PATHOGENICITY AND INFECTIVITY OF THE THAI-STRAIN DENSOVIRUS (*ATH*DNV) IN *ANOPHELES MINIMUS S.L.*

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Abstract. We report the first experimental infection of an anopheline, *An. minimus s.l.*, with the Thai-strain densovirus, *ATh*DNV. Two hundred first-instar larvae were raised in 2 virus concentrations; mortality in the low virus concentration was no different than that in controls (9.5% *vs* 7.5%, respectively). Mortality in the high virus concentraion was 17.5%. Among surviving adults, infection rates were 33.3% (low concentration) and 15.5% (high concentration), as judged by PCR-screening. Infection rates did not differ between males and females. All orally-infected females transmitted densovirus to at least some of their offspring. Vertical transmission rates ranged from 25.0-53.8%. Densovirus infection did not appear to affect fecundity, either in the number of eggs laid or the number of eggs hatched.

INTRODUCTION

Insect densoviruses (DNV) are small autonomous non-enveloped DNA viruses that belong to the family Parvoviridae and infect only arthropods. Most mosquito densoviruses are in the genus *Brevidensovirus*, characterized by having a 4kb genome, encoding all of their proteins on the same strand, and infecting all tissues of their insect hosts (Afanasiev *et al,* 1991; Bergoin and Tijssen 2000).

The first known mosquito densovirus, *Ae*DNV, was isolated from *Aedes aegypti* larvae, and infects members of the genera *Aedes*, *Culex* and *Culiseta* (Lebedeva *et al,* 1973; Buchatsky 1989; Afanasiev *et al,* 1991). The second described mosquito densovirus, *Aa*PV, was recovered from an *Ae. albopictus* (C6/36) cell line (Jousset *et al,* 1993; Boublik *et al,* 1994a). Other mosquito densoviruses have since been isolated from cell lines or laboratory strains of species of *Aedes*, *Toxorhynchites*, *Haemagogus* and *Culex*. Genomic sequence data have been obtained for all of these isolates (Gorziglia *et al,* 1980; O'Neill *et al,* 1995; Kittayapong *et al,* 1999; Jousset *et al*, 2000).

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Natural densovirus infection has been reported in *Ae. aegypti* (Afanasiev *et al,* 1991; Kittayapong *et al,* 1999), *Ae. albopictus* (Boublik *et al,* 1994a) and *Anopheles minimus* (Rwegoshora *et al,* 2000). The virus is believed to be maintained primarily through horizontal transmission in larval habitats. The life cycle of the virus is not well-studied, and vertical and venereal transmission may play a role in its maintenance (Barreau *et al,* 1997; Kittayapong *et al,* 1999).

Mosquito densoviruses are often pathogenic to their arthropod hosts. *Ae*DNV was found to efficiently kill larval *Ae. aegypti*, *Ae. caspius*, and to a lesser extent, *Culex pipiens* (Buchatsky, 1989). *Aa*PV was highly pathogenic for *Ae. aegypti*, killing up to 95% of larvae after oral infection (Barreau *et al,* 1996). A Thai-strain densovirus, *ATh*DNV, killed up to 80% of *Ae. albopictus* and 50% of *Ae. aegypti* larvae that were reared in the virus (Kittayapong *et al,* 1999). In addition to being pathogenic, mosquito densovirus can be efficiently transmitted to the offspring of infected adults. *Ae. aegypti*, after oral infection with *ATh*DNV, transmitted densovirus to 57.5% of their G_1 surviving offspring (Kittayapong *et al,* 1999).

Biological control of disease vectors, such as mosquitos, has been given priority as an approach to complement existing methods of control, largely because of its safety and relatively low cost. Because of pathogenicity to their hosts, mosquito densoviruses may be used as potential

biological control agents to reduce mosquito populations. In addition, their infectiousness and the small size of their genomes make them ideal candidates to deliver and express genes to reduce the vectorial capacity of mosquitos (Afanasiev *et al,* 1999, 2000; Ward *et al,* 2001).

We report on the infectivity, pathogenicity, and transmission of *ATh*DNV for *An. minimus s.l.*, an important vector of malaria in Thailand.

MATERIALS AND METHODS

Source of densovirus

Infected larvae from a colony of *Aedes aegypti* were used as a source of densovirus for our experiments. The colony was founded in 1993 from adult mosquitos collected from Chachoengsao Province, and was infected with the Thai-strain densovirus *ATh*DNV. It is not known how the colony first acquired its infection.

Ae. aegypti larvae were confirmed infected by PCR-screening of larval heads. They were immersed in 1% sodium hypochlorite for 20 minutes with intermittent agitation, rinsed in distilled water to remove possible virus particles on their body surface, then ground and added to the rearing trays as a viral source. Two concentrations of virus were prepared: low, consisting of 50 infected larvae, and high, consisting of approximately 500 infected larvae.

Mosquitos used in experimental infection studies

The *An. minimus s.l.* mosquitos used in the laboratory experiments were obtained from an uninfected laboratory colony at the Armed Forces Research Institute for Medical Sciences (AFRIMS) in Bangkok, Thailand. The colony had been originally collected from Tak Province and had been maintained in the laboratory for 26 generations. The colony was confirmed uninfected by PCR screening of adult mosquitos.

Virus detection using PCR

Mosquitos were screened with a PCR assay using primers designed from the densovirus ORF3 (O'Neill *et al,* 1995). Crude DNA extractions were performed by homogenizing an individual adult or larval mosquito in 100 µl of STE buffer, using the methods of O'Neill *et al* (1992). One microliter of the supernatant was used as a DNA template in the PCR reaction. Densovirus-infected *Ae. albopictus* C6/36 cell line DNA was used as a positive control.

Viral DNA was PCR amplified in 20 µl reaction volumes: 2 µl 10x buffer (Promega), 2 µl 25 mM $MgCl_2$, 0.5 µl dNTP's (10 mM each), 0.5 µl of each primer (20 mM each) and 1 unit of *Taq* DNA polymerase (Promega). The PCR thermal profile was 95°C at 1 minute, 50°C at 1 minute and 72°C at 1 minute per cycle for 35 cycles. PCR products were run on a 1% agarose gel with a 1 kb ladder (Gibco) to determine the presence and size of amplified DNA. Samples that yielded products of the expected size (350bp) were scored as positive.

Virus infectivity and pathogenicty

Anopheles minimus s.l. larvae from the laboratory-raised uninfected colony were used to determine the infectivity and pathogenicity of densovirus. Two replicates, each with 100 first-instar larvae (24 hour old), were used to test each viral concentration (low and high). Larvae were placed in autoclaved rearing trays with one liter of distilled water; virus preparation was added to the rearing trays. In addition, two pools of 100 first-instar larvae, raised under the same conditions but without the virus preparation, were used as controls.

All trays were maintained in the insectary at 26°-28°C at 70-80% relative humidity. Larvae were fed daily on autoclaved ground fish food until pupation. Dead larvae were removed daily and preserved at -20°C for later confirmation of densovirus infection. Pupae from each rearing tray were transferred to glass beakers in separate 30 cm^3 cages.

Emerged adult mosquitos from the larvae reared in low virus concentration served as the parental generation (G_0) for subsequent experiments. We refer to all mosquitos used in all our experiments relative to this parental generation.

Vertical transmission of densovirus

To determine the rate of vertical transmission, G_1 progeny were obtained from the mosquitos that had been reared in the low virus concentration. Infected adult G_0 females were bloodfed and force-mated with males from the same generation, then placed in individual vials with moist paper and a well-ventilated cap. After oviposition, G_0 females were PCR-tested to confirm densovirus infection; G_0 males were not

tested. G_1 eggs from each densovirus-positive female were hatched and reared separately to adulthood under insectary conditions, then PCR tested for the presence of densovirus

Effect of densovirus on fecundity

To determine the effect of densovirus infection on mosquito fecundity, eggs were obtained from infected G_0 females in the manner described for the vertical transmission experiments. After ovipositon, parent females were PCR-tested for densovirus infection. Eggs were counted and immersed in water; larvae were counted five days later. Uninfected females from the original *An. minimus s.l.* colony were treated identically and served as controls.

RESULTS

Virus infectivity and pathogenicity

Larval mortality in *An. minimus s.l.* raised in the low viral concentration was 9.5% (19/200); and in *An. minimus s.l.* raised in the high viral concentration was 57.5% (115/200) (Table1). Larval mortality in uninfected controls (15/200) was not significantly different from that in mosquitos raised in the low virus concentration (χ^2 = 0.51, df = 1, p = 0.473). Among those larvae that died, infection frequency was significantly greater in those raised in the high viral concentration (21/25) than in those raised in the low viral concentration (9/17) (χ^2 = 4.78, df = 1, p=0.029).

Infection rates in surviving adults were higher in mosquitos raised in the low viral concentration (14/42 = 33.3%) than in those raised in the high viral concentration (5/32 = 15.6%); that difference was bordering on statitiscal significance (χ^2 = 2.98, df = 1, p = 0.084). Infection rates in surviving adult males were not significantly different from those in adult females, for both virus concentrations (low: χ^2 = 0.20, df = 1, p=0.657; high: χ^2 =0.11, df = 1, p = 0.737).

Vertical transmission of densovirus infection

A total of 57 adult G_1 offspring, obtained from 5 densovirus-positive females that had been raised in the low virus concentration, were PCR-tested for densovirus (Table 2). All 5 females transmitted densovirus to at least some of their adult offspring. Filial infection rates ranged from

Table 1
Oral infectivity and pathogencity of *ATh*DNV for first instar *An. minimus*, as measured by positive PCR amplification.

	Virus concentration		
	None (control) (%)	Low (%)	High (%)
Total first instar larvae	200	200	200
Total dead larvae	15/200 (7.5)	19/200 (9.5)	115/200 (57.5)
Infected dead larvae	0/15 (0)	9/17 (52.9)	21/25 (84.0)
Infected surviving adults	0/34 (0)	14/42 (33.3)	5/32 (15.6)
Infected surviving males	0/17 (0)	5/17 (29.4)	2/15 (13.3)
Infected surviving females	0/17 (0)	9/25 (36.0)	3/17 (17.6)

Table 2
Vertical transmission of *ATh*DNV to G_1 progeny of orally-infected *An. minimus* females, raised in a low concentration of the virus.

Parent female	Infected female progeny (%)	Infected male progeny (%)	Total infected progeny (%)
1	4/9 (44.4)	1/4 (25.0)	5/13 (38.5)
2	5/8 (62.5)	3/7 (42.9)	8/15 (53.3)
3	1/7 (14.3)	2/5 (40.0)	3/12 (25.0)
4	6/10 (60.0)	1/3 (33.3)	7/13 (53.8)
5	2/4 (50.0)	-	2/4 (50.0)
Total	18/38 (47.4)	7/19 (36.8)	25/57 (43.9)

Table 3
Effect of *ATh*DNV on *An. minimus s.l.* fecundity.

	Infected	Uninfected
Number of parent females	18	19
Total eggs laid	1,033	1,075
Total larvae hatched	873	924
Mean number of live offspring	48.5	48.6
Live offspring/female (range)	11-82	11-84

25.0% (3/12) to 53.8% (7/13). Overall infection rates did not differ between males (7/19 = 36.8%) and females (18/38 = 47.4%) ($\chi^2 = 0.57$, df = 1, p = 0.450).

Effect of densovirus infection on mosquito fecundity

Eighteen infected *An. minimus s.l.* females, that had been raised in the low virus concentration, laid 1,033 eggs. Of those, 873 (84.5%) hatched, averaging 48.5 live offspring per female (Table 4). Nineteen uninfected *An. minimus s.l.* females laid 1,075 eggs, of which 924 (86.0%) hatched (48.6 live offspring per female). Overall, the number of live offspring was not signficantly different between infected and uninfected females ($\chi^2 = 0.871$, df = 1, p = 0.351).

DISCUSSION

Our results demonstrate that *An. minimus s.l.* is susceptible to oral infection with the Thai-strain densovirus, *ATh*DNV. The high virus concentration was more pathogenic, killing 57.5% of larvae, compared with 9.5% of larvae killed in the low virus concentration. Mortality rates among mosquitos raised in the low virus concentration were no higher than in controls. However, infection rates in surviving adult mosquitos raised in the low virus concentration were double that of those raised in the high virus concentration (33% *vs* 15.6%, respectively). Thus, there appears to be an inverse correlation between virus pathogenicity and transmission to surviving adults. This is consistent with the findings of Kittayapong *et al* (1999), who reported *ATh*DNV to be more pathogenic for *Ae. albopictus* than *Ae. aegypti*, but for infection rates among surviving adults were much higher in *Ae. aegypti* than in *Ae. albopictus* (80% *vs* 33%, respectively).

Our result suggests that *An. minimus* is not as susceptible as *Ae. aegypti* to oral infection with *ATh*DNV, nor is the virus as pathogenic for *An. minimus* as for *Ae. aegypti* or *Ae. albopictus*. Kittayapong *et al* (1999) found a mortality of 51% in *Ae. aegypti* and 82% in *Ae. albopictus*, after oral infection of first instar larvae, with a preparation made from 20 *ATh*DNV-infected larvae. Our preparation of low virus concentration was made from 50 infected larvae. However, neither our study nor that of Kittayapong *et al* (1999) estimated the viral titer, thus the differences in mortality and susceptiblity may be a result of differences in inoculating doses.

All orally-infected females that we tested passed the virus on to some of their offspring Filial infection rates in surviving adult offspring ranged from 25% to 53.8%, with no differences between males and females. Kittaypong *et al* (1999) reported somewhat higher vertical transmission rates, between 54.7% to 59.8%, for *Ae. aegypti* females that had been naturally infected with *ATh*DNV. Vertical transmission has been reported with other mosquito densoviruses. O'Neill *et al* (1995) found *He*DNV to be vertically transmitted to 20% of adult offspring of experimentally infected *Ae. albopictus*. Barreau *et al* (1997) found that after experimental infection with *Aa*PV, *Ae. aegypti* females with a high virus titer transmitted the virus to 5-7% of their surviving offspring. Thus, the Thai-strain densovirus may have a higher rate of vertical transmission than other densovirus strains, and vertical transmission may play a larger role in its maintenance in nature.

Infection with *ATh*DNV did not appear to influence *An. minimus* fecundity, in terms of number of eggs laid or number hatched. Barreau *et al* (1997) found that *Ae. aegypti* infected with *Aa*PV laid as many eggs as uninfected females. However, in contrast to our findings, infection was associated with a decreas in egg hatching.

Natural infection of *An. minimus s.l.* with *ATh*DNV has been reported, with infection rates in adult females ranging between 0-75% depending on collection month and location, and averaging 15%. Our results suggest that natural infection in *An. minimus* may be maintained by both horizontal and vertical transmission of the virus. The pathogenicity of *ATh*DNV, particularly at high virus concentrations, indicates that it may provide a possible means of biological control of

An. minimus s.l. Furthermore, our results on the vertical transmission of *ATh*DNV suggest it may serve as a useful mechanism to introduce and express genes in some *Anopheles* species.

ACKNOWLEDGEMENTS

The authors wish to thank: Ms Kathy Baisley, for editing and correcting the manuscript and for statistical assistance; Dr Ken J Linthicum of the Armed Forces Research Institute for Medical Sciences (AFRIMS), Bangkok, Thailand, for providing us with the space in his laboratory, equipment and mosquitos used in the experiments; Mr Kiti Theinthong and Ms Samnieng Planuson of the Department of Biology, Mahidol University, for their technical assistance. This study received financial support from the Swiss Development Co-operation through the National Institute for Medical Research, Tanzania, and Thailand Research Fund (RSA/08/2537) and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (ID-940196) for which we are most grateful.

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