Epizootiology and transmission of a newly discovered baculovirus from the mosquitoes *Culex nigripalpus* and *C. quinquefasciatus*

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Reports of mosquito baculoviruses are extremely uncommon and epizootics in field populations are rarely observed. We describe a baculovirus that was responsible for repeated and extended epizootics in field populations of *Culex nigripalpus* and *C. quinquefasciatus* over a 2 year period. These mosquito species are important vectors of St Louis and Eastern equine encephalitis in the United States. Our initial attempts to transmit this baculovirus to mosquitoes in the laboratory were unsuccessful. A salt mixture similar to that found in water supporting infection in the field was used in laboratory bioassays and indicated that certain salts were crucial to transmission of the virus. Further investigations revealed conclusively that transmission is mediated by divalent cations: magnesium is essential, whereas calcium inhibits virus transmission. These findings represent a major advancement in our understanding of the transmission of baculoviruses in mosquitoes and will allow characterization of the virus in the laboratory. In addition, they can explain, in great part, conditions that support epizootics in natural populations of mosquitoes that vector life-threatening diseases of man and animals.

Introduction

Baculoviruses that are pathogenic for insects have been intensively investigated owing to their potential as biological pesticides (Black *et al*., 1997; Possee *et al*., 1997) and because of their importance as gene expression vectors in invertebrate and vertebrate cells (Jarvis, 1997; Possee, 1997). They naturally infect only arthropods and most have been isolated from the Lepidoptera but they are also known from the Hymenoptera, Diptera, Trichoptera and Crustacea (Federici, 1997). Our knowledge of basic and applied baculovirology is based almost exclusively on studies of baculoviruses from the Lepidoptera with several hundred isolates reported (Martignoni & Iwai, 1986) and 16 named species (genera *Nucleopolyhedrovirus* and *Granulovirus*; Blissard *et al*., 2000). In contrast, baculoviruses from Diptera have been infrequently reported (only two tentative species in Blissard *et al*., 2000) and at very low prevalence rates in natural populations (Federici, 1985). Most baculoviruses from Diptera have been reported from the Culicidae (~ 10 isolates) and rarely from Calliphoridae, Chironomidae, Sciaridae, Tachinidae and Tipulidae (Adams & McClintock, 1991). The mosquito baculoviruses, unlike those from Lepidoptera, have been difficult if not impossible to transmit to the mosquito host and therefore basic biological studies have been greatly hindered (Federici, 1985). A newly discovered baculovirus from the mosquito *Culex nigripalpus* has now been characterized at the morphological and molecular levels and designated *Culex nigripalpus* nucleopolyhedrovirus (CuniNPV) (accompanying paper: Moser *et al*., 2001). CuniNPV infects and destroys the larval midgut causing patent infections by 48 h post-inoculation (p.i.) and death 72–96 h p.i. In this report we describe the epizootiology of CuniNPV in field populations of the mosquitoes *C. nigripalpus* and *C. quinquefasciatus* (vectors of St Louis and Eastern equine encephalitis) in North Central Florida and provide new information on environmental factors crucial to transmission of this baculovirus in mosquitoes.
Methods

Field collections. Mosquitoes were collected from two highly eutrophic ponds containing livestock effluent. One, a 30 m² man-made pond containing swine effluent, is located in Alachua County, Florida, USA, whereas the other, a 40 m² lagoon, is located at a dairy farm in Marion County, Florida. At the swine effluent site, mosquito larvae were collected from August 1996 through December 1997 once or twice a week during the peak mosquito breeding periods (April–November) and at least once a month during the off-season (December–March). At the dairy lagoon, a total of 16 larval collections were made from March 1997 through August 1998.

Larvae were concentrated at the field sites (number of dips and water volumes not standardized) by straining water through 60 mesh sieves and transferred to containers with 15–30 litres of field water for transport to the laboratory. At the laboratory, larvae were strained through a series of 10, 18, 35 and 80 mesh sieves (20, 1, 0.5 and 0.18 mm openings respectively) and the contents of each sieve washed into 300–800 ml of water. Third- and fourth-instar larvae were retained in the 35 and 18 mesh sieves while the first- and second-instar larvae remained in the 80 mesh sieve. The total volume of the sample (300–800 ml) was measured from the 18 and 35 mesh sieves. After agitation, a sample of 10–100 ml was removed, measured and the number of larvae counted to estimate the total number of larvae in each of the 18 and 35 mesh sieves. Larvae from the 18 mesh sample were examined with a stereo microscope at 20 x magnification for signs of infection and for species identification and provided the information for percentage infection by species. Infected larvae were identified by the presence of highly refractile nuclei in midgut epithelial cells, which could be viewed through the cuticle (accompanying paper: Moser et al., 2001). Larvae from the 35 mesh were examined for signs of infection (larvae from this mesh size were too small to accurately identify the species). The total number of larvae, the proportion of each species (from the 18 mesh only) and the percent infection in Culex spp. were estimated from the samples of the two sieves. The population index reflected the total number of third- and fourth-instar Culex spp. larvae collected.

Field water analysis. An estimate of the total dissolved salts was determined by recording conductivity from each field water sample. Samples of the field water were prepared for detailed chemical analysis by the following methods. Water strained through 400 mesh (38 µm openings) was centrifuged at 4800 g for 10 min and the supernatant frozen. In addition, supernatant and field water were digested in 1 M HNO₃ by heating a 1:1 mixture of water–1 M HNO₃ just below the boiling point until the volume had been reduced to 1–2 ml. The residue was reconstituted in 1 M HNO₃ and held at room temperature. In addition, whole water was filtered through a no. 41 Whatman filter and stored frozen. Water samples were submitted for elemental analysis to the University of Florida Analytical Research Laboratory. Cation concentrations were obtained by ICAP (inductive coupled argon plasma, Thermal Garrel Ash, mg/L).

Laboratory bioassay. Tissue culture production methods for the virus were unavailable and all studies were done in vivo. A colony of C. nigripalpus was unavailable until recently and therefore insects from a C. quinquefasciatus colony (Alachua County, Florida, strain 5 years in culture) were used for the majority of bioassays. Standard bioassays were done with groups of 100 3 to 4-day-old C. quinquefasciatus larvae (second-instar) exposed in 3-5 oz plastic cups in 100 ml of water with 2 ml of 2% alfalfa and potbelly pig chow mixture (2:1).

Because of the small size of the occlusion bodies (< 0.5 µm), CuniNPV concentrations were usually based on larval equivalents (LE; 10 infected third-instar larvae/ml = 10 LE) but purified virus was used in some tests and obtained and quantified according to procedures described in Moser et al. (2001). A laboratory culture of the baculovirus was initiated by amplifying field-collected virus from C. nigripalpus in colony-reared C. quinquefasciatus. Approximately 3000 3-day-old C. quinquefasciatus larvae were exposed to 100 LE virus in 14 mM MgSO₄ and harvested 48 h p.i. Groups of 50 infected larvae were frozen in deionized water and held at —80 °C. These infected larvae were used for additional amplifications or for laboratory bioassay. On the day of the assay, individual vials were removed and held at room temperature to thaw. The infected larvae were then homogenized in a glass tissue grinder and a concentration of 5–8 LE was used per exposure group. After 48 h, the larvae were removed and examined microscopically for signs of infection. Only those larvae with hypertrophied nuclei in midgut epithelial cells (Moser et al., 2001) were scored as positive.

Field water assays. Larvae were exposed to CuniNPV in whole field water strained through a 400 mesh sieve (collected from August 1996 through December 1997) and deionized water. Field water samples were held for a maximum of 5 days at 5 °C before testing. Controls without the addition of virus were included in all assays. The increase in percent infection was calculated based on paired tests with and without virus in deionized water, swine wastewater and dairy wastewater. Data were analysed by SAS general linear model and comparison among mean differences by Duncan–Waller.

Enhancement assays. Alkali pretreatment of CuniNPV prior to exposure, reported as important for the transmission of AeconNPV in the mosquito Aedes triseriatus (Federici & Love, 1972), was performed according to the previously described procedure. After neutralization, treated and untreated virus were assayed in deionized water in paired tests. Also, virus exposures were made with the addition of the optical brightener Calcoflour M2R (Sigma), which has been shown to enhance the transmission of certain baculoviruses (Shapiro & Robertson, 1992).

Deletion analysis of the principle cations present in the field water was used to determine if salts were critical for transmission of CuniNPV. A salt mixture of 1·8 mM MgCl₂, 0·5 mM CaCl₂, 0·0 mM KCl, 1·8 mM NaCl and 3 mM NH₄Cl (based on the initial swine wastewater chemical analysis) was used as an exposure medium. Bioassays were done in the complete salt mixture, mixtures with one salt deleted and in each salt individually. Based on the results of these tests, additional assays with CuniNPV were conducted in 63% serial dilutions of 20 mM MgCl₂ or MgSO₄ to determine the effect of cation concentration on infection levels. To determine possible inhibition of transmission, assays were done in 10 mM MgCl₂ with 50% serial dilutions of 20 mM CaCl₂, KCl and NaCl. Data were analysed by SAS general linear model and comparison among mean differences by Duncan–Waller.

To determine the activation potential of other cations on transmission of CuniNPV, exposures were made in the highest sublethal concentrations of BaCl₂, CoCl₂, CuCl₂, FeCl₃, KCl, MnCl₂, NaCl, NiCl₂, SnCl₂, SrCl₂ and ZnCl₂ (n = 3). The cations that did not result in increased transmission of CuniNPV were tested (at the highest sublethal concentration) to determine if they would inhibit transmission when exposures were made in the presence of 10 mM MgCl₂ (n = 3).

The role of magnesium in transmission and development of CuniNPV was investigated by exposing larvae to MgCl₂ and virus for 2, 4, 6, 8, 12, 24 and 48 h periods. All exposures were made in 10 mM MgCl₂ and CuniNPV concentrations of 5 LE or purified virus at an estimated concentration of 4·5 x 10⁸ occlusion bodies (OBs)/ml. After each exposure time, larvae were either removed from the exposure media and transferred to deionized water (n = 5) or 10 mM CaCl₂ was added to the exposure mixture to stop the infection process (n = 4). Percent infections were determined for each exposure group at 48 h p.i. and the percent infection was evaluated by log probit analysis.
Epizootiology and transmission of a mosquito baculovirus

Assays were also done with EDTA (Sigma), a strong and nonspecific divalent ion chelator and metalloprotease inhibitor and EGTA (Sigma) a chelator of divalent cations with a significantly higher affinity for calcium than magnesium. Larvae were exposed according to the protocols for the virus assay experiments (described above) with the addition of 40% serial dilutions of 1 mM EDTA or 40% serial dilutions of 1 mM EGTA (total of five concentrations).

Electron microscopy. To investigate early events in the infection process of CuniNPV, larval guts were dissected and fixed (as described below) at time intervals of 30 min, 1, 2 and 4 h p.i. Exposures were made to groups of larvae (as described above) with virus alone and with virus in the presence of 10 mM MgCl₂. Exposed larvae were prepared for ultrastructural examination by fixing dissected guts in 2.5% gluteraldehyde for 2 h, post-fixing in 2% osmium tetroxide, dehydrating in an ethanol series and embedding in Epon-Araldite. Thin sections were stained in uranyl acetate and lead citrate and examined and photographed at 75 kV.

Virus host-range. CuniNPV for these exposures was produced in C. quinquefasciatus as described above and a dose of 10 LE was used for all tests. Mosquitoes tested were from laboratory colonies of Aedes aegypti, A. albopictus, A. triseriatus, A. taeniorhynchus, Anopheles albimanus, A. quadrinaculatus and field-collected Culex-restuans, C. salinarius and Culiseta melanura. Groups of 100 48-h-old larvae were counted into cups containing 100 ml 10 mM MgCl₂ and susceptibility was determined with paired tests, one exposed group and one unexposed control. Positive controls of 100 C. quinquefasciatus larvae were exposed with each test to verify the infectivity of the virus. All larvae were examined for infection at 48 h p.i. To test the susceptibility of the predacious mosquito, Toxorhynchites ambionensis, 20 second-instar larvae were set up individually in well plates with 10 mM MgCl₂ and fed three live C. quinquefasciatus larvae infected with CuniNPV. Similar groups of T. ambionensis were fed healthy C. quinquefasciatus larvae and served as a control. Larvae were examined for signs of infection at 48, 72 and 96 h p.i. and mortality was calculated.

Results

Field studies

Agricultural wastewater can serve as a habitat for large numbers of larval mosquitoes and supports adults that are vectors of a variety of diseases of man and animals. In order to identify pathogens of larval mosquitoes, an epizootiological study was undertaken on two highly eutrophic bodies of water. These included a pond located in Alachua County, Florida, USA that contained swine effluent and a lagoon associated with a dairy farm in Marion County, Florida.

Larvae were collected at the field sites and then separated into two categories – first- and second-instar, and third- and fourth-instar – which were examined microscopically for signs of viral infection and for species identification. The two predominant mosquito species present at the swine wastewater site were C. nigripalpus during the warmer months (May–November) and C. quinquefasciatus during the cooler months (December–April). C. salinarius and C. restuans were also present during the winter but at low levels. Epizootics of CuniNPV were found in all instars of the Culex spp. larvae collected in the swine wastewater site throughout the study (Fig. 1). The highest infection levels in third- and fourth-instar larvae were found during the spring and fall months with the lowest infection levels found during the winter (Fig. 1).

C. nigripalpus was present in 91% of the samples (n = 74). Regular and extended epizootics of CuniNPV in C. nigripalpus were documented with an average infection rate of 20.1 ± 2.4% (mean ± SE, n = 43) and a maximum rate of 60%. C. quinquefasciatus was present in 49% of the collections with an average CuniNPV infection rate of 8.6 ± 2.3% (n = 9) and a maximum infection rate of 20%. C. salinarius was present in 34% of the collections with an average CuniNPV infection rate of 15.5 ± 3.1% (n = 8) and a maximum rate of 30%. C. restuans was present in 6% of the collections but was never infected with CuniNPV.

C. quinquefasciatus was the dominant species collected at the dairy wastewater site with C. nigripalpus collected infrequently. A total of 16 larval collections was made during the sampling period. Although high larval populations of C. quinquefasciatus were present (average of 19,000 ± 5000) larvae infected with CuniNPV were collected on only five occasions and never at epizootic levels (0.08 ± 0.06% infection).

Fig. 1. Population dynamics of third- and fourth-instar Culex spp. larvae in a swine wastewater site in Florida (1996–1997) and the prevalence of CuniNPV. Repeated and extended epizootics occurred throughout the spring, summer and fall of the sampling period with the highest levels in spring and fall and the lowest levels in the winter months. The population index reflected the total number of third- and fourth-instar Culex spp. larvae collected.
Table 1. Physical and chemical features of a swine effluent pond in Alachua County, Florida and a lagoon associated with a dairy farm in Marion County, Florida

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Swine (Mean ± SE)</th>
<th>Dairy (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conductivity (mS/cm)</td>
<td>1.8 ± 0.4</td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td>pH</td>
<td>7.8 ± 0.03</td>
<td>8.0 ± 0.05</td>
</tr>
<tr>
<td>Cation (mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K⁺</td>
<td>10.7 ± 0.5</td>
<td>5.8 ± 1.1</td>
</tr>
<tr>
<td>Na⁺</td>
<td>3.2 ± 0.2</td>
<td>3.3 ± 0.9</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>0.8 ± 0.05</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>1.9 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

Water analysis

The conductivity of the swine wastewater averaged 1.8 mS/cm, and the pH averaged 7.8 (Table 1). In contrast, the dairy lagoon wastewater showed a higher conductivity, (3.9 mS/cm), but the pH was similar (8.0). Although in both ponds K⁺, Na⁺, Mg²⁺ and Ca²⁺ accounted for 90% of the cations, there were significant differences in their concentrations. Whereas Na⁺ levels were similar at 3.2–3.6 mM, the concentration of K⁺ was almost twice as high in the swine pond (10.7 vs 5.8 mM) but the Mg²⁺ and Ca²⁺ concentrations were significantly lower (Mg²⁺ = 1.9 vs 3.7 mM; Ca²⁺ = 0.8 vs 3.0 mM).

Table 2. Increase in percent infection based on paired tests with and without CuniNPV in deionized water (DI), swine or dairy wastewater in laboratory bioassays

<table>
<thead>
<tr>
<th>No. of pairs</th>
<th>DI</th>
<th>Swine</th>
<th>Dairy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SE⁶</td>
<td>0.2 ± 0.1 %⁶</td>
<td>16.2 ± 2.4 %⁹</td>
<td>0.1 ± 0.1 %⁶</td>
</tr>
<tr>
<td>Max. increase</td>
<td>2.0 %</td>
<td>63.3 %</td>
<td>1.0 %</td>
</tr>
</tbody>
</table>

* Values with the same letter are not significantly different from each other.

Laboratory bioassay

Although we found that field populations of Culex spp. showed infection rates of up to 60%, repeated attempts to infect C. quinquefasciatus in deionized water produced infection rates that averaged 0.2% (Table 2). Methods reported to enhance infectivity of baculoviruses such as alkali pretreatment to dissolve the OBs and the use of optical brighteners were ineffective in increasing the infection rate. However, there was

Table 3. Percent infection in colony C. quinquefasciatus exposed to CuniNPV in mixtures of KCl, NaCl, CaCl₂ and MgCl₂ in laboratory bioassay (n = 3)

<table>
<thead>
<tr>
<th>Components of exposure medium (mM)</th>
<th>K⁺</th>
<th>Na⁺</th>
<th>Mg²⁺</th>
<th>Ca²⁺</th>
<th>% Infection*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.0</td>
<td>1.8</td>
<td>1.8</td>
<td>0.5</td>
<td>11.0 ± 3.5 %⁹</td>
</tr>
<tr>
<td>2</td>
<td>6.0</td>
<td>1.8</td>
<td>1.8</td>
<td></td>
<td>14.6 ± 10.1 %⁹</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td>0.5</td>
<td>0.8 %⁹</td>
</tr>
<tr>
<td>4</td>
<td>6.0</td>
<td>1.8</td>
<td></td>
<td>0.5</td>
<td>0.3 ± 0.3 %⁹</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>1.8</td>
<td></td>
<td></td>
<td>10.4 ± 5.2 %⁹</td>
</tr>
<tr>
<td>6</td>
<td>6.0</td>
<td></td>
<td></td>
<td>0.5</td>
<td>3.7 ± 1.9 %⁹</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>1.8</td>
<td></td>
<td></td>
<td>0.8 %⁹</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>1.8</td>
<td>1.8</td>
<td>0.5</td>
<td>6.7 ± 2.0 %⁹</td>
</tr>
<tr>
<td>9</td>
<td>6.0</td>
<td></td>
<td></td>
<td></td>
<td>0.8 %⁹</td>
</tr>
</tbody>
</table>

* Values with the same letter are not significantly different.
an 80-fold increase in infection of *C. quinquefasciatus* larvae when exposed to CuniNPV in swine but not dairy wastewater (Table 2). These results indicated that there were factors present in the swine wastewater that mediated transmission of the virus.

The addition of a salt mixture similar to that present in the swine pond water to the CuniNPV assay significantly improved infections, with an average infection rate in larvae of 11·0% (Table 3, row 1). The salts were also tested individually and in combination. The only individual salt that was essential for infectivity was magnesium, with an average larval infection rate of 10·3% (row 5). Salt mixtures without Mg\(^{2+}\) resulted in less than 1·0% infections (rows 3 and 4). Elimination of K\(^{+}\) and Na\(^{+}\) had little effect on the infection rate. With the exception of the complete salt mixture, the mixtures with Ca\(^{2+}\) tended to result in lower infection levels.

There was no difference in infection levels when exposures were made in either MgCl\(_2\) or MgSO\(_4\) and therefore MgCl\(_2\) was used in all subsequent bioassays. Serial dilutions with Mg\(^{2+}\) showed that CuniNPV percent infection was positively correlated to the Mg\(^{2+}\) concentration: as Mg\(^{2+}\) concentrations increased, CuniNPV infections increased (Fig. 2). The activation concentration\(_{50}\) (95% fiducial limits) and AC\(_{99}\) of MgCl\(_2\) was 4·3 (4·0, 4·6) mM and 13·7 (12·5, 15·3) mM respectively (log probit analysis). Conversely, as Ca\(^{2+}\) concentrations increased in the presence of Mg\(^{2+}\), infections decreased (Fig. 2). The infection concentration\(_{50}\) and IC\(_{10}\) of CaCl\(_2\) was 0·56 (0·49, 0·62) and 4·2 (3·7, 4·9) mM respectively.

Results of assays with other cations found that only divalent cations played a role as activators (Table 4) or inhibitors of infection. Activators in addition to Mg\(^{2+}\) were barium, cobalt, nickel and strontium. In each case, the higher the divalent cation concentration (within the tolerance level of the mosquito) the higher the percent infection. Nickel was a potent activator requiring a concentration of only 0·2 mM to give 95·5% infection. In addition to calcium, divalent cations that inhibited infection of CuniNPV when combined with 10 mM Mg\(^{2+}\) were copper, iron and zinc. The addition of Cu\(^{2+}\) (0·1 mM), Fe\(^{3+}\) (1 mM) or Zn\(^{2+}\) (0·5 mM) in the presence of 10 mM Mg\(^{2+}\) resulted in 100% inhibition of transmission. Manganese was neutral and tin was too toxic to determine its effect on infection. Potassium and sodium (monovalent ions) had no effect on transmission. As expected from the dependence of infection on divalent cations, EDTA is a concentration-dependent competitor that prevented transmission of CuniNPV. Levels of EDTA greater than 0·2 mM inhibited infection with CuniNPV (13·9 ± 8·5%) whereas the infection rate at EDTA levels less than 0·2 mM were not significantly different from the controls (94·0 ± 5·6, 97·4 ± 2·6% respectively). Concentrations of EDTA higher than 1·0 mM were lethal to the mosquitoes. In exposure medium with MgCl\(_2\) only, > 0·4 mM EGTA was lethal, but

![Fig. 2. Effects of Mg\(^{2+}\) and Ca\(^{2+}\) concentrations on CuniNPV infections in *C. quinquefasciatus*. The trend line from the MgCl\(_2\) concentration range fits the model% infection = 0·40 × ln([MgCl\(_2\)]) − 0·02 with an R value of 0·987. The trend line from the CaCl\(_2\) concentration range fits the model% infection = −0·2 × ln([CaCl\(_2\)]) + 0·38 with an R value of −0·975.

![Fig. 3. Effect of exposure time on the infection rate of CuniNPV in *C. quinquefasciatus*. Larvae were exposed to 5 LE of CuniNPV in 10 mm MgCl\(_2\). Infection status was determined 48 h p.i. at the time-points shown.](https://www.microbiologyresearch.org/)

**Table 4. Activation potential of divalent cations tested with CuniNPV against *C. quinquefasciatus* in laboratory bioassay (n = 3)**

<table>
<thead>
<tr>
<th>Cation</th>
<th>Dose range (mM)</th>
<th>% Infection range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba(^{2+})</td>
<td>1·25–10·0</td>
<td>1·1–12·5</td>
</tr>
<tr>
<td>Co(^{3+})</td>
<td>0·1–0·63</td>
<td>9·3–35·9</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>2·0–40·0</td>
<td>18·1–100·0</td>
</tr>
<tr>
<td>Ni(^{2+})</td>
<td>0·05–0·2</td>
<td>11·3–95·5</td>
</tr>
<tr>
<td>Sr(^{2+})</td>
<td>0·63–10·0</td>
<td>7·0–100·0</td>
</tr>
</tbody>
</table>
Fig. 4. Electron micrographs of OBs and virions of CuniNPV at various times p.i. within the peritrophic matrix (PM) of the midgut of C. quinquefasciatus larvae. (a) Intact OBs of CuniNPV 30 min p.i. in the presence of Mg\(^{2+}\). (b) OBs 60 min p.i. in the presence of Mg\(^{2+}\) indicating partial release of virions. (c) Four hours p.i. without Mg\(^{2+}\) demonstrating ‘ghosts’ within the almost completely dissolved OBs. (d, e) Virions in the gut lumen (GL) near or attached to the PM 90 min p.i. in the presence of Mg\(^{2+}\). Note the intact virion envelope on the virion embedded in the inner PM membrane (arrow). All bars represent 0.25 µm.

Electron microscopy

Examination of the larval gut contents 30 min p.i. revealed intact OBs regardless of the presence or absence of Mg\(^{2+}\) (Fig. 4a). Release of virions from OBs was first found 1 h p.i. and did not depend on the presence of Mg\(^{2+}\). Release of virions occurred prior to complete dissolution of the OBs leaving ‘ghosts’ in the OBs indicating the former location of virions (Fig. 4b, c). Numerous free virions were observed in the gut lumen 1, 2 and 4 h p.i. and were often found in close proximity to the peritrophic matrix (PM) of the insect midgut (Fig. 4d). This preliminary investigation failed to demonstrate virions crossing the PM but when Mg\(^{2+}\) was present some virions were observed embedded in the internal layer of the PM (Fig. 4e).

Discussion

The repeated and extended epizootics of CuniNPV at the swine wastewater site suggested that it is an extremely virulent pathogen with high infectivity for all instars of Culex larvae under the appropriate environmental conditions. The evidence presented here proves that divalent cations are crucial components in the transmission of CuniNPV and that Mg\(^{2+}\) and Ca\(^{2+}\) may be the main factors in field transmission. We believe that these results, in great part, explain why epizootics of CuniNPV occurred in the swine wastewater site where Mg\(^{2+}\)/Ca\(^{2+}\) ratios (1.9/0.8) mediated transmission and did not occur in the dairy wastewater site where Mg\(^{2+}\)/Ca\(^{2+}\) ratios (3.7/3.0) were unfavourable. Transmission in field water from

at concentrations < 0.4 mM, EGTA was not lethal but did not inhibit CuniNPV infections (94.7 ± 2.5%).

Infection levels in larvae exposed to CuniNPV and magnesium increased over time with the maximum infection rate achieved after a 12 h exposure (Fig. 3). The exposure time,\(\tau_{50}\) (95% fiducial limits) and ET\(_{90}\) was 7.1 (6.9, 7.3) and 13.0 (12.5, 13.6) h respectively (log probit analysis).

Virus host-range

CuniNPV infected C. salinarius (infection rate 32.9 ± 9.8%, \(n = 3\)) but not C. restuans. Species of Aedes, Anopheles, Culiseta and Toxorhynchites were refractory to infection.

Electron microscopy

Examination of the larval gut contents 30 min p.i. revealed intact OBs regardless of the presence or absence of Mg\(^{2+}\) (Fig. 4a). Release of virions from OBs was first found 1 h p.i. and did not depend on the presence of Mg\(^{2+}\). Release of virions occurred prior to complete dissolution of the OBs leaving ‘ghosts’ in the OBs indicating the former location of virions (Fig. 4b, c). Numerous free virions were observed in the gut lumen 1, 2 and 4 h p.i. and were often found in close proximity to the peritrophic matrix (PM) of the insect midgut (Fig. 4d). This preliminary investigation failed to demonstrate virions crossing the PM but when Mg\(^{2+}\) was present some virions were observed embedded in the internal layer of the PM (Fig. 4e).
Mg$^{2+}$ to overcome the high Ca$^{2+}$ levels. Despite their critical effects on CuniNPV infectivity, the mechanism underlying Mg$^{2+}$ and Ca$^{2+}$ activity remains unclear. The fact that Mg$^{2+}$ and Ca$^{2+}$ have opposite effects suggests that Ca$^{2+}$ may interfere with a critical interaction by Mg$^{2+}$ in some process involved in the initiation of infection.

A cascade of events is required for infection of a host with a baculovirus. This involves: (i) release of virions from OBs in the insect gut, (ii) the movement of virions across the PM, (iii) the attachment of virions to the midgut epithelial cells and the transfer of the nucleocapsids to the nucleus where they undergo replication, (iv) budding of virions from the midgut cell and spread of infection within the insect. To produce high levels of infection, Mg$^{2+}$ must be present during the first 8–12 h of CuniNPV exposure indicating that the activity of Mg$^{2+}$ occurs early in the process with either entry into the cells or nuclei. EM observations of OBs in the midgut lumen revealed that Mg$^{2+}$ was not required for dissolution. Other possible targets are receptors on midgut epithelial cells or PM or enzymes associated with the virion or midgut that are required for initiating infectivity. Receptors may require divalent cations for the attachment to and/or passage of virions through the PM, midgut cells or entry into nuclei.

Our observations of virions embedded in the PM indicated that specific receptors on the inner layer of the PM might be crucial for attachment but it is not known if this process is Mg$^{2+}$ dependent. However, attachment alone is insufficient for the virion to pass through the PM as the maximum size of pores in the inner PM layer is 20 nm (Werner, 1979) while the width of the virion is approximately 40 nm (Moser et al., 2001). This implies that proteins in the PM must be broken down for the virions to pass. Mg$^{2+}$ may be involved in this process. In addition, Mg$^{2+}$ could be required for the interaction of CuniNPV with receptors on cells in the posterior midgut and the gastric caeca (Moser et al., 2001).

This is only the second documented epizootic of a baculovirus in a naturally occurring mosquito population. A mixed epizootic involving a baculovirus and a cytoplasmic polyhedrosis virus (CPV) in Aedes sollicitans occurred over a 3 week period in the summer of 1971 in Louisiana (Clark & Fukuda, 1971). Although many insects were infected (56.7% at the peak), the infections were apparently due to a mixture of both viruses and therefore it was difficult to evaluate the role of each. Attempts to transmit the A. sollicitans baculovirus in the laboratory resulted in less than 15% infection, but transmission in field water or with the addition of salts was not investigated (Clark & Fukuda, 1971).

Because C. nigrispalpus is an important vector of St Louis and Eastern equine encephalitis (Nayar, 1982), our investigations demonstrating the role of Mg$^{2+}$ and Ca$^{2+}$ in facilitating the infectivity of the CuniNPV baculovirus have direct and important implications for utilizing baculoviruses for control of these insects. It may be possible to develop mosquito baculoviruses as a new type of biopesticide by micro-encapsulating the virus and Mg$^{2+}$ into formulations that would be effective regardless of the water quality. In addition, this new insight into transmission may facilitate the discovery and development of additional baculoviruses for the control of other mosquitoes species.

In contrast to employing baculoviruses for insect control, there are a number of situations where baculoviruses can cause economic harm. For example, viruses resembling baculoviruses are responsible for annual economic losses in cultured peneaids shrimp in the Americas and Hawaii due to mass mortalities that occur in the hatchery phase of production (Stuck & Overstreet, 1994). A method that could easily and economically inhibit transmission of these viruses in the shrimp farming industry would have positive and important implications. Similar methods could be employed by the silk industry to inhibit the spread of baculoviruses among silkworm larvae.

Future studies will focus on development of CuniNPV as a biopesticide and to characterize the entire genome of this baculovirus. It is hoped that this characterization will provide the knowledge necessary for manipulation of the virus to enhance desirable properties and to identify novel genes and proteins. These new constructs and proteins may prove useful in the development of improved strategies and tools for the control of mosquitoes worldwide that vector diseases of man and animals.

The authors wish to thank Dr George Rohrmann (Oregon State University) for his numerous helpful suggestions and comments on an earlier draft of the manuscript. This work would not have been possible without the support and encouragement of Dr Donald Barnard, Research Leader, USDA/ARS/CMAVE, Gainesville, FL.

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Received 12 June 2000; Accepted 26 September 2000