A nucleopolyhedrovirus from *Uranotaenia sapphirina* (Diptera: Culicidae)

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Received 29 January 2004; accepted 26 April 2004
Available online 9 June 2004

Abstract

In this report we present data on biology, gross pathology, ultrastructure, and host range studies of a naturally occurring nucleopolyhedrovirus from the mosquito *Uranotaenia sapphirina* (UrsaNPV). Development of this virus was restricted to nuclei of epithelial cells in posterior midgut and distal gastric caecum. Occlusion bodies contained numerous singly enveloped rod-shaped virions. Early occlusion bodies were irregularly shaped and seemed to subsequently coalesce to form larger polyhedra. Mature occlusion bodies had a unique dumbbell shape, and lacked a polyhedron envelope and crystalline structure. Developmental and structural features of UrsaNPV were generally similar to other mosquito NPVs, with major differences in occlusion body shape and size. Transmission tests showed that only members of *Uranotaenia* (*Ur. sapphirina* and *Ur. lowii*) were susceptible to this virus. Transmission was facilitated by magnesium. Field collected *Ur. sapphirina* larvae had a relatively high rate of dual infections with UrsaNPV and UrsaCPV (cypovirus).

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*Keywords: Uranotaenia sapphirina; Mosquito viral pathogens; Nucleopolyhedrosis; Cypovirus; Dual viral infections; Uranotaenia lowii*

1. Introduction

NPVs belong to the genus *Nucleopolyhedrovirus* (*Baculoviridae*) and are enveloped, double-stranded DNA viruses with circular genomes that are specific to arthropods. Isolated mostly from Lepidoptera, they are also known from the Hymenoptera, Diptera, Trichoptera, and Crustacea (Federici, 1997). Since the first report of a baculovirus (OcsoNPV) isolated from *Ochlerotatus sollicitans* mosquitoes in Louisiana in 1969 (Clark et al., 1969), naturally occurring NPVs were isolated from 13 more mosquito species within the genera *Aedes*, *Anopheles*, *Culex*, *Ochlerotatus*, *Psorophora*, *Uranotaenia*, and *Wyeomyia* (Murphy et al., 1995). More than 20 mosquito species were found susceptible to NPV infections (field isolates or laboratory transmitted infections). Patent infections are detected by the hypertrophied nuclei of midgut cells that appear white caused by the accumulation of the occlusion bodies. Due to the rarity of mosquito NPV isolations from the field, the difficulty of transmission in the laboratory and the impossibility to maintain colonies of some species, few morphological and transmission studies of mosquito NPVs have been reported. Most of the detailed ultrastructural studies were conducted with baculoviruses from *Oc. triseriatus* (Federici and Lowe, 1972), *Oc. epactius* (Stiles et al., 1983), *Oc. sollicitans* (Federici, 1980) and from *Cx. nigripalpus* (Moser et al., 2001). There is only one extensive study on transmission of an NPV in *Cx. nigripalpus* (Becnel et al., 2001) that demonstrates the crucial roles of Mg$^{2+}$ and Ca$^{2+}$ in this process. A nucleopolyhedrovirus was previously reported in *Ur. sapphirina* (Clark et al., 1969; Federici, 1985), and here we present the first detailed description of an NPV infection in this mosquito species. A possible, but not confirmed by electron microscopy, cypovirus (CPV) infection in *Ur. sapphirina* was reported by Clark et al. (1969); here we present ultrastructural evidence for a CPV infection in this species. This paper describes biology, gross pathology, ultrastructure and host range
studies of an NPV, as well as biology and ultrastructure of an NPV/CPV dual infections from *Ur. sapphirina* larvae collected in Florida.

*Uranotaenia sapphirina* is a common mosquito in Florida and may be found throughout most of the year. Larvae occur in permanent pools and ponds, lakes and swamps that contain emergent or floating vegetation exposed to sunlight. Although *Ur. sapphirina* feeds primarily on frogs and reptiles, has no known medical or economic importance and the females are not known to bite humans, it is nevertheless on the CDC list of mosquitoes testing positive for West Nile Virus. Recent findings (ProMed, 2003) that levels of the virus in alligators are as high as what is found in birds (high enough to infect mosquitoes), suggest that alligators might transmit the virus and *Ur. sapphirina* might play a small role in the transmission cycle of WNV and its circulation in the environment.

It should be noted that the identification of UrsaNPV and UrsaCPV at this point is based on morphological criteria alone. Without molecular data on the characterization of these viruses its identities are tentative.

2. Materials and methods

2.1. Field collections

*Uranotaenia* spp. larvae were collected in the grass surrounding a small, sunlit pond located in Cross Creek, Florida. The pond encompassed part of a pasture and a roadside ditch, however, it did go dry during prolonged drought. Although many insect predators and crayfish were present, fish were not. Other mosquitoes collected at this site included *Anopheles crucians* and *Culex erraticus*.

2.2. Gross pathology

Larvae were separated by genus, placed on a clear glass petri dish with minimal water and the ventral side examined with a dissecting scope against a black background. The larvae had a relatively clear cuticle allowing the infected cells of the midgut to be detected with a dissecting microscope.

2.3. Statistical analysis

Percent infection was estimated as the number of infected larvae divided by the number of larvae examined. This was averaged over the number of collections which had infected larvae.

2.4. Laboratory bioassay

*Uranotaenia* spp exposures: Horizontal transmission of UrsaNPV studies were limited because of the availability of field collected healthy and infected *Uranotaenia*. *Uranotaenia* larvae collected from sites which historically did not have UrsaNPV or UrsaCPV present were exposed to virus from field-infected *Uranotaenia* spp. The healthy *Uranotaenia* were primarily in the third or fourth instars and the number exposed varied from 20 to 100 larvae. They were exposed to one to five larval equivalents of infected *Ur. sapphirina* in 10–100 ml of 5 or 10 mM MgCl₂, depending on the number of larvae available, and examined two to three days after exposure.

Other mosquito exposures: Second instar larvae of *Aedes aegypti*, *Anopheles quadrimaculatus*, and *Culex quinquefasciatus*, in groups of 100 larvae, were exposed to 3–5 larval equivalents of field infected *Uranotaenia* spp. infected with UrsaNPV and UrsaCPV and exposed in 100 ml of 10–20 mM MgCl₂ and examined 2–3 days post exposure for signs of infection.

2.5. Ultrastructural studies

Guts were dissected from fourth instar larvae and transferred to 2.5% (v/v) glutaraldehyde (EM grade) in 0.1 M cacodylate buffer containing 0.1% CaCl₂ for 3h. Following fixation, samples were washed three times in 0.1 M cacodylate buffer (pH 7.2) and post-fixed in 1% (w/v) aqueous osmium tetroxide for 1h at room temperature. Samples were washed three times with water, dehydrated through a standard ethanol series (30–100% ethanol, 15 min/step) followed by two washes in acetone. Tissues were infiltrated with Epon-Araldite resin (Polybed 812, 2 g; Araldyte 502, 1 g; DDSA, 4.5 g; DMP-30 4 drops) in a stepwise fashion with ratios of acetone to resin of 3:1, 1:1, and 1:3 and finally several changes of pure resin. Resin blocks were cured overnight in a 65°C oven. Thin gold interference sections (85–100 nm thick) were collected on 200-mesh copper grids. The sections were poststained with 2.5% aqueous uranyl acetate for 10 min, followed by Standard Reynolds Lead Citrate stain for 5 min and were examined with a Hitachi H-600 electron microscope at an accelerating voltage of 75 kV.

3. Results

3.1. Field collection

The first UrsaNPV infected larvae were found in October 2001 with 8 of 152 (5.3%) *Ur. sapphirina* larvae infected. Several of these infected larvae were dually infected with a CPV. During October and November 2001, 14 of the 38 collections had patently infected larvae with either UrsaNPV or UrsaCPV or both. The maximum infection rate of UrsaNPV was 14.0% in November 2001 with an equal number dually infected. The site was totally dry by June 2002. *Uranotaenia*
sapphirina were collected again after the pond flooded in July 2002, but UrsaNPV and UrsaCPV were not recovered until October 2002. The highest infection rate of both viruses was 18.8% in November 2002. During this time, both Culex erraticus (5.4 ± 0.7%, n = 3) and Anopheles crucians (3.7 ± 2.5%, n = 3) were infected with a CPV similar to the UrsaCPV, but neither species were infected with UrsaNPV. In 2003, UrsaCPV was recovered in October, but not UrsaNPV. Table 1 gives the seasonal infection rates for both viruses.

Uranotaenia lowii were also breeding in the same site, but in much lower numbers than Ur. sapphirina. Uranotaenia lowii infected with UrsaNPV and UrsaCPV (viral identification based on light and electron microscopy) were collected in 2001. In October 2001, 0.1 ± 0.1% were infected with UrsaNPV and 1.4 ± 0.6% infected with UrsaCPV. The infection rates in November were 0.6 ± 0.3% for UrsaNPV and 2.0 ± 0.7% for UrsaCPV. Since then no infected Ur. lowii have been recovered.

3.2. Gross pathology

Virus infection affected development, feeding and behavior of the infected larvae. Patent infections developed in 2–4 days post inoculum (pi), and death occurred on 3–5 days pi. Patently infected UrsaNPV larvae had hypertrophied nuclei which were tightly packed with occlusion bodies, many exhibiting the unique “bow-tie” shape (Figs. 1–3). These nuclei appeared as white nodules in the posterior midgut and distal gastric caecum (Figs. 1 and 2). We did not observe infection in the cardia.

Midgut cells infected with UrsaCPV primarily localized to the gastric caecum and posterior stomach. UrsaCPV infections were normally characterized by an iridescent blue appearance, perhaps due to the size/arrangement of occlusion bodies. Sometimes, larvae thought to have only UrsaNPV would also be infected with UrsaCPV.

Table 1

<table>
<thead>
<tr>
<th>Year</th>
<th>Month</th>
<th>Number of collections made</th>
<th>Total larvae</th>
<th>UrsaNPV only</th>
<th>Dual UrsaNPV and UrsaCPV</th>
<th>UrsaCPV only</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>% Infection</td>
<td>% Infection</td>
<td>% Infection</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>N*</td>
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</tr>
<tr>
<td>2001</td>
<td>October</td>
<td>25</td>
<td>1642</td>
<td>4.2 ± 0.9</td>
<td>3.2 ± 1.0</td>
<td>10.8 ± 1.7</td>
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<tr>
<td></td>
<td>November</td>
<td>13</td>
<td>721</td>
<td>8.1 ± 1.9</td>
<td>7.1 ± 2.9</td>
<td>9.4 ± 0.8</td>
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<tr>
<td></td>
<td>December</td>
<td>3</td>
<td>99</td>
<td>2.0</td>
<td>2.0</td>
<td>3.0</td>
</tr>
<tr>
<td>2002</td>
<td>April–September</td>
<td>14</td>
<td>266</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>October</td>
<td>6</td>
<td>260</td>
<td>14.7 ± 3.5</td>
<td>45.6</td>
<td>7.5 ± 6.5</td>
</tr>
<tr>
<td></td>
<td>November</td>
<td>14</td>
<td>970</td>
<td>8.3 ± 4.4</td>
<td>8.8 ± 2.6</td>
<td>12.5 ± 5.5</td>
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<tr>
<td></td>
<td>December</td>
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<td>7</td>
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<td>0</td>
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<td>3665</td>
<td>24</td>
<td>16</td>
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</tr>
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</table>

N = number of collections with infected larvae.

Figs. 1–3. (1) Gross pathology of the ventral side of a fourth-instar UrsaNPV infected Uranotaenia sapphirina larva with visible hypertrophied nuclei in the midgut. (2) Dissected midgut from a fourth-instar UrsaNPV infected Uranotaenia sapphirina larva showing hypertrophied nuclei in the posterior midgut and distal gastric caecum. (3) Fresh smear of a UrsaNPV infected Ur. sapphirina larva with ruptured nuclei viewed with a phase-contrast microscope. Arrows point to numerous dumbbell shaped occlusion bodies.
3.3. Laboratory bioassays

UrsaNPV was successfully transmitted to *Ur. sapphirina* and *Ur. lowii* in the laboratory. In two paired tests, *U. sapphirina* exposed to UrsaNPV in deionized water or 10 mM MgCl₂, the addition of magnesium increased the infection rate by 12.9 ± 3.8%. In additional tests, UrsaNPV was transmitted to *Ur. lowii* (14.5 ± 9.2%, *n* = 11) in the presence of MgCl₂. Control larvae were not infected. None of the other mosquitoes exposed to UrsaNPV developed patent infections.

UrsaCPV was also transmitted to both species of *Ur. lowii* and *U. sapphirina* (9.2 ± 7.5%, *n* = 2 and 13.8 ± 6.1%, *n* = 6) respectively. Of the other mosquitoes tested, *Ae. aegypti* larvae were infected with UrsaCPV (7.8 ± 2.4%, *n* = 8) and *Cx. quinquefasciatus* were also infected with UrsaCPV (54.9 ± 12.5%, *n* = 2).

3.4. Ultrastructural studies

Electron microscopy of posterior midgut tissues revealed UrsaNPV infection in the nuclei of the epithelial cells (Figs. 4, 5) with all the nuclei approximately in the same stage of virogenesis. We did not observe infection in regenerative cells. Most of the epithelial cell nuclei were heavily infected and had developing or mature occlusion bodies. Virogenic stroma observed in some of the nuclei appeared as a loose granular material, located centrally and surrounded by virions at different stages of envelopment and occlusion. Also present were occlusion bodies of various size and maturation (Fig. 5). Empty capsids or nucleocapsids were located within the virogenic stroma with little or no evidence for nucleocapsid envelopment (Fig. 6). Occasionally, large parallel arrays of nucleocapsids and empty capsids were observed in virogenic stroma (Fig. 7). Nucleocapsids, up to 400–500 nm in length, were adjacent to shorter nucleocapsids, approximately 200 nm in length. Shorter nucleocapsids were uniform in size. Singly enveloped and non-enveloped nucleocapsids were scattered throughout the nucleus and some non-enveloped nucleocapsids were associated with the envelope precursors. These envelope precursors were spherical membranous structures (vesicles) found in the nuclei. Envelopment was initiated when either the ends or sides of nucleocapsids attached to the membrane of the vesicle (Figs. 8 and 9). Upon contact, the membrane surrounded the nucleocapsid until they were completely enveloped and some nucleocapsids moved to a position within the vesicles (Fig. 9).

A number of virions were found budding singly through the nuclear membranes and some were found in the cytoplasm. Virions budding through the nuclear membranes acquired these membranes and budded off as small vesicles containing virions (Figs. 10 and 11). These vesicles were observed only near the nuclei. Virions budding through the nuclear membranes were enveloped (Figs. 10 and 11), and among those found in the cytoplasm, some were enveloped and some were not (Figs. 11 and 13). The non-enveloped nucleocapsids found in the cytoplasm were located mostly in the part of the cell that faced the basal membrane (Fig. 13) while the enveloped ones did not seem to have any specific localization. We did not observe virions crossing plasma membranes towards neighboring cells, microvillar membranes, or the basal membrane.

At the initial stages of occlusion, virions were either occluded singly in small globular or pleomorphic occlusion bodies (OBs) or were directly incorporated into existing OBs (Fig. 14). Smaller OBs coalesced into roughly tetrahedral structures that fused producing final dumbbell, or bow-tie shaped OBs (Figs. 4, 12, and 14). Mature OBs were large, measuring up to 10–15 μm in length and 2–3 μm in diameter (Fig. 12). The OBs lacked the polyhedron envelope and the crystalline protein lattice characteristic of most NPVs. Each OB contained numerous rod-shaped virions. Each virion consisted of one nucleocapsid, an intermediate layer, and an outer envelope (Fig. 12 inset). Approximate lengths of virions and nucleocapsids were 200 and 130 nm, approximate diameters were 60 and 33 nm, respectively.

Ultrastructural studies of the posterior midgut of UrsaNPV infected *Ur. lowii* larvae (laboratory transmissions) showed viral infection in epithelial cell (Figs. 15 and 16). This was a dual UrsaNPV/UrsaCPV infection. Nuclear infection in *Ur. lowii* looked similar to what we found in the original viral host, *U. sapphirina*.

4. Discussion

UrsaNPV infection is lethal to *Uranotaenia* mosquitoes and kills the larvae within 3–5 days pi. The development and ultrastructural morphology of UrsaNPV from *U. sapphirina* were in general similar to those reported from other mosquito hosts (Federici, 1980; Federici and Lowe, 1972; Moser et al., 2001; Stiles et al., 1983). Virus development was tissue specific and was limited to the nuclei of the epithelial cells of gastric caeca and posterior portion of the midgut. No infection was observed in regenerative cells or the cardia. Virions were rod-shaped, contained one nucleocapsid and were similar in size to other mosquito NPVs. Formation of the parallel arrays of nucleocapsids of various lengths was similar to this process observed in *Oc. triseriatus* (Federici and Lowe, 1972), *Oc. sollicitans* (Federici, 1980), and *Cx. nigrispalpus* (Moser et al., 2001). Presence of short and long nucleocapsids, where the combined length of two short ones was approximately that of the long nucleocapsid was also similar to what was observed in *Oc. triseriatus* (Federici and Lowe, 1972), *Oc. sollic-
itans (Federici, 1980), in Oc. epactius (Stiles et al., 1983) and in Cx. nigripalpus (Moser et al., 2001). As suggested for Oc. sollicitans by Federici (1980), short nucleocapsids in Ur. sapphirina might be formed by cleavage of the long ones, and may be a characteristic feature of mosquito NPVs. The enveloping process was similar to the
one in *Oc. sollicitans* described by Federici (1980) and in *Oc. epactius* (Stiles et al., 1983) with a few differences. For example, the envelope precursors in *Ur. sapphirina* appeared as well defined spherical membranous vesicles while in *Oc. sollicitans* and *Oc. epactius* they were irregular ribbons or pleomorphic vesicles.
Virions of Ur. sapphirina had two phenotypes: budded virions (BVs), budding through the nuclear membranes, and occlusion derived virions (ODVs). Although we use the common term “budded virus”, the meaning is slightly different from what it stands for in lepidopteran hosts, where BVs refer to those virions that cross the plasma membrane. Budding of the virions through the nuclear membranes observed in Ur. sapphirina was generally similar to this process previously described in Cx. nigripalpus (Moser et al., 2001). Small differences could be due to the fact that in Cx. nigripalpus this process was described for the earlier stages of virogenesis, when occlusion of virions had not begun, while Ur. sapphirina material available for EM in this study was in the later stages of development. Virions of Ur. sapphirina budded singly through the nuclear membranes. They were enveloped in contrast to what was observed in Cx. nigripalpus (Moser et al., 2001) where the budded virions (budding singly and in groups) were naked nucleocapsids with no viral envelope. After budding through the nuclear membranes, the virions of Ur. sapphirina were surrounded by these membranes and budded off as vesicles containing virions. The nuclear membranes were probably dissolved soon after budding since these vesicles were observed only in the proximity of the nuclei and the majority of the virions found in the cytoplasm lacked these acquired membranes. Interestingly, some of the virions observed in the cytoplasm were naked nucleocapsids lacking the viral envelope. They were found primarily in the part of the cytoplasm that faced the basal membrane. We did not observe virions crossing plasma membranes toward neighboring cells, microvillar membranes, or the basal membrane. Similar results were found in Cx. nigripalpus (Moser et al., 2001). There is no evidence yet of lateral transmission by BVs within the midgut of mosquitoes, while in lepidopteran hosts this is a well documented event (Adams and McClintock, 1991). Thus, the mechanism by which the virus spreads from cell to cell in the mosquito midgut is not known.

ODVs were occluded only after envelopment was complete. They were occluded either singly by deposition of protein around individual virions or the virions attached to existing OBs. Formation of the OBs by attachment of the virions to existing pleomorphic occlusions and the coalescence of the OBs was similar to the process of occlusion body formation in Oc. sollicitans (Federici, 1980). This was different from that observed in Cx. nigripalpus where OBs seemed to form by condensation of protein around groups of virions without coalescence into larger OBs (Moser et al., 2001). The absence of a polyhedron envelope and crystalline protein structure in UrsaNPV are similar to other mosquito NPVs. The most distinguishing features of this virus were its large size and characteristic dumbbell or bow-tie shape exhibited by mature OBs.

Thus far, mosquito NPVs fall into two groups based on OB formation; those where small globular occlusion bodies are formed that do not coalesce (CuniNPV (Moser et al., 2001), WysmNPV (Hall and Fish, 1974)) and those that coalesce into larger OBs of various size and shape (UrsaNPV, OscoNPV (Federici, 1980)).

Transmission of UrsaNPV was enhanced by magnesium, similar to what was observed for CuniNPV, although the level of enhancement was much lower for UrsaNPV than for CuniNPV. We have no data on calcium effects on UrsaNPV transmission. We also have no evidence on the influence of a cypoviral infection on NPV transmission. A dual NPV/CPV infection was previously described in a different mosquito, Oc. sollicitans (Clark and Fukuda, 1971). In that study, the laboratory tests showed that the presence of CPV in the larvae may have predisposed them to NPV infections.

In conclusion, developmental and structural features of UrsaNPV were generally similar to other mosquito NPVs, with major differences in occlusion bodies shape and size. This study provides information on the natural history of UrsaNPV and conditions for its transmission, thus broadening our understanding of the biology mosquito NPVs and adding to the knowledge of NPVs in general.

Acknowledgments

The authors thank Dr. James Maruniak (University of Florida) for his helpful suggestions and comments on an earlier draft of the manuscript. We also appreciate the technical support of Greg Allen and Heather Furlong (USDA/ARS Gainesville).

References


