APPLICATION OF POST-PCR METHODS FOR ANALYSIS OF MOSQUITO DENSOVIRUS

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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, 1985). The Brevidensovirus/Contravirus consists of Aedes aegypti densovirus (AaeDNV) and Aedes albopictus densovirus (AalDNV). 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
plus strand with the left and mid ORFs encoding non-structural (NS) proteins and right ORF encoding a structural protein, except for AaeDNV 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 (Jouset 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 -80°C until used.

AalDNV 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 AalDNV-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 28°C for 7 days.

PCR

DNA from culture supernatant AalDNV 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 4°C. 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.
Primers used for amplification of DNV DNA were chosen from conserved sequences of both AalDNV and AaeDNV genomes (Table 1). The 25-µl PCR reaction consisted of 1X PCR buffer, 0.2 mM dNTPs, 1.5 mM MgCl₂, 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 AalDNV genomic fragment (nt 351-4025) was inserted in pUC18 plasmid (Sangdee and Pattanakit-sakul, 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 EcoRI or EcoRV at 37°C for 1.5 hours and then the DNA fragments were separated by 5% polyacrylamide gel-electrophoresis (acylamide: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.

### RESULTS

**Analysis of DNV using PCR-RFLP**

Comparisons of restriction enzyme maps of AalDNV and AaeDNV showed unique EcoRV sites at only nucleotide 580 and 3276 (within NS1 and capsid gene, respectively) in AalDNV genome. The PCR products from NS1 and capsid gene of
DNV amplified by L3-R4 and L6-R7 primers, respectively were further analyzed with EcoRV digestion of AalDNV NS1 amplicon generated fragments of 150 and 118 bp, while digestion of TsDNV revealed the undigested 269-bp DNA fragment (Fig 1A). EcoRV digestion of AalDNV capsid amplicon generated 279 and 172 bp DNA fragment and intact 452 bp DNA fragment with TsDNV (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 AalDNV and AaeDNV (Fig 2 A-C). Moreover, electrophoresis conducted at 4°C and 25°C also showed distinct patterns of these two groups of DNVs (Fig 2 A-C).

**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, AalDNV, was isolated from mosquito cell line, while AaeDNV and TsDNV were isolated from mosquitoes (Afanasiev et al, 1991; Pattanakitsakul et al, 2007; Sangdee and Pattanakitsakul, 2012). The PCR-RFLP and PCR-SSCP revealed that AaeDNV and TsDNV are more similar to each other than to AalDNV, supporting previous nucleotide sequence and phylogenetic tree analyses (Pattanakitsakul et al, 2007; Sangdee and Pattanakitsakul, 2013).
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 4°C produced a clearer discrimination between these two groups of DNVs than at 25°C. 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
AalDNV and AaeDNV genomes as they were designed from the conserved nucleotide sequences. (Pattanakitsakul et al, 2007; Sangdee and Pattanakitsakul, 2012, 2013). Both AaeDNV- and TsDNV-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 densovirus 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 TsDNV is similar to AaeDNV 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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REFERENCES


