Short Report: Comparison of the Mosquito Inoculation Technique and Quantitative Real Time Polymerase Chain Reaction to Measure Dengue Virus Concentration

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Abstract. An accurate measure of infectious dengue virus in human and mosquito tissues is critical to fully understand virus–host relationships, disease severity, viral fitness, and pathogenesis. In recent years, RNA copy number measured by quantitative real time-polymerase chain reaction has been used to measure dengue virus concentration *in vitro* and *in vivo*. In this study, we detail important differences in the measurement of viral growth kinetics in Vero and C6/36 tissue cultures, in *Aedes aegypti* mosquitoes, and in viremic human sera using RNA genomic equivalents and mosquito infectious dose 50 (MID₅₀). Although there was reasonably good correlation between the two methods, RNA copy number was 2 to 5 logs greater than infectious virus titers. These differences varied significantly depending on virus strain, viral platform, infectious virus assay, and viral growth phase. The results have important implications for the correct interpretation of biological and epidemiological data from experimental and clinical studies, and show that genomic equivalents should be interpreted with caution when used as a proxy for infectious virus in such studies.

Epidemic dengue/dengue hemorrhagic fever (DF/DHF) has emerged as the most important mosquito-borne viral disease of humans in the past 40 years with both the viruses and mosquito vectors spreading globally in the tropics.¹ This spread has been closely linked to the global trends of urbanization and globalization, combined with a lack of effective mosquito control. Most urban centers of the tropics are now hyperendemic with multiple virus serotypes co-circulating. The result has been larger and more frequent epidemics associated with more severe disease.¹

From the 1940s when dengue viruses (DENV) were first isolated^{2,3} until the 1960s, scientists relied on suckling mice for isolation and assay of DENV. In the 1960s, mammalian cell cultures were used,⁴ but both methods were highly insensitive to primary DENV isolates that had not been adapted by serial passage.⁵ Subsequent development of the mosquito inoculation technique and the C6/36 *Aedes albopictus* cell line in the 1970s provided significant improvement in sensitivity, and permitted work with unpassaged DENVs.^{5–7} However, the relatively insensitive plaque assay that measures plaque-forming units (PFU)⁸ has continued to be used to measure infectious DENV in experimental studies, and the C6/36 cell culture system has been primarily used for virus isolation^{6–9} because most virology laboratories lacked the ability to work^{7,8,10} with live mosquitoes.

The efficacy of clinical diagnosis, surveillance, prevention, and control of dengue has been limited by the lack of easy to use and sensitive diagnostic tests. Currently, laboratory diagnosis in most dengue-endemic countries relies on detecting immunoglobulin M (IgM) antibody in acute serum samples. More recently, commercial tests combining NS1 antigen and IgM antibody detection have become increasingly popular.¹¹ For DENV detection and quantitation, quantitative real time polymerase chain reaction (qRT-PCR) has become the method of choice in the past 20 years^{12–15}; this method is generally more sensitive and efficient than isolation assays, and can

provide a rapid serotype-specific diagnosis. Moreover, DENVs can be identified and quantified directly from clinical samples. Although qRT-PCR measures RNA and not infectious virus, qRT-PCR has been increasingly used in recent years to measure DENV titers.^{14,15}

Although qRT-PCR has been compared with the relatively insensitive plaque assay (PFU),^{16–20} the actual ratio of RNA copy number to infectious virus remains unclear. Moreover, it is not known to what degree the infected host, the virus strain, or time of infection may influence that ratio. To better define the quantitative and biological relationships between RNA copy number and infectious DENV, we compared qRT-PCR with the titer of infectious DENV measured by the mosquito inoculation technique (mosquito infectious dose 50, MID₅₀), which is the most sensitive biological assay available for measuring unpassaged infectious DENVs.

Quantitative comparisons were performed using viremic human sera, infected mosquitoes, vertebrate, and mosquito cell cultures. Two low passage DENV-2 strains (PR1940 and PR6913) with contrasting virus replication kinetics (Manokaran and others, 2013, manuscript submitted) isolated during a 1994 epidemic in Puerto Rico, were used in the mosquito and *in vitro* cell culture experiments. DENV-2 viremic sera were obtained from patients during a 2011 epidemic in Pakistan.²¹ The DENV-1, -3, and -4 were isolated from patients during routine surveillance in Singapore and Indonesia.^{22,23} All patient samples were collected with patient consent and institutional review board approval.

Aedes aegypti mosquitoes were obtained from a colony at the Duke-NUS Graduate Medical School. The colony was established in 2010 with specimens collected in Ang Mo Kio, Singapore, and supplemented monthly with field-collected mosquitoes (10% of colony) to maintain genetic diversity. To investigate virus kinetics, 1- to 5-day-old female *Aedes aegypti* mosquitoes were inoculated with 100 MID₅₀ of each virus⁵ and incubated at 28°C. Infected mosquitoes were harvested at Days 3, 7, 10, 14, and 17 post-infection. Surviving mosquitoes were killed by freezing and stored at –80°C until assayed. Both C6/36 and Vero cell cultures were inoculated with 0.1 multiplicity of infection of each virus. Cell culture supernatants were harvested on Days 1, 3 and 7 post-infection and stored at –80°C until assayed.

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FIGURE 1. Replication kinetics of DENV-2 (A) PR1940 and (B) PR6913 in adult female *Aedes aegypti* mosquitoes. Virus titers are measured by plaque assay (PFU/mosquito $\blacktriangle$), mosquito inoculation technique (MID₅₀/mosquito $\blacksquare$), and qRT-PCR (RNA copy number/mosquito $\bullet$). Each point represents the mean of three mosquitoes triturated individually and the error bars indicate standard error of the mean.

Three individual mosquitoes at each time point were triturated and titrated by qRT-PCR, mosquito inoculation and plaque assay, and the mean titer calculated. Cell culture supernatants at each time point were titrated by qRT-PCR and mosquito inoculation only. For the MID₅₀ assay, virus titrations were performed by making 10-fold serial dilutions of each of the virus suspensions and viremic sera in Leibovitz's L-15 medium. Dilutions were inoculated intrathoracically into six male mosquitoes and held for 10 days at 28°C, after which surviving mosquitoes were harvested and stored at -80°C⁵. Harvested mosquitoes were examined for the presence of viral antigen in brain tissues by indirect immunofluorescence on mosquito head squashes²⁴ and the MID₅₀ per mosquito or per mL was calculated by the method of Reed and

Muench.²⁵ For plaque assay,⁸ serial dilutions of each virus suspension were inoculated in triplicate onto BHK-21 cells and incubated for 1 h at 37°C. Media was aspirated and replaced with 0.8% methyl-cellulose in maintenance medium. After 6 days at 37°C, cells were fixed with 20% formaldehyde and stained with 1% crystal violet. The plates were washed and dried, and the PFU per mosquito or per mL were counted.

Viral RNA quantitation was performed following RNA extraction of viremic sera, infected-mosquito tissues, and cell culture supernatants using the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany). A one-step qRT-PCR was performed using the SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, CA). The RNA copy number was calculated by generating a standard curve

FIGURE 2. Replication kinetics of DENV-2 PR1940 and PR6913 derived in cell cultures measured by the mosquito inoculation technique (MID₅₀/mL $\blacksquare$) and quantitative real time polymerase chain reaction (qRT-PCR) (RNA copy number/mL $\bullet$). (A) PR1940 in Vero cell culture. (B) PR1940 in C6/36 cell culture. (C) PR6913 in Vero cell culture. (D) PR6913 in C6/36 cell culture. Each point represents the mean of three biological replicates and the error bars indicate standard error of the mean.

from a plasmid control containing the region of interest¹³; the primers designed for qRT-PCR target the region NS5 for DENV-1, E for DENV-2, prM for DENV-3, and E for DENV-4.

All results represent the average of three biological replicates. A two-tailed unpaired Student's *t* test was used to determine if the difference in the means was statistically significant. Linear regression analysis was used to determine if MID₅₀ titers correlated with the RNA copy number ($P < 0.05$), and calculations equivalent to analysis of covariance was used to assess differences between slopes (GraphPad Prism v5.0, GraphPad Software Inc., La Jolla, CA).

The replication kinetics of DENV in live *Aedes aegypti* mosquitoes, *Aedes albopictus* mosquito cell cultures, and Vero mammalian cell cultures showed that RNA copy number was typically 2–3 logs greater than the MID₅₀ titer, regardless of the host tissue or cell culture from which the virus was harvested (Figures 1 and 2). When titers per whole mosquito were compared, the RNA copy number was 100 to 1,000 times higher than the MID₅₀ titer, which was 100 to 1,000 times higher than the PFU measured by plaque assay (Figure 1). This difference was evident for both DENV-2 strains (PR1940 and PR6913), regardless of the maximum titers observed in all assay platforms.

In general, linear regression showed that the RNA copy number was correlated with MID₅₀ titers for DENV-2 in mosquitoes ($P < 0.0001$, $R^2 = 0.567$) and cell cultures ($P < 0.0001$, $R^2 = 0.950$) (Figure 3A). However, the slopes differ significantly ($P < 0.001$, $F = 13.95$), showing that the ratio of RNA copy number to infectious virus may differ when using differ-

TABLE 1
Comparative titration of C6/36 cell culture virus supernatants by qRT-PCR and mosquito inoculation*

DENV serotype	Copy number/mL	MID ₅₀ /mL	Log difference (<i>P</i> value)
DENV-1 EDEN2928	5.81E+10	5.88E+08	2 (0.0019)
DENV-3 Indon1219	1.57E+09	2.88E+08	0.7 (0.0006)
DENV-4 EDEN2270	1.83E+10	5.88E+07	2.5 (0.0009)

*qRT-PCR = quantitative real time polymerase chain reaction.

ent host systems to grow DENV. Although there is a relatively good general correlation between the MID₅₀ titers and RNA copy number using the same host systems to grow DENV, the accuracy of measuring infectious DENV using RNA copy number may vary based on the virus strain or time of infection as the ratio may be significantly different from one another (Figure 3B and C). Different conversion ratios were also shown for different serotypes of DENV, with 7 day old C6/36 virus supernatants for DENV-1, DENV-3, and DENV-4 showing 2.0, 0.7, and 2.5 logs higher concentrations by qRT-PCR, respectively (Table 1). Of interest, DENV-3 concentrations varied by only 0.7 log between the two methods. This small difference could be a unique replication characteristic of that virus strain or result from the specific time in viral growth when it was sampled. Clearly, more strains of all four serotypes should be tested.

A greater variation was observed when measuring DENV-2 viremias in human sera using the two methods, varying by 2–5 logs, depending on the individual serum (Figure 4). No correlation was observed between RNA copy number and infectious virus titers for human sera ($P = 0.3109$).

FIGURE 3. (A) RNA copy number versus MID₅₀ in *Aedes aegypti* mosquitoes (●) and cell cultures (○). The regression equations are DENV-2 copies = 0.653 MID₅₀ + 4.93 ($R^2 = 0.567$) and DENV-2 copies = 1.05 MID₅₀ + 2.14 ($R^2 = 0.950$), respectively. The two slopes are significantly different. (B) Ratio of genomic equivalents (GE) to MID₅₀ at different time-points for PR1940 and PR6913 in mosquitoes. (C) Ratio of genomic equivalents (GE) to MID₅₀ at different time-points for PR1940 and PR6913 in cell cultures.

FIGURE 4. Comparative titration of ten viremic (DENV-2) human sera by quantitative real time polymerase chain reaction (qRT-PCR) and mosquito inoculation.

This is the first direct comparison of RNA copy number measured by qRT-PCR, and infectious DENV titer measured by the mosquito inoculation technique, *in vitro* and *in vivo*. Our results agree with previous studies, which show positive correlations between flavivirus RNA copy number and infectious virus in cell cultures and *Aedes aegypti* mosquitoes.^{16–20} A consistently higher, but variable RNA copies to infectious virus titer ratio is likely caused by the presence of noninfectious immature virions or defective virus particles.^{16–20,26,27} However, the differences in ratio could also be caused by intrinsic variation in virus replication or translational efficiencies in different host tissues. Of importance was the lack of correlation between RNA copy number and infectious virus titers in human sera. Viremia (infectious virus) in humans is influenced by the strain of virus, the day of infection the serum was collected from the patient, and the individual's previous dengue experience, which influences the innate and adaptive immune response and thus, the production of noninfectious defective virus particles. Infectious virus titers can also be influenced by how the serum is processed after the blood draw, the number of freeze-thaw cycles, and the storage temperature and how it is shipped. It should be noted, however, that the sera used in these experiments were processed immediately after the blood draw, stored at -80°C , and shipped frozen on dry ice to Singapore; the sera were never thawed before shipping to Singapore.

In conclusion, we show that RNA genomic equivalents are not a reliable proxy for infectious virus as the host, the virus strain, and time of infection may influence the ratio of genomic equivalents to infectious DENV. Although there was a reasonably good correlation between the two methods in measuring virus concentration, caution must be exercised in generalizing about infectious virus and the interpretation of results in both clinical and experimental studies. An accurate measure of infectious virus is critical to understanding dengue virus biology and pathogenesis, and for development of effective diagnostic tests, vaccines, and therapeutics. Thus, although qRT-PCR is a highly sensitive and useful DENV diagnostic tool, quantitation of infectious DENV, especially from sera, autopsy tissues, and mosquitoes should ideally be performed using the mosquito inoculation assay, which is arguably the most sensitive quantitative assay for low passage DENVs. Realizing that this will not be possible in most dengue diagnostic and research laboratories, it is recommended that data obtained using qRT-PCR to measure infectious DENV is interpreted with caution.

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REFERENCES

- Gubler DJ, 2011. Dengue, urbanization and globalization: the unholy trinity of the 21st century. *Trop Med Health* 39: 1–9.
- Kimura R, Hotta S, 1944. Studies on dengue fever (VI). On the inoculation of dengue virus into mice (In Japanese). *Nippon Igaku* 3379: 629–633.
- Sabin AB, 1952. Research on dengue during World War II. *Am J Trop Med Hyg* 1: 30–50.
- Halstead SB, Sukhavachana P, Nisalak A, 1964. *In vitro* recovery of dengue viruses from naturally infected human beings and arthropods. *Nature* 202: 931–932.
- Rosen L, Gubler DJ, 1974. The use of mosquitoes to detect and propagate dengue viruses. *Am J Trop Med Hyg* 23: 1153–1160.
- Igarashi A, 1978. Isolation of a Singh's *Aedes albopictus* cell clone sensitive to Dengue and Chikungunya viruses. *J Gen Virol* 40: 531–544.
- Tesh RB, 1979. A method for the isolation and identification of dengue viruses, using mosquito cell cultures. *Am J Trop Med Hyg* 28: 1053–1059.
- Yuill TM, Sukhavachana P, Nisalak A, Russell PK, 1968. Dengue-virus recovery by direct and delayed plaques in LLC-MK2 cells. *Am J Trop Med Hyg* 17: 441–448.
- Vorndam AV, Kuno G, 1997. Laboratory diagnosis of dengue virus infections. Gubler DJ, Kuno G, editors. *Dengue and Dengue Haemorrhagic Fever*. New York: CAB International, 313–334.
- Gubler DJ, Kuno G, Sather GE, Velez M, Oliver A, 1984. Mosquito cell cultures and specific monoclonal antibodies in surveillance for dengue viruses. *Am J Trop Med Hyg* 33: 158–165.
- Blacksell SD, Jarman RG, Bailey MS, Tanganuchitcharnchai A, Jenjaroen K, Gibbons RV, 2011. Evaluation of six commercial point-of-care tests for diagnosis of acute dengue infections: the need for combining NS1 antigen and IgM/IgG antibody detection to achieve acceptable levels of accuracy. *Clin Vaccine Immunol* 18: 2095–2101.
- Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV, 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain-reaction. *J Clin Microbiol* 30: 545–551.
- Johnson BW, Russell BJ, Lanciotti RS, 2005. Serotype-specific detection of dengue viruses in a fourplex real-time reverse transcriptase PCR assay. *J Clin Microbiol* 43: 4977–4983.
- Guzman MG, Kouri G, 2004. Dengue diagnosis, advances and challenges. *Int J Infect Dis* 8: 69–80.
- Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, Suntayakorn S, 2000. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. *J Infect Dis* 181: 2–9.
- Richardson J, Molina-Cruz A, Salazar MI, Black WT, 2006. Quantitative analysis of dengue-2 virus RNA during the extrinsic incubation period in individual *Aedes aegypti*. *Am J Trop Med Hyg* 74: 132–141.

17. Bae HG, Nitsche A, Teichmann A, Biel SS, Niedrig M, 2003. Detection of yellow fever virus: a comparison of quantitative real-time PCR and plaque assay. *J Virol Methods* 110: 185–191.
18. van der Schaar HM, Rust MJ, Waarts BL, van der Ende-Metselaar H, Kuhn RJ, Wilschut J, Zhuang X, Smit JM, 2007. Characterization of the early events in dengue virus cell entry by biochemical assays and single-virus tracking. *J Virol* 81: 12019–12028.
19. Wang WK, Sung TL, Tsai YC, Kao CL, Chang SM, King CC, 2002. Detection of dengue virus replication in peripheral blood mononuclear cells from dengue virus type 2-infected patients by a reverse transcription-real-time PCR assay. *J Clin Microbiol* 40: 4472–4478.
20. Colton L, Biggerstaff BJ, Johnson A, Nasci RS, 2005. Quantification of West Nile virus in vector mosquito saliva. *J Am Mosq Control Assoc* 21: 49–53.
21. Khan MA, Ellis EM, Tissera HA, Alvi MY, Rahman FF, Masud F, Chow A, Howe S, Dhanasekaran V, Ellis BR, Gubler DJ, 2013. Emergence and diversification of dengue 2 cosmopolitan genotype in Pakistan. *PLoS ONE* 8: e56391.
22. Low JG, Ooi EE, Tolfvenstam T, Leo YS, Hibberd ML, Ng LC, 2006. Early Dengue infection and outcome study (EDEN)—study design and preliminary findings. *Ann Acad Med Singapore* 35: 783–789.
23. Gubler DJ, Suharyono W, Lubis I, Eram S, Sulianti Saroso J, 1979. Epidemic dengue hemorrhagic fever in rural Indonesia. I. Virological and epidemiological studies. *Am J Trop Med Hyg* 28: 701–710.
24. Kuberski TT, Rosen L, 1977. A simple technique for the detection of dengue antigen in mosquitoes by immunofluorescence. *Am J Trop Med Hyg* 26: 533–537.
25. Reed LJ, Muench HA, 1938. A simple method of estimating fifty percent endpoints. *Am J Hyg* 27: 493–497.
26. Wang S, He R, Anderson R, 1999. PrM- and cell-binding domains of the dengue virus *E* protein. *J Virol* 73: 2547–2551.
27. Murray JM, Aaskov JG, Wright PJ, 1993. Processing of the dengue virus type 2 proteins prM and C-prM. *J Gen Virol* 74: 175–182.