Acta virologica **57**: 379 – 381, 2013 doi:10.4149/av_2013_03_379

# LETTER TO THE EDITOR

## A microneutralization assay for dengue viruses using mosquito C6/36 cells

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*Received January 9, 2013; accepted July 15, 2013*

**Keywords:** dengue virus; microneutralization assay; C6/36 cells

Dengue fever and dengue hemorrhagic fever, which are caused by the dengue viruses (DENVs), have become major worldwide public health problem during recent decades. DENVs are arthropod-borne viruses that belong to the *Flavivirus* genus in the *Flaviviridae* family and have four known serotypes, DENV-1, -2, -3, and -4. During a 1942 epidemic in Japan, the first DENV was identified. Plaque reduction neutralization test (PRNT) to measure DENV plaques using neutral red staining was first reported in 1967 (*1*). PRNT has been used to determine serum titers capable of reducing dengue virus plaques by 50% ($PRNT_{50}$) by comparing with a control standard (*2*). Consequently, PRNT has been widely used in vaccine development, related academic research, and in public health laboratories for measuring DENVs. Though PRNT is useful for assessing the number of infectious DENV PFUs, there are two critical limitations. PRNT should be performed in six-well plates and requires a large volume of immune serum, potentially imposing a limitation on experiments with low-titer sera (*3*). Additionally, the fact that clinical DENV isolates do not form clear plaques in cultured cell monolayers highlights the difficulty in using PRNT to detect DENVs (*4*). Due to these limitations, many studies have concentrated on developing more efficient and convenient protocols to detect DENVs and to measure the neutralizing activity of DENV-specific antibodies.

An *in vitro* microneutralization assay, based on an enzyme-linked immunospot (ELISPOT) protocol, represents an improved and modified PRNT procedure (*2, 3, 5*). Many cell types, including BHK-21, LLC-MK2 (rhesus monkey kidney cell line), Vero and CV-1 (African green monkey-derived epithelial cell lines) are being studied for development of an *in vitro* microneutralization assay. However, use of the C6/36 cell line derived from whole hatched larva of *Stegomyia albopicta* (*Aedes albopictus*), which is a natural vertical transmission host of DENVs, has not been previously reported in the microneutralization assay. However, these cells are used for propagation of all of DENVs and PRNT. In the present study, we developed a microneutralization assay for assessing DENV infection using a propagation host C6/36 mosquito cell line grown as a monolayer in 96-well plates. This method represents an improved and modified PRNT performed in 96-well ELISPOT plates (Millipore), thereby requiring substantially less serum than the classic PRNT.

We initially monitored the infection of all clinical DENV isolates through confocal laser scanning microscopy (LSM 510 META; Carl Zeiss). C6/36 cells were successfully infected with clinical DENV isolates as evidenced by positive staining with DENV-antibody (AbD Serotec) compared with isotype control staining (Fig. 1a). To develop the microneutralization plaque assay using C6/36 cells, we confirmed the spot formation in C6/36 cells by infection with clinical DENV isolates. Initially, 1 x $10^4$ per well C6/36 cells were inocu-

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**Abbreviations**: DENV = dengue virus; ELISPOT = enzyme-linked immunospot; EDIII = envelop domain III; PRNT = plaque reduction neutralization test

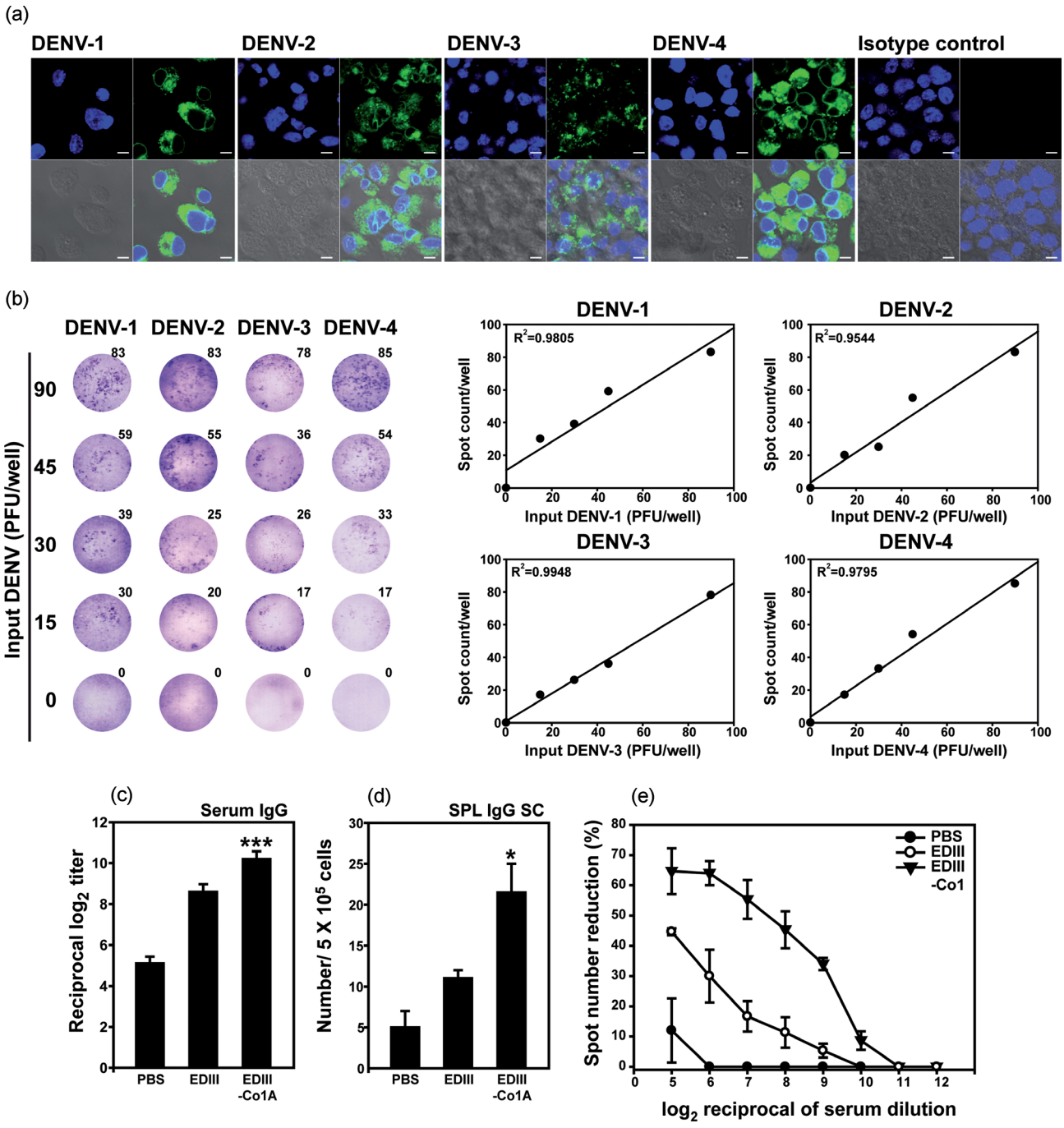


**Fig. 1**

(a) Infection of monolayered C6/36 cells with all four serotypes of clinical DENV isolates. DENVs were labeled with DENV-antibody followed by a FITC-conjugated secondary antibody (green). Nuclei were counter-stained with DAPI (blue). (b) Correlation between the number of plaques formed by infection with each serotype and estimated by end-point plaque titration in microplate wells. (c) The level of EDIII-specific serum IgG and (d) the number of EDIII-specific IgG-secreting cells in splenocytes in the mice immunized with each indicated antigen. (e) A microplate plaque reduction neutralization test with sera from mice immunized with each indicated antigen using C6/36 cells against DENV-2. *p <0.05 and ***p <0.001 indicate significant differences compared with the control EDIII-immunized group.

lated onto 96-well PVDF ELISPOT plates and cultured for two days. The monolayer of C6/36 cells was infected with each serially diluted serotype of DENVs at 28°C for 90 min. After removing the excess diluted DENVs, each plate was overlaid with 0.75% methylcellulose (Sigma) and incubated for four days. Finally, after fixing with 4% paraformaldehyde, permeabilizing with 0.5% Triton X-100 for 10 min, and blocking with 5% BSA, the spots were detected with a DENV-antibody followed by an AP-conjugated secondary antibody (Fig. 1b, left panel). The number of spots that developed after adding substrate solution correlated with the inoculated DENV PFUs with a correlation coefficient greater than 0.95 (Fig. 1b, right panel). These results suggest that all DENVs can be correctly detected by spot formation using this system in which 96-well ELISPOT plates and DENV clinical isolates were used.

Next, we applied the *in vitro* microneutralization assay to an oral dengue vaccine model using recombinant envelop domain III (EDIII) protein conjugated with M cell-targeting ligand, Co1 (*6*). After oral immunization with each indicated antigen for four weeks, ligand conjugation significantly increased the EDIII-specific serum IgG level and the number of EDIII-specific IgG-secreting cells in splenocytes (SPL IgG SC) (Fig. 1c and d). To further analyze the neutralizing activity of the induced EDIII-specific antibodies, we applied the microneutralization method established in this study using C6/36 cells (Fig. 1e). The $PRNT_{50}$ titers against DENV-2 were nearly eight-fold greater with the serum from the mice immunized with ligand-conjugated EDIII than those immunized with EDIII alone. Based on these data, we suggest that the proposed *in vitro* microneutralization assay in a 96-well ELISPOT plate using C6/36 cells can be used to accurately characterize clinical isolates of DENV and analyze the effectiveness of the DENV vaccine.

**Acknowledgements.** This study was supported by the Korea Institute of Planning and Evaluation for Technology of Food, Agriculture, Forestry and Fisheries. The authors thank Dr. Ninh's group at the National Institute of Hygiene and Epidemiology in Vietnam for providing valuable DENV clinical isolates.

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