Infectivity and Pathogenicity of a Novel Baculovirus, CuniNPV from *Culex nigripalpus* (Diptera: Culicidae) for Thirteen Species and Four Genera of Mosquitoes

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ABSTRACT The infectivity and pathogenicity of newly discovered baculovirus, CuniNPV (family *Baculoviridae*, genus *Nulclepolyhedrovirus*) originally isolated from the mosquito *Culex nigripalpus* Theobald, was evaluated in laboratory bioassys against thirteen species and four genera of mosquitoes native to the northeastern U.S. Purified virus at a dosage rate of 1.6×10^7 occlusion bodies/ml with 10 mM Mg^{2+} added was used in exposures with second through fourth instars at temperatures ranging from 17 to 27°C. High infection rates and accompanying mortality were achieved in *Cx. pipiens* L. (83.0–14.4%), *Cx. pipiens* f. *molestus* (80.4% infection), and *Cx. salinarius* Coquillett (48.0–43.1%). *Cx. restuans* Theobald was also susceptible but infection rates were lower (21.3–12.5%). The gross pathology associated with infection was identical to that reported in *Cx. nigripalpus.* Infected larvae were lethargic and were often suspended at the water surface. Development of CuiNPV was observed in the nuclei of the midgut epitheial cells in the gastric caeca and posterior region of the stomach of host larvae. One hundred percent mortality was observed in all larvae that exhibited gross symptoms of infection within 4-d p.i. *Cx. territans* Walker (subgenus *Neoculex* Dyar) was the only *Culex* mosquito that was not susceptible. No infections were obtained with any species of *Aedes* [*Ae. vexans* (Meigen)], *Culiseta* [*Culiseta morsitans* (Theobald)] or *Ochlerotatus* [*Ochlerotatus canadensis* (Theobald), *Oc. cantator* (Coquillett), *Oc. communis* (De Geer), *Oc. excrucians* (Walker), *Oc. japonicus* (Theobald), *Ochlerotatus stimulans* (Walker), and *Ochlerotatus triseriatus* (Coquillett)]. The host range of CuniNPV appears to be restricted to *Culex* mosquitoes within the subgenus *Culex.* An inhibitory effect on transmission of CuniNPV was observed when a liver powder/Brewer's yeast mixture was used as a source of food reinforcing the critical role of Mg^{2+} and sensitivity of the infection process to the presence other divalent cations (Cu^{2+}, Fe^{2+}, and Zn^{2+}) in the larval medium that interfered with the infection process. The high infectivity and pathogenicity of CuniNPV for the principal vectors of West Nile virus in North America make CuniNPV an attractive candidate for future development as a biopesticide.

KEY WORDS CuniNPV, Baculoviridae, infectivity, mosquito, *Culex*

CuniNPV IS A NOVEL baculovirus (family *Baculoviridae*) that was recently isolated from a larval population of the mosquito *Culex nigripalpus* Theobald (Becnel et al. 2001, Moser et al. 2001). The virus has been tentatively placed within the genus *Nucleopolyhedrovirus* (NPV) but possesses a number of morphological, developmental and molecular characteristics that distinguish it from other NPVs and the closely related *Granulovirus* (GV) (Moser et al. 2001).

Development of CuiNPV is restricted to the nuclei of the midgut epitheial cells in the gastric caeca and posterior region of the stomach of host larvae (Moser et al. 2001). The virus has two virion phenotypes, an occluded form that initiates infection in the midgut epithethial cells, and a budded form that spreads the infection within the midgut. The occlusion bodies (OBs) are found exclusively within the nuclei of infected cells, but unlike NPVs from lepidopteran hosts, they are globular in shape, not polyhedral, and they lack the polyhedron envelope. The OBs of CuniNPV are similar in size to those of GVs (average 400 nm in diameter), but unlike GVs, they typically contain four to eight singly enveloped nucleocapsids (virions) rather then one or two. The OBs of other NPVs typically contain multiple virions.

Phylogenetic analysis of the amino acid sequences of p74 and DNA polymerase, place CuniNPV as a separate clade distinct from lepidopteran NPVs and GVs, suggesting that this virus may represent a new genus (Moser et al. 2001). Analysis of the complete genome sequence of CuniNPV (108,252 bp) (Afonso et al. 2001) also reveal striking differences in genome

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organization, a low level of conservation of homologous genes, and a lack of many genes conserved in other baculoviruses, further suggesting a large evolutionary distance between CuniNPV and lepidopteran baculoviruses.

Repeated and extended epizootics of CuniNPV have been documented in larval populations of *Cx. nigripalpus* (avg. = 20.1%) at a swine wastewater site in Florida, USA (Becnel et al. 2001). Natural infections have also been noted in cohabitating larval *Cx. quinquefasciatus* Say (avg. = 8.6%) and *Cx. salinarius* Coquillett (avg. = 15.5%). Laboratory bioassays (Becnel et al. 2001) with *Cx. quinquefasciatus* have conclusively shown that oral transmission of the virus to mosquito larvae is mediated by certain salts present in the swine pond water, most critically those containing the divalent cation Mg^{2+}. High levels of infection and associated pathology are achieved only when Mg^{2+} is present during the first 8–12 h of exposure. The discovery of the essential role of Mg^{2+} in facilitating the transmission of CuniNPV provides a unique opportunity to evaluate the infectivity and pathogenicity of this virus for other mosquitoes, especially *Culex* spp., and assess its potential as a biological control agent. In an initial assessment with selected species from the southeastern U.S., Becnel et al. (2001) found *Cx. quinquefasciatus* and *Cx. salinarius* to be susceptible to infection, consistent with the field observations, but found *Cx. restuans* Theobald, *Culiseta melanura* (Coquillett), *Ae. aegypti* (L.), *Ae. albopictus* (Skuse), *Anopheles albimanus* Weidemann, *An. quadrimaculatus* Say, *Oc. taeniorhynchus* (Weidemann), *Oc. triseriatus* (Coquillett), and *Toxorhynchites ambionensis* (Doleschall) to be refractory. In this investigation we now report on the infectivity and pathogenicity of CuniNPV to 13 species and four genera of mosquitoes native to the northeastern United States.

Materials and Methods

Virus Culture Production. The isolate of CuniNPV used in this study was obtained from field-collected *Cx. nigripalpus* larvae found at the original swine wastewater site in Gainesville, Alachua County, FL in 1997 (Becnel et al. 2001, Moser et al. 2001). An in vivo culture of the virus was established by amplifying field-collected virus from *Cx. nigripalpus* in a laboratory colony of *Cx. quinquefasciatus* (established mid-1980s, Alachua Co., FL) following the procedures of Becnel et al. (2001). Production was modified from the previous protocol by using purified virus as inoculum rather than whole infected larvae (larval equivalents). Moser et al. (2001) estimated the concentration of purified OBs spectrophotometrically by establishing a standard curve at an OD of 260 nm. Occlusion bodies were quantified initially with a Petroff-Hauser counting chamber and darkfield microscopy optics. The absorbance at OD_{260} was measured for different concentrations, and a regression analysis done on the data. The resulting regression equation was used to calculate the occlusion body concentration based on OD_{260}. Groups of ≈3000 3-d old (second instar) *Cx. quinquefasciatus* larvae were exposed at an estimated dose of 2×10^8 OBs per ml in 14 mM $MgSO_4$ and harvested 2-d postinoculation (p.i). These infected larvae were used as inoculum for virus culture maintenance and for obtaining purified virus for use in the laboratory bioassays.

Virus Purification. Viral OBs were purified from infected 5-d old (2 d p.i.) *Cx. quinquefasciatus* larvae as described by Moser et al. (2001). Larvae were ground with a Tekmar tissuemizer in 0.1% aqueous SDS and filtered though polyester to remove large parts. The filtrate was spun at 1,470 × *g* for 5 min and the supernatant filtered though a 5-μm hydrophobic filter. The filtrate was further purified by centrifugation on a continuous Ludox gradient or through 30% Ludox (16,320 × *g* for 30 min). The OBs banded at a density of ≈1.14–1.18 g/ml on the continuous gradient and pelleted in 30% Ludox (density of 1.09 g/ml). They were washed in 0.1 mM NaOH, pH 10.0 (2×) followed by deionized water (2×) and stored at 4°C. The concentration of OBs was estimated spectrophotometrically as described above.

Virus Standardization Bioassay. A standard exposure bioassay was conducted with purified virus against 3–4 d old *Cx. quinquefasciatus* larvae obtained from a laboratory colony to verify the infectivity of the virus and to determine the dose to be used in the exposure trials with the other mosquito species. In these initial trials, groups of 100 *Cx. quinquefasciatus* larvae were exposed to three estimated viral concentrations: 1.6×10^7 OBs/ml, 1.6×10^6 OBs/ml, and 1.6×10^5 OBs/ml. Trials were conducted at 27°C in 150 ml plastic cups containing 10 mM Mg^{2+}, 100 ml of water and 2 ml of 2% alfalfa and potbelly pig chow mixture (2:1) for food. Larvae were examined with a stereo microscope (400–600×) against a black background for signs of infection after 2-d p.i. Only those larvae with hypertrophied nuclei in midgut epithelial cells were scored as positive. Percent infection was determined for each exposure group at 2-d p.i., and the percent infection was evaluated by log probit analysis (Finney 1971). Groups without the addition of the virus served as controls.

Virus Host Range Bioassays. Thirteen mosquito species were evaluated for susceptibility to CuniNPV. Larval specimens for exposure were obtained from local field populations or established laboratory colonies as denoted: *Cx. pipiens* L. (laboratory colony, established 2000, New Haven, CT), *Cx. pipiens* f. *molestus* (autogenous laboratory colony, established 2001, New York, NY), *Cx. restuans* (field-collected eggs, New Haven, CT), *Cx. salinarius* (field-collected eggs, West Haven, CT), *Cx. territans* Walker (field-collected larvae, Chester, CT), *Culiseta morsitans* (Theobald) (field-collected larvae, Stafford, CT), *Ae. vexans* (Meigen) (field-collected larvae, Waterford, CT), *Ochlerotatus canadensis* (Theobald) (field-collected larvae, Hamden, CT), *Oc. cantator* (Coquillett) (field-collected larvae, Waterford, CT), *Oc. communis* (De Geer) (field-collected larvae, Barkhamsted, CT), *Oc. excrucians* (Walker) (field-collected larvae, Barkhamsted, CT), *Oc. japonicus* (Theobald) (field-

collected larvae, Kent, CT), *Ochlerotatus stimulans* (Walker) (field-collected larvae, Hamden, CT), and *Oc. triseriatus* (Coquillett) (laboratory colony, established mid-1990s, Waterford, CT). The identity and larval stage of the field-collected mosquitoes were determined using descriptions in Means (1979, 1987) and Vinogradova (2000). An exposure trial with laboratory colony *Cx. quinquefasciatus* was also conducted as a positive control. Purified virus at a dosage rate of 1.6×10^7 OBs/ml was used in all trials as described above. Exposures were conducted with second through fourth instars at temperatures ranging from 17 to 27°C depending on the species. The infectivity of the virus at each temperature was verified against second instar *Cx. pipiens* from the laboratory colony. All of the bioassays were carried out in 150-ml plastic cups containing 10 mM Mg^{2+}, 100 ml of distilled water and 2 ml of 2% alfalfa and potbelly pig chow mixture (2:1) for food. The number of larvae exposed in each trial was variable (24–127) depending on the availability of larval specimens. Larvae were examined microscopically as described previously for viral infection after 2 d, 3 d, and 4 d p.i., and only those larvae with hypertrophied nuclei in the midgut epithelial cells were scored as positive. Controls consisted of an equal number of larvae obtained from the same egg raft (*Culex* spp.) or larval sample (*Aedes*, *Culiseta*, and *Ochlerotatus* spp.) that were maintained under the same conditions with 10 mM Mg^{2+}, but were not exposed to the virus.

Impact of Food Type. The effect of food type on the infectivity of CuniNPV was additionally evaluated with *Cx. pipiens* and *Cx. quinquefasciatus.* Second instars were exposed to purified virus at a dosage rate of 1.6×10^7 OBs/ml in 150-ml plastic cups containing 10 mM Mg^{2+}, and 100 ml of distilled water as described in the previous bioassys. One group was fed 2 ml of a 2% alfalfa and potbelly pig chow mixture (2:1), while the other group was fed 2 ml of a 2% dried liver powder and Brewer's yeast mixture (3:2). Larvae were examined microscopically for signs of viral infection after 2-d p.i. and data were subjected to chi-square analysis (Jandel Corporation, 1995).

Results

Virus Standardization Bioassay. The results of the standard exposure bioassay conducted with purified virus against 3–4 d old *Cx. quinquefasciatus* larvae obtained from a laboratory colony are shown in Fig. 1. Percent infection was significantly correlated ($r^2 = 0.95$, $P < 0.001$) with dosage. Over 80% infection was achieved at an estimated viral concentration of 1.6×10^7 OB's/ml, while mean percent infection was 62.2% at 1.6×10^6 OBs/ml, and 27.5% at 1.6×10^5 OBs/ml.

Virus Host Range Bioassays. Results of the experimental bioassays are summarized in Table 1. CuniNPV was transmitted to *Cx. pipiens*, *Cx. pipiens* f. *molestus*, *Cx. restuans*, and *Cx. salinarius.* The pathology associated with infection was identical to that reported in *Cx. quinquefasciatus* and *Cx. nigripalpus.* Infected larvae were noticeably lethargic and were often suspended at the water surface. The nuclei of cells located in the proximal region of the gastric caeca, and the posterior portion of the stomach appeared opaque to white in color because of the proliferation of OBs when viewed against a black background (Fig. 2). One hundred percent mortality was observed in all larvae that exhibited gross symptoms of infection in the midgut and all infected larvae died within 4-d p.i.

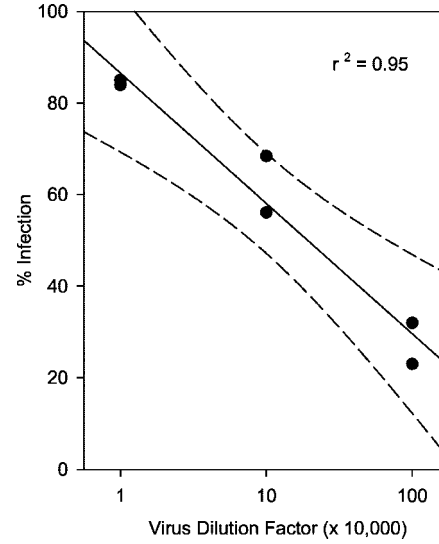

Fig. 1. Results of 2-d post inoculation standard bioassay of CuniNPV against second instar *Cx. quinquefasciatus* showing 99% CL. Estimated undiluted virus concentration = 1.6×10^{11} OBs/ml.

Although there was some variation in the individual tests, the overall infection rates achieved in *Cx. pipiens* (range, 83.0% to 14.4%, mean = 50.1%, $n = 7$) and *Cx. pipiens* f. *molestus* (80.4%, $n = 1$) were comparable to those obtained in the standard bioassay with *Cx. quinquefasciatus.* However, in all instances where larvae of *Cx. pipiens* were examined for infection at daily intervals, maximum infection rates were not obtained until 4-d p.i. Fourth instar *Cx. pipiens* were also found to be susceptible (75.0% and 26.0% infection in two trials), as were larvae that were exposed to CuniNPV in cooler water temperatures of 17°C (78.6%) and 20°C (26.0%).

Infection rates obtained with *Cx. restuans* were notably lower; maximum infection rates of 21.3%, 20.0%, and 12.5% were achieved in three successive trials with second instars. The maximum infection rate observed in a single feeding trial with *Cx. salinarius* was 48.0%. No CuniNPV infections were achieved in bioassays with *Cx. territans*, *Cs. melanura*, *Ae. vexans*, *Oc. canadensis*, *Oc. cantator*, *Oc. communis*, *Oc. excrucians*, *Oc. japonicus*, *Oc. stimulans*, or *Oc. triseriatus.* No infections or unusual mortality were observed in any field-collected or laboratory-reared control larvae that were not exposed to the virus.

Table 1. Results of bioassays experiments showing susceptibility of 14 mosquito species exposed to 1.6×10^7 OBs/ml of CuniNPV virus in 10 mM $MgCl_2$

Species	Date	Source[a]	Exposure group			Cumulative % infection (days postinoculation)		
			No.	Stage[b]	Temp.	2	3	4
Cx. pipiens	2/13	L	97	L_2	27°C	13.4	—	14.4
	3/11	L	99	L_4	27°C	6.0	10.0	26.0
	3/18	L	88	L_2	27°C	23.6	26.8	48.0
	3/18	L	127	L_4	27°C	28.4	40.9	75.0
	3/28	L	103	L_2	17°C	—	75.7	78.6
	5/23	L	100	L_2	20°C	—	—	26.0
	7/10	F_e	100	L_2	27°C	76.0	—	83.0
Cx. pipiens f. *molestus*	2/26	L	92	L_2	27°C	80.4	—	—
Cx. quinquefasciatus	2/7	L	193	L_2	27°C	84.5	—	—
Cx. restuans	4/17	F_e	96	L_2	27°C	12.5	12.5	12.5
	7/15	F_e	75	L_2	27°C	8.0	18.7	21.3
	7/22	F_e	100	L_2	27°C	3.0	11.0	20.0
Cx. salinarius	7/10	F_e	102	L_2	27°C	43.1	47.1	48.0
Cx. territans	7/10	F_1	100	L_2	27°C	0	0	0
Cs. morsitans	5/23	F_1	63	L_2	20°C	0	0	0
Ae. vexans	4/12	F_1	66	L_2	17°C	0	0	0
Oc. canadensis	3/18	F_1	99	L_2	27°C	0	0	0
	4/12	F_1	24	L_2	17°C	0	0	0
Oc. cantator	4/12	F_1	100	L_2	17°C	0	0	0
Oc. communis	4/12	F_1	100	L_2	17°C	0	0	0
Oc. excrucians	3/26	F_1	101	L_2	17°C	0	0	0
Oc. japonicus	4/5	F_1	100	L_2	17°C	0	0	0
Oc. stimulans	4/5	F_1	100	L_2	17°C	0	0	0
Oc. triseriatus	2/15	L	100	L_2	27°C	0	0	0

[a] L = lab colony, F_e = field-collected eggs, F_1 = field-collected larvae
[b] $L_{2,4}$ = Larval instar.

Impact of Food Type. An inhibitory effect on transmission of CuniNPV was observed when a liver powder/Brewer's yeast mixture was used a source of food (Table 2). In concurrent bioassays with second instar *Cx. pipiens* and *Cx. quinquefasciatus*, significantly ($P < 0.001$) lower infection rates were achieved in larvae fed the liver powder/Brewer's yeast mixture (7.8% and 5.0%, respectfully) when compared with the standard alfalfa/hog chow mixture (91.0% and 100%, respectfully).

Discussion

In this report we have unequivocally demonstrated infectivity and pathogenicity of CuniNPV in the presence of Mg^{2+}, for *Cx. pipiens*, *Cx. pipiens* f. *molestus*, and *Cx. restuans*, and reaffirmed the susceptibility of *Cx. salinarius*. We have also demonstrated infectivity of CuniNPV to fourth instars, transmission to second instars at temperatures as low as 17°C, and 100% mortality in all larvae that exhibit discernible infections in

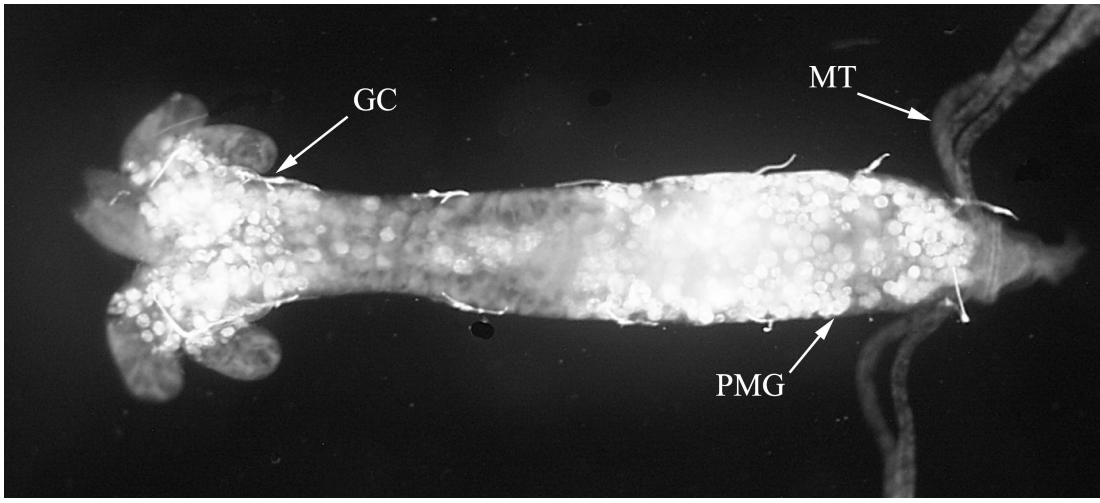

Fig. 2. Dissected midgut from a third instar *Cx. pipiens* showing CuniNPV infection in the gastric caeca (GC) and posterior portion of the stomach (PMG). MT, Malpighian tubules.

Table 2. Effect of larval food on the susceptibility of second instar *Cx. pipiens* and *Cx. quinquefasciatus* to CuniNPV virus in 10 mM $MgCl_2$ at an estimated dosage rate of 1.6×10^7 OBs/ml

Species	Larval food			
	Alfalfa/hog chow		Liver/yeast	
	No.	% infection	No.	% infection
Cx. pipiens	100	91.0	102	7.8[a]
Cx. quinquefasciatus	102	100	100	5.0[a]

[a] Significantly lower than % infection with alfalfa/hog chow by Chi-square analysis ($P < 0.001$).

the midgut. This now brings to five the number of mosquito species that have exhibited susceptibility to this virus. The others include *Cx. nigripalpus* (original host) and *Cx. quinquefasciatus* (Becnel et al. 2001). All five susceptible species belong to the subgenus *Culex* L (Darsie and Ward 1981).

Culex territans, a member of the subgenus *Neoculex* Dyar, was the only *Culex* mosquito that we were not able to infect in the bioassays. No infections were similarly obtained with any species of *Aedes* (*Ae. vexans*), *Culiseta* (*Cs. morsitans*), or *Ochlerotatus* (*Oc. canadensis*, *Oc. cantator*, *Oc. communis*, *Oc. excrucians*, *Oc. japonicus*, *Oc. stimulans*, or *Oc. triseriatus*). The latter observations were in agreement with the previous findings of Becnel et al. (2001) who reported species of *Aedes*, *Anopheles*, *Culiseta*, *Ochlerotatus*, and *Toxorhynchites* from the southeastern U.S. to be refractory. Although further testing with other species, especially those in the *Culex* subgenera *Neoculex*, *Melanoconium* Theobald and *Tinolestes* Coquillett should be conducted, it appears that susceptibility to CuniNPV is restricted to *Culex* mosquitoes, and quite likely, species within the subgenus *Culex*.

The consistently lower levels of infection achieved in repeated bioassays with *Cx. restuans* indicate that this species is not as susceptible to CuniNPV as are *Cx. pipiens*, *Cx. quinquefasciatus*, and *Cx. salinarius*. However, once infected, equivalent pathology and mortality ensues. These observations suggest that the reduced susceptibility of this species is likely associated with initial viral entry into the midgut epithelial cells. Further bioassays with higher concentrations of Mg^{2+} within the tolerance level of *Cx. restuans*, should be explored as one possible means for increasing the infectivity of CuniNPV for this species. We further note that our results with *Cx. restuans* differ from those of Becnel et al. (2001) who were not able to infect field-collected second instar *Cx. restuans* larvae from Florida in a standard 2 d p.i. bioassay with nonpurified virus. The reasons for this discrepancy are unclear, as we do not know if the apparent difference in susceptibility reflects a difference in the midgut physiology of these two geographically separated populations, or is a result of some other factor.

The inhibitory effect on the infectivity of CuniNPV for two highly susceptible species, *Cx. quinquefasciatus* and *Cx. pipiens* when a liver powder/Brewer's yeast suspension was used as a source of food was initially enigmatic, but wholly consistent with the observations of Becnel et al. (2001), who reported 100% inhibition of transmission with the addition of the divalent cations Ca^{2+} (5 mM), Cu^{2+} (0.1 mM), Fe^{2+} (1 mM), and/or Zn^{2+} (0.5 mM) in the presence of 10 mM Mg^{2+}. Analysis of the desiccated liver powder (Miller, 2003.) reveals high concentrations of each of the aforementioned minerals that undoubtedly interfere with the infection process. These findings reinforce the critical role of Mg^{2+} and sensitivity of the infection process to the presence other divalent cations in the larval medium. The latter has important implications that could potentially negate the infectivity of CuniNPV to susceptible mosquito larvae, even in the presence of high concentrations of Mg^{2+}.

The recent introduction (Anderson et al. 1999, Lanciotti et al. 1999) and unprecedented spread of West Nile (WN) virus (family *Flaviviridae*, genus *Flavivirus*) in North America (CDC 2002a; 2002b), and recognition of the role of *Culex* mosquitoes in enzootic maintenance and epizootic amplification of this virus (Andreadis et al. 2001, Bernard and Kramer 2001, Kulasekera et al. 2001, White et al. 2001), have drawn increased attention to the control of *Culex* mosquitoes (Gubler et al. 2000). Current control strategies in urban and suburban locales have relied heavily on the use of larvicides, most notably the biopesticides *Bacillus sphaericus* Neide and to a lesser degree *Bacillus thuringiensis* subsp. *israelensis* de Barjac, and the growth regulator methoprene. However, environmental concerns (real or perceived) with the effect of methoprene on nontarget aquatic organisms and recent reports of resistance in field populations of *Cx. pipiens* and *Cx. quinquefasciatus* to *B. sphaericus* (Rao et al. 1995, Silva-Filha et al. 1995, Yuan et al. 2000, Sinegre et al. 1994) indicate a need to examine additional products. The high infectivity and pathogenicity of CuniNPV for the principal vectors of WN virus in North America (*Cx. pipiens*, *Cx. quinquefasciatus*, *Cx. nigripalpus*, *Cx. salinarius*) make CuniNPV an attractive candidate for future development as a biopesticide. However, it is clear that any formulation of this product will require a sufficient concentration of Mg^{2+} that will ensue its efficacy in organically polluted water habitats (e.g., storm drains. catch basins) typical for *Culex* species.

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