Effect of the viral preparation viroden on the mosquito *Aedes aegypti* L. under experimental condition (in Russian). *Med Parazitol (Mosk)* **2**, 79-83.

Lebedinets, N. N. & Kononko, A. G. (1989). [Experimental study of densovirus infection transmission in blood-sucking mosquito populations]. *Med Parazitol (Mosk)*, 79-83.

Lightner, D. V. (1996). A handbook of p*ATh*ology and diagnostic procedures for diseases of penaeid shrimp. Baton Rouge, LA: World Aquaculture Society.

Lightner, D. V. & Redman, R. M. (1985). A parvo-like virus disease of penaeid shrimp. *J. Invertebr. PAThol.* **45**, 47-53.

Lightner, D. V., Redman, R. M. & Bell, T. A. (1983a). Detection of IHHN virus in *Penaeus stylirostris* and *P. vannamei* imported into Hawaii. *J World Mariculture Soc* **14**, 212-225.

Lightner, D. V., Redman, R. M. & Bell, T. A. (1983b). Infectious hypodermal and hematopoietic necrosis, a newly recognized virus disease of penaeid shrimp. *J Invertebr PAThol* **42**, 62-70.

Lightner, D. V., Redman, R. M., Poulos, B. T., Mari, J. L., Bonami, J. R. & Shariff, M. (1994). Distinction of HPV-type virus in *Penaeus chinensis* and *Macrobrachium rosenbergii* using a DNA probe. *Asian Fish Sci* **7**, 267-272.

Little, T. J. & Kraaijeveld, A. R. (2004). Ecological and evolutionary implications of immunological priming in invertebrates. *TrenDs Ecol Evol* **19**, 58-60.

Little, T. J., O'Connor, B., Colegrave, N., Watt, K. & Read, A. F. (2003). Maternal Transfer of Strain-Specific Immunity in an Invertebrate. *Curr Biol* **13**, 489-492.

Lukashov, V. V. & Gou*Ds*mit, J. (2001). Evolutionary relationships among parvoviruses: virus-host coevolution among autonomous primate parvoviruses and links between adeno-associated and avian parvoviruses. *J Virol* **75**, 2729-2740.

Macdonald, W. W. (1962a). The genetic basis of susceptibility to infection with semi-periodic *Brugia malayi* in *Aedes aegypti*. *Ann Trop Med Parasitol.* **56**, 373-382.

Macdonald, W. W. (1962b). The selection of a strain of *Aedes aegypti* susceptible to infection with semi-periodic *Brugia malayi*. *Ann Trop Med Parasitol.* **56**, 368-372.

Macdonald, W. W. (1963). A preliminary cross-over value between the gene f^n filarial susceptibility, *Brugia malayi*, and the sex locus in *Aedes aegypti*. *Ann Trop Med Parasitol.* **57**, 461-465.

McCue, L. A., Thompson, W., Carmack, C. S. & Lawrence, C. E. (2002). Factors influencing the identification of transcription factor binding sites by cross-species comparison. *Genome Res* **12**, 1523-1532.

Moret, Y. & Schmid-Hempel, P. (2001). Immune defence in bumble-bee offspring. *Nature* **414**, 506.

Moret, Y. & Siva-Jothy, M. T. (in press). Adaptive innate immunity? Responsive-mode prophylaxis in the mealworm beetle Tenebrio molitor. *Proc R Soc Lond B Biol Sci.*

Moss, S. M. (2002). Marine Shrimp Farming in the Western Hemisphere: Past Problems, Present Solutions, and Future Visions. *Rev Fish Sci* **10**, 601-620.

Munstermann, L. E. & Craig G.B., J. (1979). Genetics of *Aedes aegypti*. Updating the linkage map. *J Hered.* **70**, 291-296.

Myers, E. W. & Miller, W. (1988). Optimal alignments in linear space. *Comput Appl Biosci.* **4**, 11-17.

Namikoshi, A. W., Jin Lu Yamashita, Takayoshi Nishizawa, Toyohiko Nishioka, Toyohiro Arimoto, Misao Muroga, Kiyokuni (2004). Vaccination trials with *Penaeus japonicus* to induce resistance to white spot syndrome virus. In *Aquaculture*, pp. 25.

O'Neill, S. L., Kittayapong, P., Braig, H. R., Andreadis, T. G., Gonzalez, J. P. & Tesh, R. B. (1995). Insect densoviruses may be widespread in mosquito cell lines. *J Gen Virol* **76**, 2067-2074.

Owens, L., De Beer, S. & Smith, J. (1991). Lymphoidal parvovirus-like particles in Australian penaeid prawns. *Dis Aquat Org* **11**, 129-134.

Owens, L., Haqshenas, G., McElnea, C. & Coelen, R. (1998). Putative spawner-isolated mortality virus associated with mid-crop mortality syndrome in farmed *Penaeus monodon* from northern Australia. *Dis Aquat Org* **34**, 177-185.

Owens, L. & McElnea, C. (2000). Natural infection of the redclaw crayfish *Cherax quadricarinatus* with presumptive spawner-isolated mortality virus. *Dis Aquat Org* **40**, 219-223.

Page, R. D. (1996). TreeView: an application to display phylogenetic trees on personal computers. *Computer Applications In The Biosciences: CABIOS* **12**, 357-358.

Pijlman, G. P., van den Born, E., Martens, D. E. & Vlak, J. M. (2001). *Autographa californica* baculoviruses with large genomic deletions are rapidly generated in infected insect cells. *Virol* **283**, 132-138.

Rhode, S. L., . (1978). Defective interfering particles of parvovirus H-1. *J Virol* **27**, 347-356.

Rosen, L. (1987). Sexual transmission of dengue viruses by *Aedes albopictus*. *Am J Trop Med Hyg* **37**, 398-402.

Sahtout, A. H., Hassan, M. D. & Shariff, M. (2001). DNA fraGmentation, an indicator of apoptosis, in cultured black tiger shrimp *Penaeus monodon* infected with white spot syndrome virus (WSSV). *Dis Aquat Org* **44**, 155-9.

Salzet, M. (2001). Vertebrate innate immunity resembles a mosaic of invertebrate immune responses. *TrenDs Immunol* **22**, 285-288.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). Molecular cloning: A laboratory manual., 2 edn. New York: Cold Spring Harbor Laboratory.

Santamaria, T. G. (1999). Evaluacion de la resistencia de diferentes poblaciones del camarin marino *Litopenaeus vannamei* (Boone 1931) al virus del sindrome del Taura (TSV) bajo condiciones controladas. In *Facultad de Biologia Marina*. Cartagena de Indias D.T. Columbia: Universidad Jorge Tadeo Lozano.

Schmid-Hempel, P. & Ebert, D. (2003). On the evolutionary ecology of specific immune defence. *TrenDs Ecol Evol* **18**, 27-32.

Severson, D. W., Brown, S. E. & Knu*D*son, D. L. (2001). Genetic and physical mapping in mosquitoes: molecular approaches. *Annual Rev Entomol* **46**, 183-219.

Severson, D. W., Knu*D*son, D. L., Soares, M. B. & Loftus, B. J. (2004). *Aedes aegypti* genomics. *Insect Biochem Mol Biol* **34**, 715-721.

Shike, H., Dhar, A. K., Burns, J. C., Shimizu, C., Jousset, F. X., Klimpel, K. R. & Bergoin, M. (2000). Infectious hypodermal and hematopoietic necrosis virus of shrimp is related to mosquito brevidensoviruses. *Virol* **277**, 167-77.

Siegl, G., Bates, R. C., Berns, K. I., Carter, B. J., Kelly, D. C., Kurstak, E. & Tattersall, P. (1985). Characteristics and taxonomy of Parvoviridae. *Intervirol* **23**, 61.

Stairs, G. R. (1965). Quantitative differences in susceptibility to nuclearpolyhedrosis virus among larval instars of the forest tent caterpillar, *Malacosoma disstria*. *J Invertebr PAThol* **7**, 427-429.

Sukhumsirichart, W., Wongteerasupaya, C., Boonsaeng, V., Panyim, S., Sriurairatana, S., Withyachumnarnkul, B. & Flegel, T. W. (1999). Characterization and PCR detection of hepatopancreatic parvovirus (HPV) from *Penaeus monodon* in Thailand. *Dis Aquat Org* **38**, 1-10.

Sumanochitrapon, W., Strickman, D., Sithiprasasna, R., Kittayapong, P. & Innis, B. L. (1998). Effect of size and geographic origin of *Aedes aegypti* on oral infection with dengue-2 virus. *Am J Trop Med Hyg* **58**, 283-286.

Superti-Furga, A., Rocchi, M., Schafer, B. W. & Gitzelmann, R. (1993). Complementary DNA Sequence and Chromosomal Mapping of a Human Proteoglycan-Binding Cell-Adhesion Protein (Dermatopontin). *Genomics* **17**, 463-467.

Tabachnick, W. J. (1991). Evolutionary genetics and the yellow fever mosquito. *Am Entomol.* **37**, 14-24.

Tang, K. F. J., Durand, S. V., White, B. L., Redman, R. M., Mohney, L. L. & Lightner, D. V. (2003). Induced resistance to white spot syndrome virus infection in *Penaeus*

stylirostris through pre-infection with infectious hypodermal and hematopoietic necrosis virus-a preliminary study. *Aquaculture* **216**, 19-29.

Tardieux, I., Poupel, O., Lapchin, L. & Rodhain, F. (1990). Variation among strains of *Aedes aegypti* in susceptibility to oral infection with dengue virus type 2. *Am J Trop Med Hyg.* **43**, 308-313.

Teakle, R. E., Jensen, J. M. & Giles, J. E. (1985). Susceptibility of *Heliothis armigera* to a commercial nuclear polyhedrosis virus. *J Invertebr PAThol* **46**, 166-173.

Teakle, R. E., Jensen, J. M. & Giles, J. E. (1986). Age-related susceptibility of *Heliothis punctiger* to a commercial formulation of nuclear polyhedrosis virus. *J Invertebr PAThol* **47**, 82-92.

Teetor-Barsch, A. & Roberts, D. W. (1983). Entoogenous *Fusarium* species. *MycopAThologia* **43**, 423-429.

Thomas, J. W., Touchman, J. W., Blakesley, R. W., Bouffard, G. G., Beckstrom-Sternberg, S. M., Margulies, E. H., Blanchette, M., Siepel, A. C., Thomas, P. J., McDowell, J. C., Maskeri, B., Hansen, N. F., Schwartz, M. S., Weber, R. J., Kent, W. J., Karolchik, D., Bruen, T. C., Bevan, R., Cutler, D. J., Schwartz, S., Elnitski, L., Idol, J. R., Prasad, A. B., Lee-Lin, S.-Q., Maduro, V. V. B., Summers, T. J., Portnoy, M. E., Dietrich, N. L., Akhter, N., Ayele, K., Benjamin, B., Cariaga, K., Brinkley, C. P., Brooks, S. Y., Granite, S., Guan, X., Gupta, J., Haghighi, P., Ho, S.-L., Huang, M. C., Karlins, E., Laric, P. L., Legaspi, R., Lim, M. J., Maduro, Q. L., Masiello, C. A., Mastrian, S. D., McCloskey, J. C., Pearson, R., Stantripop, S., Tiongson, E. E., Tran, J. T., Tsurgeon, C., Vogt, J. L., Walker, M. A., Wetherby, K. D., Wiggins, L. S., Young, A. C., Zhang, L.-H., Osoegawa, K., Zhu, B., Zhao, B., Shu, C. L., De Jong, P. J., Lawrence, C. E., Smit, A. F., Chakravarti, A., Haussler, D., Green, P., Miller, W. & Green, E. D. (2003). Comparative analyses of multi-species sequences from targeted genomic regions. *Nature* **424**, 788-793.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). ClustalW: improving the sensitivity of progressive multiple sequence ali*Gm*ent through sequence weighting, position specific gap penalties and weight matrix choice. *Nucl AciDs Res* **22**, 4673-4680.

Thornqvist, P. O. & Soderhall, K. (1997). Crustacean immune reactions, a short review. In *Diseases in Asian Aquaculture III*, pp. 203-218. Edited by T. W. Flegel & I. H. MacRae. Manila: Fish Health Section, Asian Fisheries Society.

Tsai, M. F., Kou, G. H., Liu, H. C., Liu, K. F., Chang, C. F., Peng, S. E., Hsu, H. C., C.H., W. & Lo, C. F. (1999). Long-term presence of white spot syndrome virus (WSSV) in a cultivated shrimp population without disease outbreaks. *Dis Aquat Org.* **38**, 107-114.

van Munster, M., Dullemans, A. M., Verbeek, M., van den Heuvel, J. F. J. M., Reinbold, C., Brault, V., Clerivet, A. & van der Wilk, F. (2003). Characterization of a new densovirus infecting the green peach aphid *Myzus persicae*. *J Invertbr PAThol* **84**, 6-14.

Van Regenmortel, M. H. V., Fauquet, C. M., Bishop, D. H. L., Carstens, E. B., Estes, M. K., Lemon, S. M., Maniloff, J., Mayo, M. A., McGeoch, D. J., Pringle, C. R. & Wickner, R. B. (2000). Virus taxonomy: seventh report of the international committee on taxonomy of viruses: Academic Press.

Vasconcelos, S. D. (1996). Alternative routes for the horizontal transmission of a nucleopolyhedrosisvirus. *J Invertebr PAThol* **68**, 269-274.

Venegas, C. A., Nonaka, L., Mushiake, K., Nishizawa, T. & Muroga, K. (2000). Quasi-immune response of Penaeus japonicus to penaeid rod-shaped DNA virus (PRDV). *Dis Aquat Org* **42**, 83-89.

Watanabe, H. (1986). Resistance of the silkworm, *Bombyx mori*, to viral infections. *Agri Ecosyst & Env* **15**, 131-139.

Whitlock, V. H. (1977). Effect of larval maturation on mortality induced by nuclear polyhedrosis and granulosis virus infections of *Heliothis armigera*. *J Invertebr PAThol* **30**, 80-86.

WHO (2002). Dengue and dengue haemorrhagic fever. Fact sheet No. 117, World Health Organization, Geneva.

Wongprasert, K., Khanobdee, K., Glunukarn, S., Meeratana, P. & Withyachumnarnkul, B. (2003). Time-course and levels of apoptosis in various tissues of black tiger shrimp *Penaeus monodon* infected with white spot syndrome virus. *Dis Aquat Org* **55**, 3-10.

Yoganandhan, K., Narayanan, R. B. & Sahul Hameed, A. S. (2003). Larvae and early post-larvae of *Penaeus monodon* (Fabricius) experimentally infected with white spot syndrome virus (WSSV) show no significant mortality. *J Fish Dis* **26**, 385-391.

Zhang, X., Huang, C. & Qin, Q. (2004). Antiviral properties of hemocyanin isolated from shrimp *Penaeus monodon*. *Antivir Res* **61**, 93-99.

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1.1 วารสาร VIRUS RESEARCH ปี ค.ศ. 2002

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Comparison of penaeid shrimp and insect parvoviruses suggests that viral transfers may occur between two distantly related arthropod groups

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1.2 วารสาร DEVELOPMENTAL AND COMPARATIVE IMMUNOLOGY ปี ค.ศ. 2006

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Challenging successive mosquito generations with a densonucleosis virus yields progressive survival improvement but persistent, innocuous infections

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Comparison of penaeid shrimp and insect parvoviruses suggests that viral transfers may occur between two distantly related arthropod groups

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Abstract

The DNA and putative amino acid sequences of representative insect and shrimp parvoviruses (subfamily *Densovirinae*) were analyzed using computer programs. Shrimp viruses included hepatopancreatic parvovirus (HPV) of *Penaeus monodon* (HPVmon) and *P. chinensis* (HPVchin), spawner-isolated mortality virus from *P. monodon* (SMVmon) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) from *P. vannamei*. Insect viruses included *Aedes aegypti* densovirus (*Aae*DNV), *Aedes 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 diameter and ssDNA genome length was between 4 and 6 kb. Using BLAST or Clustal W with the sequence fragments 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.

Keywords: Insect; Crustacean; Shrimp; Parvovirus; *Densovirinae*; Phylogeny

1. Introduction

Viruses in the Family *Parvoviridae* consist of small icosahedral, non-enveloped particles 18–26 diameter (Afanasiev et al., 1991). The genome

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(*Parvovirinae* or *Densovirinae*) is single stranded, linear DNA with only two overlapping genes that generate two sets of structurally related but biologically distinct polypeptides called nonstructural (NS) protein and the viral capsid polypeptide (VP) (Shike et al., 2000). The family *Parvoviridae* includes two subfamilies, *Parvovirinae* with viruses that infect vertebrates, and *Densovirinae* with viruses that infect invertebrates, mainly insects. Boublik et al. (1994) suggested that the overall organization of three densoviruses, *Bombyx mori* densovirus (*Bm*DNV), *Aedes* DNV and the *Junonia coenia* densovirus (*Jc*DNV), revealed striking differences.

Of the parvoviruses in crustaceans, only those of shrimp have been studied in any detail. These include hepatopancreatic parvovirus (HPV) in *P. chinensis* (HPVchin) (Lightner and Redman, 1985) and *Penaeus monodon* (HPVmon) (Sukhumsirichart et al., 1999; Phromjai et al., 2001), spawner-isolated mortality virus from *P. monodon* (SMVmon) (Fraser and Owens, 1996; Owens et al., 1998, 2000; Owens and McElnea, 2000) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Lightner et al., 1983; Bonami et al., 1990; Shike et al., 2000). These viruses have caused disease in cultivated shrimp in many countries all over the world (Flegel, 1997). Shike et al. (2000) recently reported that IHHNV of shrimp and the mosquito densoviruses *Aae*DNV and *Aal*DNV shared similar overall genomic organization. Specifically, the left ORF of these viruses most likely encoded the major NS protein (NS1) since it contained conserved replication initiator motifs and NTP-binding and helicase domains similar to those in NS-1 from all other parvoviruses, and the putative NS1 of IHHNV shared amino acid sequence homology with NS1 of the mosquito densoviruses.

Spawner-isolated mortality virus (SMV) of *P. monodon* was described as a parvovirus based on its morphological characteristics by transmission electron microscopy (TEM) and its specific gravity (Fraser and Owens, 1996). It has also been recently reported from freshwater crayfish in Australia (Owens and McElnea, 2000). It was of interest to know the relationship amongst the three types of shrimp parvovirus and also their relationship to known insect parvoviruses. To this end, recent sequence information from these three shrimp virus types was compared with that published for the insect parvoviruses.

2. Materials and methods

2.1. DNA sequences

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 1.

2.2. 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 the CLUSTAL W software contained in the BIOEDIT program (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 by 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 PHYLIP package (NEIGHBOR), based on the Kimura two-parameter distance estimation method or the proportion of differences (*p* distance). The program TreeView was also used to view the phylograms (Page, 1996).

The phylogenetic tree for DNA was constructed based on 696 bp of aligned DNA sequences. 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 BLAST 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 LALIGN program version 2.0 (http://www.ch.embnet.org/software/LALIGN

–form.html) was used to calculate global alignment of sequences (Myers and Miller, 1988).

3. Results and discussion

3.1. 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 et al., 1983, 1994; Lightner and Redman, 1985; Owens et al., 1991; Sukhumsirichart et al., 1999), virion size for insect and penaeid shrimp viruses ranged between 18 and 30 nm in diameter and all had genomes of ssDNA that ranged between 4 and 6 kb (Table 2). 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.

3.2. Sequences comparisons of insect and penaeid shrimp parvoviruses

Using BLAST program 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.

Table 1
Source and origin of parvoviruses sequences used

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

Table 2
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 (nm)	Length of genome	References
IHHNV = Infectious Hypodermal and Hematopoietic Necrosis Virus (PstDNV)	Densovirus	*P. aztecus*, *P. californiensis*, *P. chinensis*, *P. duorarum*, *P. esculentus*, *P. japonicus*, *P. monodon*, *P. ocidentalis*, *P. schmitti*, *P. semisulcatus*, *P. setiferus*, *P. stylirostris*, *P. vannameli*	22	4075 bp (IHHNV)	Lightner et al., 1983; Bonami et al., 1990
				3873 bp (PstDNV)	Shike et al., 2000
HPV = Hepatopancreatic Parvovirus	Parvovirus	*P. chinensis*, *P. esculentus*, *P. indicus*, *P. japonicus*, *P. mergulensis*, *P. monodon*, *P. penicillatus*, *P. schmitti*, *P. semisulcatus*, *P. styltrostris*, *P. vannamei*	22–24	4–4.3	Lightner and Redman, 1985; Bonami et al., 1995
				5.8 kb	Sukhumsirichart, 1998; Sukhumsirichart et al., 1999
SMV = Spawner-Isolated Mortality Virus	Parvovirus	*P. monodon*	20	ND	Fraser & Owens, 1996
*Aae*DNV = *Aedes aegypti* Densonucleosis Virus	Densovirus	Mosquitoe: *Aedes aegypti*	18-26	4009 bp	Afanasiev et al., 1991
*Aal*DNV = *Aedes albopictus* Parvovirus	Contravirus	1. Mosquitoe: *Aedes aegypti*; 2. *Aedes albopictus* C6/36 cell line	22	4176 bp	Boublik et al., 1994
*Bm*DNV = *Bombyx mori* Densovirus	Densovirus	Silkworm: *Bombyx mori*	22	5048 bp	Bando et al., 1987a,b, 1990
*Ds*DNV = *Diatraea saccharalis* Densovirus	Densovirus	Sugarcane borer (insects or their larvae)	ND	5941 bp	Boublik et al., 1997 (GenBank NC_001899)
*Gm*DNV = *Galleria mellonella* densovirus	Densovirus	Lepidoptera: Pyralidae *Galleria mellonella* Larvae	ND	6039 bp	Simpson et al., 1998
*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	*P. monodon*, *P. esculentus*, *P. mergulensis*	25-30	ND	Owens et al., 1991
*Pf*DNV = *Periplaneta fuliginosa* densovirus	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

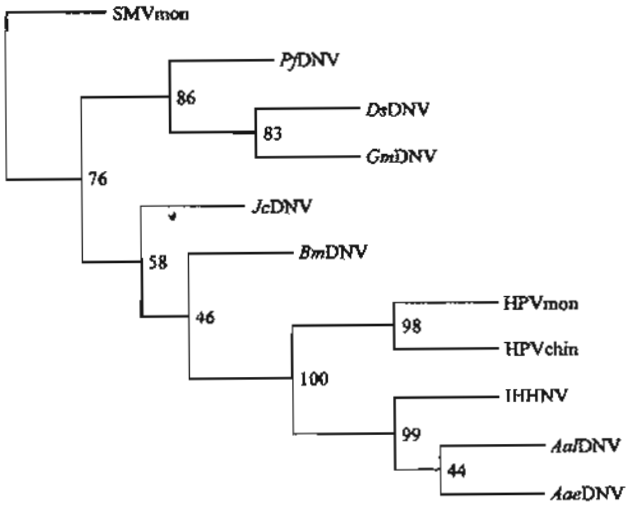

Fig. 1. The neighbor-joining phylogenetic tree generated from a heuristic search of aligned insect and shrimp parvoviruses DNA sequences. The phylogenetic tree was constructed based on 696 bp of aligned DNA sequences. The branch lengths are scaled according to the number of base substitutions. The scale 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). For the sources of DNA sequences data are shown in Table 1.

A phylogenetic tree based on genomic DNA of shrimp and insect parvoviruses (Fig. 1) 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. The grouping of IHHNV with the mosquito brevidensoviruses *Aae*DNV and *Aal*DNV was in agreement with the study of Shike et al. (2000) who found the same relationship in a phylogenetic tree based on a 199-aa conserved region characteristic of the NTP-binding and helicase domains of the nonstructural protein (NS1) of IHHNV, of insect parvoviruses and vertebrate parvoviruses. However, the clustering of SMV with a different group of insect densoviruses and the separation of HPV into yet another group suggests that the shrimp parvoviruses studied are of diverse origin and that they are not closely related. Shike et al. (2000) reported that IHHNV was the first example of a densovirus with a host range outside the class Insecta that diverged from the Crustacea over 500 million years ago. Our analysis suggests that SMVmon and HPVmon/HPVchin may represent two other groups of distantly related densoviruses in the class Crustacea.

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 (Fig. 2A). Other proteins that showed homology were capsid protein of HPVchin and unknown protein of HPVmon (15.3%) and proteins of insect parvoviruses (Fig. 3A). It was interesting that the capsid protein of IHHNV showed very low similarity to capsid proteins of *Aae*DNA 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 (Fig. 2B).

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 (Fig. 3A). The results of the comparison were used to generate another phylogenetic tree (Fig. 3B). 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 (Fig. 2B) and NS1 or related sequences (Fig. 3B) were somewhat different. The phylogenetic tree based on NS1 was similar to that described by Shike et al. (2000) with the closest relationship found between IHHNV and *Aae*DNV or *Aal*DNV. However, when capsid proteins were used, IHHNV fell closer to *Bm*DNV and *Pf*DNV than to *Aae*DNV or *Aal*DNV. The phylogram based on NS1 sequences corresponded most closely to the phylogram constructed based on DNA sequences and it suggested that NS1 might be more conserved than the capsid protein.

Jousset et al. (2000) reported that there was a lack of sequence homology between *Cp*DNV and *Aal*DNV genomes, and that lack of antigenic

cross-reactivity between capsid polypeptides indicated that the two mosquito viruses *Aae*DNV and *Aal*DNV were also phylogenetically distant. They also reported that *Cp*DNV appeared to be related to *Junonia coenia* densovirus (*Jc*DNV) both serologically and genomically. Due to lack of appropriate sequence information we were unable to include *Cp*DNV in our analysis, but based on the work of Jousset et al. (2000), it might eventually cluster with *Jc*DNV, distant from *Aal*DNV. In contrast to their work, however, our results indicated that *Aae*DNV and *Aal*DNV were closely related.

(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				
*Jc*DNV orf1	5.4	0.5	0.1	1.1	0.6	*Jc*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	
*Pf*DNV orf5	2.5	0.5	0.2	2.0	0.2	8.6	9.7	4.8	*Pf*DNV orf5
*Ds*DNV VP1	0.1	0.5	0.0	1.0	0.2	83.1	78.7	14.9	8.6

(B)

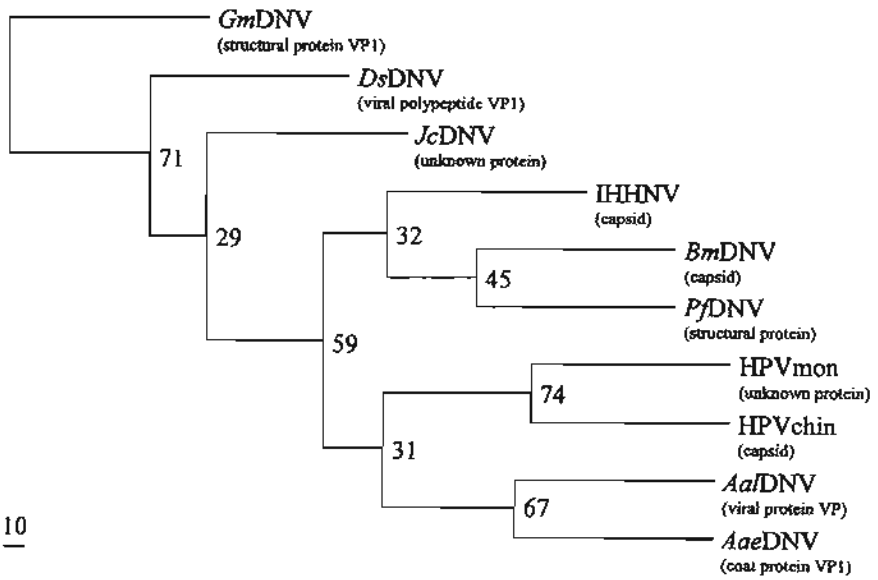

Fig. 2. Comparisons based on putative protein sequences of capsid and structural proteins using a global alignment method (LALIGN). (A) Overall parvoviral protein similarities. (B) The neighbor-joining phylogenetic tree generated from a heuristic search of aligned insect and shrimp parvoviruses deduced 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	IHHNV NS1	AaeDNV NS	AalDNV NS1	JcDNV orf2	BmDNV NS_orf1	PfDNV NS	DsDNV NS1
HPVmon Unk								
IHHNV NS1	1.6							
AaeDNV NS	1.3	18.2						
AalDNV NS1	1.5	20.4	73.8					
JcDNV Orf2	0.3	13.7	11.7	12.1				
BmDNV NS_orf1	0.5	0.1	0.2	0.4	17.7			
PfDNV NS	1.1	0.2	0.4	11.2	28.0	18.0		
DsDNV NS1	0.3	14.3	29.7	11.5	92.8	17.8	29.7	

(B)

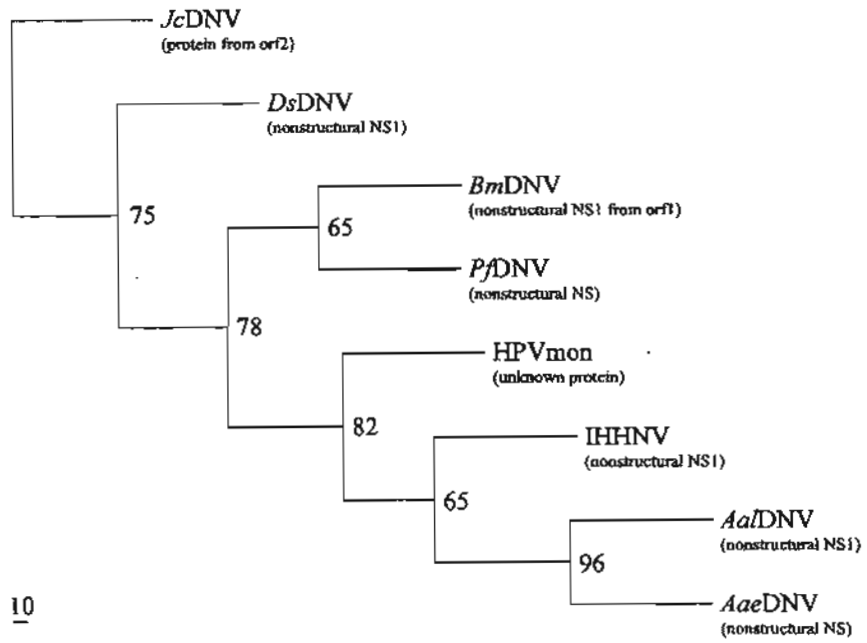

Fig. 3. Comparisons based on putative protein sequences of insect virus nonstructural protein (NS) and most similar proteins using global alignment method (LALIGN). (A) Overall parvoviral protein similarities. (B) The neighbor-joining phylogenetic tree generated from a heuristic search of aligned insect and shrimp parvoviruses deduced amino acid sequences. The 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).

Based on DNA sequence comparisons, we were surprised to find that the shrimp parvoviruses did not form one cluster and the insect parvoviruses another. The fact that the three shrimp viruses fell into two different parvovirus clades, including two different insect virus groups suggests that there may have been and may still be viral transfers between these distantly related arthropods. Indeed, Lo et al. (1996) found that a PCR probe for white spot syndrome virus of penaeid shrimp gave positive results with aquatic insect larvae and suggested that they were possible reservoir hosts. Since this work was not followed up with more detailed tests such as in situ DNA hybridization

assays, it may have been that the insect larvae were simply mechanical carriers of the shrimp virus. On the other hand, cultivated penaeid shrimp larvae and some insect larvae are carnivorous and known by shrimp farmers to eat each other when sizes are appropriate and opportunity arises. It would be worthwhile looking in the shrimp environment to determine whether any aquatic insect larvae there can be infected with shrimp parvoviruses and act as reservoirs for them.

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References

Afanasiev, B.N., Galyov, E.E., Buchatsky, L.P., Kozlov, Y.V., 1991. Nucleotide sequence and genomic organization of *Aedes* Densonucleosis virus. Virology 185, 323–336.

Bando, H., Kusuda, J., Gojobori, T., Maruyama, T., Kawase, S., 1987a. Organization and nucleotide sequence of a densovirus genome imply a host-dependent evolution of the parvoviruses. J. Virol. 61, 553–560.

Bando, H., Kusuda, J., Kawase, S., 1987b. Molecular cloning and characterization of Bombyx densovirus genomic DNA. Arch. Virol. 93, 139–146.

Bando, H., Choi, H., Ito, Y., Kawase, S., 1990. Terminal structure of a densovirus implies a hairpin transfer replication which is similar to the model for AAV. Virology 179, 57–63.

Bonami, J.R., Brehelin, M., Mari, J., Trumper, B., Lightner, D.V., 1990. Purification and characterization of IHHN virus of penaeid shrimps. J. Gen. Virol. 71, 2657–2664.

Bonami, J.R., Mari, J., Poulos, B.T., Lightner, D.V., 1995. Characterization of hepatopancreatic parvo-like virus, a second unusual parvovirus pathogenic for penaeid shrimps. J. Gen. Virol. 76, 813–817.

Boublik, Y., Jousset, F.X., Bergoin, M., 1994. Complete nucleotide sequence and genomic organization of the *Aedes albopictus* Parvovirus (AaPV) pathogenic for *Aedes aegypti* lavae. Virology 200, 752–763.

Boublik, Y., Kouassi, K.N., Cavallaro, C., Bergoin, M., 1997. Complete nucleotide sequence and genome organization of an infectious clone of *Diatraea saccharalis* densovirus (*Ds*DNV). Lab. de Pathologie Comparee, Universite Montpellier II, Place Eugene Bataillon, Montpellier F-34095, France, GenBank Accession Number NC_001899.

Dumas, B., Jourdan, M., Pascaud, A.M., Bergoin, M., 1992. Complete nucleotide sequence of the cloned infectious genome of *Junonia coenia* densovirus reveals an organization unique among parvovirus. Virology 191, 202–222.

Fraser, C.A., Owens, L., 1996. Spawner-isolated mortality virus from Australia *Penaeus monodon*. Dis. Aquat. Org. 27, 141–148.

Flegel, T.W., 1997. Special topic review: major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. World J. Microbiol. Biotechnol. 13, 433–442.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41, 95–98.

Hu, Y., Zheng, J., Iizuka, T., Bando, H., 1994. A densovirus newly isolated from the smoky brown cockroach *Periplaneta fuliginosa*. Arch. Virol. 138, 365–372.

Jousset, F.X., Baquerizo, E., Bergoin, M., 2000. A new densovirus isolated from the mosquito *Culex pipiens* (Diptera: Culicidae). Virus Res. 67, 11–16.

Lightner, D.V., Redman, R.M., 1985. A parvo-like virus disease of penaeid shrimp. J. Invertebr. Pathol. 45, 47–53.

Lightner, D.V., Redman, R.M., Bell, T.A., 1983. Infectious hypodermal and hematopoietic necrosis (IHHN), a newly recognized virus disease of penaeid shrimp. J. Invertebr. Pathol. 42, 62–70.

Lightner, D.V., Redman, R.M., Poulos, B.T., Mari, J.L., Bonami, J.R., Shariff, M., 1994. Distinction of HPV-type virus in *Penaeus chinensis* and *Macrobrachium rosenbergii* using a DNA probe. Asian Fish Sci. 7, 267–272.

Lo, C.F., Ho, C.H., Peng, S.E., Chen, C.H., Hsu, H.E., Chiu, Y.L., Chang, C.F., Liu, K.F., Su, M.S., Wang, C.H., Kou, G.H., 1996. White spot syndrome associated virus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. Dis. Aquat. Org. 27, 215–225.

Myers, E.W., Miller, W., 1988. Optimal alignments in linear space. Comput. Appl. Biosci. 4, 11–17.

Owens, L., McElnea, C., 2000. Natural infection of the red-claw crayfish *Cherax quadricarinatus* with presumptive spawner-isolated mortality virus. Dis. Aquat. Org. 40, 219–223.

Owens, L., De Beer, S., Smith, J., 1991. Lymphoidal parvo-like virus in Australian prawns. Dis. Aquat. Org. 11, 129–134.

Owens, L., Haqshenas, G., McElnea, C., Coelen, R., 1998. Putative spawner-isolated mortality virus associated with mid-crop mortality syndrome in farmed *Penaeus monodon* from northern Australia. Dis. Aquat. Org. 34, 177–185.

Owens, L., Lightner, D.V., Pantoja, C., 2000. Construction of the spawner-isolated mortality virus (SMV) clone and development of diagnostic assays, unpublished data.

Page, R.D.M., 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12, 357–358.

Phromjai, J., Sukhumsirichart, W., Pantoja, C., Lightner, D.V., Flegel, T.W., 2001. Different reactions obtained using the same DNA detection reagents for Thai and Korean hepatopancreatic parvovirus (HPV) of penaeid shrimp. Dis. Aquat. Org. 46, 153–158.

Shike, H., Dhar, A.K., Burns, J.C., Shimizu, C.and, Jousset, F.X., 2000. Infectious hypodermal and hematopoietic necrosis virus of shrimp is related to mosquito brevidensoviruses. Virology 277, 167–177.

Simpson, A.A., Chipman, P.R., Baker, T.S., Tijssen, P., Rossmann, M.G., 1998. The structure of an insect parvovirus (*Galleria mellonella* densovirus) at 3.7 A resolution. Structure 6, 1355–1367.

Sukhumsirichart, W., 1998. Genome analysis and development of a simple and specific detection of hepatopancreatic parvovirus in *Penaeus monodon* using polymerase chain reaction. Ph.D..Thesis (Biochemistry), Faculty of Graduate Studies, Mahidol University, Bangkok.

Sukhumsirichart, W., Wongteerasupaya, C., Boonsaeng, V., Panyim, S., Sriurairatana, S., Withyachumnarnkul, B., Flegel, T.W., 1999. Characterization and PCR detection of hepatopancreatic parvovirus (HPV) from *Penaeus monodon* in Thailand. Dis. Aquat. Org. 38, 1–10.

Tatusova, T.A., Madden, T.L., 1999. Blast 2 sequences—a new tool for comparing protein and nucleotide sequences. FEMS Microbiol. Lett. 174, 247–250.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids Res. 22, 4673–4680.

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Challenging successive mosquito generations with a densonucleosis virus yields progressive survival improvement but persistent, innocuous infections

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Abstract

Research on cultivated shrimp suggests that they have the capability to tolerate viral pathogens 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 yr and lack continuous cell lines. Thus, we developed a mosquito-densovirus model to examine whether similar phenomena occur in insects. Serial challenge of five generations with a stock densovirus (*ATh*DNV) resulted in progressive survival increases from 15% to 58%. 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. 36% in F2) but they grew normally to establish each succeeding generation. At the end of five generations, comparison of deduced amino acid sequences from genome fragments revealed a significantly higher ($p = 0.02$) estimated prevalence of defective targets in the survivor virus population (29.7% ± 10.0 SD) than in the stored viral population (3.3% ± 5.8 SD). The results paralleled those reported for serially passaged C6/36 mosquito cell cultures infected with a densovirus. 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; Tolerance; Adaptation

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1. Introduction

Global field experience with cultivated shrimp has shown that catastrophic introductions of exotic viral pathogens are followed within a few years by the appearance of natural, native shrimp that are grossly normal carriers of the exotic virus [1]. The virus is transmissible and can be lethal to naïve shrimp. Results from using a fry of mixed captured spawners to stock cultivation ponds suggests that tolerance depends on factors related to individual production batches of shrimp (i.e., it is adaptive in nature) [1]. This adaptive pattern cannot be explained easily by currently accepted knowledge or theories regarding shrimp or crustacean defense. The natural phenomenon has been repeated experimentally by genetic selection of families on the basis of high survival upon viral challenge for the two shrimp pathogens—infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Supershrimp Co. Ltd., now closed) and Taura syndrome virus (TSV) [2]. Tests with TSV tolerant families genetically selected on the basis of external challenge tests suggest that tolerance may be specific to each pathogen and not cross-protective [3,4]. Specificity is such that even small changes in the viral genome of TSV, for example, have been shown to greatly alter virulence [5] or to overcome tolerance developed through breeding selection [6].

Detailed study of host–viral interaction in shrimp is hindered by long shrimp generation times (1–2 yr) 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 pathogens 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 pathogens [7]. For example, 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 [8–17]. However, a problem with many published studies has been the tendency to measure "resistance" by survival rate, and the general failure to determine whether or not survivors are infected.

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* and densovirus (*ATh*DNV) model to examine the effect of challenging successive generations of an arthropod population with a viral pathogen.

2. Materials and methods

2.1. Cell line

A cell line of the C6/36 clone of *A. albopictus* was used [18]. The densovirus-free line was grown in minimal essential medium at pH 7.0 (MEM, GIBCO™ Invitrogen), supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% non-essential amino acids, penicillin (100 unit/ml) and streptomycin (0.1 mg/ml) that accompanied the commercial preparation. 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 [19]. The live C6/36 cells were counted using 0.4% Trypan Blue and a hemocytometer under a light microscope every time before propagation.

2.1.1. Virus and viral stock preparation

The Thai strain of densonucleosis virus used in this study was detected in a colony of *A. albopictus* and *A. aegypti* in 1999 by Dr. Pattamaporn Kittayapong from Center For Vectors and Vector-Borne Diseases (CVVD), Faculty of Science, Mahidol University, Thailand [20]. It was the sixth mosquito densovirus described, and was designated *ATh*DNV. About 50 moribund infected mosquito larvae from an *A. aegypti* colony were homogenized with 1.5 ml of GIBCO™ minimum essential medium (MEM) at pH 7.2, with addition of the accompanying 1% non-essential amino acids, 100 unit/ml penicillin and 0.1 g/ml streptomycin, but without the heated, inactivated fetal bovine serum (FBS). The homogenized viral solution was filtered through a 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 two times with phosphate buffered saline (PBS) pH 7.2 before inoculation with 2 ml of the viral suspension for 2 h 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 second passage of infected C6/36 cells was scraped

from the flasks and aliquoted in 0.5 ml lots for storage in microcentrifuge tubes at −80 °C.

2.1.2. Mosquitoes

An initial batch of mosquito eggs (several thousand) was provided by the Ministry of Public Health of Thailand, from a domestic colony of *A. aegypti* that has been maintained in captivity for more than 10 yr. 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 to avoid contamination. Lavae were reared in 2 l 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 g body weight provided by the National Laboratory Animal Center, Mahidol University, Thailand.

2.1.3. Challenge of mosquito lavae

First instar mosquito larvae were challenged with *ATh*DNV following a protocol slightly modified from that described by Barreau et al. [19,21]. Four hours after hatching, 500 first instar larvae were washed in sterile distilled water two 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 followed by incubation at 30 °C. Control larvae were treated in the same manner except that they were mock-challenged with an equal volume of harvested, densovirus-free C6/36 cells. After 48 h, the larvae had molted to second instar and were transferred to 2 l water bowls, where they were 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. Survivors were considered those larvae that reached the adult stage.

2.1.4. DNA extraction

To begin experiments, the starting mosquito population was screened twice for freedom 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 [22]. The larvae were pooled and homogenized with a glass rod in 5 ml 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. [23] with commercial phenol saturated with Tris–HCL. DNA in the final upper phase was precipitated with two 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 the 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 one mosquito specimen.

2.1.5. AThDNV 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 [24] yielding an amplicon of 350 bp. DNA extract (150 µl) was used for the PCR reaction. DNA extract from an *ATh*DNV infected *A. albopictus* C6/36 cell culture was used as the positive control template, while sterile double-distilled water and DNA extract from *A. 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 10 × buffer (Promega, Madison, WI), 2 µl of 25 mM $MgCl_2$, 0.5 µl of dNTPs (10 mM each), 0.5 µl of each primer (20 mM 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 1.5 + 100 bp molecular weight marker (Promega). During the course of the investigation a nested primer pair *ATh*DNV_213F 5′ CTT CGG ATT AGC ATC ACC 3′ and *ATh*DNV_213R 5′ GGG ATC AAC CTT CTA AAG 3′ were developed to obtain higher sensitivity, and these yielded a product of 213 bp using the same PCR protocol as with the outer

primers. Samples that yielded products of the expected size (350 bp by one-step PCR or 213 bp by nested PCR) were scored as positive for *ATh*DNV.

2.1.6. AThDNV detection by fluorescent 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 fragment from open reading frame 3 [24] to amplify a 350 bp DNA fragment. Labeling was carried out using fluorescein-12-dUTP (Roche Molecular Biochemicals, Germany) according to the product manual (version 3, September 1999). The labeling mixture included sterile redistilled water, 10 × PCR buffer without $MgCl_2$, 4 mM $MgCl_2$, 200 μM dNTP (PCR Fluorescein labeling mix), 1 μM primers, 1 U 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.

Mosquito samples were fixed in Davison's AFA for 2–4 h before being dehydrated and embedded in paraffin using standard histological methods [25]. 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 [25]. 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 followed by washing three times with de-ionized water for 5 min each and addition of one drop of anti-fade solution (Verstashield, H-1000) 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 negative hybridization.

2.1.7. Normal histology and electron microscopy

Preparation of adult mosquitoes for normal histology was done according to Lightner [25]. Larval specimens for transmission electron microscopy (TEM) were fixed for 2 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.8 and then washed twice in 0.1 M cacodylate buffer, pH 7.8 at 4 °C. The samples were post-fixed for 2–3 h in 1% osmium tetroxide in 0.1 M 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 sections were cut in a Reichert ultramicrotome, mounted on formvar-coated copper grid and examined in the TEM (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.

2.1.8. Cloning and sequencing of DNA

PCR products were amplified from template DNA using three primer pairs designed for *ATh*DNV (Dr. Pahol Kosiyachinda) and covering a total of 1.5 kb of the genome from positions 454 to 2096 relative to the *Aa*PV genome. These were 5′-RPT-0794 (5′ TAA CGC GTC ACA GGC AAG 3′) and 3′-RPT-1384 (5′ GTG ATA GTC GCT TCT GCA C 3′) for relative positions 464–1959 (amplicon 799 bp), 5′-RPT-0582 (5′ AAA CCG TTG GTG ACC TCT ACC 3′) and 3′-RPT2081 (5′ CTG GTT TTA CCA TGG CCA ATA AG 3′) for relative positions 603–1401 (amplicon 1495 bp) and 5′-RPT-0794 and 3′-RPT2081 for relative positions 603–2096 (amplicon 1393 bp). The amplification protocol was the same as described before in *ATh*DNV screening by PCR. DNA fragments purified from agarose gels were ligated to pDrive cloning vector (QIAGEN) following the QIAGEN manual protocol. The ligated product was transformed into *Escherichia coli* JM109. Transformed cells were spread together with 50 μl of 20 mg/ml X-gal in dimethylformamide solution on Luria Bertani (LB) agar (LB broth, Difco, Detroit, MI, USA, plus 1.5% gar) containing 50 μg/ml of ampicillin. White colonies were selected as positive transformants. Recombinant plasmids were then purified, DNA concentrations measured at OD_{260} and 150 ng used to check insertion of PCR fragments. For sequencing, an additional two primers were used 5′-RPT-1281 (5′ CAG GAG GAA ACA GCA CAA GAG TCA 3′) and 5′-RT-1758 (5′ GAA AGA ACA CGTATA CAA 3′), together with those above, to break up long sequences. All clone regions were sequenced at least twice and some up to four times for verification. Sequencing was done using an ABI PRISM dye terminator cycle ready reaction kit (Perkin Elmer) with and ABI PRISM 377 automated DNA sequencer by Macrogen Company

Limited (Chongro-Ku, South Korea) or using an Amersham dye terminator kit with a MegaBACE DNA500 automated sequencer at the Institute of Molecular Biology and Genetics, Mahidol University, Bangkok.

2.1.9. Computer analysis

BIOEDIT software (version 5.0.6) was used to manipulate the sequences [26]. The alignment of sequences was performed by using the CLUSTAL W software contained in the BIOEDIT program [27].

2.1.10. Serial challenge protocol

The scheme for serial *ATh*DNV challenge of successive generations of *A. aegypti* is shown in Fig. 1. 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, five replicates were used, while two 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 two 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. To check for potential loss of virulence in the aliquoted stock virus, a special batch of 500 larvae produced from the maintained parental mosquito stock (F0) was challenged at the end of the experimental period of 6 months.

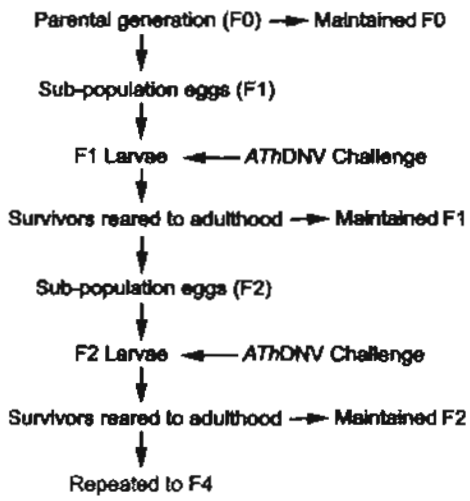

Fig. 1. *ATh*DNV-*Aedes aegypti* mosquito adaptation test.

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 three 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 third–fourth instars; 100 to examine for the presence of *ATh*DNV by semi-thin sections and by TEM in first instars within 2 h after hatching (to avoid infections by horizontal transmission). For PCR analysis, it was necessary to rear to the third–fourth instar larvae stage in order to have sufficient DNA for the assays.

3. Results

3.1. Host–viral adaptation

Challenge of successive generations of mosquitoes with *ATh*DNV according to the protocol in Fig. 1 resulted in a survival increase from 15±4% at F1 to 58±6% at F4, while the mean survival of the mock challenged control mosquito groups was 94±4% throughout (Table 1). Survival for a batch of 500 larvae produced from the maintained parental mosquito stock and challenged at the end of the experimental period of 6 months was 18%. This was comparable to survival at the beginning of the experiments (i.e., 15±4%), indicating that virulence of the stored virus had not diminished. Although survival of the viral-challenged mosquitoes increased with each generation, the number of eggs

Table 1
Percent survival of four generations of *A. aegypti* successively challenged with *ATh*DNV aliquots of the same frozen stock

Mosquito generation	Mosquitoes challenged with *ATh*DNV		Mock-challenged mosquitoes	
	Survivors to adulthood (from replicates of 100)	% Survival ± SD	Survivors to adulthood (from replicates of 100)	% Survival ± SD
First generation (F1) (naïve population)	75/500[a] (18, 15, 10, 20, 12)	15 ± 4	191/200 (96, 95)	96 ± 1
Second generation (F2) (offspring of F1 survivors)	74/300 (20, 25, 29)	25 ± 5	182/200 (92, 90)	91 ± 1
Third generation (F3) (offspring of F2 survivors)	119/300 (47, 40, 32)	40 ± 8	181/200 (87, 94)	91 ± 5
Fourth generation (F4) (offspring of F3 survivors)	109/200 (56, 59)	58 ± 6	187/200 (97, 90)	94 ± 5

[a]Because this number of survivors was too low to produce the next generation and also provide sufficient specimens for various tests, a second, single batch of 500 larvae was challenged and the survivors (approximately 75) were pooled.

produced gradually decreased from 500 at F0 to 300 at F2 and F3 and 200 at F4.

3.1.1. *AThDNV infection status of challenge survivors*

To determine whether mosquitoes that survived *ATh*DNV challenge were infected or not, 109 F1 surviving, adult females (collected after egg laying to generate F2) were tested by PCR and infections were verified by FISH and histology for the presence of *ATh*DNV. All of these mosquitoes exhibited grossly normal appearance and behavior. Of 94 tested by PCR assay, 13 were *ATh*DNV positive. Of the remaining 15 used for histology and FISH (see below), two showed evidence of *ATh*DNV infection, giving an overall confirmed prevalence of 15/109 = 14% in the surviving F1 adults. The two of 15 adult F1 mosquitoes found to be *Ath*DNV positive by normal histology using H and E staining (Fig. 2) showed many enlarged nuclei containing typical, eosinophilic to basophilic parvovirus inclusions. 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 with these specimens confirmed the presence of *ATh*DNV nucleic acid in the enlarged nuclei (Fig. 3).

Of two batches of 100 F1 generation eggs hatched (i.e., F2 generation larvae) and tested by PCR in sets 50 and 42 larvae, 23 and nine positive larvae were detected, respectively. This was a total of 33 positive in 92 (36%) taken as a sub-sample of 200 larvae. Application of epidemiological sampling software [22] showed that the maximum estimated prevalence was 43% in the sampled population of 200.

Presence of *Ath*DNV was also confirmed by TEM with first instar F2 larvae that survived *Ath*DNV challenge. Semi-thin sections (Fig. 4) prepared prior to TEM revealed intranuclear parvovirus inclusions similar to those revealed by H and E staining and FISH. The sections also revealed lack of tissue disruption, suggesting that normal functions had been maintained despite viral infection. TEM revealed large numbers of 22–24 nm parvovirus virions free in the nucleoplasm and collected into paracrystalline arrays. It also showed lack of tissue disruption. Of six blocks prepared from 100 specimens, only one (16%) showed evidence of viral particles. Application of epidemiological sampling software [22] showed that the maximum estimated prevalence was 57% in the sampled population of 100.

Once the initial PCR results for *ATh*DNV had been confirmed by histology, FISH and TEM, we considered that PCR testing only was sufficient to confirm the presence of *ATh*DNV in the succeeding F3 and F4 generations. In the F3 generation, a pool of 50 mosquito larvae was positive for *Ath*DNV by one-step PCR. Of five pools of 50 larvae each from the F4 generation, two were positive for *Ath*DNV by one-step PCR and two by nested PCR. Only one pool was negative. Although prevalence could not be estimated, the results indicated that the virus persisted in the surviving mosquito populations.

3.1.2. *Genomic analysis of AThDNV in stock and surviving mosquitoes*

A total of 50 clones of inserted fragments were sequenced and used for analysis. These sequences

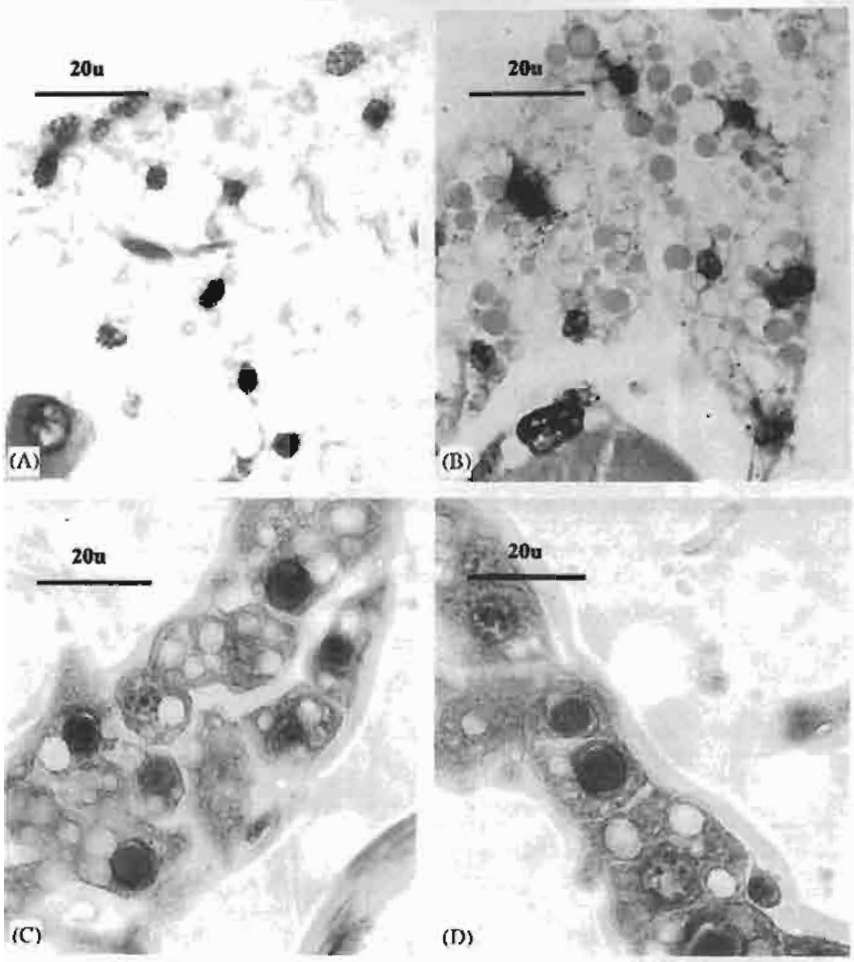

Fig. 2. Hematoxlyhn and Eosin (H&E) stained sections of *ATh*DNV-infected (A and B) and normal (C and D) *A. aegypti* adults. Parvotypes inclusions or hypertrophied nuclei were usually found in infected nuclei with densoviruses and/or parvoviruses. *ATh*DNV forms large intranuclear inclusions (C and D) that are dark blue with H and E staining. The inclusions start out reddish in color and often push the nucleolus against the nuclear membrane where it eventually looks like a blue crescent beside the blue *ATh*DNV inclusion. They contrast sharply with smaller normal nuclei (A and B).

have been deposited at GenBank under the accession numbers shown in Table 2. Each clone was considered to have originated from a different target gene fragment and was translated into a deduced amino acid sequence based on alignment with two amplicons from the NS1/NS2 overlap region and one amplicon from the NS1 region by comparison to the full sequence of *A. albopictus* densonucleosis virus (*Aal*DNV) genome (GenBank accession number NC_004285) as shown in Figs. 5–7. The *Aal*DNV sequence was used as a reference since there was no complete sequence available for *Ath*DNV and since there is high sequence identity between *Aal*DNV and *Ath*DNV. Alignments within the NS1 and NS2 regions, 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 Figs. 5 and 7 are illustrated for NS2 and NS1, respectively, transcription products would be defective for the reading of both NS1 and NS2 proteins, because the single base deletions and insertions occurred after the start codons for both proteins. Calculating from the percentage of putative defective sequences in each of the three sampled regions, the mean prevalence of defective sequences in the freezer group was 3.3% ± 5.8 SD, while that in the F4 group was

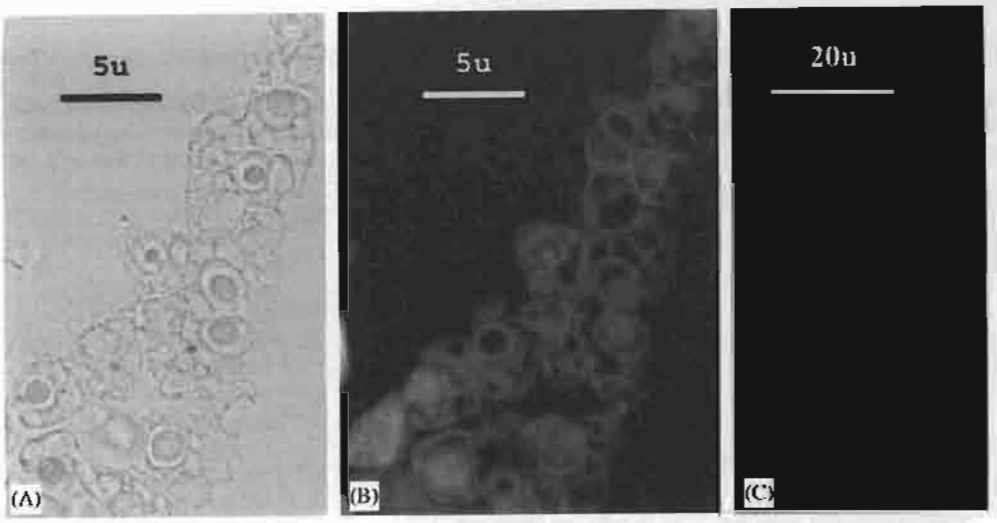

Fig. 3. Fluorescene in situ hybridization (FISH) for *ATh*DNV in adult *A. aegypti* mosquitoes. The bright field micrograph (A) shows enlarged nuclei that give green fluorescence positive for the presence of *ATh*DNV (B). Red fluorescence from propidium iodide at >620 nm is shown for normal nuclei from uninfected mosquitoes (C).

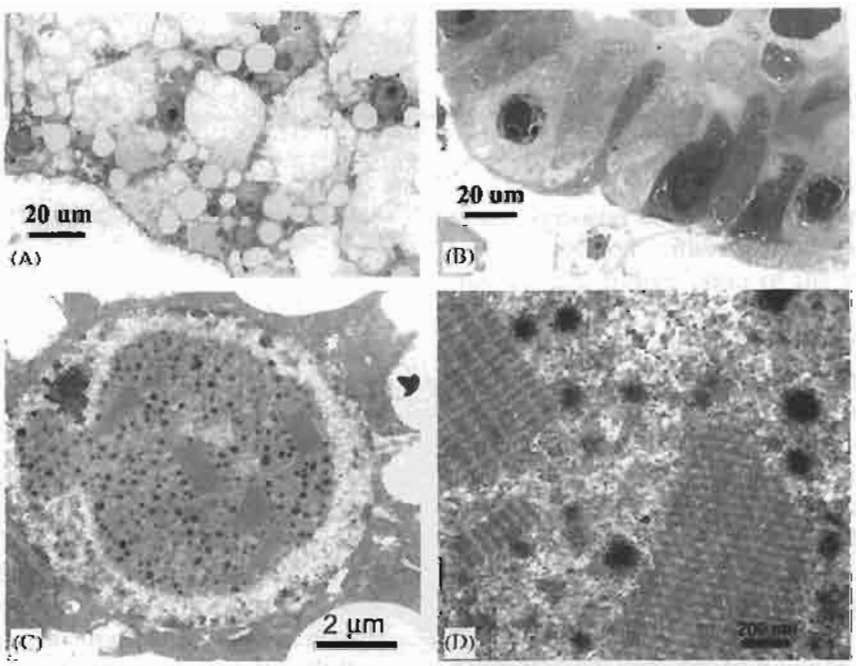

Fig. 4. Light microscopy and ultrastructure of normal and *ATh*DNV-infected mosquitoes. Light microscopy of semi-thin sections (A and B) and electron microscopy of ultra-thin sections (C and D) of Epon-araldite-embedded mosquito larva of the third (F3) generation (i.e., F2 offspring). (A) Normal control mosquito larva showing no intranuclear inclusions in fat body tissue. (B) Enlarged nuclei in gut epithelial cells of mosquito larvae. Low magnification (C) and high magnification (D) electron micrographs of fat body nuclei of mosquito larva showing intranuclear inclusions containing large numbers of 22–24 nm viral particles either free or in para-crystalline arrays. Note also the marginated chromatin in C.

Table 2
GenBank accession numbers for the sequences of the *ATh*DNV clones amplified from freezer stocks of *ATh*DNV or from *ATh*DNV-infected survivors of F4

Name of *ATh*DNV densovirus fragments cloned from freezer stock	GenBank accession number	Name of *ATh*DNV densovirus fragments cloned from surviving mosquitoes	GenBank accession number
F0, 2a	AY633752	F4S, 1a	AY649573
F0, 1a	AY649564	F4S, 2a	AY649574
F0, 3a	AY649565	F4S, 3a	AY649575
F0, 4a	AY649566	F4S, 4a	AY649576
F0, 5a	AY649567	F4S, 5a	AY649577
F0, 6a	AY649568	F4S, 6a	AY649578
F0, 7a	AY649569	F4S, 7a	AY649579
F0, 8a	AY649570	F4S, 8a	AY649580
F0, 9a	AY649571	F4S, 9a	AY649581
F0, 10a	AY649572	F4S, 10a	AY649582
F0, 1b	AY751374	F4S, 3b	AY751381
F0, 2b	AY751375	F4S, 4b	AY751382
F0, 3b	AY751376	F4S, 5b	AY751383
F0, 5b	AY751377	F4S, 6b	AY751384
F0, 6b	AY751378	F4S, 7b	AY751385
F0, 7b	AY751379	F4S, 1c	AY751386
F0, 8b	AY751380	F4S, 2c	AY751387
F0, 1c	AY751394	F4S, 4c	AY751388
F0, 2c	AY751395	F4S, 5c	AY751389
F0, 3c	AY751396	F4S, 6c	AY751390
F0, 4c	AY751397	F4S, 7c	AY751391
F0, 5c	AY751398	F4S, 8c	AY751392
F0, 6c	AY751399	F4S, 9c	AY751393
F0, 7c	AY751400		
F0, 8c	AY751401		
F0, 9c	AY751402		
F0, 10c	AY751403		

29.7% ± 10.0 SD. The difference was statistically significant ($p = 0.017$).

4. Discussion

4.1. Surviving mosquito populations are infected with ATh DNV

Using this relatively simple mosquito–virus model, we have been able to demonstrate increasing survival (i.e., 15–58%) in successive generations challenged with a fixed viral stock of *ATh*DNV, as described for shrimp in the introduction to this manuscript. Also, similar to shrimp, we have shown that many challenged survivors are actually infected with *ATh*DNV, although they show no gross signs of disease and are capable of successful maturation and reproduction.

The high number of infected cells and the presence of paracrystalline arrays of viral particles in transmission electron micrographs of first instar larvae collected within 2 h of hatching suggested that vertical transmission of the virus was possible by the transovarial route. Vertical transmission was previously reported for *Aal*DNV but not confirmed [28]. On the other hand, transovarial transmission was demonstrated for *Aae*DNV, since surface sterilization of the ova did not prevent larval infection [29]. We do not exclude the possible horizontal transmission amongst larvae and adults at later stages, although study of the possibility and rate was not included in our work. Horizontal viral transmission amongst larvae has been reported. Barreau et al. [21,28] described transmission via defecation or regurgitation, while Vasconcelos [30] described nucleopolyhedrovirus transmission with *Mamestra brassicae* lavae via both cannibalism and release of virus prior to death. Rosen [31] described horizontal transmission of dengue-2 virus by *A. albopictus* males. From the viewpoint of our

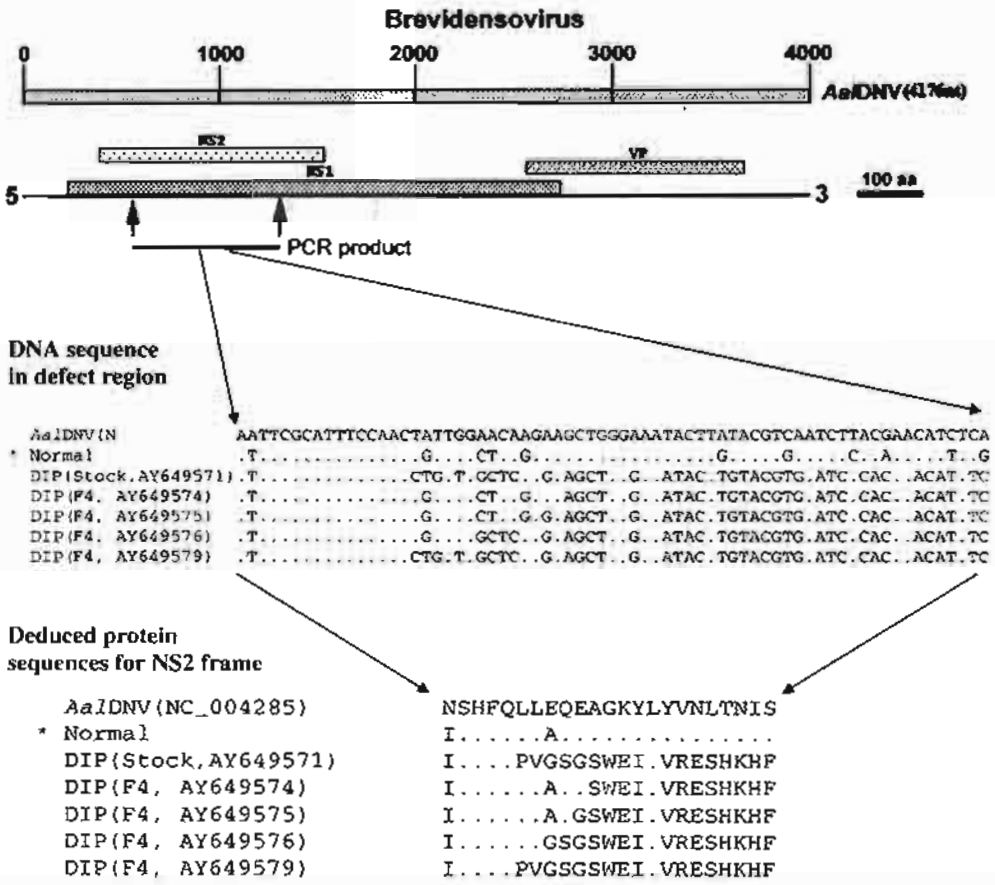

Fig. 5. Diagrammatic representation of defective genome sequence analysis (cf., defective interfering particles or DIP) based on reference to the complete AaPV gene sequence that has very high homology to the *Ath*DNV sequence. This allowed alignment of the NS1 and NS2 reading frames. In this figure, the PCR product targeted positions 603–1401 relative the the AaPV genome and yielded a 799 bp fragment, with clone defects in the illustrated sub-region ranging from relative positions 663 to 731. Note that since the start codons for NS1 and NS2 both fall before the defect region, analysis following the NS1 reading frame would also result in a frame shift occurring in the defect region. *Normal identical sequence of 9/10 freezer clones (AY649564, AY649565, AY649566, AY649567, AY649568, AY649569, AY649570, AY649572, AY633752) and 6/10 F4 generation clones (AY649573, AY649577, AY649578, AY649580, AY649581, AY649582).

study, the route of transmission to successive generations was not particularly relevant. It was sufficient to show that, although grossly normal, challenge survivors were infected in significant numbers and capable of transmitting that infection to future generations.

We found that the number of eggs produced with each succeeding generation of mosquitoes decreased. Although this could have been the result of *ATh*DNV infection, it could have resulted also from inbreeding in the groups of survivor mosquitoes. Barreau et al. [28] reported that fecundity of *Aal*DNV-infected females was not significantly different from *Aal*DNV-free females. By contrast, Kuznetsova & Buchatsky [32] found that some *A. aegypti* females infected with *Aae*DNV did not suck blood, so that both fertility and number of eggs laid were reduced [33]. Specifically designed tests would be needed to distinguish between infection and inbreeding effects with our model system. However, if the infection effect turns out to be real, then it suggests that dependence on vertical transmission only would probably result in rapid decline of viral prevalence in successive generations of natural mosquito populations. This concurs with results from laboratory trials by Barreau et al. [28] who showed that vertical transmission alone did not last beyond two generations.

The fact that *ATh*DNV challenged survivors and survivors from other viral-challenged arthropods generally become persistently infected is a highly significant general difference from vertebrates.

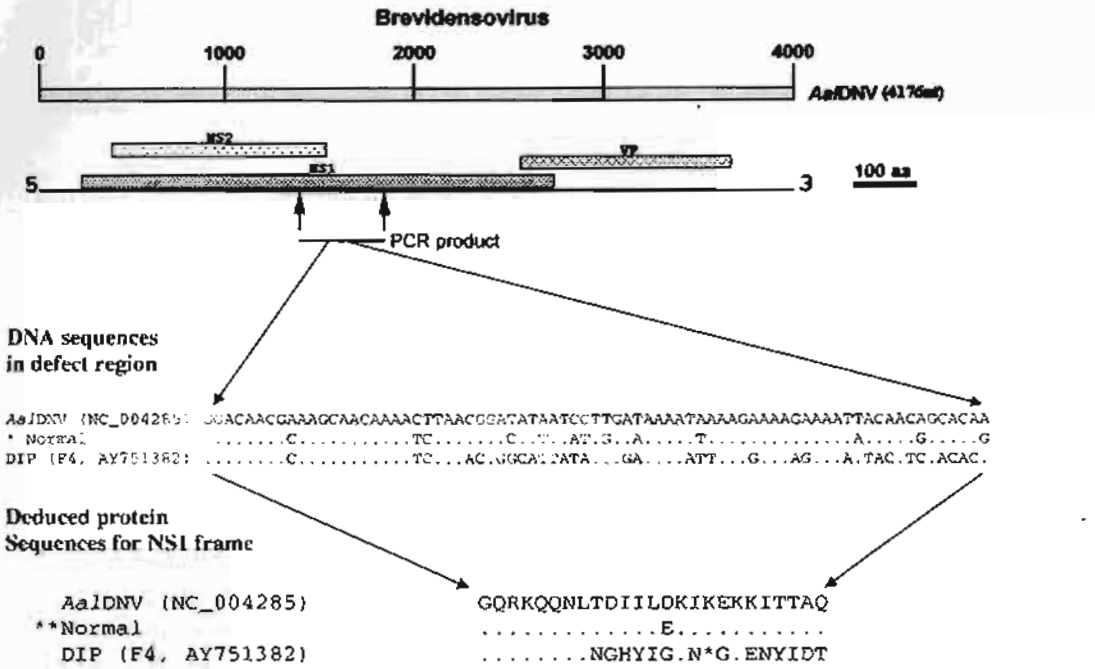

Fig. 6. As with Fig. 5, this diagramatic representation of defective genome sequence analysis (cf., defective interfering particles, DIP) is based on reference to the complete AaPV gene sequence. In this figure, the PCR product targeted positions 464–1959 relative the the AaPV genome and yielded approximately 1.5 kp fragment, with clone defects in the illustrated sub-region ranging from relative positions 1622 to 1696. Note that the defect would not effect NS2 since it occurred outside of the NS2 coding region. *Stop condon; **normal identical sequence of 7/7 freezer clones (AY751374, AY751375, AY751376, AY751377, AY751378, AY751379, AY751380) and 4/5 F4 generation clones (AY751381, AY751383, AY751384, AY751385).

In our opinion, this difference should be emphasized by referring to the survivors as "tolerant" rather than "resistant" to infection [1]. We clearly saw tolerance by LM and TEM as a lack of host response and tissue disruption when *AThDNV*-infected cells held positions in the epidermis and gut epithelium. It parallels the situation with persistent viral infections in shrimp, where even large numbers of infected cells cause no tissue disruption and elicit no mobilization of hemocytes [1]. It contrasts sharply with the shrimp response to bacterial and fungal pathogens [34,35] where host response usually leads to pathogen clearance, and where long-term chronic infections are characterized by hemocytic aggregation leading to encapsulation and granuloma formation [25,36].

4.2. Surprising increase in proportion of defective viral genomes

Based on previous experience with shrimp, we expected that the viral population in the surviving mosquitoes would not differ significantly from the stock viral population used to challenge successive mosquito populations. The reason for this expectation was the common field and experimental observations that shrimp survivors of viral challenges were able to transmit lethal infections to naïve shrimp (see introduction section). Contrary to our expectations, there was a higher proportion of defective genome copies in the surviving mosquito population. However, this did not conflict with the previous experience, since a sufficient number of normal copies were still present to allow for successful transmission.

4.3. Significance of persistent infections and defective genomes

We were left to explain the possible reason for the *Ath*DNV tolerance of the surviving mosquitoes and the reason for the high number of defective genomes in their viral population. Tolerance to a densovirus infection can also occur in insect cell lines and has been likened to superinfection exclusion [37]. Specifically, serial passage of C6/36 cells infected with the densovirus *Aal*DNV resulted in a gradual decline in the percentage of infected cells from

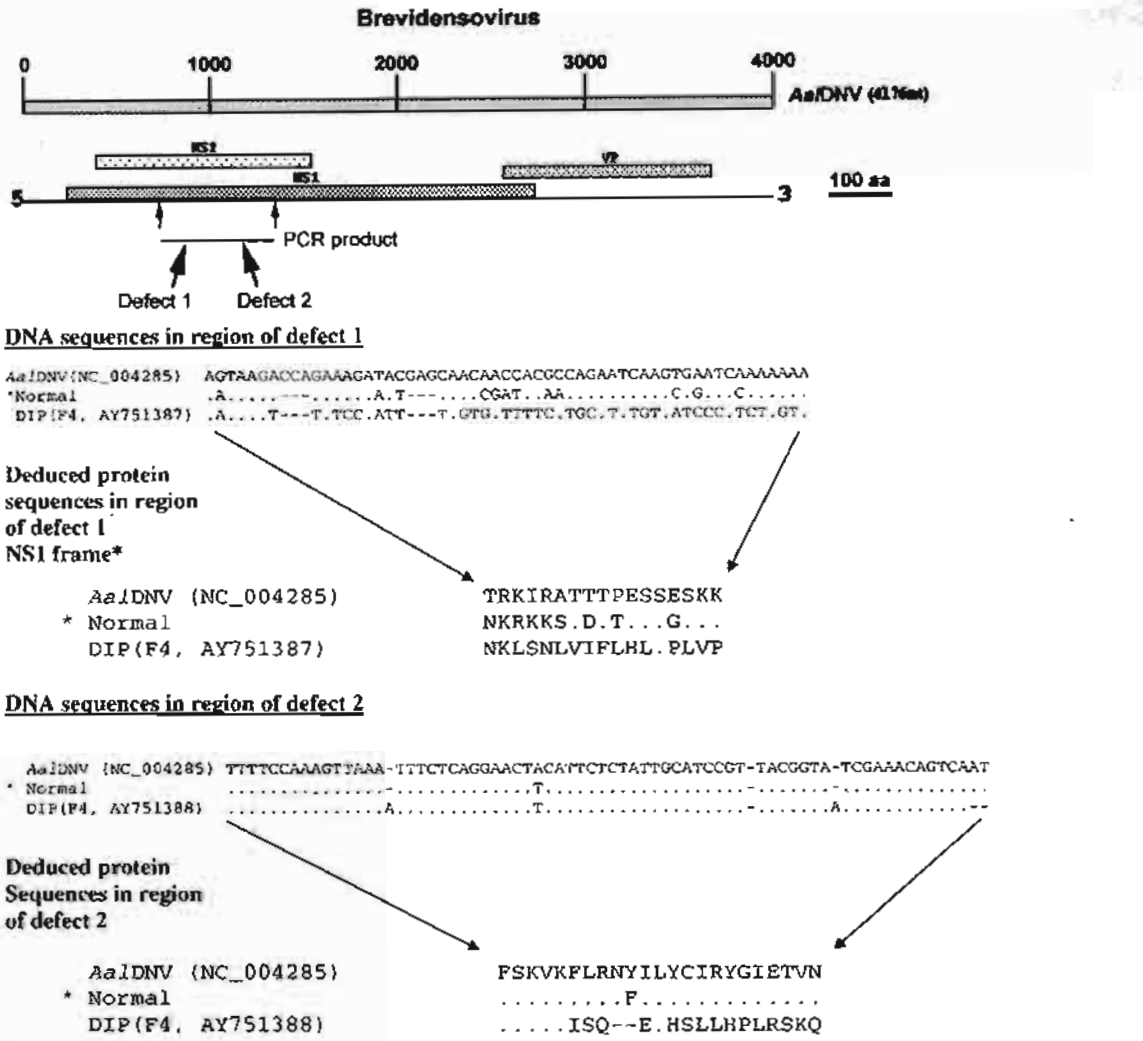

Fig. 7. As with Figs. 5 and 6, this diagrammatic representation of defective genome sequence analysis (cf., defective interfering particles, DIP) is based on reference to the complete AaPV gene sequence. In this figure, the PCR product targeted positions 603–2096 relative the AaPV genome and yielded a fragment of approximately 1.5 kp, that was split into two clones that had defects in widely separated regions. For spatial reasons, the illustration shows the two sub-regions separately. Note that since the start codons for NS1 and NS2 fall before the defect regions, analysis using the NS2 reading frame would also result in defects for NS2. *Normal identical sequence of 10/10 freezer clones (AY751394, AY751395, AY751396, AY751397, AY751398, AY751399, AY751400, AY751401, AY751402, AY751403) and 6/8 F4 generation clones (AY751386, AY751389, AY751390, AY751391, AY751392, AY751393).

around 90% to a steady state of approximately 20% from the tenth passage onwards. This effect was attributed to defective interfering particles (DIP) that had previously been suggested to reduce viral production or to compete with infective particles for cell surface receptors [38–40]. The results of our experiments are consistent with this concept. Populations that survived *ATh*DNV challenge were infected, showed less severe disease and possessed high levels of defective *ATh*DNV genomes that could represent DIP similar to those reported in insect cell cultures. It could be argued that the persistent viral infections act as a kind of "specific memory" that can serve to reduce the severity of disease. Such memory would satisfy the memory requirement in the viral accommodation theory proposed by Flegel and Pasharawipas [41].

Briefly, this theory proposes that crustaceans actively accommodate viral pathogens and that this results in a specific block to viral-triggered apoptosis (programmed cell death) [1,41]. Specificity entails a memory requirement. Since 1998, tests of the theory have shown that moribund shrimp for two major shrimp viruses do exhibit increasing levels of

apoptosis as lethal viral infections progress [42,43]. It has also been found that viral challenges often result in cryptic infections [44–46]. Specific memory provided by persistent infections can satisfy the memory requirement of the viral accommodation theory and improve its alignment with current research results. Updated, it would propose "that crustaceans and other arthropods actively accommodate viral pathogens as persistent infections that function specifically to reduce the severity of disease and to dampen viral triggered apoptosis".

If high production of defective viral genomes in persistent infections is a general arthropod method of reducing disease severity, then the mechanism of their formation is important. Early proposals suggested that DIP arose from production of randomly truncated viral genomes that retained an origin of replication, and that they could outcompete full genome sequences because of their small size [39,40]. However, recent work with a baculovirus indicates that DIP production involves more complex, rapid generation of internal deletions that may require somewhat sophisticated cellular machinery [47]. Our results also revealed internal (albeit small) deletions or insertions in the *Ath*DNV genome. Given the small size of the densovirus genome and the fact that it generates only three proteins, any machinery involved in defective genome formation must be of host cell origin.

Altogether, a picture begins to emerge of a concerted process that may have arisen by evolutionary advantage in the arthropod line. Using the virus itself as the template for production of defective genomes (= DIP?) would allow the host to immediately respond to any viral variation that might arise. By accommodating viruses in persistent infections without mortality, there would be positive selection of viral variants with the least negative effect on the host, and positive selection for host variants least affected by presence of the virus. The latter has clearly been accomplished by breeding selection for TSV tolerance in *P. vannamei* [3,4] indicating an important host genetic component in specific tolerance. By contrast, there would be strong negative selection against increased virulence in the viral population and against high disease in the host population. The process would lead to rapid co-existence of the host and virus. This appears to have occurred with infectious hypodermal and hematopoeitic necrosis virus (IHHNV) in the black tiger shrimp *Penaeus monodon* where negative effects from IHHNV infection are very rarely seen [1].

A second advantage of persistent infections may be a reduction in severity of disease from superinfection by a second virus. This has been reported for shrimp [48] and for mosquito cells [37,49], although the molecular basis for the process is unknown. In both cases, it is important to understand that the second infection is successful but does not lead to mortality. It is possible that presence of a resident virus can help to dampen viral triggered apoptosis that would normally lead to death.

One predicted outcome of viral accommodation would be a slower rate of evolution in arthropod viruses than in vertebrate viruses that are under strong selective pressure to evade host defenses. Indeed, Holland [50] has stated, "...the rates of evolution (mutation fixation) for the flaviviruses, the alphaviruses and other aboviruses are generally lower by an order of magnitude or more than are the rates for many other (non-arthropod-borne) viruses." He suggested that the lower rate of evolution might have arisen from the need for arboviruses to maintain efficient replication capacity in quite different selective environments (i.e., vertebrate and arthropod hosts). Alternatively, it could as easily be that the process of viral accommodation in the arthropod host populations tends to continually pull the viruses back to the most stably accommodated types.

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References

[1] Flegel TW. The shrimp response to viral pathogens. In: Browdy CL, Jory DE, editors. The new wave. Proceedings of the special session on sustainable shrimp aquaculture, World Aquaculture 2001. Orlando: Boca Raton, World Aquaculture Society; 2001. p. 190–214.

[2] Gitterle T. Evaluacion de la resistencia de diferentes poblaciones del camarin marino *Litopenaeus vannamei* (Boone 1931) al virus del sindrome del Taura (TSV) bajo condiciones controladas. Cartagena de Indias D.T. Columbia:

Facultad de Biologia Marina, Universidad Jorge Tadeo Lozano; 1999.
[3] Moss SM. Marine shrimp farming in the western hemisphere: past problems, present solutions, and future visions. Rev Fish Sci 2002;10(3–4):601–20.
[4] Moss SM, Doyle RW, Lightner DV. Breeding shrimp for disease resistance: challenges and opportunities for improvement. In: Walker PJ, Lester RG, Bondad-Reantaso MG, editors. Diseases in Asian aquaculture V. Proceedings of the fifth symposium on diseases in Asian aquaculture. Manila: Asian Fisheries Society; 2005. p. 379–93.
[5] Erickson HS, Zarain-Herzberg M, Lightner DV. Detection of Taura syndrome virus (TSV) strain differences using selected diagnostic methods: diagnostic implications in penaeid shrimp. Dis Aquat Org 2002;52:1–10.
[6] Erickson HS, Poulos BT, Tang KFJ, Bradley-Dunlop D, Lightner DV. Taura syndrome virus from Belize represents a unique variant. Dis Aquat Org 2005;64(2):91–8.
[7] Watanabe H. Resistance of the silkworm, *Bombyx mori*, to viral infections. Agric Ecosyst Environ 1986;15:131–9.
[8] Stairs GR. Quantitative differences in susceptibility to nuclearpolyhedrosis virus among larval instars of the forest tent caterpillar, *Malacosoma disstria*. J Invertebr Pathol 1965;7:427–9.
[9] Magnoler A. Bioassay of nucleopolyhedrosis virus against larval instars of *Malacosoma neustria*. J Invertebr Pathol 1975;25:343–8.
[10] Boucias DG, Nordin GL. Interinstar susceptibility of the fall webworm, *Hyphantria cunea*, to its nucleopolyhedrosis and granulosis viruses. J Invertebr Pathol 1977;30:68–75.
[11] Boucias DG, Johnson DW, Allen GE. Effects of host age, virus dosage, and temperature on the infectivity of a nucleopolyhedrosis virus against the velvetbean caterpillar, *Anticarsia gemmatalis*, larvae. Environ Entomol 1980;9:59–61.
[12] Whitlock VH. Effect of larval maturation on mortality induced by nuclear polyhedrosis and granulosis virus infections of *Heliothis armigera*. J Invertebr Pathol 1977;30:80–6.
[13] Evans HF. The influence of larval maturation on responses of *Mamestra brassicae* L. (Lepidoptera: Noctuidae) to nuclear polyhedrosis virus infection. Arch Virol 1983;75(2):163–70.
[14] Evans HF. Quantitative assessment of the relationship between dosage and response of the nuclear polyhedrosis virus of *Mamestra brassicae*. J Invertebr Pathol 1981;37:101–9.
[15] Teakle RE, Jensen JM, Giles JE. Susceptibility of *Heliothis armigera* to a commercial nuclear polyhedrosis virus. J Invertebr Pathol 1985;46:166–73.
[16] Teakle RE, Jensen JM, Giles JE. Age-related susceptibility of *Heliothis punctiger* to a commercial formulation of nuclear polyhedrosis virus. J Invertebr Pathol 1986;47:82–92.
[17] Engelhard EK, Volkman LE. Developmental resistance in fourth instar *Trichoplusia ni* orally inoculated with *Autographa californica* M nuclear polyhedrosis virus. Virology 1995;209:384–9.
[18] Igarashi A. Isolation of a Singh's *Aedes albopictus* cell clone sensitive to dengue and Chikungunya viruses. J Gen Virol 1978;40:531–44.
[19] Barreau C, Jousset FX, Cornet M. An efficient and easy method of infection of mosquito larvae from virus-contaminated cell cultures. J Virol Method 1994;49:153–6.
[20] Kittayapong P, Baisley KJ, O'Neill SL. A mosquito densovirus infecting *Aedes aegypti* and *Aedes albopictus* from Thailand. Am J Trop Med Hyg 1999;61:612–7.
[21] Barreau C, Jousset F-X, Bergoin M. Pathogenicity of the *Aedes albopictus* parvovirus (AaPV), a denso-like virus, for *Aedes aegypti* mosquitoes. J Invertebr Pathol 1996;68(3):299–309.
[22] Cameron A. Survey toolbox for aquatic animaldiseases; a practical manual and software package. Canberra: Australian Centre for International Agricultural Research; 2002.
[23] Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory; 1989.
[24] O'Neill SL, Kittayapong P, Braig HR, Andreadis TG, Gonzalez JP, Tesh RB. Insect densoviruses may be widespread in mosquito cell lines. J Gen Virol 1995;76:2067–74.
[25] Lightner DV. A handbook of pathology and diagnostic procedures for diseases of penaeid shrimp. Baton Rouge, LA: World Aquaculture Society; 1996.
[26] Hall TA. BioEdit: a user-friendly program for Windows 95/98/NT. Nucl Acid Symp Ser 1999;41:95–8.
[27] Thompson JD, Higgins DG, Gibson TJ. ClustalW: improving the sensitivity of progressive multiple sequence aligment through sequence weighting, position specific gap penalties and weight matrix choice. Nucl Acid Res 1994;22:4673–80.
[28] Barreau C, Jousset FX, Bergoin M. Venereal and vertical transmission of the *Aedes albopictus* parvovirus in *Aedes aegypti* mosquitoes. Am J Trop Med Hyg 1997;57:126–31.
[29] Lebedinets NN, Kononko AG. Experimental study of densovirus infection transmission in blood-sucking mosquito populations. Med Parazitol (Mosk) 1989;2:79–83.
[30] Vasconcelos SD. Alternative routes for the horizontal transmission of a nucleopolyhedrosis virus. J Invertebr Pathol 1996;68:269–74.
[31] Rosen L. Sexual transmission of dengue viruses by *Aedes albopictus*. Am J Trop Med Hyg 1987;37:398–402.
[32] Kuznetsova MA, Buchatsky LP. Effect of the viral preparation viroden on the mosquito *Aedes aegypti* L. under experimental condition (in Russian). Med Parazitol (Mosk) 1988;2:79–83.
[33] Buchatsky LP. Densonucleosis of blood sucking mosquitoes. Dis Aquat Org 1989;6:145–50.
[34] Thornqvist PO, Soderhall K. Crustacean immune reactions, a short review. In: Flegel TW, MacRae IH, editors. Diseases in Asian aquaculture III. Manila: Fish Health Section, Asian Fisheries Society; 1997. p. 203–18.
[35] Johansson MW, Keyser P, Sritunyalucksana K, Soderhall K. Crustacean haemocytes and haematopoiesis. Aquaculture 2000;191(1–3):45–52.
[36] Flegel TW, Fegan DF, Kongsom S, Vuthikornudomkit S, Sriurairatana S, Boonyaratpalin S, et al. Occurrence, diagnosis and treatment of shrimp diseases in Thailand. In: Fulks W, Main KL, editors. Diseases of cultured penaeid shrimp in Asia and the United States. Honolulu, HI: Oceanic Institute; 1992. p. 57–112.
[37] Burivong P, Pattanakitsakul S-N, Thongrungkiat S, Malasit P, Flegel TW. Markedly reduced severity of Dengue virus infection in mosquito cell cultures persistently infected with

Aedes albopictus densovirus (AalDNV). Virology 2004; 329(2):261–9.

[38] Rhode SL. Defective interfering particles of parvovirus H-1. J Virol 1978;27:347–56.

[39] Kirkwood TBL, Bangham CRM. Cycles, chaos, and evolution in virus cultures: a model of defective interfering particles. Proc Nat Acad Sci USA 1994;91:8685–9.

[40] Frank SA. Within-host spatial dynamics of viruses and defective interfering particles. J Theoret Biol 2000;206(2):279–90.

[41] Flegel TW, Pasharawipas T. Active viral accommodation: a new concept for crustacean response to viral pathogens. In: Flegel TW, editor. Advances in shrimp biotechnology. Bangkok: National Center for Genetic Engineering and Biotechnology; 1998. p. 245–50.

[42] Khanobdee K, Soowannayan C, Flegel TW, Ubol S, Withyachumnarnkul B. Evidence for apoptosis correlated with mortality in the giant black tiger shrimp *Penaeus monodon* infected with yellow head virus. Dis Aquat Org 2002;48(2):79–90.

[43] Wongprasert K, Khanobdee K, Glunukarn S, Meeratana P, Withyachumnarnkul B. Time-course and levels of apoptosis in various tissues of black tiger shrimp *Penaeus monodon* infected with white spot syndrome virus. Dis Aquat Org 2003;55(1):3–10.

[44] Yoganandhan K, Narayanan RB, Sahul Hameed AS. Larvae and early post-larvae of *Penaeus monodon* (Fabricius) experimentally infected with white spot syndrome virus (WSSV) show no significant mortality. J Fish Dis 2003; 26(7):385–91.

[45] Hsu HC, Lo CF, Lin SC, Liu KF, Peng SE, Chang YS, et al. Studies on effective PCR screening strategies for white spot syndrome virus (WSSV) detection in *Penaeus monodon* Brooders. Dis Aquat Org 1999;39(1):13–9.

[46] Tsai MF, Kou GH, Liu HC, Liu KF, Chang CF, Peng SE, et al. Long-term presence of white spot syndrome virus (WSSV) in a cultivated shrimp population without disease outbreaks. Dis Aquat Org 1999;38:107–14.

[47] Pijlman GP, van den Born E, Martens DE, Vlak JM. *Autographa californica* Baculoviruses with large genomic deletions are rapidly generated in infected insect cells. Virology 2001;283(1):132–8.

[48] Tang KFJ, Durand SV, White BL, Redman RM, Mohney LL, Lightner DV. Induced resistance to white spot syndrome virus infection in *Penaeus stylirostris* through pre-infection with infectious hypodermal and hematopoietic necrosis virus—a preliminary study. Aquaculture 2003; 216(1–4):19–29.

[49] Burivong P. Densovirus: its kinetics of growth and interaction with infectivity of Dengue virus in mosquito cells. Medical science. Bangkok: Mahidol University; 2003. p. 79.

[50] Holland JJ. Evolving virus plagues. Proc Natl Acad Sci USA 1996;93:545–6.