Isolation and Characterization of Densonucleosis Virus from *Aedes aegypti* Mosquitoes and Its Distribution in India

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Key Words

Densonucleosis virus · Mosquitoes · *Aedes aegypti* ·
Phylogenetic analysis

Abstract

Objectives: Mosquito densonucleosis viruses (DNVs) are known to persistently infect the insect cell line and mosquito population in nature, causing mortality in mosquitoes. Here we report the isolation and characterization of a DNV from *Aedes aegypti* and its distribution among different *Ae. aegypti* populations from India. **Methods:** We screened *Ae. aegypti* mosquito populations from different states of India by PCR. Virus isolation and purification was performed using a cesium chloride gradient from a positive mosquito colony. Characterization of this isolate was carried out by electron microscopy, Western blot and sequencing. **Results:** Electron microscopy showed the presence of parvovirus-like particles, and Western blot showed the presence of 2 viral proteins of 40 and 41 kDa. A total of 3,776 bases of genome were sequenced, which included a 3'UTR of 128 bases, a coding region of 3,507 bases and a 5'UTR of 141 bases. Three open reading frames (ORFs) were identified and characterized. The NIVDNV genome showed 95% similarity with *Culex pipiens pallens* DNV and 93% similarity with *Ae. aegypti* DNV.

Conclusion: Phylogenetic analysis of all 3 ORFs showed that this new isolate falls in the lineage of Brevidensovirus along with other mosquito DNVs.

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Introduction

Densonucleosis viruses (DNVs) are the etiological agents of the insect disease known as densonucleosis [1] and belong to the family Parvoviridae. They infect and replicate only within insects. Densonucleosis disease leads to either death or the loss of vital functions in all stages of infected organisms. Densonucleosis was first discovered in the caterpillar *Galleria mellonella*, resulting in the isolation of *G. mellonella* DNV. Since then, DNVs have been isolated from a wide range of economically or medically important insects including the orders Lepidoptera, Diptera, Orthoptera and Dictyoptera. The first mosquito densovirus (MDV) was isolated from an infected laboratory colony of *Aedes aegypti* and was named *Ae. aegypti* DNV (AeDNV) [2]. DNVs have also been isolated from other mosquitoes such as *Ae. albopictus* [3], *Culex pipiens* [4], *Toxorhynchites splendens* [5] and *Anopheles minimus* [6] as well as from mosquito cell lines [7].

DNVs are single-stranded linear DNA viruses belonging to the genus *Brevidensovirus* of the subfamily *Densovirinae*. Each DNV virion packages either a plus or a minus DNA strand. The virion appears as an icosahedral, nonenveloped particle with a diameter of 18–26 nm. Sequence analysis and comparison showed that the nucleotide and amino acid sequences of *Ae. albopictus* C6/36 cell DNV (C6/36DNV) are highly homologous to the AeDNV genome, but the genomic organization and some repeating sequences of C6/36DNV are more closely related to those of *Ae. albopictus* DNV (AalDNV) [8].

Both AeDNV and AalDNV are highly pathogenic for mosquito cell lines and all mosquito tissues, killing up to 100% of early-stage larvae [9, 10]. However, the rate of mortality decreases if the virus infects during the later stages of larval development, as the larvae can survive infection and develop into adults. Infected mosquitoes can also transmit the virus vertically to the next generation [9, 11, 12].

DNVs have recently gained a lot of scientific interest because of their potential as a system for delivering foreign genes to mosquitoes and for the biological control of the activity of mosquitoes. It has also been reported that the infection of *Aedes* mosquitoes with DNV greatly reduces their susceptibility to dengue virus [13, 14]. With the growing threat of emerging viruses, especially arboviruses, the role played by such endogenously infected viruses in vector dynamics and transmission constitutes a need-of-the-hour research question.

During the recent outbreak of chikungunya virus in India, mosquitoes were received by our laboratory from various states in India for the detection of arboviruses. Further investigations showed the presence of DNV in certain populations. The present communication reports the first isolation and molecular characterization of a DNV from India and its distribution among various *Ae. aegypti* mosquito populations across different states.

Materials and Methods

Development of the DNV-Infected Colony of Ae. aegypti

The *Ae. aegypti* mosquito colony used for these experiments was from an insectary maintained at this institute for the last 25 years. The mosquitoes were maintained at $28 \pm 2^\circ$ and a relative humidity of 70–80%. The larvae were fed on yeast tablets and dog biscuits mixed in the proportion of 70 and 30%, respectively. The adults were reared in cages and were fed on 10% glucose. Females were fed with chicken blood meal every third day to obtain eggs.

Ae. aegypti mosquitoes caught in the field were screened for DNV infection by polymerase chain reaction (PCR) [14]. The mosquitoes found to be infected with DNV were triturated in

phosphate-buffered saline (PBS) and centrifuged at 5,000 rpm to remove the cell debris, and the supernatant was added to the uninfected larval pan containing the first-instar *Ae. aegypti* larvae. When these larvae reached the fourth instar, they were tested for DNV infection using the protocol described by Wei et al. [14]. This process was repeated to generate the DNV-infected mosquito colony.

Isolation and Purification of DNV

Approximately 10,000 fourth-instar larvae from the infected colony were triturated in 150 ml of PBS using a grinder (Fisher Scientific). The slurry was centrifuged at 10,000 rpm for 30 min to remove the cell debris. The supernatant was divided into 1-ml aliquots and stored at -80° . This was used as the viral stock throughout the study. Four milliliters of the virus stock was filtered using 0.22- μ m filters. It was then centrifuged at 25,000 rpm for 2 h at 4° in an ultracentrifuge on a cushion of 30% glycerol in NTE buffer (NaCl 100 mM, Tris HCl 10 mM, EDTA 4 mM, pH 7.4). The pellet obtained was dissolved in 1 ml of PBS and was centrifuged at 40,000 rpm for 4 h at 4° on a 30–60% discontinuous cesium chloride (CsCl) gradient. Each fraction was separated and analyzed by transmission electron microscopy, PCR and sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE).

Raising Immune Sera and Purification of IgG

The purified fraction of the DNV was used to immunize adult Swiss albino mice via the intraperitoneal route. Three booster doses were given at intervals of 1 week. One week after the final dose, blood from immunized mice was collected by orbital bleeding and immune sera were separated. The immune sera were adsorbed against fresh *Ae. aegypti* larvae from the normal colony to remove the antibodies raised against the mosquito proteins. The adsorption was performed by incubating 500 μ l of immune sera with 500 μ l of larval suspension for 2 h at room temperature in a rocker. This was then centrifuged at 10,000 rpm for 20 min. The pellet was discarded and the supernatant was used to purify IgG with the Protein G column (Sigma-Aldrich, St. Louis, Mo., USA) following the manufacturer's instructions.

SDS-PAGE and Western Blot

The different fractions of gradient purification and the immunoprecipitate were analyzed by SDS-PAGE using 4% stacking and 12% resolving acrylamide gels, with a constant voltage of 100 V. The gels were stained by the silver staining method [15]. The gradient centrifuge fraction that showed the bands for the suspected proteins in SDS-PAGE was used for immunoprecipitation with the purified IgG. For immunoprecipitation, 50 μ l of a 10^{-1} dilution of IgG was incubated with 50 μ l of purified virus (from the gradient centrifuge fraction) on a rocker for 2 h at 4° . This was then centrifuged at 14,000 rpm for 30 min. The supernatant was stored separately and the pellet was suspended in 30 μ l of NTE buffer. The proteins were transferred to a nitrocellulose membrane. The nitrocellulose membrane was blocked using blocking buffer composed of 4% bovine serum albumin and 0.05% Tween-20 in PBS. The purified IgG raised against the DNV was diluted in PBS (1:200) and used to probe the virus. The reaction was determined using antimouse antibodies tagged with alkaline phosphatase (1:25,000) and 5-bromo-4-chloro-3-indonyl phosphate as substrate (Sigma).

Table 1. Genomic location of the primers and their sequences

No.	Primer name	Genomic location	Sequence
1	DNV 32 F	32–55	TTTCGTGATACGGATACTGTAGAT
2	DNV 232 F	232–255	CTTAATTTCCACTACCACATGGTC
3	DNV 570 F	570–592	GGTGACCTCTACCCACAATTAC
4	DNV 1462 F	1462–1481	ACAGAAGCGATGGATACG
5	DNV 1745 F	1745–1768	ACGCATCGAAAAGAACACGTATAC
6	DNV 2688 F	2688–2713	GACCTAATATGAGCGAAATGGTACC
7	DNV 3401 F	3401–3428	ACATCATATCCTAGAATGCATATGGCA
8	DNV 302 R	302–322	AATGTTCAACGCAGACTGAA
9	DNV 648 R	648–670	GTACAAGTATTTCCCAGCTTCC
10	DNV 805 R	805–826	ATCTTTGGACAGCGTCTCGTA
11	DNV 2238 R	2238–2256	TGTGATCCACGTTGGTGT
12	DNV 3239 R	3239–3258	CTGCAGCGCTAATGTCTGT
13	DNV 3754 R	3754–3771	AGGTGGAATACGGAGGTG

Transmission Electron Microscopy

CsCl gradient-purified virus was examined by negative staining in a transmission electron microscope. Briefly, 100 µl of the fraction was adsorbed on a carbon-coated Formvar support on 400 mesh copper grids (Polysciences, Sunnyvale, Calif., USA) for 20 min, excess fluid was removed by capillary blotting and the grids were negatively stained with 1% phosphotungstic acid. Specimens were then examined in a transmission electron microscope under a 120-kV operating voltage (Tecnai 12 Biotwin; FEI Co., Eindhoven, The Netherlands). Images were digitally recorded at a resolution of 1,028 × 768 pixels using a Megaview II (SIS Germany).

Screening of Different Geographical Populations of *Ae. aegypti* for the Presence of DNV

Various populations of *Ae. aegypti* mosquitoes from different states of India were tested for the presence of DNV by PCR as described above. From the southern part of India, mosquitoes screened included 3 populations from the state of Kerala, 5 from Andhra Pradesh and 8 from Karnataka. The western part of India was covered by screening 11 populations from the state of Maharashtra and 2 each from Goa and Gujarat. One population each from Daman, Assam and West Bengal, which are located in eastern India, and 1 from Delhi, which represented the northern part of India, were also analyzed.

Amplification, Sequencing and Phylogenetic Analysis of the MDV

Viral DNA was extracted from virus stock using DNAzol (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's protocol. Overlapping primers were designed from the known sequences of AeDNV (accession No. M37899) and *C. pipiens pallens* DNV (CpPDNV; accession No. EF579771). The complete coding region was amplified in overlapping fragments using different primer sets (table 1). The amplification was performed using Platinum Taq DNA polymerase (Invitrogen). The PCR conditions for amplification were 94° for 3 min, 93° for 30 s, 53° for 1 min, 72° for 30 s, repeat for 35 cycles and final extension of 72° for 5 min. The amplification of the 3' palin-

dromic sequence was a challenge due to formation of secondary structure. To amplify this region, the DNA was denatured at 98° for 10 min and was snap cooled on ice. Then the master mix was added to the denatured sample and was subjected to amplification. The amplicons were analyzed on 1.5% agarose gel and the amplified PCR products were gel eluted using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. The purified PCR products were used for cyclic PCR using a Big Dye terminator kit (Applied Biosystems Inc., Foster City, Calif., USA). The conditions used for cyclic PCR were 96° for 5 min, 96° for 10 s, 50° for 5 s, 60° for 4 s, repeat for 40 cycles and extension at 60° for 5 min. Cyclic PCR products were purified and products were sequenced using the ABI 3100 automated DNA sequencer.

The sequences obtained were analyzed by the Basic Local Alignment Search Tool (BLAST) and were manually curretted using the software Kodon. The curretted sequences were analyzed by GeneRunner software to determine the open reading frames (ORFs) and their properties. The curretted sequences of each individual ORF were aligned by Clustal W (as implemented in Mega 3.1), and phylogenetic analysis was performed by the neighbor joining method with 1,000 bootstrap replicates using Mega 3.1 [16]. The sequences of other MDVs available in the NCBI database such as CppDNV [17], AeDNV [18], C6/36DNV [8] and *Haemagogus equinus* DNV [19] were also used in phylogenetic analysis. Sequences of DNVs isolated from other arthropods, namely hepatopancreatic parvovirus of *Penaeus monodon* [20], *Casphalia extranea* DNV [21] and *Dendrolimus punctatus* DNV [22], were used as out-group sequences.

Results

Development of the DNV-Infected Mosquito Colony

The DNV-infected *Ae. aegypti* mosquito colony was tested randomly for the presence of DNV by PCR. The colony was found to be positive for the presence of DNV.

Fig. 1. Western blotting of the immunoprecipitated purified virus. Lane 1: pellet after 30% glycerol cushion; lane 2: supernatant after immunoprecipitation; lane 3: pellet after immunoprecipitation; lane 4: IgG included as negative control; lane 5: protein marker.

The specific band of 1,097 bases as reported for this primer pair was observed (data not shown).

DNV Isolation and Purification

All the fractions obtained from the CsCl gradient were found to be positive when screened by PCR, but the electron microscopy and Western blot studies showed that the 30% fraction had the highest purity and maximum concentration of DNV.

SDS-PAGE and Western Blotting

When samples collected from different fractions of the CsCl gradient were subjected to SDS-PAGE, it was found that the 30% fraction had bands of proteins suspected to be DNV. This fraction was used for immunoprecipitation. When the immunoprecipitated virus was subjected to SDS-PAGE and Western blotting, virus-specific bands of 40 and 41 kDa were seen, along with IgG bands (fig. 1). This duplex of bands was similar to that reported for DNV [8].

Transmission Electron Microscopy

When subjected to negative staining, morphologically distinct parvovirus-like particles with a size range of 18–22 nm could be detected at moderate to high frequency in the fields scanned (fig. 2).

Presence of MDV in Different Geographical Populations

Three *Ae. aegypti* populations from Kerala, 2 from Andhra Pradesh and 1 from Karnataka were found to be positive for this MDV. Five populations from Maharash-

Fig. 2. Transmission electron microscopy showing icosahedral, nonenveloped particles of about 20 nm similar to other DNVs.

tra were positive and 1 each from Goa and Gujarat. The population from Assam was found to be positive, while those from West Bengal and Daman were found to be negative. The sample from Delhi was also positive for the MDV.

Amplification, Sequencing and Phylogenetic Analysis of the MDV

A total of 3,776 bases of the DNV genome were sequenced. This included 128 bases of the 3' palindrome,

Fig. 3. Phylogenetic relationship between NIVDNV and other MDVs using the left ORF. CppDNV (EF579771), AeDNV (M37899), C6/36DNV (AY095351), *Haemagogus equinus* DNV (HeDNV; AY605055), *Dendrolimus punctatus* DNV (DpDNV; NC_006555), *Casphalia extranea* DNV (CeDNV; NC_004288) and hepatopancreatic parvovirus of penaeid shrimp (Hpv; NC_007218) were used for Clustal W and phylogenetic analysis of ORFs of NIVDNV.

3,507 bases of coding region and 141 bases of the 5'UTR. This sequence consists of 21.2% adenine, 41.3% thymidine, 19.5% guanine and 18.0% cytosine and it encodes 3 overlapping ORFs. BLAST analysis showed 95% similarity between this sequence and CppDNV and 93% similarity with AeDNV. The palindromic region at the 5'UTR end could not be sequenced because of the difficulty in amplifying it.

The left and right ORFs stretch in a consecutive manner along one strand of DNA. They almost cover the entire length of the plus strand. These ORFs overlap at their extremities. Another small ORF referred to as the mid-ORF is completely located within the left ORF, in a different reading frame. The minus-strand ORF found in AeDNV [23] was not observed here. Several putative promoter sequences were observed in the positive strand. Eight AATAAA sequences, which are signals for polyadenylation, were also found in the plus strand. They fall at positions 1020, 1443, 1566, 1976, 2214, 2391, 2906 and 3680. The consensus promoter sequence TATAA was found at 12 sites in the genome. Sequences related to the consensus promoter sequence were also found at many places.

Left ORF

The start codon of the left ORF falls at nucleotide 129 and the ORF ends at 2676. This creates a protein of 849 amino acids (aa) with a molecular weight of approximately 97.6 kDa. This ORF has 92% amino acid homology with the NS1 protein of AeDNV. The encoded amino acid sequence also consists of a GTP/ATP binding motif with the conserved amino acid sequence GITNAGKSL. The amino acid stretch from positions 594 to 749 showed similarity with the superfamily 3 helicase of DNA viruses' domain profile according to prediction using the soft-

ware Expert Protein Analysis System (Swiss Institute of Bioinformatics). A stretch of around 200 aa which is highly conserved among different DNVs was found to be located at amino acid positions 328–505.

Mid ORF

The mid ORF falls completely within the left ORF, though in a different reading frame. The putative protein of this ORF has 362 aa if the translation begins at the first AUG at nucleotide position 376 and ends at nucleotide position 1462. The protein has a molecular mass of 41.1 kDa. This putative protein had 94% similarity with the NS2 protein of AeDNV. However, no similarity was seen with other parvoviruses.

Right ORF

This ORF begins at nucleotide position 2561 and ends at 3635. Thus, the left and right ORFs overlap at their extremities. If translation begins at the first AUG, this ORF creates a protein of 358 aa with a molecular weight of 40.6 kDa. This protein was found to have a high degree of analogy with the viral capsid proteins of other MDVs. This ORF has 85% amino acid homology with the capsid protein of AeDNV. The putative protein contains the highly conserved sequence RGTKRKR at amino acid positions 14–20 within the glycine-rich region as reported for other MDVs. There is a glycosaminoglycan attachment site at the 27th aa, which reinforces the possibility that the right ORF may synthesize the viral capsid protein. This motif contains the consensus sequence SGAG, where the glycosaminoglycan will attach to the serine molecule.

Phylogenetic analyses using each ORF showed that this DNV isolate falls in the lineage of Brevidensovirus. A phylogenetic tree (fig. 3) was constructed for each ORF,

Table 2. Percentage homology of the newly isolated *Ae. aegypti* DNV with other MDVs

Organism	Left ORF, %		Mid ORF, %		Right ORF, %	
	aa	nt	aa	nt	aa	nt
CypDNV	96	97	97	98	94	95
AeDNV	92	95	94	96	85	85
HeDNV	67	81	74	81	77	69
C6/36DNV	67	81	73	80	77	68

but as those trees showed similar topology, only the NS1 gene (left ORF) is presented here. This virus shows maximum homology with CypDNV. The percentage homology of nucleotides and amino acids for each ORF (left, mid and right) was compared with known MDVs and is presented in table 2.

Discussion

This is the first report of the isolation and characterization of MDV from India. The present study shows infection in various geographical populations of *Ae. aegypti* mosquitoes from different states of India, indicating that MDV is widely distributed. It has been observed by earlier workers that mosquito strains may differ in their susceptibility and response to DNV infections [24]. Other studies have shown seasonal and spatial variation in natural DNV infection in *Anopheles* mosquitoes [6].

AeDNV isolated from *Ae. aegypti* mosquitoes showed only 2 proteins of 38 and 40 kDa [18]. Similarly, in our study, immunoprecipitation and SDS-PAGE in conjunction with Western blot studies showed the presence of 2 proteins of 40 and 41 kDa. It is well known that insect DNVs are small, 25- to 28-nm-diameter, nonenveloped, icosahedral viruses. These features could be visualized on an electron microscope and hence, further quick characterization became possible. After confirmation of DNV by SDS-PAGE, Western blotting and partial PCR amplification, attempts were made to amplify the complete genome of the virus, but due to a palindrome sequence, only the 3'UTR, the complete coding region and part of the 5'UTR were sequenced.

The sequencing data confirmed that this virus encodes 3,507 bases of coding region and 3 ORFs, as reported earlier. ORF1 (left ORF) is 2,547 nt long and encodes a nonstructural protein (NS1) of 849 aa. ORF2 (mid

ORF) is 1,086 nt and encodes the NS2 protein, which is 358 aa long. ORF3 (right ORF) is 1,074 nt long and encodes 362 aa for a capsid protein (VP). The genome organization is very similar to the genome of AeDNV. The minus-strand ORF found in AeDNV [18] was not observed here. The right ORF of our virus has 6 N glycosylation sites, while AeDNV has 5 [25]. VP of the new isolate contains the highly conserved sequence RGTKRKR as reported for other DNVs from mosquitoes at a similar amino acid position [14–20] within the glycine-rich region.

Phylogenetic analyses performed for all 3 ORFs showed that the isolated DNV falls in the lineage of MDV. Our analysis suggests separation of AeDNV and AalDNV based on all 3 ORFs and that NIVDNV is closer to AeDNV than the AalDNV genome. This virus shows maximum homology with CypDNV of 96, 97 and 94% similarity for the left, mid and right ORFs, respectively, at the amino acid level. The genome similarity of this virus with CypDNV, which was isolated in China [17], indicates the possibility that this virus evolved during a similar time period in this continent and can infect different species of mosquitoes. Widespread distribution of DNV in *Ae. aegypti*, the principal vector of dengue and chikungunya in this country, calls for in-depth studies on how the presence of this virus affects the vector competence of mosquitoes.

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