NEW GENETIC VARIATION OF *AEDES ALBOPICTUS* DENSOVIRUS ISOLATED FROM MOSQUITO C6/36 CELL LINE

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Abstract. Densovirus (DNV) is a small single-stranded DNA, non-enveloped virus belonging to the subfamily Densovirinae of the Parvoviridae family. This group of invertebrate viruses infects exclusively insects. Two of the major densoviruses, *Aedes aegypti* (*Aae*DNV) and *Ae. albopictus* (*Aal*DNV), infect mosquitoes that carry viruses responsible for two important public health diseases, namely, dengue hemorrhagic fever and yellow fever. The present study describes cloning, sequencing and phylogenetic analysis of a new densovirus, *Aal*DNV-4, from infected *Ae. albopictus* C6/36 cell line. The total nucleotide sequence (3.9 kb) of *Aal*DNV-4 was obtained from sequencing of DNA fragments, and is 98% homologous to the initial *Aal*DNV previously isolated, and distinguishable from other *Aal*DNVs reported earlier. This full-length viral genome contains a 40-bp deletion at the left terminal region, 12 substitutions and 3 indels. Phylogenetic analysis of *Aal*DNV-4 genome indicates that this virus is more closely related to the original *Aal*DNV found in C6/36 cell line than to *Aae*DNV isolated from other mosquitoes. It was concluded that *Aal*DNV-4 may have been derived from the original DNV found in the C6/36 cell line and has transferred worldwide from the exchange of this cell line among laboratories.

Keywords: *Aedes albopictus*, Densovirus, C6/36, genetic variation

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INTRODUCTION

Densovirus (DNV), a non-enveloped icosahedral viral particle of 18-20 nm in diameter, contains single-stranded DNA and belongs to the family Parvoviridae (Kurstak, 1972; Bachmann *et al*, 1975; Siegl *et al*, 1985). Two subfamilies, Parvovirinae and Densovirinae, are classified in this family, with viruses in the subfamily Parvovirinae infecting vertebrates and those in Densovirinae infecting invertebrates, mostly insects. All densoviruses infect only closely related insects and are fatal

to their host larvae.

Within the subfamily Densovirinae, three genera are known: *Densovirus* (infecting cockroaches), *Iteravirus* (infecting silk worms) and *Brevidensovirus* or *Contravirus* (infecting mosquitoes). The latter genus consists of *Aedes aegypti* (*Aae*DNV) and *Aedes albopictus* (*Aal*DNV) densovirus (Afanasiev *et al*, 1991; Jousset *et al*, 1993; Boublik *et al*, 1994). These two densoviruses share 77.3% similarity in genome sequences, which have palindromic structures that form stable hairpin structures at both termini. These structures have been suggested to play a role in DNA replication (Afanasiev *et al*, 1991, 1994; Boublik *et al*, 1994). Both densoviruses genomes are 4.0-4.2 kb in size, and encode for three open reading frames (ORF) on plus strand with the upstream and middle ORFs encoding for non-structural proteins (NS) and the downstream ORF encoding a structural protein. In *Aae*DNV, there is an extra ORF in the minus strand of the genome encoding an anonymous protein. The genome of densoviruses can be encapsidated separately with a plus or minus strand (Afanasiev *et al*, 1991; Boublik *et al*, 1994).

The mosquitoes that were infected with *Aedes* densovirus are important vectors of dengue virus, which causes dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) in humans worldwide, especially within tropical and subtropical regions (Hayes and Gubler, 1992; Rigau-Perez *et al*, 1998; Rodriguez-Tan and Weir, 1998). *Aedes* densovirus is an attractive model to develop as a biological control of mosquitoes because densovirus genomes are small facilitating molecular manipulations. *Junonia coenia* denovirus (*Jc*DNV) and the closely related *Galleria mellonella* densovirus (*Gm*DNV) have been exploited as expression vectors for insect cells (Jousset *et al*, 1990; Dumas *et al*, 1992; Giraud *et al*, 1992). Infectious clones of *Jc*DNV and *Aae*DNV have also been used to express reporter β-galactosidase gene in insect cell lines and larvae (Giraud *et al*, 1992; Afanasiev *et al*, 1994).

We report here a new densovirus isolated from *Ae. albopictus* C6/36 cells. This virus is named *Aal*DNV-4 after the order of isolation. Molecular characterization of this virus revealed that *Aal*DNV-4 is similar to the other three *Aal*DNVs, namely, *Aal*DNV-1, -2 and -3, as regards genome size, nucleotide sequence and immunoreactivity to specific antibodies.

MATERIALS AND METHODS

Densovirus

*Aal*DNV was first isolated from the *Aedes albopictus* C6/36 cell line and kindly provided by Dr Pattamaporn Kittayapong, Faculty of Science, Mahidol University, Bangkok, Thailand. This densovirus has been maintained in C6/36 cells at the Division of Medical Molecular Biology, Faculty of Medicine Siriraj Hospital, Mahidol University.

Cultivation of *Aal*DNV in *Aedes albopictus* C6/36 cell line

The cultivation of DNV was prepared in a 500 ml spinner flask by infection of 10^8 C6/36 cells/ml attached to 1.5 g of microcarrier beads with 10 ml of viral suspension (from C6/36 cell culture). The cell culture was maintained for 7 days in 300 ml of Leibowiz's medium (L-15) containing 10% fetal bovine serum (FBS) (Gibco BRL, Gaithersberg, MD) and 10% tryptose phosphate broth (TPB). Then the microcarrier beads were centrifuged at 800*g* for 10 minutes and the supernatant was used for virion DNA preparation. C6/36 cells were removed from the microcarrier beads by incubating with 2.5 mM

EDTA in phosphate-buffered saline pH 7.4 (PBS) at room temperature for 5 minutes and used for isolation of the replicative form of viral DNA. In addition 10^7 C6/36 cells were infected with 5 ml of viral supernatant for 7 days as described above.

Isolation of *Aal*DNV replicative form (RF) DNA

Isolation of RF of viral DNA was carried out using a modified Hirt's extraction procedure (Hirt, 1967). In brief, infected C6/36 cells (10^8) were resuspended with 1 ml lysis buffer containing 0.32 M sucrose, 3 mM $CaCl_2$, 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris-HCl pH 8.0, 0.1% nonidet P-40, 1 mM DTT and 0.25 mg RNase A, and incubated at 28ºC for 30 minutes before centrifugating at 650*g* for 10 minutes at 4ºC. The supernatant was added with 2 ml solution containing 0.2 M NaOH and 1% SDS and incubated on ice for 15 minutes. Then, 1 ml of 3.0 M potassium acetate pH 5.5 was added and the solution was incubated on ice for 15 minutes, centrifuged at 11,257*g* for 15 minutes at 4ºC before mixing with 3 ml of isopropanol and incubating at room temperature for 15 minutes. DNA was sedimented at 11,257*g* for 15 minutes at room temperature, and washed with 50 µl of 5 M LiCl to remove residual proteins attached to DNA. DNA pellet was extracted once with phenol : chloroform : isoamylalcohol (25:24:1) solution and precipitated with absolute ethanol. RF *Aal*DNV DNA was resuspended in 100 µl of 10 mM Tris-HCl pH 8.0/1 mM EDTA (TE) buffer and concentration was estimated by observing the EtBr-stained gel under UV transilluminator compared with known amounts of standard molecular weight DNA.

Cloning and sequencing of *Aal*DNV genome

About 0.1 µg of RF *Aal*DNV DNA was treated with 3 U of Klenow DNA polymerase (New England BioLabs, Ipswich, MA) and 5 mM dNTPs in 50 µl reaction volume at 37ºC for 30 minutes in prior to making blunt end DNA. The reaction mixture was heated at 75ºC for 10 minutes before extracting with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) solution and precipitating with 2 volumes of absolute ethanol at -70ºC for 20 minutes. DNA was sedimented at 11,257*g* for 20 minutes and washed twice with 70% ethanol. Then DNA was dissolved in 10 µl of distilled water and ligated with 50 ng of linearized and dephosphorylated pUC18 cloning vector using 3U T4 DNA ligase (New England BioLabs) at 16ºC for 15 hours and transfected into *E. coli* DH5α competent cells.

For site specific cloning of the RF *Aal*DNV DNA, the nucleotide sequences of densovirus (*Aae*DNV and *Aal*DNV) in GenBank database were analyzed for restriction endonuclease sites, and *Eco*R I sites were selected because this enzyme could clearly differentiate between the two densovirus genomes (Fig 1). Digestion of RF *Aal*DNV DNA with *Eco*R I generated fragments of 0.6, 0.8, 1.1 and 1.6 kb, which was compatible with *Aal*DNV genome. The 3.7 kb *Pst* I-*Hin*d III DNA fragment of *Aal*DNV was chosen for cloning and sequencing. Three hundred ng of *Aal*DNV DNA were digested sequentially with *Pst* I and *Hin*d III at 37ºC for 2 hours. Approximately 90 ng of *Pst* I-*Hin*d III digested DNA were ligated with 50 ng of pUC18 digested with the same two enzymes at 16ºC for 15 hours and used to transfect *E.coli* DH5α as described above.

Nucleotide sequencing was performed using BigDye™ Terminators Cycle Sequencing kit (PE-Applied Biosystems, Foster City, CA) together with ABI PRISM 310 Genetic Analyzer according to the

supplier's protocol. Raw electrophoregram was collected using Data Collection software (PE-Applied Biosystems) and the nucleotide sequences obtained were verified using the Sequence Navigator software (PE-Applied Biosystems). Nucleotide sequencing was performed in both directions.

Screening for recombinant *Aal*DNV clone by colony hybridization

The *E. coli* transformants were plated on nitrocellulose membrane on Luria-Bertani (LB) agar plate containing 50 µg/ml ampicillin and incubated at 37ºC for 11 hours. Then the nitrocellulose membrane was lifted up and bacterial colonies were transferred to new membrane by replica technique (Frederick *et al*, 1992). The nitrocellulose membrane containing bacterial colonies were incubated further on LB plate containing 100 µg/ml chloramphenicol at 37ºC for 11 hours. The nitrocellulose membrane was treated twice by laying on filter paper soaked with 0.5 M NaOH for 5 minutes, then the same membrane was treated twice by laying down on filter paper soaked with 1 M Tris-HCl pH 7.4 for 5 minutes. The final step was laying down on filter paper soaked with 1 M Tris-HCl pH 7.4 and 1.5 M NaCl for 5 minutes.

The radiolabeled probe was performed by using random primer labeling kit (Amersham Biosciences, San Diego, CA). In brief, 30 ng of 381-bp *Aal*DNV DNA fragment (nt 3447-3827) was reacted in 50 µl reaction volume containing 10 U Klenow DNA polymerase I, 100 µCi α-^{32}P dCTP and 10 µl dNTP at 37ºC for 1 hour. This radiolabeled probe was used to hybridize with 10 sheets of pretreated nitrocellulose membrane in 10 ml hybridization solution containg 1% bovine serum albumin (BSA), 1% Ficoll 400 and 1% polyvinylpyrrolidone (PVP) at 65ºC for 15 hours. Then the hybridized membrane was washed twice with 300 ml of washing solution containing 0.088% NaCl, 0.041% sodium citrate and 0.1% sodium dodecyl sulfate (SDS) at 65ºC for 30 minutes, then the air-dried nitrocellulose was exposed to x-ray film for 2-6 hours. The positive signal appeared as black spot after developing the film.

Production of antibodies against *Aal*DNV structural protein

Rabbit polyclonal antibodies were prepared by injection of 100 µg of purified recombinant fusion protein of *Aal*DNV structural protein and maltose binding protein (MBP) (New England BioLabs) three times during a 2-week interval. One week after the last immunization, rabbit serum was collected and checked for the presence of antibodies against *Aal*DNV structural protein by western blot analysis.

Western blot analysis

Transfected C6/36 cells ($1x10^5$) were collected for SDS-PAGE analysis. Proteins in the acrylamide gel were transferred to nitrocellulose membrane (0.45 µm, Sartorius) using a semi-dry blotter (Amersham Biosciences, San Diego, CA) at a constant current of 0.8 mA/cm^2 for 1 hour. Membrane then was incubated with blocking buffer of 5% skimmed milk in PBS for 1 hour at room temperature, followed by incubation with rabbit immune serum against *Aal*DNV structural protein at a dilution of 1:100 in blocking buffer for 2 hours at room temperature. Then the nitrocellulose membrane was incubated with swine anti-rabbit IgG antibodies conjugated with horseradish peroxidase (dilution of 1:1,000) for 1 hour at room temperature. The chromogenic substrate of 0.4% 3, 3' diaminobenzidine (Sigma, St Louis, MO) and 0.02% H_2O_2 were added

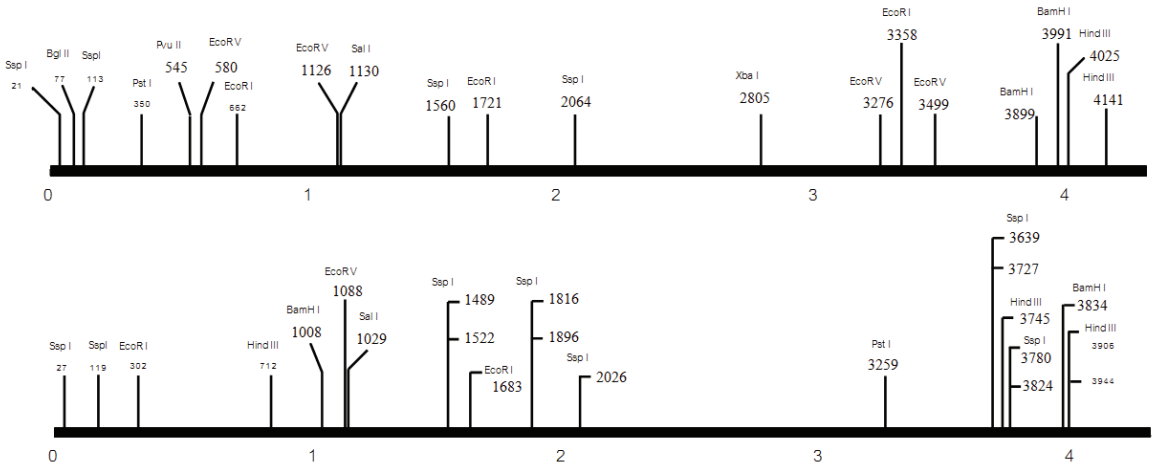

Fig 1–Restriction maps of *Aal*DNV (upper) and *Aae*DNV (lower) genome. Genome size is marked in kb under the dark line and enzyme site with nucleotide location in bp is shown above the dark line.

for 5 minutes in order to develop visible bands (dark brown color) and reaction was terminated by rinsing the membrane in distilled water.

Phylogenetic analysis of *Aal*DNV genome

The nucleotide sequence of *Aal*DNV was aligned with those of DNV isolated from various mosquitoes and insects viz. *Aedes, Culex, Haemagogus, Bombyx, Galleria, Periplaneta, Diatraea* and mosquito C6/36 cell line deposited in the GenBank database. Multiple sequence alignment (MUSCLE) was used for sequence comparison, and molecular evolutionary genetic analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods (MEGA5) were employed to assess these sequences and to calculate the evolutionary distances. Phylogenetic trees were constructed using Neighbor-Joining method with 1000 bootstrap replications. The evolutionary distances were computed using Kimura 2-parameter method and are reported in units of the number of base substitutions per site.

RESULTS

Isolation and characterization of *Aal*DNV DNA

DNV DNA observed as a 4.2 kb band in 1% agarose gel-electrophoresis (Fig 2A) was isolated from an infected *Ae. albopictus* C6/36 cell line. To differentiate whether DNV genome was derived from *Aal*DNV or *Aae*DNV, the sample was digested with *Eco*R I because this enzyme provides DNA fragments that can differentiate between the two types of DNV. The DNA bands of 0.6, 0.8, 1.1 and 1.6 kb (Fig 2B) demonstrated that DNV was that of *Aal*DNV (Fig 1).

In order to clone *Aal*DNV genome, terminal ends of the double-stranded DNA was blunt-ended with Klenow DNA polymerase I and then cloned into pGEM4Z digested with *Sma* I for transfection into *E. coli* DH5α. About 10^4 *E. coli*

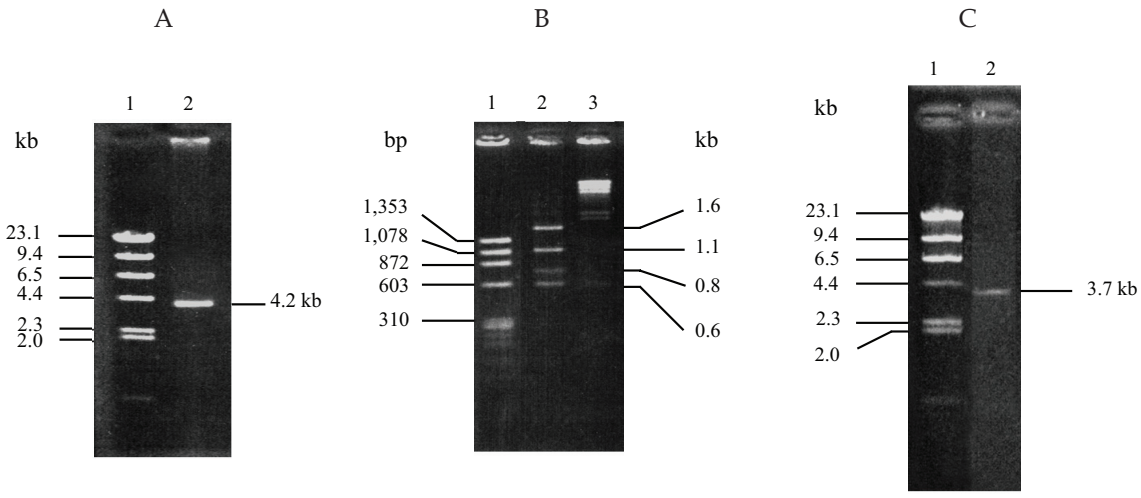

Fig 2–Agarose gel-electrophoresis of *Aal*DNV RF DNA isolated from cytoplasmic fraction of infected *Ae. albopictus* C6/36 cells. (A) Undigested *Aal*DNV DNA (lane 2). Lane 1 contains *Hin*d III digested λ DNA marker. (B) *Eco*R I-digested *Aal*DNV RF DNA (lane 2). Lane 1 and lane 3 is *Hae* III-digested ϕx174 DNA and *Hin*d III digested λ DNA marker, respectively. DNA bands of 1.6, 1.1, 0.8 and 0.6 kb are marked with arrows. (C) *Aal*DNV DNA digested with *Pst* I-*Hin*d III (lane 2). Lane 1 is *Hin*d III-digested λ DNA marker.

transformants were screened by colony hybridization with ^{32}P-labeled *Aal*DNV DNA probe. No positive recombinant clones were observed after hybridization indicating that no full-length blunt-ended DNA clones were obtained. Then the double-stranded DNV DNA was subsequently digested with *Pst* I-*Hin*d III, subcloned into pUC 18 vector and transfected into *E. coli* DH5α. After screening the recombinant clones with radiolabeled *Aal*DNV DNA, positive clones containing insert size of 3.7 kb (Fig 2C) were chosen for nucleotide sequencing, which revealed that this fragment was derived from nts 351-4025 of *Aal*DNV genome deposited in GenBank database (accession no.X74945). There were 9 missense mutations, 2 silent mutations, 1 indel mutation and 1 mutation in the non-coding region.

*Eco*R I-digested *Aal*DNV genome produced 4 DNA fragments while the restriction map of *Aal*DNV reported by Boublik *et al* (1994) showed only fragments of 0.65, 0.8, 1.1 and 1.6 kb, indicating that approximately 50 bp is missing in this *Aal*DNV genome of our study. Nucleotide sequencing of 0.6 kb DNA fragment showed a 40-bp indel (nt 154-193) of the viral genome (Fig 3). Another *Ssp* I-*Eco*R I DNA fragment corresponding to nt 113-662 of *Aal*DNV also showed the same 40-bp deletion.

Phylogenetic analysis of *Aal*DNV

As this *Aal*DNV was isolated after discovery of three *Aal*DNVs, we applied the name '*Aal*DNV-4'. Phylogenetic tree analysis comparing with other DNVs isolated from mosquitoes and cell lines showed *Aal*DNV-4 was similar to the original isolate of *Aal*DNV-1 (Boublik *et al*, 1994) (Fig 4). Other insect DNVs, *viz*. *Bm*DNV, *Gm*DNV, *Ds*DNV and *Pf*DNV, served as outlier group for this comparison.

154–**TGGAACACACGGACGTGGAAATTTAGAAAGTATTTTTCTG**–193

. . . . . .

194–TGGAACACACGGACGTGGAAATTTAGAAAGTATTTTTCTGTGGAAA–239

Fig 3–Direct repeats in the terminal noncoding sequence of *Aal*DNV genome. The 40-bp deletion, nt 154-193, of *Aal*DNV-4 is shown in bold letters. The underlined nucleotide sequence represents the core sequence of SV40 transcription enhancer.

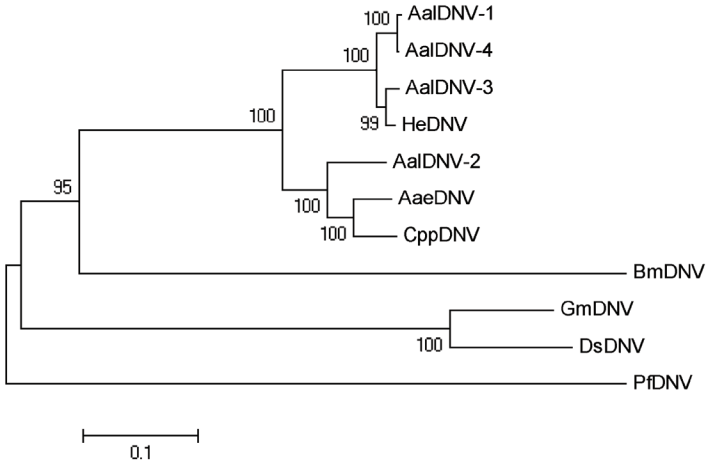

Fig 4–Phylogenetic tree of *Aal*DNV-4 and other DNVs. The phylogenetic tree is based on a comparison of 3.9-kb sequence between nucleotide 113- 4025. It was analyzed with the Molecular Evolutionary Genetic Analysis program using maximum likelihood, evolutionary distance, and maximum parsimony methods. DNV sources are *Aae*DNV (M 37899), *Aal*DNV-1 (X 74945), *Aal*DNV-2 (AY 095351), *Aal*DNV-3 (AY 310877), *Cpp*DNV (EF 579771), *He*DNV (AY 605055), *Bm*DNV (AB 042597), *Gm*DNV (L 32896), *Pf*DNV (NC_000936) and *Ds*DNV (NC_001899) from the GenBank database. Numbers above the branches refer to percentage of bootstrap values for 1,000 replicates.

Analysis of *Aal*DNV structural protein

Western blot analysis performed on mock- and *Aal*DNV-infected C6/36 cells using rabbit immune serum against structural protein of *Aal*DNV showed the presence of 41 kDa protein only in *Aal*DNV-infected C6/36 cells, while no band was detected with pre-immune serum (Fig 5).

DISCUSSION

The source of *Aedes* DNV in this study was *Ae. albopictus* mosquito C6/36 cell line. Viral DNA (4.2 kb) was isolated from cytoplasmic fraction of DNV-infected C6/36 cells suggesting that it is in the form of double-stranded rather than a single-stranded DNA. This may be the result of extraction of virion DNA under a high-salt condition, which is usually used for preparation of double-stranded DNA (Bando *et al*, 1992; Hu *et al*, 1994). The size of *Aal*DNV-4 DNA in this study was similar to that described for *Aal*DNV and

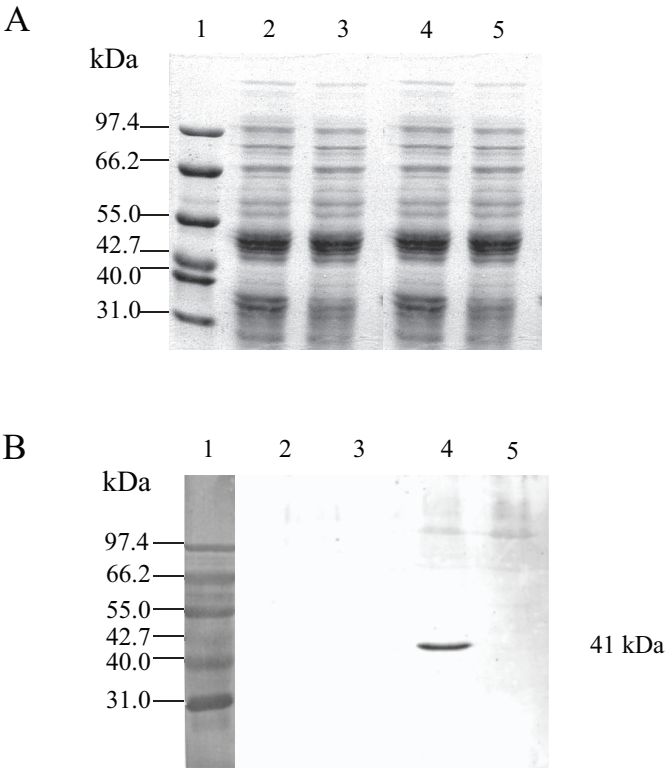

Fig 5–Western blot analysis of *Aal*DNV structural protein. *Aal*DNV-infected C6/36 cell lysate was analyzed by 10% SDS-PAGE and proteins were stained with Coomassie blue (A) or reacted with rabbit antibody against 41 kDa structural protein (B). Lane 1, standard protein size marker; lanes 2 and 4, *Aal*DNV-infected C6/36 cell lysate; lanes 3 and 5, mock-infected cell lysate. Lanes 2-3 and lanes 4-5 were incubated with pre-immune and immune rabbit serum, respectively.

*Aae*DNV (Afanasiev *et al*, 1991; Boublik *et al*, 1994). As no full-length *Aal*DNV-4 DNA clone was obtained using colony hybridization, subcloning of DNA fragment was performed. The longest fragment obtained was 3.7 kb *Pst* I-*Hind* III DNA. Nevertheless, this fragment of *Aal*DNV-4 was still missing both terminal regions needed for generating the infectious DNA (Jourdan *et al*, 1990; Afanasiev *et al*, 1991; Dumas *et al*, 1992; Boublik *et al*, 1994). There have been no reports of single full-length clones from *Aedes* DNV, and the full-length clones described by Afanasiev *et al* (1991) and Boublik *et al* (1994) came from the ligation of partial fragments. *Aal*DNV contained a 40-bp deletion located in the direct repeat region following the left T-shaped structure. The 40-bp deletion may present a unique structure of the *Aal*DNV-4 genome because DNA fragments generated from digestion of the genome with *Eco*R I were approximately 50 bp shorter in the 5′ terminal fragment than the length of reported *Aal*DNV. The 40-bp deletion also was detected after sequencing in both directions suggesting that this is not due to cloning artifact.

This 40-bp deletion was located in the non-coding region and did not affect translation of the coding sequence. However, in other *Aal*DNVs this left terminal region contains 3 TATA-like boxes (between nt 154-239 region), but in *Aal*DNV-4 there remains only 2 TATA-like boxes due to the deletion of one TATA-like box in the 40-bp deletion region, but this did not have an effect on the viral replication and viral infectivity as this virus was pathogenic to C6/36 cells (Burivong *et al*, 2004). The observation of 12 mutations and 3 indels did not produce any frameshift of the coding sequence. The important issue derived from the cloning and nucleotide sequencing of *Aal*DNV-4 genome was the high similarity (98%) of the nucleotide sequence compared with that of reported *Aal*DNV (Boublik *et al*, 1994). *Aal*DNV was first

Table 1
Nucleotide and amino acid substitutions of *Ae. albopictus* densovirus (*Aal*DNV).

Nucleotide Position	*Aal* DNV (Boublik *et al*, 1994)	*Aal* DNV (this study)	
		Nucleotide substitution	Amino acid substitution
1014	T	A	I ⟶ K
1383	A	G	N ⟶ S
1997	C	A	N
2324	C	G	H ⟶ D
3109	G	A	E ⟶ K
3110	A	C	E ⟶ A
3271	T	C	L
3331[a]	-	ACA[a]	T
3341	A	C	E ⟶ A
3367	T	G	Y ⟶ D
3407	G	A	R ⟶ K
3617	A	C	E ⟶ A
3896	T	C	Non-coding

[a]Nucleotide insertion

isolated and cloned from *Ae.albopictus* C6/36 cell line (Jousset *et al*, 1993; Boublik *et al*, 1994). *Aae*DNV has been isolated also from a laboratory-reared colony of *Ae. aegypti* mosquitoes (Lebedeva *et al*, 1973; Afanasiev *et al*, 1991). These two DNVs revealed a 77% similarity in nucleotide level, but the origin of these *Aal*DNVs is from C6/36 cell line that has been distributed to laboratories around the world. This virus has not been found so far in indigenous *Ae. albopictus* mosquitoes (Kittayapong *et al*, 1999).

Surprisingly, *Aal*DNV-4 discovered in this study was obtained from cultivation of *Ae. albopictus* C6/36 cell line with *Ae. aegypti* mosquito homogenate. From the high similarity in nucleotide sequence of this *Aal*DNV, it is suggested that *Aal*DNV-4 might be contamination from C6/36 cell line used for cultivation of DNVs. Several investigators also have described the widespread presence of insect DNVs in mosquito cell lines distributed in several laboratories (Boublik *et al*, 1994; O'Neill *et al*, 1995; Chen *et al*, 2004; Paterson *et al*, 2005). Phylogenetic analysis of several DNVs in comparison also suggested this new *Aal*DNV-4 is similar to *Aal*DNV-1 (Jousset *et al*, 1993; Boublik *et al*, 1994). Thus, it appears that chronic infection of mosquito cell lines by DNVs is a common phenomenon, probably as a result of contamination by manipulations and exchanges of cell cultures between laboratories.

Moreover, this *Aal*DNV-4 has similar characteristics with *Aal*DNV described earlier, in particular the lack of cytopathic effect in C6/36 cell line (Burivong *et al*, 2004; Kanthong *et al*, 2008, 2010). Despite the lack of cytopathic effect, DNVs isolated from mosquito cell lines have proven to be pathogenic for mosquito larvae by oral infection and are able to replicate and transmit in adult mosquitoes by inoculation (Jousset *et al*, 1993; Barreau *et al*, 1994, 1996, 1997). However, DNVs

have been shown to be present in C6/36 cells as persistent infection together with other dengue or Japanese encephalitis viruses (Burivong *et al*, 2004; Kanthong *et al*, 2008, 2010). DNV-infected cells also have demonstrated an effect on dengue infection by reducing dengue viral severity (Burivong *et al*, 2004; Mosimann *et al*, 2011). DNVs have been successively used for the expression of heterologous genes and packaging into infectious particles in two insect DNVs, *Jc*DNV and *Aae*DNV (Giraud *et al*, 1992; Kimmick *et al*, 1998; Afanasiev *et al*, 1999; Allen-Miura *et al*, 1999). We could not observe any deleterious effects from the deletion of 40-bp (nt 154-193) of a direct repeat near the NS1 gene (starts at nt 335) on viral DNA replication and packaging of virions.

If densovirus is to become an important tool for biological control of insect-borne pathogens, we need to explore the molecular structure and pathophysiology in their hosts, especially as several new DNVs have been reported recently in *Culex pipiens pallens* and *Ae. aegypti* from China and India, respectively (Zhai *et al*, 2008; Sivaram *et al*, 2010).

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