APPLICATION OF POST-PCR METHODS FOR ANALYSIS OF MOSQUITO DENSOVIRUS

Ubonwan Jotekratok[1,2], Kobporn Boonnak[1]*, Aroonroong Suttitheptumrong[1], and Sa-nga Pattanakitsakul[1]

[1]Division of Molecular Medicine, Office for Research and Development, [2]Graduate Program in Immunology, Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

Abstract. Two clades of *Aedes* densovirus, *Aedes aegypti* densovirus and *Aedes albopictus* densovirus, were classified according to the origin of isolation. These two densoviruses were isolated from indigenous mosquitoes and mosquito cell lines, respectively. This group of invertebrate viruses belongs to the subfamily Densovirinae of the Parvoviridae family and infects only insects. Several types of densoviruses have been isolated from mosquitoes especially *Aedes aegypti* and *Aedes albopictus*, which are important vectors of dengue hemorrhagic fever and yellow fever in humans. We describe applications of post-PCR techniques, restriction fragment length polymorphism (RFLP) and single-strand conformation polymorphism (SSCP) to classify these two clades of *Aedes* densoviruses isolated from different origins. These methods are simple and rapid and are applicable to identify other groups of densoviruses isolated from biological samples.

Keywords: densovirus, mosquito, post-PCR technique, RFLP, SSCP

INTRODUCTION

The subfamily invertebrate Densovirinae of Parvoviridae family is divided into three genera: *Densovirus*, *Iteravirus* and *Brevidensovirus* or *Contravirus* (Kurstak, 1972; Bachmann *et al*, 1975; Siegl *et al*, 1985). The *Brevidensovirus*/*Contravirus* consists of *Aedes aegypti* densovirus (*Aae*DNV) and *Aedes albopictus* densovirus (*Aal*DNV). Other densoviruses have been reported in several other mosquito species and cell lines, such as *Culex pipiens*, *Toxorhynchites splendens* and *Haemagogus equines* (O'Neill *et al*, 1995; Pattanakitsakul *et al*, 2007; Zhai *et al*, 2008).

DNV is an 18-20 nm non-enveloped icosahedral particle containing a single-stranded DNA of 4.0-4.2 kb (Afanasiev *et al*, 1991; Jousset *et al*, 1993; Boublik *et al*, 1994). Its genome contains a unique palindromic hairpin structure at both termini, which have been suggested to be involved in DNA replication (Afanasiev *et al*, 1991, 1994; Boublik *et al*, 1994). There are 3 open reading frames (ORFs) on the

Correspondence: Dr Sa-nga Pattanakitsakul, Division of Molecular Medicine, Office for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, 4th Fl, SiMR Bldg, Wang Lang Road, Bangkok 10700, Thailand.
Tel: +66 (0) 2419 2755; Fax: 66 (0) 2411 0169
E-mail: sanga.pat@mahidol.ac.th, sispn@yahoo.com
*Present address: Department of Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchawithi Road, Bangkok 10400, Thailand.

plus strand with the left and mid ORFs encoding non-structural (NS) proteins and right ORF encoding a structural protein, except for *Aae*DNV that has an extra ORF in the minus strand coding a protein of unknown function. DNV genome is usually encapsidated as a plus or minus strand in its virion (Afanasiev *et al*, 1991; Boublik *et al*, 1994).

Of these DNVs, *Aedes* DNVs have attracted more attention because these mosquitoes are important vectors of dengue virus-causing diseases in humans, such as including dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Hayes and Gubler, 1992; Rigau-Perez *et al*, 1998; Rodriguez-Tan and Weir, 1998). On the other hand, *Aedes* DNV has been suggested to be an attractive model for development as a biological agent for control of insects because of its small genome size and thereby facilitating gene manipulation and transfection into insects (Jousset *et al*, 1990; Dumas *et al*, 1992; Giraud *et al*, 1992). Thus *Aedes* DNV is a more specific microorganism for infecting mosquitoes, and there has been no report to date of it causing deteriorate effects to humans, making this virus potentially applicable for biological control of mosquito-borne diseases.

We report here a simple post-PCR technique (PCR-restriction fragment length polymorphism (RFLP) and PCR-single strand conformation polymorphism (SSCP)) for analysis of two clades of mosquito DNV genomes from biological specimens, including culture supernatants and mosquitoes.

MATERIALS AND METHODS

DNV samples

DNV-infected *Ae. aegypti* and *Toxorhynchites splendens* (Pattanakitsakul *et al*, 2007) mosquitoes were kindly provided by Dr Pattamporn Kittayapong, Department of Biology, Faculty of Science and Dr Supatra Thongrungkiat, Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Bangkok, respectively. The mosquitoes were kept at -80ºC until used.

*Aal*DNV from culture supernatant of infected C6/36 cell line was propagated by infecting C6/36 cells with culture stock of DNV as described previously (Sangdee and Pattanakitsakul, 2012). The *Aal*DNV-infected C6/36 cells were cultivated in T-75 flask containing 10 ml of Leibovitz's medium (L-15) containing 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY) and 10% tryptose phosphate broth (TPB) at 28ºC for 7 days.

PCR

DNA from culture supernatant *Aal*DNV and from infected *Ae. aegypti* mosquitoes were isolated using DNAzol reagent (GibcoBRL, Grand Island, NY). In brief, 100 μl of culture supernatant was added to 250 μl of DNAzol solution followed by gentle mixing and allowed to stand at room temperature for 5 minutes, then centrifuged at 11,600g for 20 minutes at 4ºC. The DNA was precipitated by adding 125 μl of cold absolute ethanol, then stand at room temperature for 5 minutes before centrifugation again, washed twice with 500 μl of 70% ethanol. The DNA pellet was dried and dissolved with 10 μl of distilled water (Pattanakitsakul *et al*, 2007). Each mosquito was homogenized using a glass homogenize in a 1.5-ml microcentrifuge tube containing 300 μl of Leibovitz's medium (L-15) containing 1% fetal bovine serum (FBS) (Gibco BRL) and a 10 μl aliquot was removed for DNA isolation. The extracted DNA was dissolved in 10 μl of sterile distilled water.

Table 1
PCR primers used for amplification of densovirus genome.

Primer	Nucleotide number	Gene	Primer sequence (5′- 3′)	Amplicon size (bp)
L3	430-452	NS1	CAGGAGGAGAGAATTGGATTTGG	269
R4	698-677		CCCAGCTTCTTGTTCCAATAGT	
L7	2178-2199	NS1-Capsid	CGATGATTACACCAGTAAACGT	436
R3	2613-2592		AGTGCTGTCTGC CATTCCTCTG	
L6	2997-3017	Capsid	AACAAGACAGAGACTGCTAAC	452
R7	3448-3427		GCATTCTTGGATATGATGTTCT	

Primers used for amplification of DNV DNA were chosen from conserved sequences of both *Aal*DNV and *Aae*DNV genomes (Table 1). The 25-μl PCR reaction consisted of 1X PCR buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 10 pmol of each primer pair, 0.5 U *Taq* DNA polymerase and 5 μl of template DNA. The thermocycling conditions conducted in DNA Thermal Cycler 480 (Perkin Elmer-Applied Biosystems, Foster City, CA) for 30 cycles were as follows: 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute.

In addition, a 3.7 kb *Aal*DNV genomic fragment (nt 351-4025) was inserted in pUC18 plasmid (Sangdee and Pattanakitsakul, 2012), and the recombinant plasmid was propagated in *E. coli* DH5α, purified using QIAprep Miniprep kit (Qiagen, Hilden, Germany) and was employed as a reference control in PCR amplification and in subsequent RFLP experiments.

RFLP

A 5 μl aliquot of PCR solution was digested with 5 U *Eco*RI or *Eco*RV at 37°C for 1.5 hours and then the DNA fragments were separated by 5% polyacrylamide gel-electrophoresis (acrylamide:bisacrylamide, 30:1) (PAGE) at 150 volts for 40 minutes. Finally the DNA fragments were stained with ethidium bromide and visualized under a UV transilluminator.

SSCP

SSCP analysis was carried out as described previously with slight modification (Orita *et al*, 1989; Bannai *et al*, 1994). A 1 μl aliquot of PCR solution was mixed with 7 μl of denaturing solution containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF, and the mixture was heated at 95°C for 5 minutes, followed by immediate cooling on ice before 5 μl aliquot of the mixture was subjected to 10% PAGE (acrylamide:bisacrylamide = 49:1) containing 5% glycerol in 45 mM Tris-borate (pH 8.0)/1 mM EDTA buffer at 4°C or 25°C. The electrophoresis was performed at 20 mA for 4-6 hours in a minigel electrophoresis apparatus equipped with a constant temperature control system (AE-6410, ATTO, Tokyo, Japan). Single-strand DNA fragments were visualized by silver staining (Daiichi Pure Chemicals, Tokyo, Japan).

RESULTS

Analysis of DNV using PCR-RFLP

Comparisons of restriction enzyme maps of *Aal*DNV and *Aae*DNV showed unique *Eco*RV sites at only nucleotide 580 and 3276 (within NS1 and capsid gene, respectively) in *Aal*DNV genome. The PCR products from NS1 and capsid gene of

DNV amplified by L3-R4 and L6-R7 primers, respectively were further analyzed with *Eco*RV digestion of *Aal*DNV NS1 amplicon generated fragments of 150 and 118 bp, while digestion of *Ts*DNV revealed the undigested 269-bp DNA fragment (Fig 1A). *Eco*RV digestion of *Aal*DNV capsid amplicon generated 279 and 172 bp DNA fragment and intact 452 bp DNA fragment with *Ts*DNV (Fig 1B).

Analysis of DNV using PCR-SSCP

The principle of SSCP analysis relies on the heterogeneity of nucleotide sequence of single strand DNA to form its secondary structure under non denaturing conditions in gel-electrophoresis. PCR-SSCP of PCR products derived from capsid and from the junction between NS1 and capsid displayed different mobility patterns between *Aal*DNV and *Aae*DNV (Fig 2 A-C). Moreover, electrophoresis conducted at 4ºC and 25ºC also showed distinct patterns of these two groups of DNVs (Fig 2 A-C).

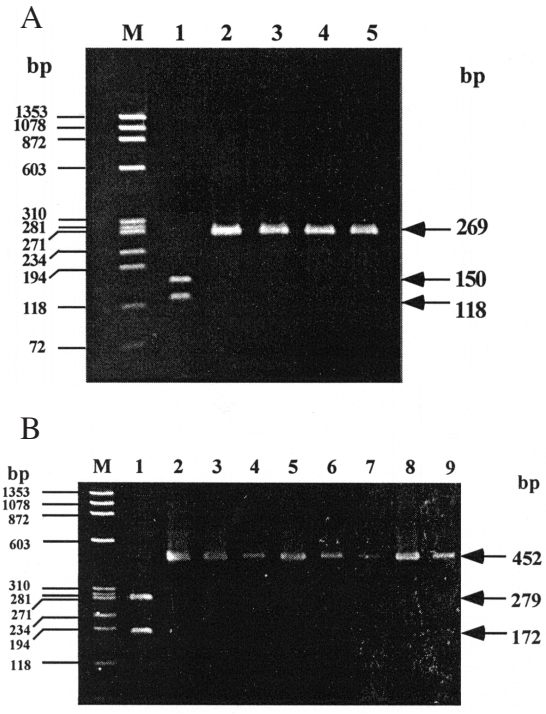

Fig 1–PCR-RFLP analysis of mosquito DNVs. PCR products amplified using L3/R4 (NS1 gene) (A) and L6/R7 (capsid gene) (B) primers (Table 1) were digested with *Eco*RV, separated by 5% PAGE, and stained with ethidium bromide staining. (A) Lane M: ΦX-174 *Hae*III-digested DNA size markers; lane 1: *Aal*DNV DNA; lanes 2-5: DNV-infected *T. splendens* DNA. (B) Lane M: ΦX-174 *Hae*III-digested DNA size markers; lane 1: *Aal*DNV DNA; lanes 2-9: DNV-infected *T. splendens* DNA.

DISCUSSION

Post-PCR methods including nested PCR, PCR-RFLP and PCR-SSCP have been applied for the analysis of gene transcripts and gene mutations (Orita *et al*, 1989), routine determination of HLA typing (Bannai *et al*, 1994; Xu *et al*, 2010) and recently for analysis of single nucleotide polymorphism (Chen *et al*, 1995). In the present study we used PCR-RFLP and PCR-SSCP to discriminate between two groups of mosquito DNVs. Two clades of mosquito DNVs derived from different origins have been reported and classified based on their distinct nucleotide sequences and suggested to be important DNVs that widely found in important mosquitoes, especially *Aedes* species (Sangdee and Pattanakitsakul, 2013). The clade, *Aal*DNV, was isolated from mosquito cell line, while *Aae*DNV and *Ts*DNV were isolated from mosquitoes (Afanasiev *et al*, 1991; Pattanakitsakul *et al*, 2007; Sangdee and Pattanakitsakul, 2012). The PCR-RFLP and PCR-SSCP revealed that *Aae*DNV and *Ts*DNV are more similar to each other than to *Aal*DNV, supporting previous nucleotide sequence and phylogenetic tree analyses (Pattanakitsakul *et al*, 2007; Sangdee and Pattanakitsakul, 2013).

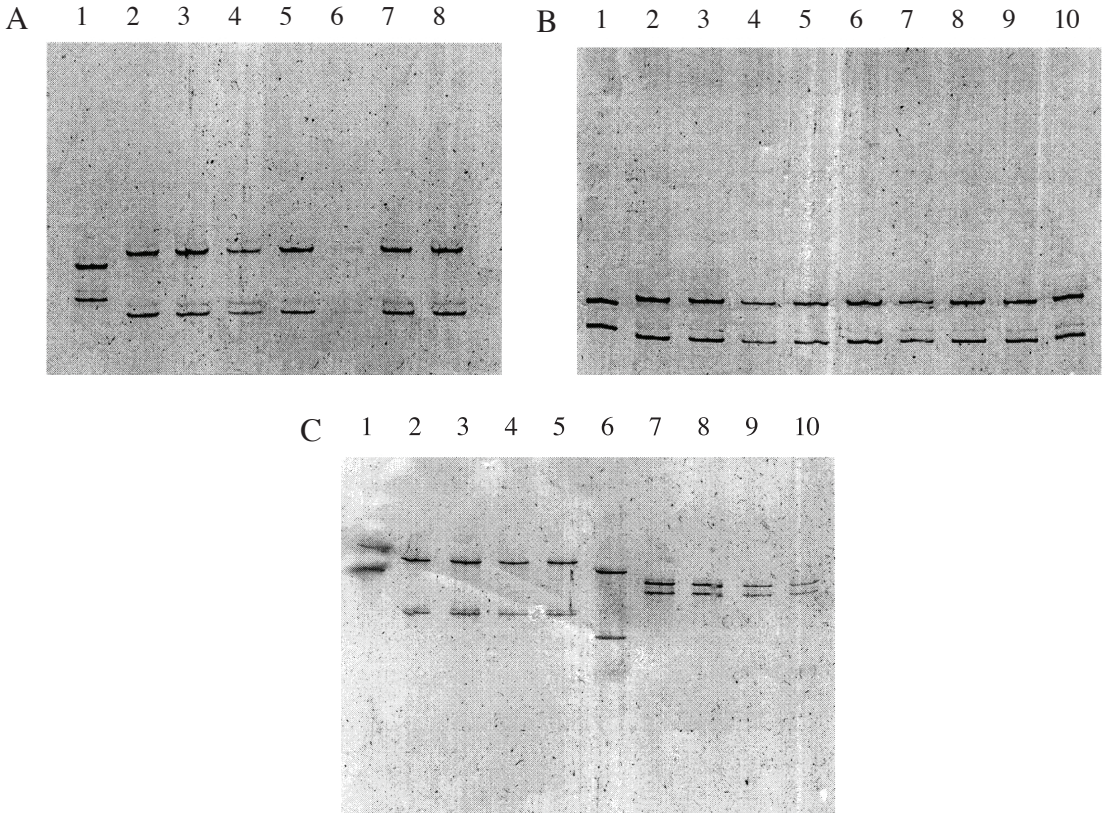

Fig 2–PCR-SSCP analysis of mosquito DNVs. PCR products amplified using L6/R7 (capsid gene) (A, B), and L6/R7 (capsid gene) and L7/R3 (NS1-capsid gene) (C) primers (Table 1) were subjected to SSCP analysis on 10% PAGE at 4ºC (A, C) and 25ºC (B). (A) Lane1: *Aal*DNV DNA; lanes 2-8: *Aae*DNV-infested mosquito DNA. (B) Lane 1: *Aal*DNV; lanes 2-10, *Aae*DNV-infected mosquito DNA. (C) Lane 1: *Aal*DNV capsid DNA; lanes 2 - 5: *Aae*DNV-infected mosquito capsid DNA; lane 6: *Aal*DNV NS1-capsid DNA; lanes 7-10: *Aae*DNV-infected mosquito NS1-capsid DNA.

Although PCR-RFLP is rapid and easy to perform, but it may not be suitable for analyzing unknown DNVs containing genetic variations that may result in changes of restriction sites. On the other hand, PCR-SSCP is more appropriate in overcoming the latter problem as this method is able to discriminate among only one nucleotide change (Bannai *et al*, 1994). However, during electrophoresis SSCP pattern is affected by temperature because it affects formation of intra-molecular base pairing and hence the formation of single-strand DNA (Chen *et al*, 1995). At low temperatures, single-stranded DNA can form more stable structures and migrate according to their conformation differences. Thus PCR-SSCP at 4ºC produced a clearer discrimination between these two groups of DNVs than at 25ºC. Although a faint SSCP analysis was observed in one sample (Fig 2A), but this may be due to low amount of DNV in this mosquito sample.

All primer pairs used in the study were appropriate for amplification of both

*Aal*DNV and *Aae*DNV genomes as they were designed from the conserved nucleotide sequences. (Pattanakitsakul *et al*, 2007; Sangdee and Pattanakitsakul, 2012, 2013). Both *Aae*DNV- and *Ts*DNV-infected mosquitoes have been studied previously by PCR and nucleotide sequencing (Kittayapong *et al*, 1999; Pattanakitsakul *et al*, 2007). Although other insect densoviruses were not be analyzed in this study, it is possible to explore this using the same procedure.

Several isolated insect densoviruses have been reported from mosquito cell lines distributed among several laboratories (Boublik *et al*, 1994; O'Neill *et al*, 1995; Chen *et al*, 2004; Paterson *et al*, 2005). *Toxorhynchites splendens* mosquitoes become infected by ingestion of DNV when feeding on infected *Culex* larvae (Pattanakitsakul *et al*, 2007). Thus *Ts*DNV is similar to *Aae*DNV group and is classified in the same group based on similarity of nucleotide sequence and phylogenetic tree analysis (Pattanakitsakul *et al*, 2007). These mosquito DNVs infect the same mosquito species that carry viruses causing dengue hemorrhagic fever and yellow fever, but *Aedes* DNV has not been reported to infect humans.

In summary, post-PCR such as PCR-RFLP and PCR-SSCP is appropriate for discrimination of clades of mosquito DNVs and can be adopted for using in the study of other insect DNVs. These post-PCR methods were rapid and require no advanced instrument to carry out in the laboratory. Although these methods could be applied for screening genetic variations of mosquito DNVs in natural mosquitoes and insects, but they may need several pairs of primers to cover the variation in nucleotide sequences of DNVs.

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