APPLICATION OF QUANTITATIVE PCR FOR QUANTIZATION OF DENSOVIRUS GENOME

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Abstract. Densovirus is classified as invertebrate virus belonging to the subfamily Densovirinae of Parvoviridae family. This group of viruses infects only insects and several densoviruses have been isolated from indigenous mosquitoes and mosquito cell lines. A number of mosquitoes, especially *Aedes aegypti* and *Ae. albopictus* are important vectors of viruses, which are the major causes of dengue hemorrhagic fever and yellow fever in humans. As densoviruses do not cause any pathology in humans, these viruses have been proposed to be a potential vector for use in biological control of mosquitoes and insects. We report the application of quantitative (q)PCR to determine the amount of densovirus genome in mosquito cell culture supernatant and mosquito. This method is simple, rapid and has a wide dynamic range, and therefore is likely to be useful and applicable in the determination of viral load of other viruses in a variety of biological specimens.

Keywords: densovirus, qPCR, quantization, mosquito

INTRODUCTION

The family Parvoviridae is composed of 2 subfamilies of Parvovirinae and Densovirinae. Viruses in Parvovirinae subfamily infect vertebrates while those in Densovirinae infect invertebrates, mostly insects (Kurstak, 1972; Bachmann *et al*, 1975; Siegl *et al*, 1985). Densovirinae are divided into three genera: *Densovirus* (infecting cockroaches), *Iteravirus* (infecting silk worms) and *Brevidensovirus* or *Contravirus* (infecting mosquitoes), the latter genus consisting of *Aedes aegypti* densovirus (*Aae*DNV) and *Aedes albopictus* densovirus (*Aal*DNV). In addition to these two densoviruses, several other densoviruses have been reported infecting various 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). Mosquitoes that are infected with *Aedes* DNV are important vectors of dengue virus, the causative agent of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) in human populations worldwide (Hayes and Gubler, 1992; Rigau-Perez *et al*, 1998; Rodriguez-Tan and Weir, 1998).

Densovirus is a non-enveloped and

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icosahedral viral particle of 18-20 nm in diameter containing single-stranded DNA of 4.0-4.2 kb (Afanasiev *et al*, 1991; Boublik *et al*, 1994; Jousset *et al*, 1993). DNV genome contains palindromic structures forming stable hairpin structures at both termini, which have been suggested to play a role in DNA replication (Afanasiev *et al*, 1991,1994; Boublik *et al*, 1994), and encodes 3 open reading frames (ORF) on plus strand with the up-stream and mid ORFs encoding non-structural proteins (NSs) and down-stream ORF encoding a structural protein, except *Aae*DNV, which contains an extra ORF in the minus strand encoding a protein of unknown function. The DNV genomes can be separately encapsidated as a plus or minus strand in the virions (Afanasiev *et al*, 1991; Boublik *et al*, 1994).

Aedes DNV is an attractive model with which to develop as a biological control of insects because DNV genomes are small, which facilitate cloning and development of expression vectors for insects (Jousset *et al*, 1990; Dumas *et al*, 1992; Giraud *et al*, 1992). As *Aedes* DNV is specific for mosquitoes, with no report of causing harmful effects to humans, this virus is feasible to be used as a potential biological control agent of mosquitoes carrying insect-borne diseases.

We report a quantitative (q)PCR technique to measure the amounts of DNV genome in culture supernatants and mosquitoes. This method can be applied to monitor DNV load in biological specimens, such as serum and mosquito.

MATERIALS AND METHODS

Recombinant *E. coli* clone of *Aal*DNV

Recombinant *E. coli* clone containing 3.7 kb *Aal*DNV genome inserted in pUC18 plasmid vector has been described previously (Sangdee and Pattanakitsakul, 2012). DNV genome in pUC18 contained nucleotide sequence 351-4025 and the recombinant plasmid was transfected into *E. coli* DH5α and purified using QIAprep Miniprep kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The recombinant plasmid was used for optimization of qPCR and for construction of standard curve for determining amounts of DNV genome.

Cultivation of *Aal*DNV

*Aal*DNV was cultivated in C6/36 (*Aedes albopictus*) cell line according to previous report (Sangdee and Pattanakitsakul, 2012). In brief, stock virus at various multiplicity of infection (MOI) was used to infect C6/36 cell line for study of the kinetics of viral replication.

Mosquitoes

Toxorhynchites splendens mosquitoes were kindly provided by Dr Supatra Thongrungkiat, Department of Tropical Medicine, Mahidol University, Bangkok, Thailand (Pattanakitsakul *et al*, 2007) and were kept at -80ºC until used.

Quantification of DNV DNA in culture supernatant and mosquito by qPCR

DNA from *Aal*DNV-infected C6/36 cell culture supernatant (Sangdee and Pattanakitsakul, 2012) and *Tx. splendens* were isolated using DNAzol reagent (GibcoBRL, Grand Island, NY). For the latter, a mosquito was individually homogenized in a glass homogenizer containing 300 µl of Leibovitz's medium (L-15) and 1% fetal bovine serum (FBS). Then 10 µl aliquot was used for isolation of DNA as described previously (Pattanakitsakul *et al*, 2007). DNA was dissolved with 10 µl of sterile distilled water for use as template for qPCR.

Primers used for amplification of DNV DNA were forward primer (5′ AAC

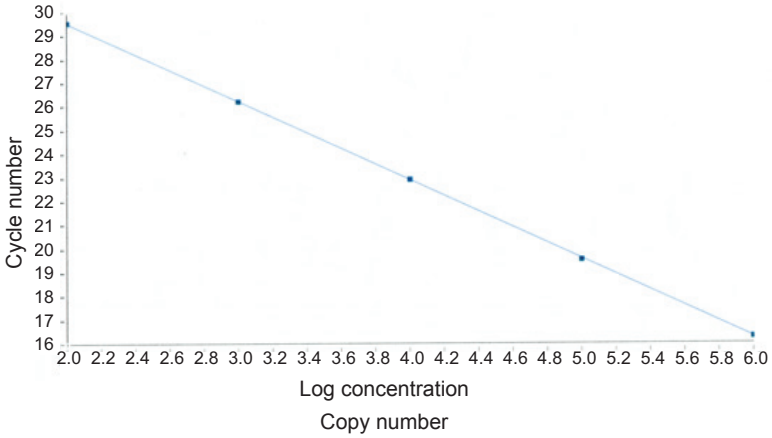

Fig 1–DNV kinetics in C6/36 cell culture supernatant. DNV copy number was plotted against time post-infection in C6/36 cells. Virus kinetics at MOI of 0.1, 1.0 and 10 are indicated in each graph.

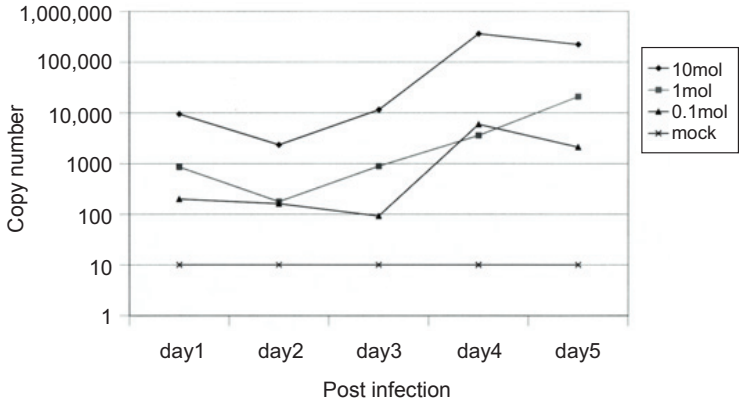

Fig 2–Standard curve for quantification of DNV DNA by qPCR. The standard curve was constructed by plotting threshold cycle against copy number of DNA genome.

AAG ACA GAG ACT GCT AAC 3′, nt 2968 to 2988) and reverse primer (5′ GCA TTC TTG GAT ATG ATG TTC T 3′, nt 3422 to 3401) in order to generate an amplicon from a conserved sequence of the structural gene of both *Aal*DNV and *Ts*DNV genomes and the nucleotide positions corresponded to the sequence of *Aae*DNV (GeneBank accession number M37899).

Reaction mixture contained 2 µl of DNA, and 18 µl of master mix containing 2 µl of LightCycler–FastStart DNA Master SYBR Green I, 5 mM $MgCl_2$, 2 µM each primer pair and water. Thermocycling was conducted in a LightCycler instrument equipped with software version 3.5 (Roche Applied Sciences, Mannheim, Germany) as follows: 95°C for 15 seconds, 50°C for 5 seconds and 72°C for 20 seconds, with temperature transition rate of each step set at 20°C/second. In order to verify the specific products, a melting curve analysis was performed after the final step of amplification in order to confirm specificity of the primer pair and absence of primer dimers. Recombinant DNV plasmid with known amounts of DNA was 10-fold serially diluted (10^2 - 10^6 copies) and used to generate a standard curve. DNA from *Aal*DNV was also amplified in parallel with mosquito samples as a positive control of amplification, while distilled water was replaced to DNA for negative control.

RESULTS

The key parameter that needed to be optimized in qPCR was the concentration of $MgCl_2$ to which *Taq* DNA polymerase is

Table 1
Quantification of *Ts*DNV genome in *Toxorhynchites splendens* mosquitoes.

Tx. splendens no.	Copy number of *Ts*DNV genome
1	2.25×10^7
2	9.42×10^5
3	4.42×10^7
4	7.60×10^5

highly sensitive. Comparison of 1-5 mM $MgCl_2$ showed that optimal concentration was 5 mM under the conditions used (data not shown).

Quantification of DNV genome content of a sample was obtained from a calibration curve. This consisted of determining SYBR Green fluorescence (excitation at 480 nm and emission at 520 nm) threshold cycle of a series of solutions containing known DNV genome copy numbers (Fig 1). The amount of DNV genome was calculated from the size of DNA template plasmid (6.361 kb) and 1 µg of this plasmid is equal to 0.23 pmol or 1.385×10^{11} molecules. The standard curve was linear with high correlation coefficient of $r^2 \geq 0.99$.

DNA isolated from C6/36 cell culture supernatant was measured at various times of cultivation with *Aal*DNV at various MOIs. Viral copy number increased in a time-dependent fashion from day 1 to day 5 post-infection. Viral DNA of MOI from 1-10 decreased slightly on day 2 of cultivation but then increased up to day 5, while viral DNA from viral culture with MOI of 0.1 was inconsistent with cultivation time (Fig 2). No viral DNA was detected in the mock-control supernatant.

Applying qPCR to quantitate *Aal*DNV in infected *Tx. splendens* revealed that the numbers of viruses per mosquito varied from 7.60×10^5 to 4.42×10^7 copies (Table 1).

DISCUSSION

SYBR Green I, a minor groove double-stranded DNA binder, has more frequently used in qPCR because of the simple and economical procedure (Wong and Medrano, 2005). These include measurements of gene expression (Bustin, 2002; Huggett *et al*, 2005), pathogens loads (Espy *et al*, 2006; Watzinger *et al*, 2006) and of viral kinetics as genome copy numbers of some viruses can not be determined due to unavailability of plaque formation or antibody detection technique.

The primer pair used in qPCR of this study has previously been demonstrated to be appropriate for amplification of both groups of DNV genome (*Aal*DNA and *Aae*DNV), which are derived from different isolated origins (Pattanakitsakul *et al*, 2007; Sangdee and Pattanakitsakul, 2012, 2013). DNV in C6/36 culture supernatant and mosquito is *Aal*DNV and *Ts*DNV, respectively (Pattanakitsakul *et al*, 2007; Sangdee and Pattanakitsakul, 2012). *Aedes* densovirus is specific for mosquito infection and there is no report of infection in humans. Virus infection of C6/36 cells at MOI of 1 and 10 began to show presence of virus in culture supernatant from day 2, while at MOI of 0.1 this was observed at day 3 but the amounts were inconsistent. During the early phase of infection the low MOI of virus was not enough to infect all cells in the culture and could not produce more progeny virus to infect remaining cells in the culture. This may affect the viral replication during in the first 3 days of infection. After the increasing of viral progeny the viral replication increased rapidly after day 3 post-infection.

Several insect densoviruses have been reported to be isolated from mosquito cell lines distributed in several laboratories (Boublik *et al*, 1994; O'Neill *et al*, 1995; Chen *et al*, 2004; Paterson *et al*, 2005). We have reported previously that DNV in these mosquito cell lines was from ingestion of predated larvae used in the laboratory during routine feeding mosquitoes (Pattanakitsakul *et al*, 2007). This contaminating DNV is similar to *Aae*DNV group based on phylogenetic analysis of the nucleotide sequences (Pattanakitsakul *et al*, 2007). The qPCR technique used in this study allowed determination of the amounts of contaminating DNV without having to perform a virus titer assay. Although these mosquitoes were reared in a laboratory insectaria and although DNV infections of natural indigenous mosquitoes are uncommon, they could be found (Kittayapong *et al*, 1999).

In summary, results of the present study demonstrate that qPCR can be applied for quantification of viral genome in the study of viral kinetics and of viral load in mosquitoes. This technique should be applicable to other viruses isolated from a variety of biological samples.

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REFERENCES

Afanasiev BN, Galyov EE, Buchatsky LP, Kozlov YV. Nucleotide sequence and genomic organization of Aedes densonucleosis virus. *Virology* 1991; 185: 323-36.

Afanasiev BN, Kozlov YV, Carlson JO, Beaty BJ. Densovirus of *Aedes aegypti* as an expression vector in mosquito cells. *Exp Parasitol* 1994; 79: 322-39.

Bachmann PA, Hoggan MD, Melnick JL, Pereira HG, Vago C. Parvoviridae. *Intervirology* 1975; 5: 83-92.

Boublik Y, Jousset FX, Bergoin M. Complete nucleotide sequence and genomic organization of the *Aedes albopictus* parvovirus (AaPV) pathogenic for *Aedes aegypti* larvae. *Virology* 1994; 200: 752-63.

Bustin SA. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J Mol Endocrinol* 2002; 29: 23-39.

Chen S, Cheng L, Zhang Q, *et al*. Genetic, biochemical and structural characterization of a new densovirus isolated from a chronically infected *Aedes albopictus* C6/36 cell line. *Virology* 2004; 318: 123-33.

Dumas B, Jourdan M, Pascaud AM, Bergoin M. Complete nucleotide sequence of the cloned infectious genome of *Junonia coenia* densovirus reveals an organization unique among parvoviruses. *Virology* 1992; 191: 202-22.

Espy MJ, Uhl JR, Sloan LM, *et al*. Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clin Microbiol Rev* 2006; 19: 165-256.

Giraud C, Devauchelle G, Bergoin M. The densovirus of *Junonia coenia* (*Jc* DNV) as an insect cell expression vector. *Virology* 1992; 186: 207-18.

Hayes EB, Gubler DJ. Dengue and dengue hemorrhagic fever. *Pediatr Infect Dis J* 1992; 11: 311-7.

Huggett J, Dheda K, Bustin S, Zumla A. Real-time RT-PCR normalisation; strategies

and considerations. *Genes Immun* 2005; 6: 279-84.

Jousset FX, Barreau C, Boublik Y, Cornet M. A parvo-like virus persistently infecting a C6/36 clone of *Aedes albopictus* mosquito cell line and pathogenic for *Aedes aegypti* larvae. *Virus Res* 1993; 29: 99-114.

Jousset FX, Jourdan M, Compagnon B, Mialhe E, Veyrunes JC, Bergoin M. Restriction maps and sequence homologies of two densovirus genomes. *J Gen Virol* 1990; 71: 2463-6.

Kittayapong P, Baisley KJ, O'Neill SL. A mosquito densovirus infecting *Aedes aegypti* and *Aedes albopictus* from Thailand. *Am J Trop Med Hyg*. 1999; 61: 612-7.

Kurstak E. Small DNA densonucleosis virus (DNV). *Adv Virus Res* 1972; 17: 207-41.

O'Neill SL, Kittayapong P, Braig HR, Andreadis TG, Gonzalez JP, Tesh RB. Insect densoviruses may be widespread in mosquito cell lines. *J Gen Virol* 1995; 76: 2067-74.

Pattanakitsakul S, Boonnak K, Auethavornanan K, *et al*. A new densovirus isolated from the mosquito *Toxorhynchites splendens* (Wiedemann) (Diptera:Culicidae). *Southeast Asian J Trop Med Public Health* 2007; 38: 283-93.

Paterson A, Robinson E, Suchman E, Afanasiev B, Carlson J. Mosquito densonucleosis viruses cause dramatically different infection phenotypes in the C6/36 *Aedes albopictus* cell line. *Virology* 2005; 337: 253-61.

Rigau-Perez JG, Clark GG, Gubler DJ, Reiter P, Sanders EJ, Vorndam AV. Dengue and dengue haemorrhagic fever. *Lancet* 1998; 352: 971-7.

Rodriguez-Tan RS, Weir MR. Dengue: a review. *Tex Med* 1998; 94: 53-9.

Sangdee K, Pattanakitsakul S. New genetic variation of *Aedes albopictus* densovirus isolated from mosquito C6/36 cell line. *Southeast Asian J Trop Med Public Health* 2012; 43: 1122-33.

Sangdee K, Pattanakitsakul S. Comparison of mosquito densoviruses: two clades of viruses isolated from indigenous mosquitoes. *Southeast Asian J Trop Med Public Health* 2013; 44: 586-93.

Siegl G, Bates RC, Berns KI, *et al*. Characteristics and taxonomy of Parvoviridae. *Intervirology* 1985; 23: 61-73.

Watzinger F, Ebner K, Lion T. Detection and monitoring of virus infections by real-time PCR. *Mol Aspects Med* 2006; 27: 254-98.

Wong ML, Medrano JF. Real-time PCR for mRNA quantitation. *Biotechniques* 2005; 39: 75-85.

Zhai YG, Lv XJ, Sun XH, *et al*. Isolation and characterization of the full coding sequence of a novel densovirus from the mosquito *Culex pipiens pallens*. *J Gen Virol* 2008; 89: 195-9.