ANTIGEN SANDWICH ELISA PREDICTS RT-PCR DETECTION OF DENGUE VIRUS GENOME IN INFECTED CULTURE FLUIDS OF *AEDES ALBOPICTUS* C6/36 CELLS

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Abstract. Antigen detection by sandwich ELISA was evaluated to predict RT-PCR detection of dengue viral genome in infected culture fluid of *Aedes albopictus* clone C6/36 cells. Serum specimens collected from dengue patients within 5 days from onset of fever in 2 hospitals in Metro Manila, Philippines, were inoculated into C6/36 cells, and incubated at 28ºC. A total of 282 infected culture fluid specimens were harvested and examined by sandwich ELISA and RT-PCR to detect dengue viral antigen and genome, respectively. In the sandwich ELISA, the P/N ratio was calculated by dividing optical density (OD) of a given test specimen by the OD of the standard negative specimen. Samples with a P/N ratio ≥ 4.001 were positive for viral genome detection by RT-PCR. The sensitivity and specificity of antigen sandwich ELISA with RT-PCR as the standard, were 90.4% and 100%, respectively. Although antigen sandwich ELISA is less sensitive than RT-PCR, its usefulness lies in its capability to screen a large number of samples at a minimum cost, especially during an outbreak. Samples that meet a set cutoff value can undergo confirmation by RT-PCR for further epidemiological studies.

INTRODUCTION

Dengue virus, a mosquito-borne flavivirus, is the causative agent of dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome. There are four serologically related but antigenically distinct serotypes of the virus, namely DEN-1, DEN-2, DEN-3 and DEN-4. These viruses possess a single-stranded, positive-sense RNA genome, and can be propagated in a wide range of host cells, including those of mammalian and insect origin (Henchal and Putnak, 1990). A high virus yielding clone C6/36 was isolated from an *Aedes albopictus* cell line (Igarashi, 1978), and has been widely used to isolate dengue virus from patient serum specimens (Igarashi *et al*, 1982; Gubler *et al*, 1984; Roche *et al*, 2000). Dengue viruses replicate in infected C6/36 cells, are released into the culture fluid and undergo multiple cycles of replication.

Successful virus isolation is important for several reasons. It is the most accurate method to identify the infecting virus since most of the serologic tests used in the diagnostic laboratory are not reliably type-specific (Vorndam and Kuno, 1997). In the case of

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dengue virus, determination of serotype can be conveniently done after virus propagation in C6/36 cells. Furthermore, characterization of viral genomes and genetic analysis for epidemiological studies make use of viral RNA isolated from infected culture fluid. RT-PCR of the viral genes provides an invaluable tool for rapid analysis.

Although RT-PCR is a rapid and powerful tool for dengue virus analysis, it is expensive and requires technical expertise. Thus, the objective of the present study was to evaluate if antigen sandwich ELISA a more economical and simpler technique can predict the outcome of viral genome detection by RT-PCR using culture fluid (ICF) from infected C6/36 cells. This was done by obtaining the P/N ratios from the ELISA-OD of the test (infected culture fluid) and standard negative (uninfected culture fluid) specimens.

MATERIALS AND METHODS

Infected culture fluids

Two hundred eighty-two available ICFs were used for antigen sandwich ELISA and RT-PCR. These were prepared from serum samples of 232 clinically diagnosed dengue patients admitted from April to November 1995 at San Lazaro Hospital and St Luke's Medical Center in Metro Manila, Philippines. Serum samples were taken within 5 days of the onset of fever and were aliquoted and stored at -86ºC until use.

Aedes albopictus C6/36 cells were grown to confluence in 2 ml of Eagle's medium in Earle's saline supplemented with 0.2 mM each of non-essential amino acids and 10% heat-inactivated fetal bovine serum (FBS). After three days of incubation at 28ºC, 10 μl of patient serum was inoculated with a two hour adsorption. Then, 2 ml of maintenance medium (same medium as above but with 2% FBS) was added to each culture tube. Infected culture fluid (ICF) was harvested after one week and stored at -86ºC until used for antigen sandwich ELISA and RT-PCR. In some cases, after collection of ICF from culture tubes, some ICF were used as inocula to further propagate the virus for one more week in C6/36 cells.

Antigen sandwich ELISA

Viral antigen present in the ICF was detected by micro-sandwich ELISA following the method of Voller *et al* (1976). Each of the 96 flat-bottom wells on a microplate was coated with 100 μl of antiflavivirus IgG (20 μg/ml in coating buffer) overnight at 4ºC. This antibody was prepared from high-titered dengue patient sera by chromatography on a DEAE Sephacel column (Pharmacia, Sweden). The plates were washed with PBS-Tween (0.05% Tween 20 in phosphate-buffered saline, pH 7.4) 3 times at 3-minute intervals. One hundred microliters of ICF, and the standard negative specimen were pipetted into duplicate wells and incubated at 37ºC for 1 hour. The plates were washed as above and each well was reacted with 100 μl of 400 times diluted in-house prepared horseradish peroxidase (HRPO)-conjugated antiflavivirus IgG in PBS-Tween. The plates were washed as above and the color reaction was carried out by adding 100 μl of substrate solution [0.5mg/ml *o*-phenylenediamine dihydrochloride (OPD) and 0.02% of H_2O_2 in 0.05 M citrate phosphate buffer, pH 5]. After one hour of incubation in the dark at room temperature, the reaction was stopped by adding 100 μl of 1N sulphuric acid to each well. The optical density at 450 nm wavelength (OD_{450}) on each well was measured by an ELISA reader with 620 nm as the reference wavelength. Optical density obtained from infected culture fluid (P) was divided by the OD of the uninfected culture fluid (N), which served as a negative control. The P/N ratios were then correlated with the results of the RT-PCR.

Reverse transcription polymerase chain reaction

The rapid RT-PCR protocol of Morita *et al* (1991) was used to detect dengue viral genome from the ICF without prior RNA extraction. The primers used were the universal dengue virus primers YF3-DENS (5′-TGGATGACCACTG AAGATATGCT-3′) and DEN 3′UR (5′-GGAGC TACAGGCTGCACGGTTT-3′) (Chanyasanha *et al*, 1995). Five microliters of ICF were incubated with an equal volume of detergent mix [1% Nonidet P-40, 10 U of RNase inhibitor (Takara, Kyoto, Japan) in phosphate-buffered saline] in a 500 μl Eppendorf tube for one minute at room temperature to release viral RNA from the virus particles. This was followed by adding 90 μl of RT-PCR mix [100 pmol of each primer, 0.2 mM dNTPs, 10 mM Tris (pH 8.9), 1.5 mM $MgCl_2$, 80 mM KCl, 0.5 mg/ml of BSA, 0.1% sodium cholate, 0.1% Triton X-100, 10 U of avian myeloblastosis reverse transcriptase (Life Science, USA) and, 2 U of thermostable Tth DNA polymerase (Toyobo, Osaka, Japan)]. The reaction mixture was covered with 2 drops of mineral oil, and the tube was incubated for 10 minutes at 53ºC for the first strand reverse transcription reaction. Then, PCR amplification (denaturation at 92ºC for 60 seconds, annealing at 53ºC for 60 seconds and extension at 72ºC for 60 seconds) by thermal cycler (Iwaki Tokyo, Japan) was started immediately and repeated 35 times. Five microliters of PCR product (438 bp fragment) were subjected to agarose gel electrophoresis, and amplified DNA fragments were visualized by ethidium bromide staining.

RESULTS

A total of 282 infected culture fluid samples with the original inocula coming from 232 patients were used in the present study. The results of antigen sandwich ELISA and RT-PCR performed on the 282 infected culture fluid samples are given in Table 1. All 47 samples (100%) with P/N ratios ≥ 4.001 were positive for RT-PCR. For this group, the lowest P/N ratio obtained was 4.08, while the highest was 20.658. All 35 samples (100%) with P/N ratios ≤ 1.000 were negative by RT-PCR. The highest ratio in this group was 1.000 and the lowest was 0.585. In the groups of samples with a P/N ratio between 1.000 to 2.000, 2.000 to 3.000, and 3.000 to 4.000, the percentages positive by PCR correspondingly increased with increasing P/N ratios as follows: 0.77% (1.001≤2.000), 4.92% (2.001≤3.000) and 11.11% (3.001≤4.000).

Table 1
Correlation of P/N values from antigen sandwich ELISA with RT-PCR results.

Antigen sandwich ELISA Range of P/N[a]	No. of ICFs	RT-PCR (+)	RT-PCR (-)	% of positive samples
4.001≤P/N≤21.000	47	47	0	100.0
3.001≤P/N≤4.000	9	1	8	11.1
2.001≤P/N≤3.000	61	3	58	4.9
1.001≤P/N≤2.000	130	1	129	0.8
0.001≤P/N≤1.000	35	0	35	0.0
Total	282	52	230	

[a]P/N is the ratio of the optical density of the infected culture fluid (P or test sample) to the optical density of the uninfected culture fluid (N or negative control).

Table 2
Comparison of results of antigen sandwich ELISA with RT-PCR as standard.

		RT-PCR		
		Positive	Negative	Total
Antigen sandwich ELISA	Positive P/N ≥ 4.001	47	0	47
	Negative P/N ≤ 4.000	5	230	235
	Total	52	230	282

Sensitivity: 47/52 x 100 = 90.4%; Specificity: 230/230 x 100 =100%

Based on these data, samples with a P/N ratio≥ 4.001 were 100% positive.

If a P/N ratio≥ 4.001 is set as the cutoff value for positive detection of dengue virus, the sensitivity and specificity of the antigen sandwich ELISA using RT-PCR as the standard were 90.4% and 100%, respectively (Table 2).

DISCUSSION

In this study, serum samples were collected from 232 clinically diagnosed dengue patients within 0-5 days from the onset of fever. All 232 serum samples were inoculated into C6/36 *Aedes albopictus* cells to culture for dengue virus. This yielded a total of 282 infected culture fluids. The additional 50 (282 less 232) ICFs came from using the first ICF to further propagate the virus. The first ICF refers to the culture fluid that was collected after one week of incubation.

All 282 ICFs were subjected to antigen sandwich ELISA and the values of the P/N ratio were determined.

Regardless of the results of the ELISA, the same samples were subjected to RT-PCR, which was used as the gold standard for the presence of the virus. Fifty-two out of 282 ICFs were positive by RT-PCR. These positive ICFs came from only 25 patients, which means there were either 1, 2 or 3 ICFs derived from a single positive case.

These 2 methods that detect either the presence of virus (RT-PCR) or the antigen (ELISA) were used in the present study to correlate dengue virus detection rates from infected C6/36 cell culture fluids. We determined a specific cutoff value for the P/N ratio from antigen sandwich ELISA to predict a positive result on RT-PCR. When we set the cutoff value of the P/N to be greater than or equal to 4.001, the detection rate by RT-PCR was 100%. Our results show that all 47 samples with P/N ratios ≥ 4.001 were positive on RT-PCR. In contrast, only 5 of 235 samples with P/N ratios ≤ 4.000 were positive on RT-PCR.

Chanyasanha *et al* (1995) compared detection of dengue virus in 162 culture fluid samples after 7 days of incubation with patient sera using 3 different strategies, namely: RT-PCR for viral genome, antigen sandwich ELISA for viral antigen, or inoculation of BHK-21 cells for infectious virus. Their results showed the positivity rate was highest using RT-PCR (13/162 or 8.02%), followed by the immunoperoxidase staining of the infected BHK-21 cells (8/162 or 4.93%), then the antigen sandwich ELISA (5/162 or 3.09 %). In their study, the P/N ratio for positive detection was equal to or greater than 1.5.

In the present study, the sensitivity of antigen sandwich ELISA using RT-PCR as the gold standard was 90.4% while its specificity was 100%. It is advantageous to use antigen sandwich ELISA to predict true positive cases,

especially for epidemiological studies during outbreaks since it can be used to screen a large number of samples at a minimum cost. Only those samples that meet a set cutoff P/N value need to undergo further confirmation by RT-PCR.

ACKNOWLEDGEMENTS

We thank St Luke's Medical Center (SLMC), the Japan Society for the Promotion of Science, and the Japan International Cooperation Agency for funding this research; San Lazaro Hospital and SLMC for providing serum samples; and Ms Cynthia A Mapua for some helpful comments. This research was part of the work done under the Dengue Research Program of the Research and Biotechnology Division, St Luke's Medical Center.

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