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).

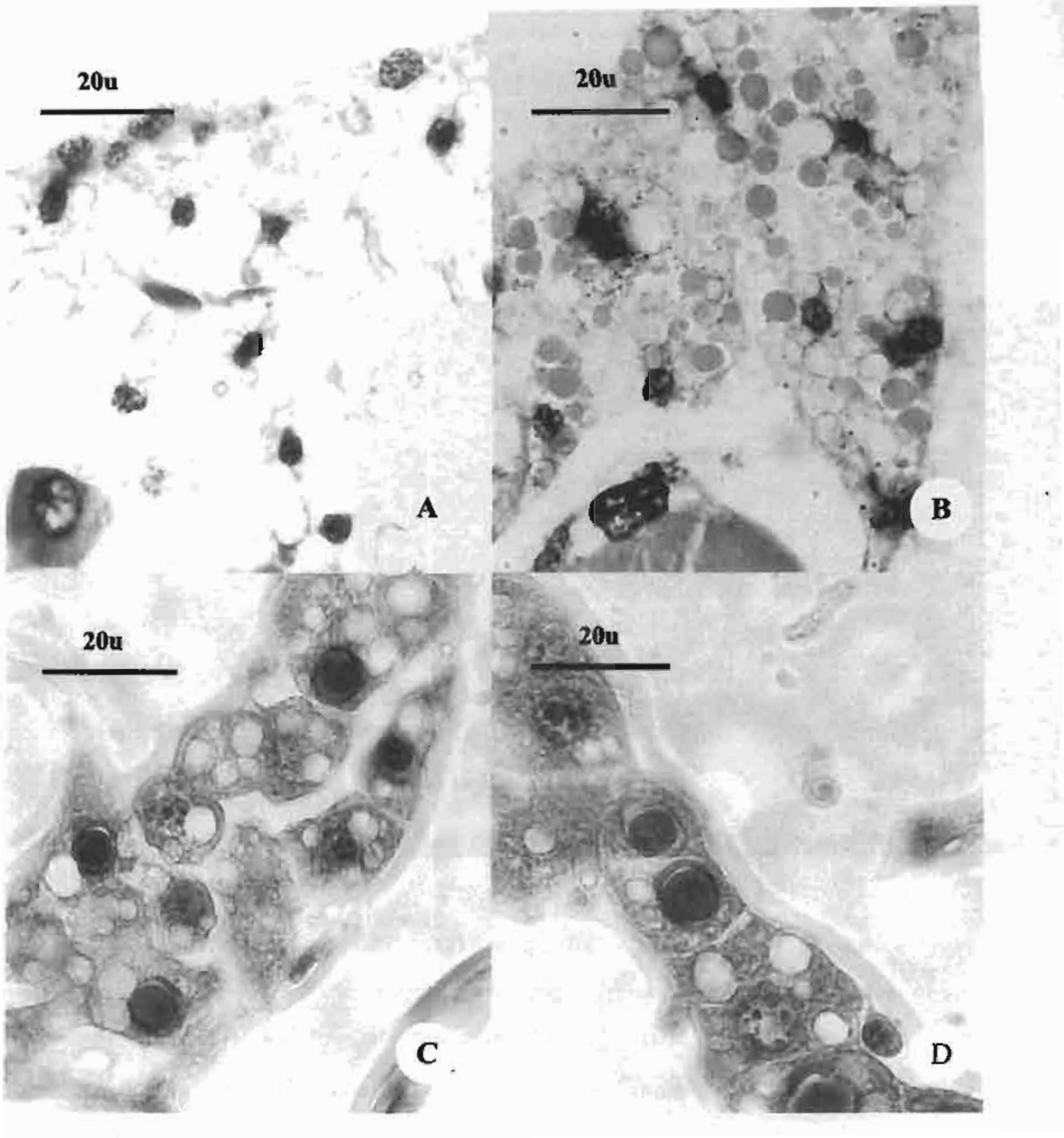

Figure 14. Hematoxylin and Eosin (H&E) staining with sections of normal (A&B) *Ae. aegypti* adults and *ATh*DNV-infected (C&D). Parvovirus-type inclusions in fat body tissues were seen as large, basophilic intranuclear inclusions in nuclei with marginated chromatin (C&D). These nuclei contrast sharply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

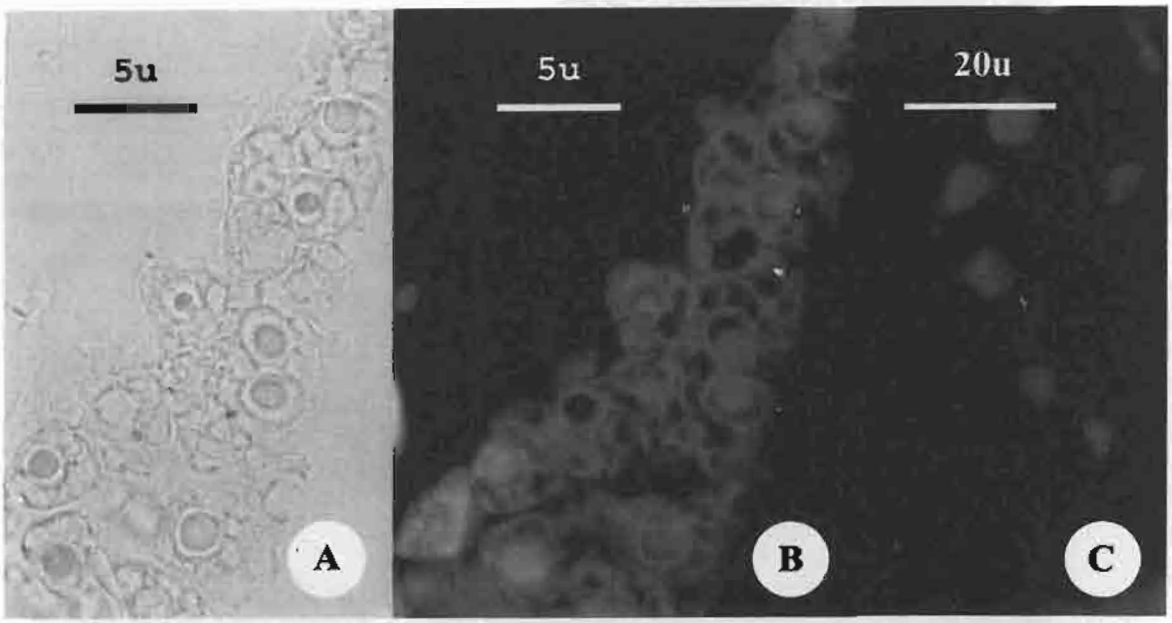

Figure 15. Fluorescene *in situ* hybridization for *ATh*DNV in adult *Ae. aegypti* mosquitoes. The bright field micrograph (A) shows enlarged nuclei that give green fluorescence positive for the presence of *ATh*DNV (B). Red fluorescence from propidium iodide at >620 nm indicated a normal nuclei reaction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

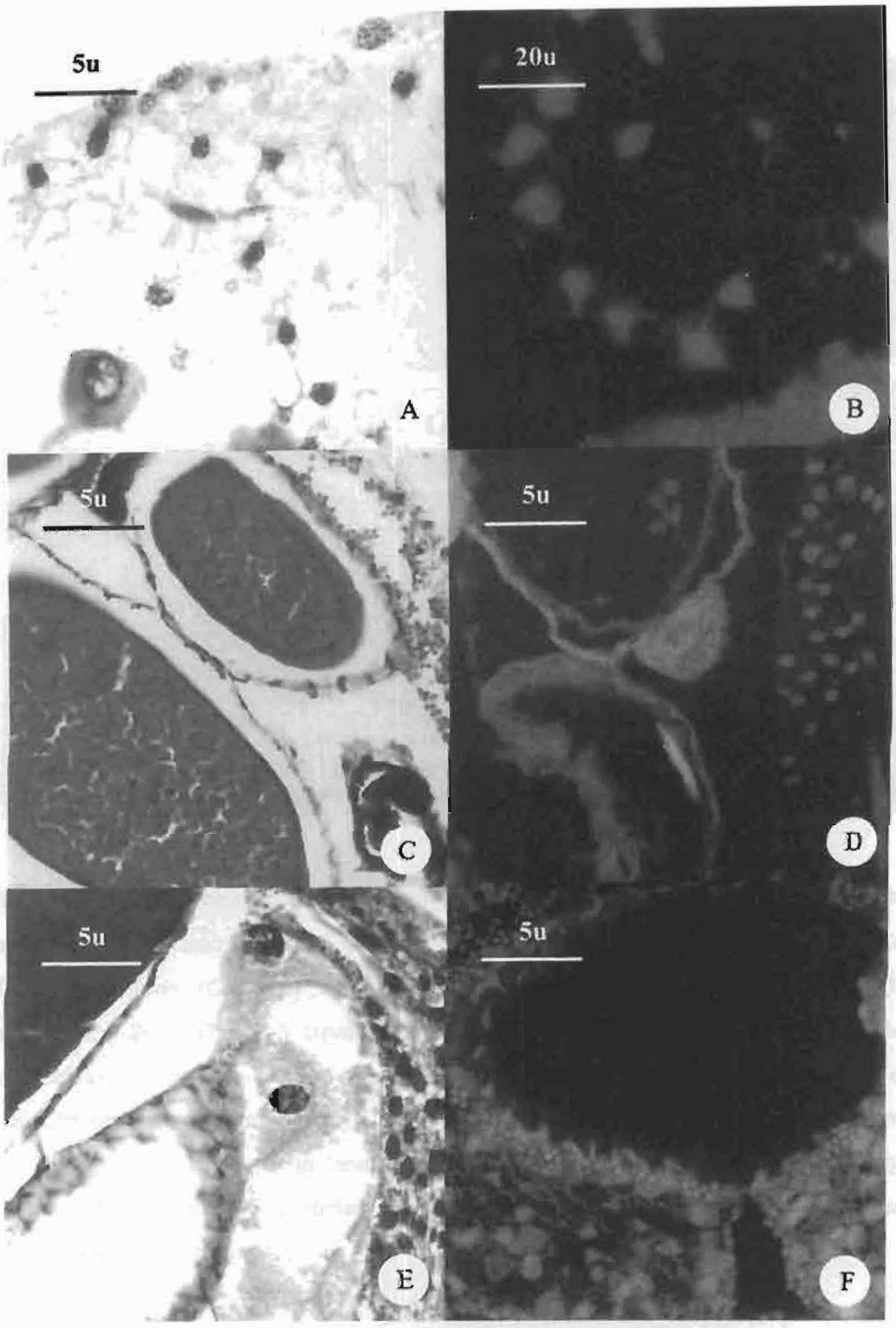

Figure 16. Fluorescene *in situ* hybridization (B,D&F) and H&E staining (A,C&E) for *ATh*DNV infected tissue and normal tissue in adult *Ae. aegypti* mosquitoes. Normal tissues were shown in A, B, C&D while *ATh*DNV infected tissues were shown in E&F.

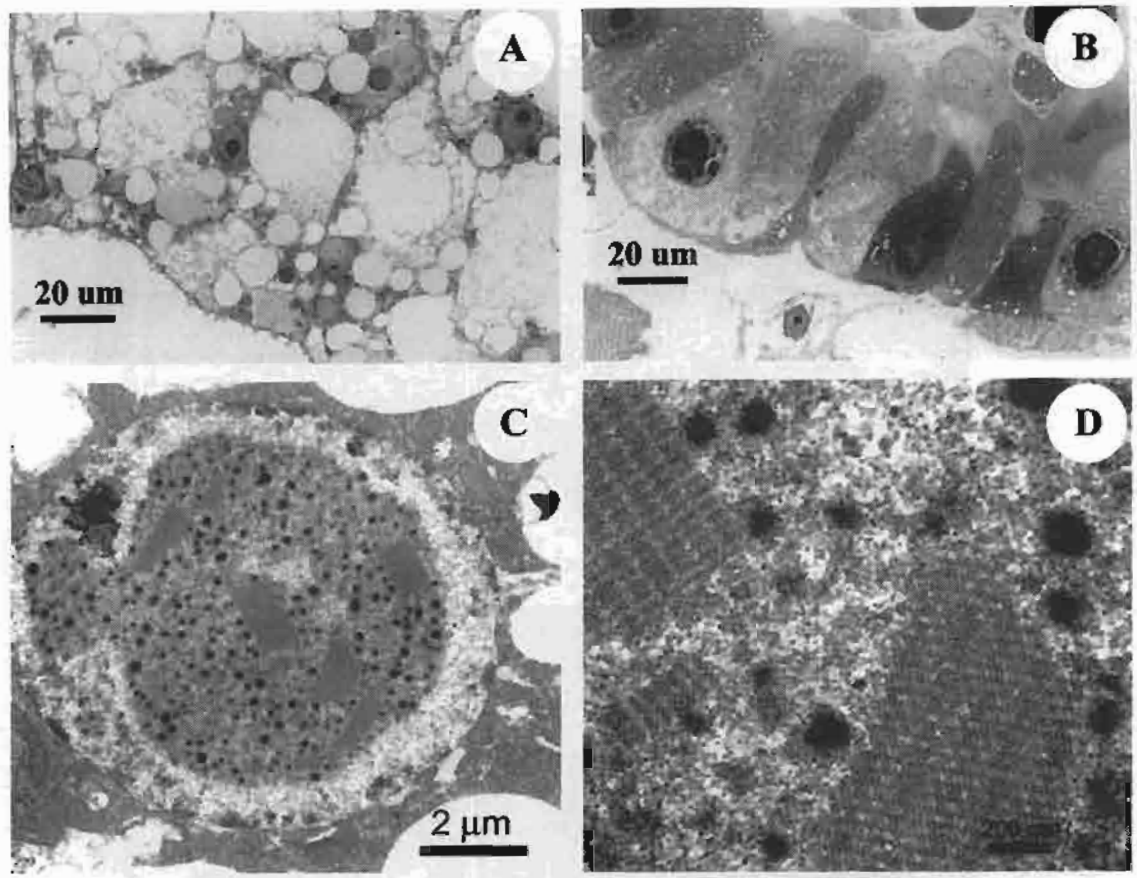

Figure 17. Light microscopy and ultastructure of normal and *ATh*DNV-infected mosquitoes. Light microscopy of semi-thin sections (A&B) and electron microscopy of ultra-thin sections (C&D) of Epon-Aradite embedded mosquito larva of the 3rd generation (F2) of Group 3 larvae. (A) Normal control mosquito 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.

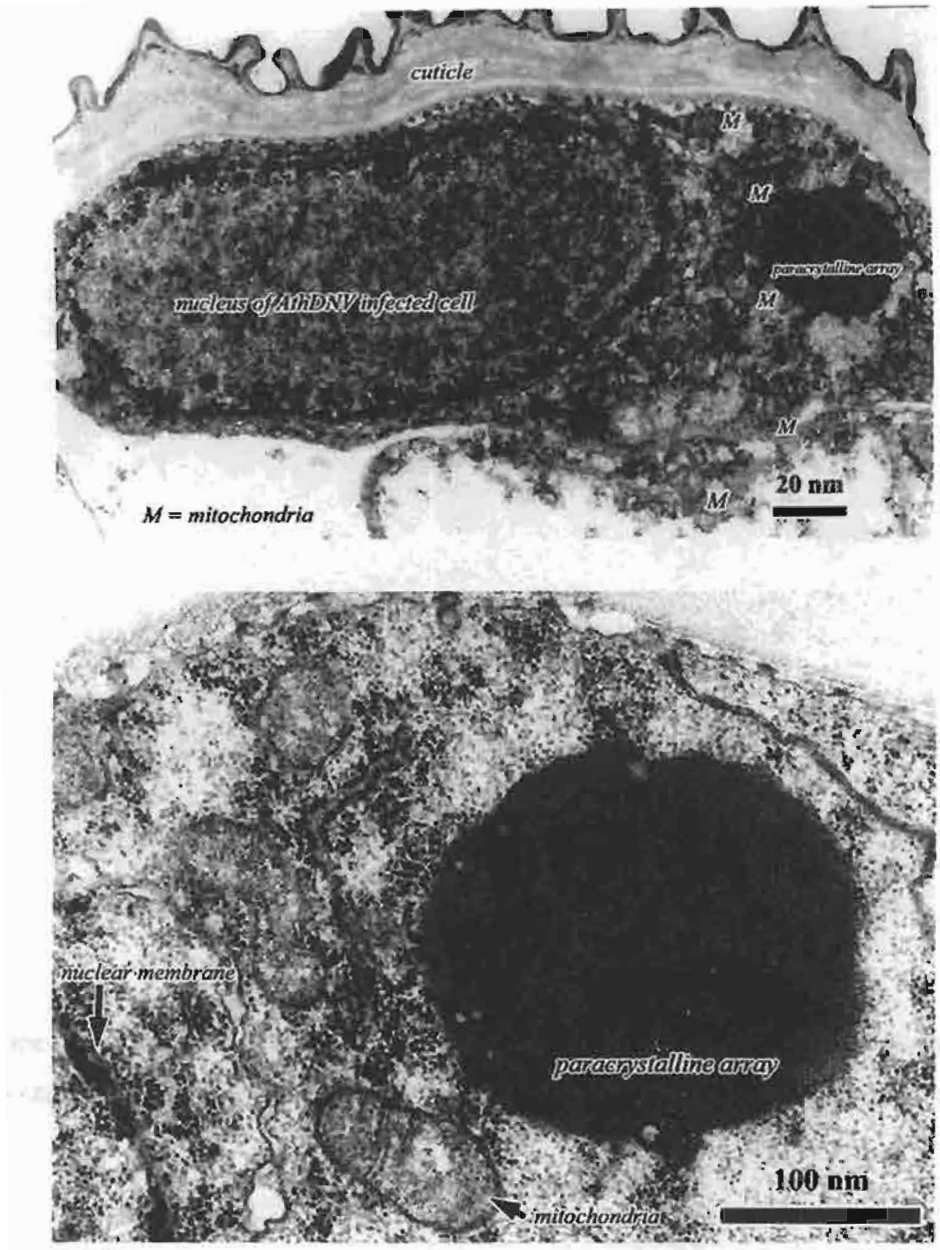

Figure 18. Electron micrographs at low (upper) and high (lower) magnification showing a subcuticular epithelial cell infected with *ATh*DNV. Note that the nucleus contains large numbers of free virions while the pracrystalline array is contained in the cell cytoplasm.

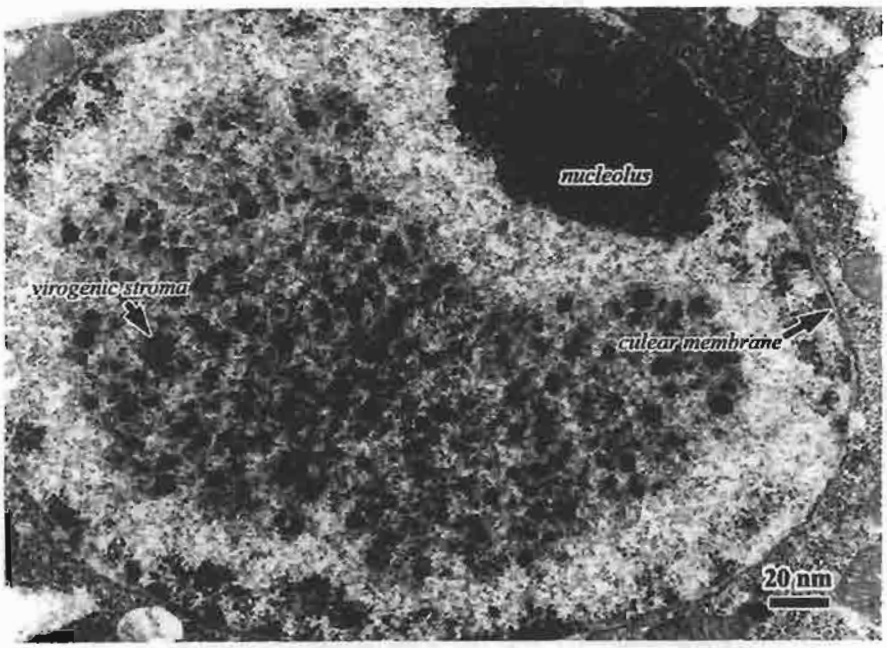

Figure 19. Electron micrograph of the nucleus of an *ATh*DNV-infected subcuticular epithelial cell showing large numbers of free virions. Note the marginaged chromatin.

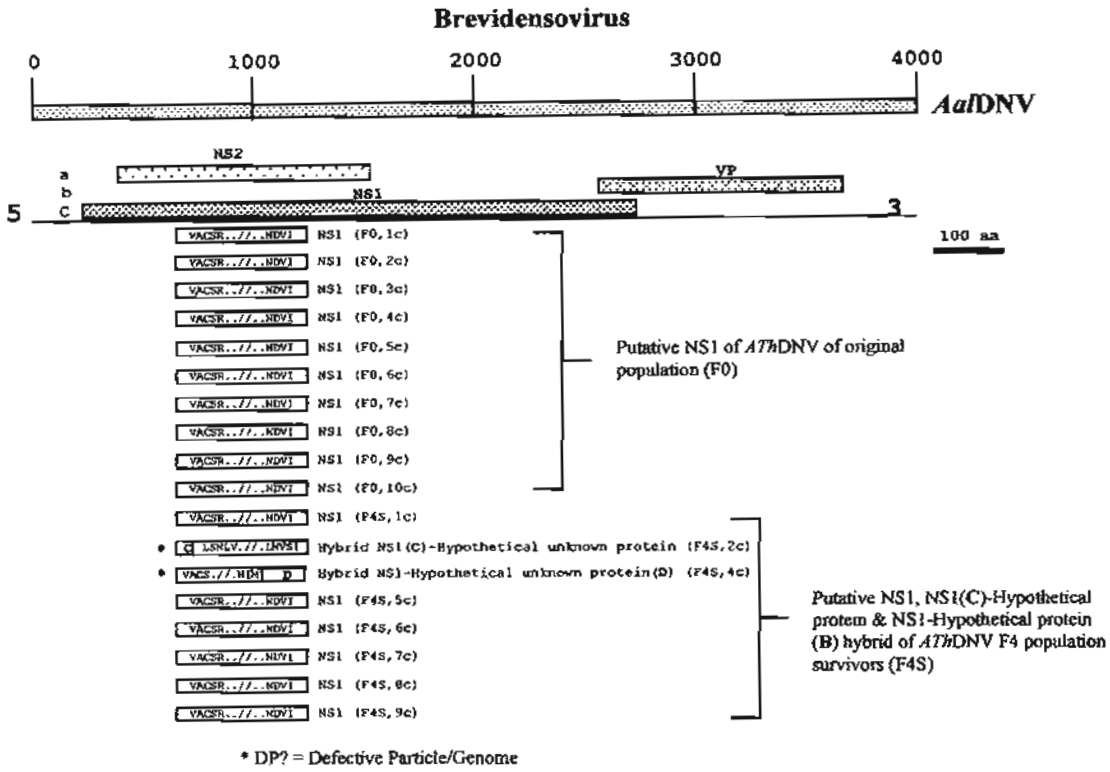

Figure 20. Descriptive model comparing region 1 of deduced NS1 protein sequences from the freezer virus and the F4 survivor virus populations. Putative defective sequences are marked with an asterisk.

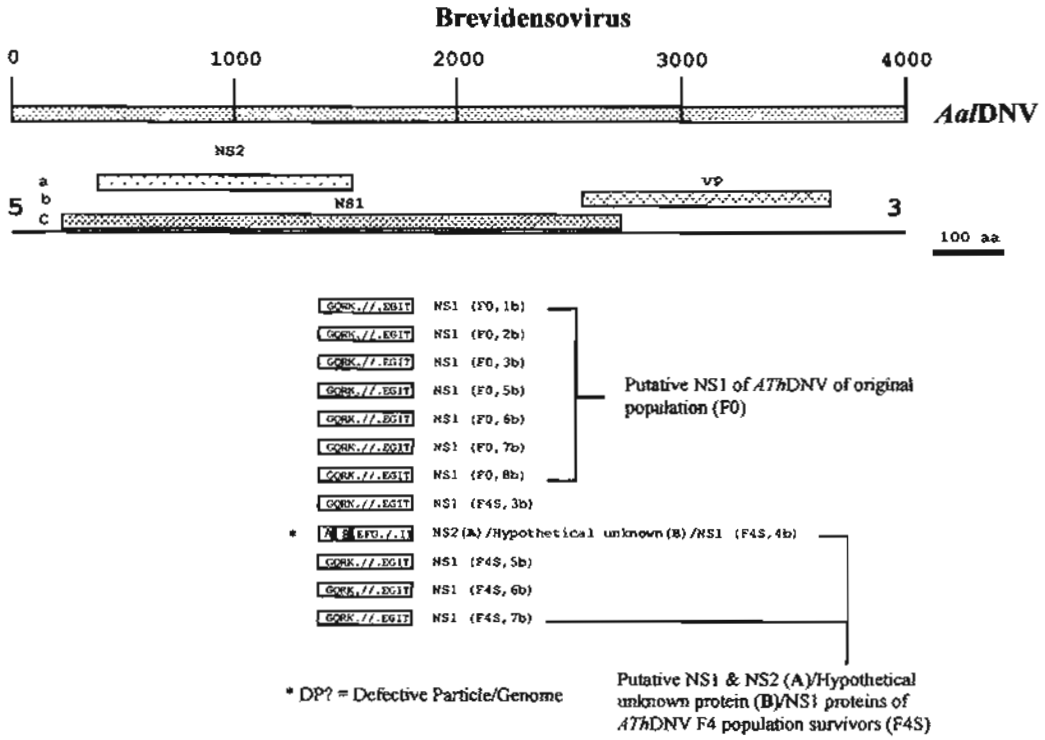

Figure 21. Descriptive model comparing region 2 of deduced NS1 protein sequences from the freezer virus and the F4 survivor virus populations. Putative defective sequences are marked with an asterisk.

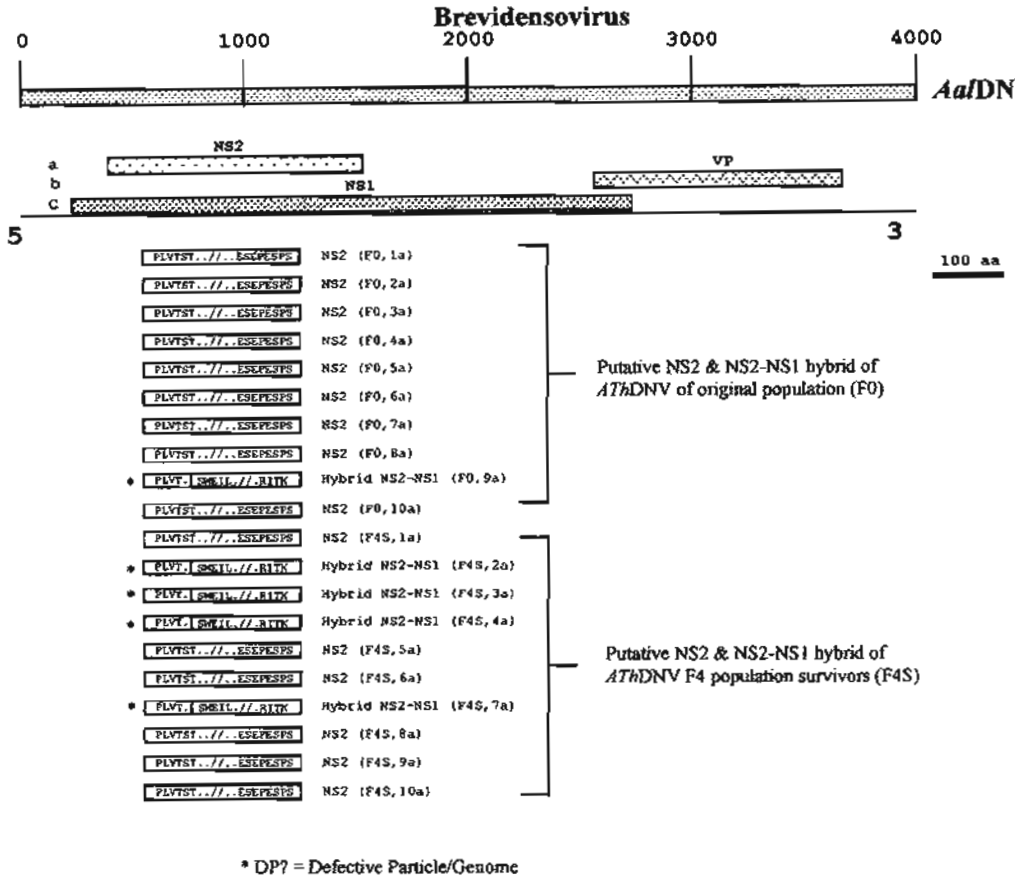

Figure 22. Descriptive model comparing deduced NS2 protein sequences from the freezer virus and the F4 survivor virus populations. Putative defective sequences are marked with an asterisk.

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.

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BIOGRAPHY

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RESEARCH GRANTS	1. Thailand Research Fund, Royal Golden Jubilee (RGJ), Ph.D. program 2. Basic Research Grant for the Royal Golden Jubilee Ph.D. program

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

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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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Received 23 August 2001; received in revised form 29 April 2002; accepted 29 April 2002

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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Received 1 March 2005; received in revised form 9 November 2005; accepted 8 December 2005

2. การเสนอผลงานในที่ประชุมวิชาการ

2001 International Commemorative Symposium. 70th Anniversary of the Japanese Society of Fisheries Science (JSFS). October 1-5, Pacifico Yokohama, Yokohama, Japan

2001 The JSPS-NRCT International Symposium on Sustainable Shrimp Culture and Health Management, Diseases and Environment. Tokyo University of Fisheries, Tokyo, Japan, September 30.

2002 5th Symposium on Diseases in Asian Aquaculture (DAA5), The Gold Coast International Hotel in Surfers Paradise, Queensland, Australia, November 22 -December 4.

2003 Asian-Pacific Aquaculture 2003, Ambassador Bangkok Hotel, Bangkok, Thailand, September 22-25.

2004 The 5th International Symposium on Marine Shrimp, Bangkok, Thailand, March 29-30.

2004 7th Asian Fisheries Forum 2004, Penang, Malaysia, November 30, 2004-December 4.

ภาคผนวก

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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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0168-1702/02/$ - see front matter

PII: S0168-1702(02)00084-9

(*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

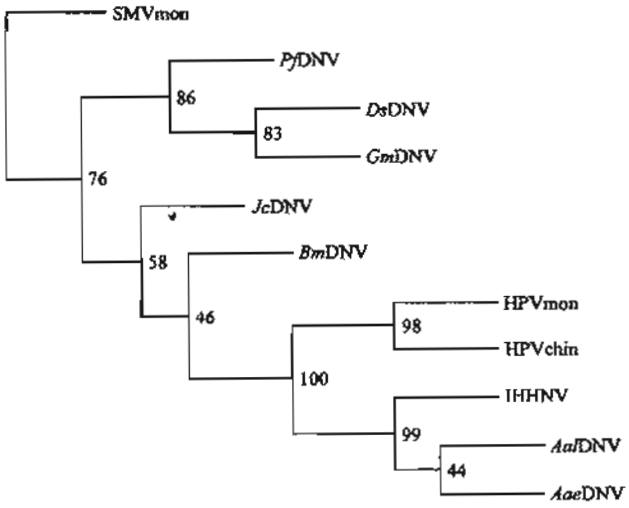

Fig. 1. The neighbor-joining phylogenetic tree generated from a heuristic search of aligned insect and shrimp parvoviruses DNA sequences. The phylogenetic tree was constructed based on 696 bp of aligned DNA sequences. The branch lengths are scaled according to the number of base substitutions. The scale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

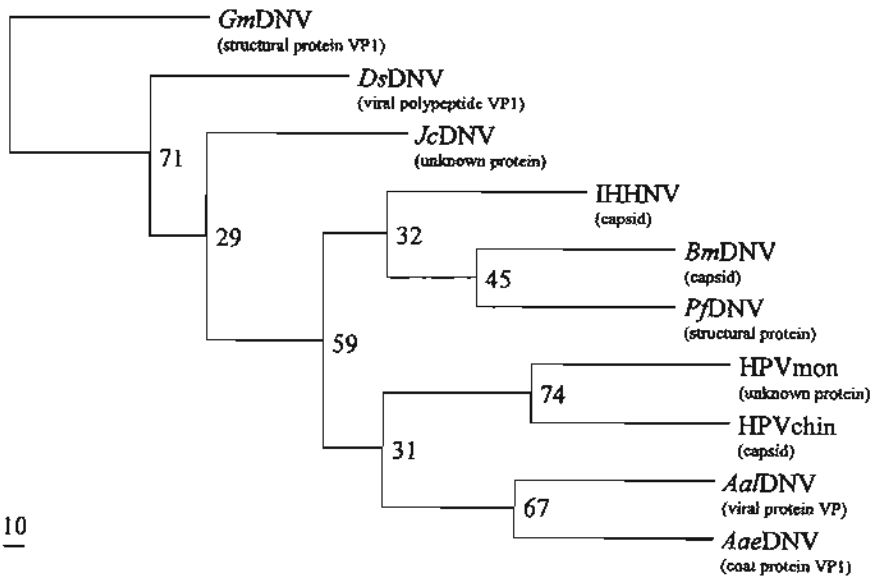

Fig. 2. Comparisons based on putative protein sequences of capsid and structural proteins using a global alignment method (LALIGN). (A) Overall parvoviral protein similarities. (B) The neighbor-joining phylogenetic tree generated from a heuristic search of aligned insect and shrimp parvoviruses deduced 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)

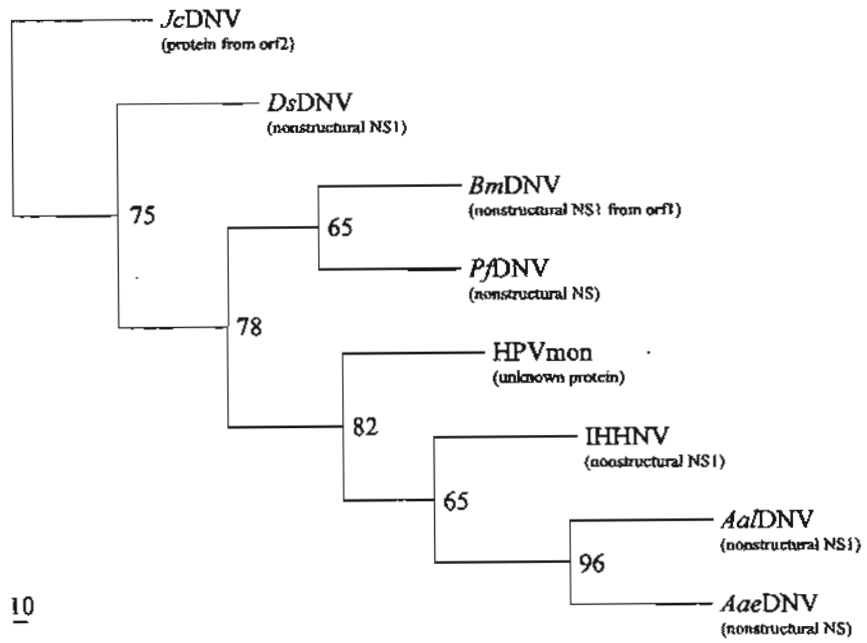

Fig. 3. Comparisons based on putative protein sequences of insect virus nonstructural protein (NS) and most similar proteins using global alignment method (LALIGN). (A) Overall parvoviral protein similarities. (B) The neighbor-joining phylogenetic tree generated from a heuristic search of aligned insect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

Acknowledgements

The authors would like to thank the Thai National Center for Genetic Engineering and Biotechnology (BIOTEC) and the Thailand Research Fund under the Royal Golden Jubilee (RGJ) program for support to carry out this work.

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

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Received 1 March 2005; received in revised form 9 November 2005; accepted 8 December 2005

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]The Center of Excellence for Shrimp Molecular Biology and Biotechnology (Centex Shimp) is jointly supported by Mahidol University and the National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Science Park, 113 Paholyothin Road,Klong, Klong Luang, Phtthumthani 12120, Thailand.

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doi:10.1016/j.dci.2005.12.006

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.

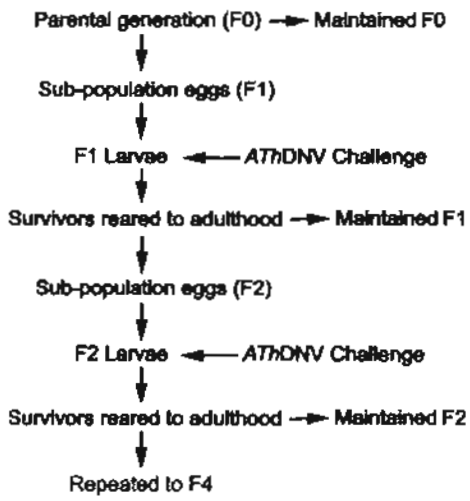

Fig. 1. *ATh*DNV-*Aedes aegypti* mosquito adaptation test.

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

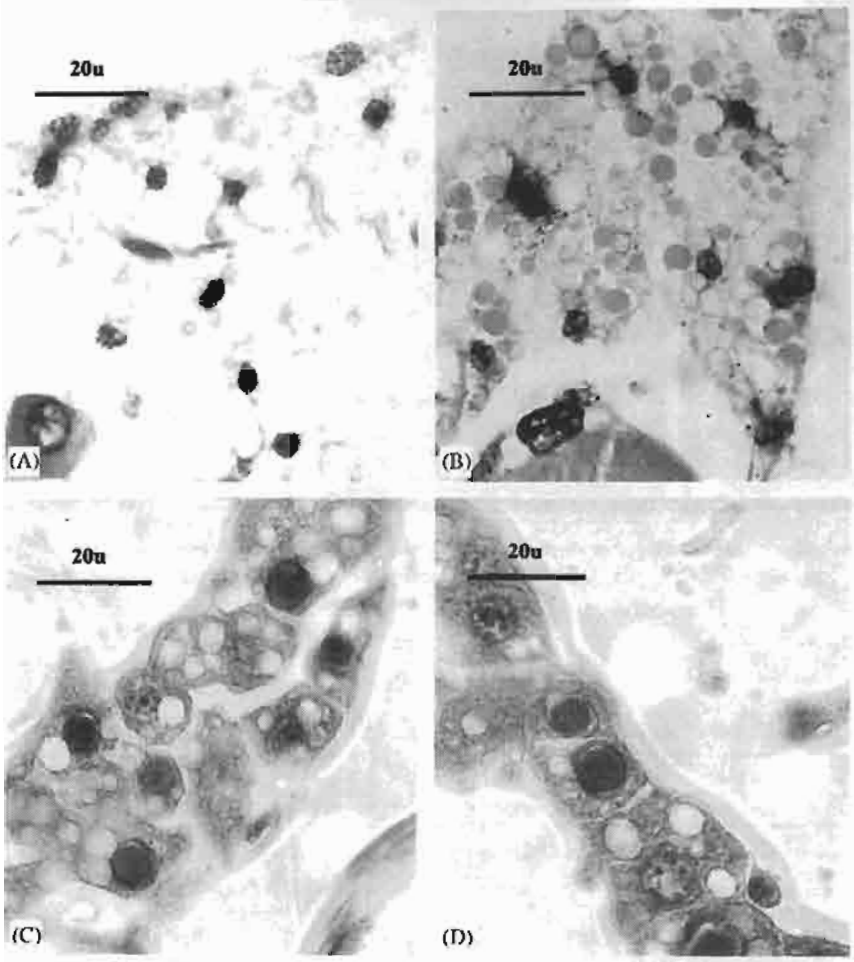

Fig. 2. Hematoxlyhn and Eosin (H&E) stained sections of *ATh*DNV-infected (A and B) and normal (C and D) *A. aegypti* adults. Parvotypes inclusions or hypertrophied nuclei were usually found in infected nuclei with densoviruses and/or parvoviruses. *ATh*DNV forms large intranuclear inclusions (C and 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

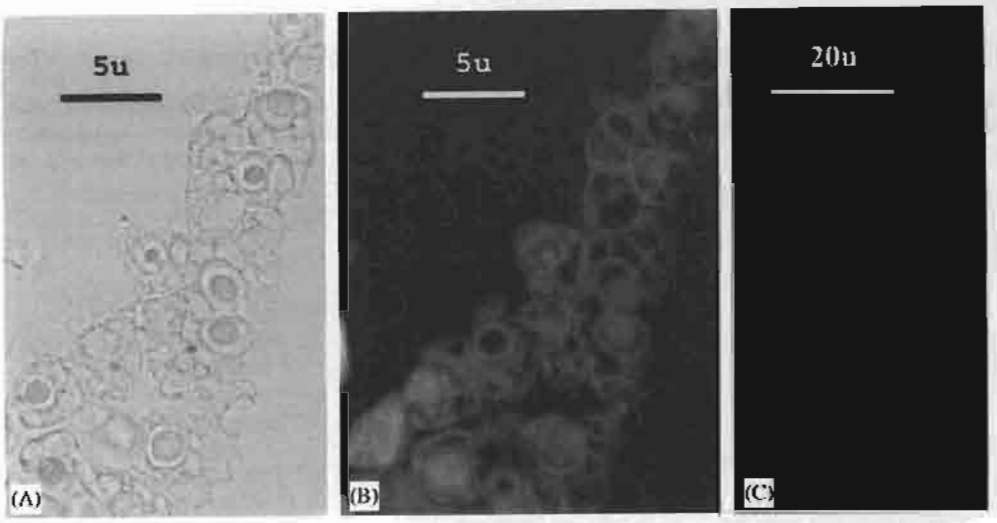

Fig. 3. Fluorescene in situ hybridization (FISH) for *ATh*DNV in adult *A. aegypti* mosquitoes. The bright field micrograph (A) shows enlarged nuclei that give green fluorescence positive for the presence of *ATh*DNV (B). Red fluorescence from propidium iodide at >620 nm is shown for normal nuclei from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

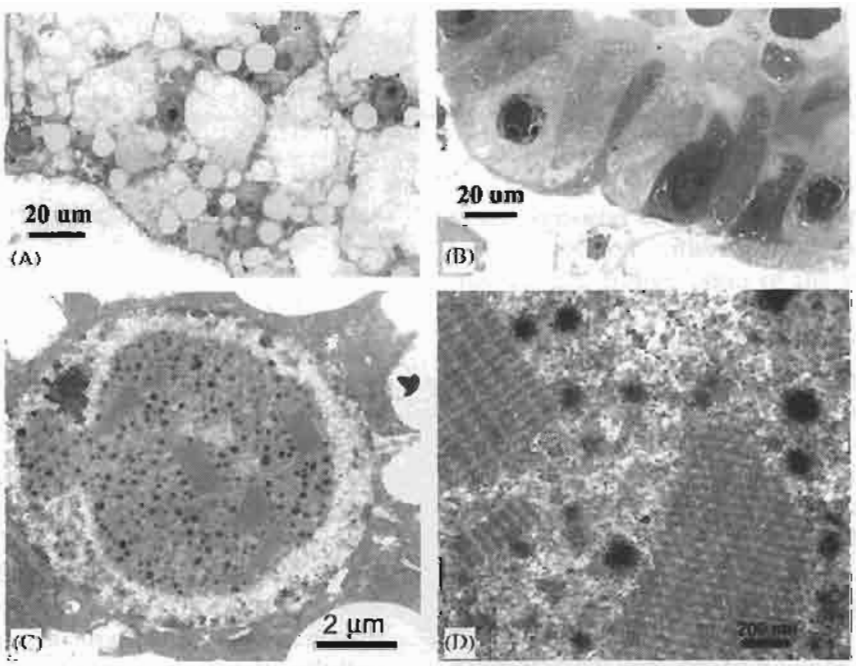

Fig. 4. Light microscopy and ultrastructure of normal and *ATh*DNV-infected mosquitoes. Light microscopy of semi-thin sections (A and B) and electron microscopy of ultra-thin sections (C and D) of Epon-araldite-embedded mosquito larva of the third (F3) generation (i.e., F2 offspring). (A) Normal control 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

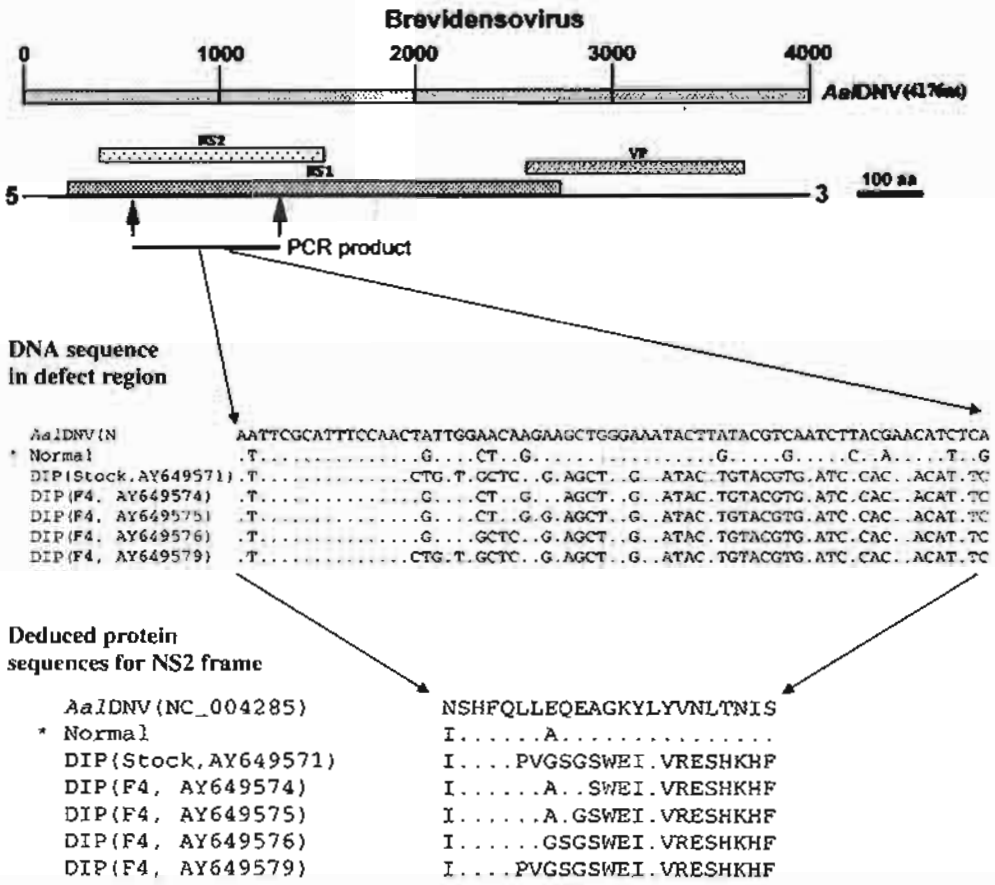

Fig. 5. Diagrammatic representation of defective genome sequence analysis (cf., defective interfering particles or DIP) based on reference to the complete AaPV gene sequence that has very high homology to the *Ath*DNV sequence. This allowed alignment of the NS1 and NS2 reading frames. In this figure, 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.

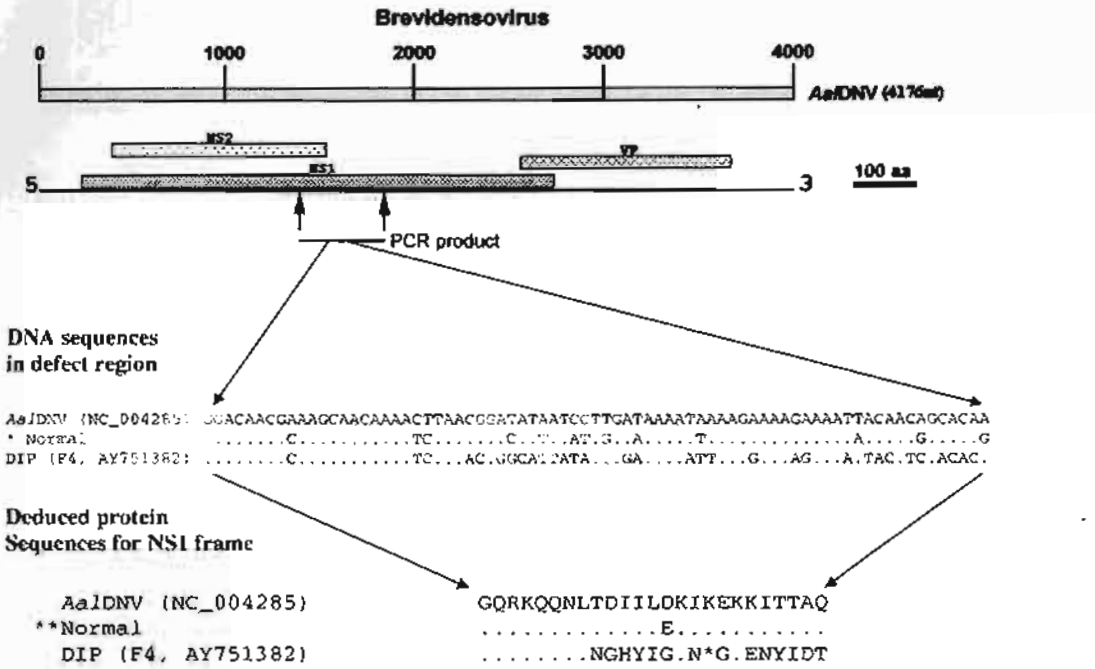

Fig. 6. As with Fig. 5, this diagramatic representation of defective genome sequence analysis (cf., defective interfering particles, DIP) is based on reference to the complete AaPV gene sequence. In this figure, the PCR product targeted positions 464–1959 relative the the AaPV genome and yielded approximately 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

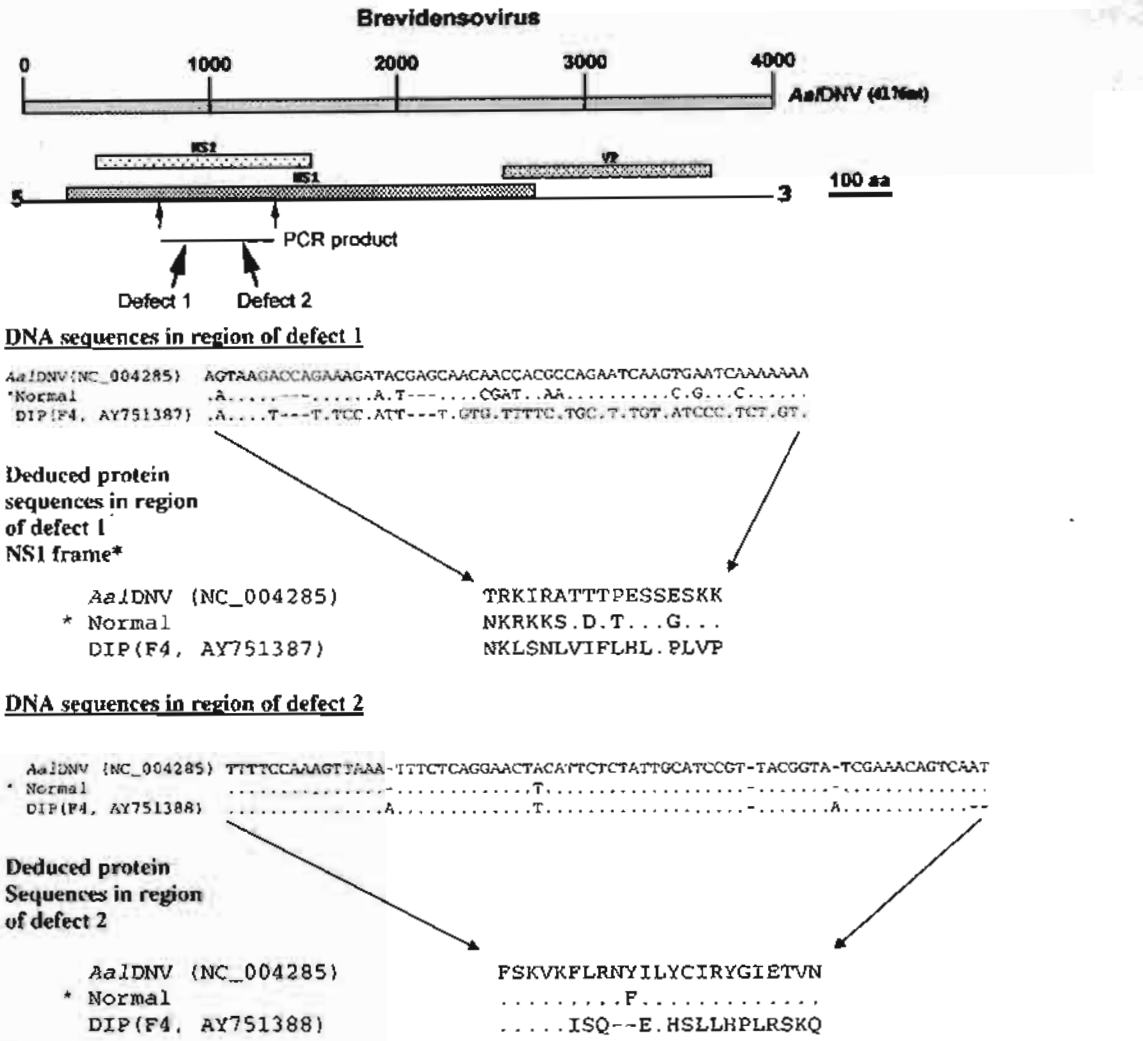

Fig. 7. As with Figs. 5 and 6, this diagrammatic representation of defective genome sequence analysis (cf., defective interfering particles, DIP) is based on reference to the complete AaPV gene sequence. In this figure, the PCR product targeted positions 603–2096 relative the AaPV genome and yielded 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.

Acknowledgements

The authors would like to thank the Thai Research Fund for research support to Songsak Roekring in the form of a Royal Golden Jubilee (RGJ) Scholarship and a Basic Research Grant for the Royal Golden Jubilee (BGJ). Partial support was also provided by the Thai National Center for Genetic Engineering and Biotechnology and Mahidol University (SCBI-47-T-217).

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