Insect densoviruses may be widespread in mosquito cell lines

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A diagnostic PCR assay was designed based on conserved regions of previously sequenced densovirus genomic DNA isolated from mosquitoes. Application of this assay to different insect cell lines resulted in a number of cases of consistent positive amplification of the predicted size fragment. Positive PCR results were subsequently confirmed to correlate with densovirus infection by both electron microscopy and indirect fluorescent antibody test. In each case the nucleotide sequence of the amplified PCR fragments showed high identity to previously reported densovirus isolated from mosquitoes. Phylogenetic analysis based on these sequences showed that two of these isolates were examples of new densovirus. These viruses could infect and replicate in mosquitoes when administered orally or parenterally and these infections were largely avirulent. In one virus/mosquito combination vertical transmission to progeny was observed. The frequency with which these viruses were detected would suggest that they may be quite common in insect cell lines.

The family Parvoviridae is currently split into two subfamilies, the Parvovirinae and the Densovirinae. Members of the first subfamily infect a wide range of vertebrates and are well characterized; in contrast, members of the genus Densovirus (DNV), subfamily Densovirinae, infect only arthropods and relatively little is known of their biology. DNVs are typically small autonomous DNA viruses characterized by the presence of inverted terminal repeat sequences and the separate encapsidation of complementary single-stranded DNA. Two subgroups (I and II) of the insect DNVs have been proposed recently, based primarily on information obtained from lepidopteran isolates (Tijssen & Arella, 1991). According to this classification, Group I DNVs are characterized by a 6 kb genome and code their structural and non-structural proteins from separate strands; they infect all cells of the insect except those of the midgut. Group II DNVs are smaller, 4.9 kb, are thought to code all their proteins from one strand and, in contrast to their Group I counterparts, only infect insect midgut cells. All described DNVs have narrow host ranges, infecting only closely related insects (Buchatsky, 1989).

To date, much of our understanding of DNVs has come from studies of lepidopteran isolates. However, in recent years three mosquito viruses have been described; one isolated from Aedes aegypti larvae (ADNV) (Afanasiev et al., 1991; Lebedeva et al., 1973), a second recovered from an Aedes albopictus (C6/36) cell line (AaPV) (Barreau et al., 1992; Boublik et al., 1992, 1994) and a possible third densovirus observed by electron microscopy in an Aedes pseudoscutellaris (AP-61) cell line (Gorziglia et al., 1980). Genomic sequence data have been obtained for both ADNV and AaPV (Afanasiev et al., 1991; Boublik et al., 1994), and they are reported to share over 77% identity at the nucleotide level (Boublik et al., 1994). They also appear to be quite different from previously described DNVs. Both have genomes of 4 kb and are polytropic, infecting all tissues of their respective insect hosts. The ADNV genome has open reading frames (ORFs) primarily on the plus strand with one ORF present from the minus strand. AaPV has three ORFs present on the plus strand only. As yet little information is available about the utilization of these ORFs. DNVs of lepidopterans typically encapsidate both plus and minus strands with equal frequency, while approximately 85% of purified ADNV virions contain minus strands only. As such, the two sequenced mosquito DNVs do not fit easily into the classification proposed by Tijssen & Arella (1991).

The high level of identity between the two described mosquito DNVs allowed us to design oligonucleotides

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from ORF3 that would potentially amplify an approximately 350 bp fragment of the viral genome from infected insects or infected insect cell lines (5' AGACARMGR-TGCTAAC; 5' GATTTTGTATATACTCCTTC). These primers were predicted to be capable of amplifying fragments only from the aforementioned two mosquito DNVs and not from the previously described DNVs of other insects that have been classified as either Group I or II densoviruses.

We initially screened a number of continuous insect cell lines (Table 1) with this primer set. Insect cells were grown in 25 cm² flasks, harvested by vigorous shaking or scraping, washed in PBS and resuspended in 200 µl of STE buffer (Sambrook et al., 1989), and incubated with Proteinase K (200 µg/ml) for 30 min at 37 °C followed by 10 min at 95 °C. Cells were pelleted at 14000 g for 1 min and 1 µl of the supernatant was used in 20 µl total volume PCRs with a temperature profile of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, for 35 cycles, as previously described (O'Neill et al., 1992). Amplification of a 350 bp fragment occurred in four of the 17 lines tested (Table 1). These PCR positive cell lines were derived from quite divergent mosquito species: *Aedes albopictus, Culex theileri, Haemagogus equinus* and *Toxorhynchites amboinensis*.

In order to confirm that the positive PCR amplifications represented the identification of real viral genomes and not fortuitous PCR amplification of genomic DNA, the PCR fragments from the four mosquito cell lines were cloned and sequenced. For cloning, 100 µl PCRs were concentrated by ethanol precipitation and ligated into Bluescript KS phagemid vector that had first been cut with EcoRV and t-tailed according to Holton & Graham (1991). *E. coli* DH5α cells were transformed and the resulting clones were sequenced with Sequenase version 2.0 (USB). The sequence of the PCR fragments revealed high identity in each instance to the previously reported mosquito DNVs, ADNV and AaPV. For the region sequenced, all clones were found to be greater than 90% identical to each other as well as to the previously reported sequences of ADNV and AaPV.

In addition, insect cell lines that were identified as being positive for DNV infection by PCR were also examined by electron microscopy. Cells were fixed in 2.5% gluteraldehyde solution containing 0.1% CaCl₂ and 1% sucrose in 100 mN-sodium cacodylate, post-fixed in 1% OsO₄ and en bloc stained in 2% uranyl acetate. Thin sections were post-stained in 5% uranyl acetate followed by Reynold’s lead citrate. Each cell line was confirmed to be infected with virus particles with morphology typical for insect densoviruses (Fig. 1). These particles were nonenveloped, homogeneous in appearance and measured 10–12 nm in diameter. They were most often observed within membrane bound vesicles (up to 5 µm in diameter) located within the cytoplasm of the host cell and were often arranged in dense crystalline arrays.

Experiments were performed to examine whether filtered lysates from PCR-positive cell cultures could be used to infect other insect cell lines. Stocks of each of the

### Table 1. Insect cell lines used in this study

<table>
<thead>
<tr>
<th>Insect species</th>
<th>Cell designation</th>
<th>Origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aedes albopictus</em></td>
<td>C6/36</td>
<td>Larvae</td>
<td>Igarashi (1978)</td>
</tr>
<tr>
<td><em>Aedes pseudocutellaris</em></td>
<td>AP-61</td>
<td>Larvae</td>
<td>Varma et al. (1974)</td>
</tr>
<tr>
<td><em>Aedes aegypti</em></td>
<td>RML-12</td>
<td>?</td>
<td>Grace (1966)</td>
</tr>
<tr>
<td><em>Aedes aegypti</em> (albopictus?)</td>
<td>AA-1042 (ATC-10?)</td>
<td>?</td>
<td>Chao &amp; Ball (1976)</td>
</tr>
<tr>
<td><em>Culex tarsalis</em></td>
<td>Chao/Ball</td>
<td>Embryonated eggs</td>
<td></td>
</tr>
<tr>
<td><em>Culex quinquefasciatus</em></td>
<td>Hsu</td>
<td>Adult ovary</td>
<td>Hsu (1971)</td>
</tr>
<tr>
<td><em>Culex theileri</em></td>
<td>Line 1</td>
<td>Embryonated eggs</td>
<td>Oelofsen et al. (1990)</td>
</tr>
<tr>
<td><em>Anopheles gambiae</em></td>
<td>MOS 55</td>
<td>Larvae</td>
<td>Marhoul &amp; Pudney (1972)</td>
</tr>
<tr>
<td><em>Toxorhynchites amboinensis</em></td>
<td>TRA-284</td>
<td>Larvae</td>
<td>Kuno (1982)</td>
</tr>
<tr>
<td><em>Toxorhynchites amboinensis</em></td>
<td>TRA-171</td>
<td>Larvae</td>
<td>Kuno (1980)</td>
</tr>
<tr>
<td><em>Phlebotomus papatasi</em></td>
<td>PP-9</td>
<td>Embryonated eggs</td>
<td>R. B. Tesh, unpublished</td>
</tr>
<tr>
<td><em>Lutzomyia longipalpis</em></td>
<td>LL-5</td>
<td>Embryonated eggs</td>
<td>Tesh &amp; Modi (1983)</td>
</tr>
<tr>
<td><em>Drosophila melanogaster</em></td>
<td>S2</td>
<td>Larvae</td>
<td>Schneider (1972)</td>
</tr>
<tr>
<td><em>Spodoptera frugiperda</em></td>
<td>SF-9</td>
<td>Ovary</td>
<td>Summers &amp; Smith (1987)</td>
</tr>
<tr>
<td><em>Antheraea eucalypti</em></td>
<td>CCI-80</td>
<td>Ovary</td>
<td>Grace (1962)</td>
</tr>
<tr>
<td><em>Heliotis zeae</em></td>
<td>IPLB-HZ1075/UND-K</td>
<td>Ovary</td>
<td>Goodwin et al. (1982)</td>
</tr>
</tbody>
</table>

* Cell lines that gave positive PCR amplification of the predicted size with the degenerate DNV primers.
† The origin of AA-1042 is unclear but there is evidence that it may be synonymous with ATC-10, a line of Singh’s *Aedes aegypti* cell culture which was contaminated with *Aedes albopictus* cells (Brown & Knudson, 1987).
Fig. 1. (a) Electron micrograph of a DNV-infected cell from a *Toxorhynchites amboinensis* (TRA-284) cell line showing dense accumulation of virus particles within a membrane bound vesicle (arrow). N, host cell nucleus; bar, 2 μm. (b) Electron micrograph of DNV from a *Toxorhynchites amboinensis* (TRA-284) cell line. Note the crystalline arrays of virus particles. Bar, 0.2 μm.
four putative densoviruses were prepared directly from the respective infected mosquito cells, except the Haemagogus virus which was first inoculated into DNV-free C6/36 cells (as described below), and then prepared as a virus stock. Passage of this latter virus was necessary due to the poor growth rate of the infected Haemagogus equinus cell line. All cells were grown in 25 cm² plastic tissue culture flasks at 25 °C. After formation of a complete monolayer, the flasks containing the cells were placed in a freezer at −70 °C. The frozen cells were subsequently thawed; the resultant lysate was centrifuged at 2000 g for 10 min, and the supernatant was passed through a 0.22 µm filter. The filtrate was mixed with fetal bovine serum (30%) and dispensed in aliquots as the virus stock. These stocks were stored at −70 °C and were used in subsequent infectivity experiments.

Insect cells were grown in 25 cm² plastic tissue culture flasks with the appropriate growth media. When cell growth was abundant, the medium was decanted off; then each culture was inoculated with 300 µl of one of the virus stocks noted above. After incubation at 25 °C for 2–3 h, 5 ml of fresh growth medium was added to each flask, and cultures were again maintained at 25 °C. The growth medium was changed every 2 or 3 days. Six to eight days after inoculation, the cells were harvested, washed with PBS and subjected to the PCR assay as described above.

The four viruses were inoculated into each of the 12 insect cell lines listed in Table 1 that were not already infected with DNVs. Subsequent PCR analysis of each of the inoculated insect cell lines indicated that viral genomes could be amplified from each one. To rule out the possibility that we were simply detecting the original inoculum, virus infection and replication were confirmed by demonstrating viral antigen in several of the insect cell lines by indirect fluorescent antibody test (IFAT), using densovirus-specific mouse hyperimmune ascitic fluids and a commercial FITC-conjugated goat anti-mouse immunoglobulin (Tesh & Andreadis, 1992) (Fig. 2). Specific viral antigen was observed intracellularly in infected cells 6–10 days after inoculation.

Of the 12 insect cell lines that were infected with these viruses, moderate cytopathic effect (CPE) was only observed with the Haemagogus isolate (HeDNV) in the Aedes aegypti cell line RML-12 and in the Aedes albopictus cell line C6/36. The other cell lines were persistently infected, as shown by PCR and IFAT, but showed no CPE.

Live insects were also experimentally infected either orally or parenterally with the two new densoviruses and
were subsequently assayed for the presence of viral genome with the PCR assay. Adult mosquitoes were infected parenterally by intrathoracic injection, following the method described by Rosen & Gubler (1974). Each mosquito was inoculated with approximately 0.2–0.3 μl of undiluted virus stock. After inoculation, the insects were held at approximately 26–28 °C in a humid environment and were maintained on 30% sucrose solution. Mosquitoes were examined daily for signs of illness or death. Samples were taken for testing from 6–16 days after inoculation. Inoculation of adult mosquitoes with the virus isolated from the Haemagogus (HeDNV) and Toxorhynchites (TaDNV) cell lines resulted in positive PCR signals from nearly 100% of individuals from each species for up to 16 days post-injection (none were tested after 16 days). No signs of pathogenesis were seen in the inoculated mosquitoes except when HeDNV was injected into Aedes albopictus. In this case some of the inoculated mosquitoes developed signs of illness within a week. The affected mosquitoes had difficulty flying and were reluctant to blood feed; once blood fed they had trouble digesting the meal, and many subsequently died. It was possible, however, to obtain eggs from some of the infected mosquitoes; their G1 progeny were subsequently reared to adults and a sample was assayed for the virus. Of the sampled G1 progeny 20% were found to be similarly infected although no sign of disease was noted in this generation (Table 2). This indicates that HeDNV is capable of being transmitted vertically by inoculated females.

Table 2. Infectivity of TaDNV and HeDNV for mosquitoes as measured by positive PCR amplification of adult individuals*

<table>
<thead>
<tr>
<th>Species</th>
<th>Oral (%)</th>
<th>Inoculated (%)</th>
<th>Transovarial (%)</th>
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<tbody>
<tr>
<td>TaDNV (TRA-284)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aedes albopictus</td>
<td>15% (2/13)</td>
<td>100% (5/5)</td>
<td>0% (0/10)</td>
</tr>
<tr>
<td>Culex quinquefasciatus</td>
<td>18% (7/40)</td>
<td>90% (9/10)</td>
<td>0% (0/10)</td>
</tr>
<tr>
<td>Anopheles gambiae</td>
<td>0% (0/5)</td>
<td>100% (5/5)</td>
<td></td>
</tr>
<tr>
<td>HeDNV (GML-HE-12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aedes albopictus</td>
<td></td>
<td>100% (6/6)†</td>
<td>20% (2/10)</td>
</tr>
<tr>
<td>Aedes aegypti</td>
<td>21% (10/47)†</td>
<td>100% (10/10)</td>
<td>0% (0/10)</td>
</tr>
<tr>
<td>Culex quinquefasciatus</td>
<td>9% (1/11)</td>
<td>100% (10/10)</td>
<td>0% (0/10)</td>
</tr>
</tbody>
</table>

* Oral infectivity was measured as the percentage of adults giving positive PCR amplification with viral primers after allowing them to feed upon crushed, infected mosquito carcasses or larvae. Inoculated adults were assayed up to 16 days after thoracic injection of filtered virus stock. Transovarial transmission was measured as the percentage of PCR positive adult offspring of inoculated females.

† Experiments in which infected individuals displayed varying degrees of illness.

produced higher levels of oral infection. Under the experimental conditions used, we observed that up to 20% of the exposed insects carried the virus as adult mosquitoes (Table 2). Infected individuals showed no signs of illness with TaDNV and only Aedes aegypti showed symptoms of pathogenesis when infected orally with HeDNV. In the latter case up to 10% of larvae died as fourth instars. Dead larvae were assayed for the virus and all gave positive PCR amplifications.

The results provide further confirmation that the cell lines that were determined to be infected by PCR were indeed carrying autonomous infectious DNVs. These viruses could infect mosquitoes when administered orally or through inoculation, and HeDNV was capable of being transmitted vertically by inoculated females. Furthermore, the frequency with which new viruses were detected in mosquito cell lines with limited sampling suggests that they may be widespread in mosquitoes and may form an as yet unrecognized group of insect densoviruses.

In order to examine the phylogenetic relationships of the putative viruses identified by PCR, genomic sequences were subjected to maximum parsimony analysis. The sequences of cloned PCR fragments were manually aligned (Fig. 3) and subjected to the Branch and Bound algorithm of PAUP version 3.0 (Swofford, 1990). The most parsimonious tree generated from this algorithm is shown in Fig. 4.

The sequenced PCR fragments provide phylogenetic information which indicates that all of the putative viruses are more closely related to the described Aedes albopictus DNV (AaPV) than to the Aedes aegypti DNV (ADNV). Of the different clones, those from cell lines 1042 (Aedes aegypti) and CL3-5 (Culex theileri) appear to be the same as AaPV which was originally isolated from C6/36 Aedes albopictus cells (Fig. 4). At first this seemed curious, since these cells are from other mosquito species.
Fig. 3. Nucleotide sequences of cloned DNV fragments compared to the previously reported viruses AaPV and ADNV.
However, a previous report based on allozyme data has suggested that the 1042 (ATC-10) cell line may be of *Aedes albopictus* origin and not from *Aedes aegypti* as claimed (Brown & Knudson, 1987). Interestingly, the *Culex theileri* cell line was initially established using filtered spent media from C6/36 (*Aedes albopictus*) cell cultures (the same cell line from which AaPV was originally isolated). These observations potentially explain why the clones derived from these diverse cell lines appear to be identical to the described *Aedes albopictus* DNV (AaPV).

Sequence data from clones of viral DNA from the other two mosquito cell lines, TRA-284 (*Toxorhynchites amboinensis*) and GML-HE-12 (*Haemagogus equinus*), suggest that these two cell lines are infected with DNVs that are intermediate in sequence to AaPV and ADNV, although more closely aligned to AaPV. These clones are more closely related to each other and form a distinct monophyletic grouping within the tree (Fig. 4). As such, TaDNV and HeDNV appear to represent new DNVs of mosquitoes.

Although the two new DNVs were both infectious orally to mosquitoes, the observed infection rates were lower than those reported previously with ADNV and AaPV. This could be due to the fact that both HeDNV and TaDNV were isolated from persistently infected insect cell lines and as such have reduced infectivity for live mosquitoes. Alternatively, the relatively low infection rates may be due to the use of unnatural mosquito hosts. HeDNV and TaDNV were originally recovered from persistently infected *Haemagogus equinus* and *Toxorhynchites amboinensis* cell lines, respectively. Presumably, the two viruses originated from those respective mosquito species. It is possible that higher oral and vertical transmission rates would occur in the natural mosquito hosts.

There is considerable interest at present in the prospect of introducing and expressing in mosquitoes foreign genes that might modulate the ability of these insects to transmit various disease agents to humans (Aldhous, 1993; Crampton et al., 1994). However, a convenient means to introduce and express foreign genes in mosquitoes has yet to be found. In this context, natural mosquito viruses potentially could be of great utility. The recent identification of densovirus infections in two commonly used mosquito cell lines (C6/36 and AP-61) as well as in a laboratory colony of *Aedes aegypti* suggests that mosquito DNV infections may be more common than is currently appreciated. In this study we were able to identify new examples of DNVs from mosquito cell lines by utilizing a PCR based assay. On the basis of their nucleotide sequence two of these isolates appear to be quite divergent from previously described mosquito DNVs and as such lend support to the notion that these viruses may be quite widespread.

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