Comparison of Whole Blood and Plasma for Dengue Virus RNA Detection by Reverse Transcriptase - PCR

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ABSTRACT

Aiming at the improvement of the molecular diagnosis of dengue infection, whole blood and plasma samples from 31 suspected dengue patients admitted at San Lazaro Hospital (Manila, Philippines) were examined using dengue RT-PCR. Using TRIzol® for whole blood and TRIzol® LS for its corresponding plasma in the RNA extraction phase, 26 (84%) whole blood samples were positive while its only 15 (48%) for its corresponding plasma (*P*=0.001). In contrast to screening with SD BIOLINE® dengue NS1 antigen test of which 24 (77%) was positive, plasma RT-PCR have significantly lower results (*P*=0.035) while there is no significant difference for whole blood RT-PCR (*P*=0.727). In contrast to SD BIOLINE® dengue antibody test (IgM and/or IgG) of which 13 (42%) were positive, whole blood RT-PCR obtained a significantly higher result (*P*=0.004) while there is no significant difference with plasma RT-PCR (*P*=0.832). These results indicate that the better specimen for RNA extraction for dengue diagnosis by RT- PCR is the whole blood. Aside from rapid detection and quantification of viral load, increasing the success rate of RNA extraction can be useful for molecular epidemiological studies involving classification of dengue into different serotypes and genotypes, and characterization of dengue strains to reveal markers of virulence.

Key words: Dengue RT-PCR, Whole Blood, Plasma.

INTRODUCTION

Dengue virus is a mosquito-borne pathogen that can cause asymptomatic to severe infections.[1] In the past decades, dengue virus through its vectors (*Aedes aegypti* and *Aedes albopictus*) has spread in tropics and subtropics, placing risk of infection to more than 2.5 billion people worldwide. Dengue virus causes a wide range of diseases from more than 100 million cases of classical dengue fever to more than 250,000 cases of dengue hemorrhagic fever. This has led to more than 20,000 dengue related deaths each year.[2-3]

To date, there are no vaccines or specific treatments available; therefore, sensitive methods for case detection are of utmost importance for the clinical management of the disease.[4] For the early diagnosis of dengue infection, current methods of detection rely on: virological detection of dengue virus RNA and serological determination of dengue NS1 antigen and dengue IgM or IgG antibody in patients whole blood, serum or plasma.[5,6]

Various protocols for the highly predictive diagnostic test of dengue has been investigated especially the RT-PCR, but varied results were obtained thus far.[7] There is still a need for optimization of: (1) inclusion/exclusion criteria of probable cases; (2) timing of blood sample collection; (3) most appropriate method of RNA extraction and; (4) list of test systems, primer design, equipment and reagents. Equally pressing concern is the study on the blood sample component that would best yield the desired viral component during early stage of dengue virus infection.

Thereby, to extend the diagnostic window and total sensitivity of dengue RT-PCR, we compared the use of whole blood and its corresponding plasma by extracting its RNA by TRIzol® or TRIzol® LS methods, respectively. Both methods are optimized protocols based on the guanidine isothiocyanate extraction of RNA from cellular samples and body fluids, such as whole blood and plasma, respectively. We then focused on their ability to provide enough dengue RNA templates for amplification by a conventional RT-PCR protocol.[8] In addition, we also compared the performance of these methods in the presence of acute (NS1 antigen) and the convalescent phase markers (dengue IgM and/or IgG antibodies) in plasma. Finally, this study was patterned to an approach that is useful in a clinical setting by which all the specimens were collected from suspected patients, admitted in the emergency room of San Lazaro Hospital (Manila, Philippines).

MATERIALS AND METHODS

Clinical Samples

We identified and obtained EDTA whole blood samples from 31 patients presenting at the San Lazaro Hospital (Manila, Philippines). All patients recalled that they are within day 1 to 7 of fever onset and were suspected for dengue infection by the emergency room physicians. From the specimens collected, aliquots of whole blood and plasma were prepared and stored at -20°C before testing. In addition, dengue serologic tests (NS1, IgM and IgG) were also used to screen these samples. Whole blood and plasma negative controls were also obtained from patients that were negative for dengue infection by serology and RT-PCR. This work was approved by the San Lazaro Hospital Research and Ethics Review Board.

Specimen Preparation

Four milliliters (4 mL) of EDTA whole blood was drawn from each patient by venipuncture. From each sample, 250 µL of whole blood were transferred to a 1.5 mL microcentrifuge tube. Plasma samples were then isolated from the remaining blood by centrifugation at $1,000 \times g$ for 10 min. From the EDTA tube, a 110 µL of plasma was directly used for serological testing while another 250 µL of plasma was transferred to a 1.5 mL microcentrifuge tube. Both the 250 µL whole blood and its corresponding 250 µL plasma were sent to the laboratory for storage at −20°C until use.

Serological Screening

The 110 µL of plasma were initially screened using the SD Bioline® Dengue Duo according to manufacturer's instruction. Briefly, the test device was placed on a flat surface. For Dengue NS1 antigen test, 100 µL of plasma was placed into the sample well of NS1 test device. For Dengue IgM/IgG antibodies, 10 µL of plasma was placed into the sample well followed by 100 µL of diluent to the assay diluent well of IgM/IgG test device. Results were interpreted within 15 to 20 min. The presence of two color lines, "T" band and "C" band within the result window indicates a positive result.

Preparation of Positive Controls

In addition to dengue positive plasma samples, infected culture fluid (ICF) was also used as positive controls. The virus were replicated in C6/36 cells grown at 28°C in Eagle's medium containing Earle's saline supplemented with 0.2 mM each of non-essential amino acids and 10% heat-inactivated fetal calf serum. After harvest, an ICF with the titer of ≥ 9.6 units was used.

RNA extraction

The 250 µL of whole blood and plasma aliquots were then subjected to RNA extraction by TRIzol® and TRIzol® LS (GIBCO BRL, USA), respectively. Briefly, both the method consisted of addition of 750 µL of the TRIzol® or TRIzol® LS reagent to a microcentrifuge tube followed by 250 µL of each sample (whole blood or plasma). This solution was vortexed for 15 s and incubated at room temperature for 5 min. After this procedure, 200 µL of chloroform was added to the tube and the solution was centrifuged at $12,000 \times g$ for 15 min. The aqueous phase was transferred to a new tube, precipitated with 500 µL isopropanol, and centrifuged at $12,000 \times g$ for 10 min. The resulting pellet was washed with absolute ethanol and precipitated at $7500 \times g$ for 5 min at 4°C. The pellet was dried at room temperature for 10 min and resuspended in 20 µL of elution buffer. All RNA extracts in microcentrifuge tubes were stored temporarily in crushed ice.

cDNA synthesis

The cDNA strand was synthesized immediately from the RNA extracts of both whole and plasma. cDNA synthesis was carried out on a 20 µL final volume containing 10 µL of RNA template and 10 µL of RT mixture consisting of 1 µL random primers (50 µM), 1 µL dNTP mix (10 mM), 1 µL DEPC treated H_2O, 4 µL 5× RT buffer, 1 µL DTT (0.1M), 1 µL RNAse OUT™ RNAse Inhibitor (40 U/µL) and 1 µL Super Script III® Reverse Transcriptase (200U/µL). The sample was incubated at 37°C for 1 hr, and was stored at -4°C until further use.

Reverse Transcription - PCR

PCR was carried out on a 25 µL final volume containing 5 µL of cDNA and 20.0 µL of PCR mixture consisting of 5.5 µL Vivantis® 2x Taq, 13.5 µL DEPC-treated water and 0.5 µL each of 50 µM DC1 and DC2 primer (according to Lanciotti *et al.*), which anneal to a conserved region on the C/prM genes. RT-PCR was performed according to the following cycles: 3 min denaturation step at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min; 56°C for 1.5 min; and 72°C for 2 min, and ended with final extension at 72°C for 5 min. The amplicons were detected on a 1.5% agarose gel electrophoresis prepared on TBE buffer, stained with 20 µL/mL of Ethidium Bromide and then visualized by the Biorad 2D® UV Transilluminator. Positive (ICF and dengue positive plasma) and Negative (dengue negative plasma) controls were included for each step of the process.

Statistical analysis

The comparison of positivity rates of RNA isolation between whole blood and plasma and serological screening was analyzed by the McNemar test for paired samples, with a 5% level used to establish a statistically significant difference. Statistical comparisons were made by using SPSS (version 16) software.

RESULTS

Positivity rates of dengue serologic markers and RT-PCR of whole blood and plasma RNA extracts

The 31 collected samples consist of 21 acute phase (collected 1-4 days of fever onset) and 10 convalescent phase specimens (collected 5-7 days after fever onset). All samples was collected in average of 4 days after fever onset and was positive to at least one of the three tests within all phases. Among the serological tests, 24 (77%), 13 (42%) and 30 (97%) were positive for dengue NS1 antigen, IgM/IgG antibody and the combined NS1/IgM/IgG, respectively. Among the virological tests, 26 (84%) and 15 (48%) were positive for the dengue RT-PCR of whole blood and plasma RNA extracts, respectively (Table 1).

Comparison of positivity rates of dengue serologic markers and dengue RT-PCR of whole blood and plasma RNA extracts

In comparison to the dengue NS1 antigen positivity, the positivity rate of whole blood dengue RT-PCR has no significant difference (77% vs. 84%, $p=0.727$), while plasma RT-PCR was significantly lower (77% vs. 48%, $p=0.035$). In comparison to Dengue IgM/IgG antibody positivity, the positivity rate of whole blood was significantly higher (42% vs. 84%, $p=0.004$) while for plasma, there is no significant difference (42% vs. 48%, $p=0.832$). In comparison to the combined NS1/IgM/IgG antibody positivity, the positivity rate of whole blood has no significant difference (97% vs. 84%, $p=0.219$) while plasma were significantly lower (97% vs. 48%, $p=<0.01$) (Table 1).

Positivity rates of serologic markers and RT-PCR in phases of infection

When the dengue NS1 antigen, dengue IgM/IgG antibody, combined NS1/IgM/IgG and the RT-PCR positivity rates of whole blood and plasma RNA extraction were examined among the 21 acute phase samples: 19 (91%), 7 (34%), 21 (100%), 19 (91%) and 12 (57%) were positive, respectively. Among the 10 convalescent phase samples: 5 (50%), 6 (60%), 9 (90%), 7 (70%) and 3 (30%) were positive, respectively (Table 2).

Comparison of positivity rates of serologic markers and RT-PCR in phases of infection

In comparison to dengue NS1 antigen positivity among acute phase samples, a significantly lower positivity rate was obtained by plasma RT-PCR (91% vs. 57%, $p=0.039$), while for whole blood RT-PCR, there was no significant difference (91% vs. 91%, $p=1.000$). In comparison to IgM/IgG antibody positivity among acute phase samples, a significantly higher positivity rate was obtained with whole blood (33% vs. 91%, $p=0.002$) while with plasma, there was no significant difference (33% vs. 57%, $p=0.302$). In comparison to the combined positivity of serological screening among acute phase samples, a significantly lower success rate was obtained with plasma (100% vs. 57%, $p=0.008$) while for whole blood, there is no significant difference (100% vs. 91%, $p=0.479$). No significant differences among the positivity of NS1, IgM/IgG and the combined NS1/IgM/IgG in comparison to whole blood or plasma RT-PCR were found from convalescent samples ($p=>0.05$) as shown in Tables 2 and 3.

Comparison of dengue RT-PCR positivity rates between whole blood and plasma RNA extracts

A significantly lower positivity rate was obtained by plasma RT-PCR when compared directly to whole blood RT-PCR (48% vs. 84%, $p=0.001$). This is due to the significantly lower positivity rate of plasma RT-PCR versus whole blood RT-PCR in the acute phase of infection (57% vs. 91%, $p=0.016$) while no significant differences were found in the convalescent phases of infection (70% vs. 30%, $p=0.125$) (Table 3).

Table 1: Serological Positivity and the RT-PCR Detection Rates of Dengue virus RNA in Whole Blood and Plasma Extracts.

Results	Number of Dengue positive samples (%)				
	SD Bioline®			RT-PCR	
	NS1	IgM/IgG	NS/IgM/IgG	WB	Plasma
Positive	24 (77)	13 (42)	30 (97)	26 (84)	15 (48)
Negative	7 (23)	18 (58)	1 (3)	5 (16)	16 (52)

	p value for NS1 vs.		P value for IgM/IgG vs.		p value for NS1/IgG/IgM vs.	
	WB	Plasma	WB	Plasma	WB	Plasma
	NS*a*	0.035	0.004	NS	NS	<0.001

*a*NS: not significant

Table 2: Serological Positivity and the RT-PCR Detection Rates of Dengue RNA in the Acute and Convalescent Phase of Infection.

Results	Number of Dengue positive samples (%)				
	SD Bioline®			RT-PCR	
	NS1	IgM/IgG	NS/IgM/IgG	WB	Plasma
Acute	19 (91)	7 (33)	21 (100)	19 (91)	12 (57)
Convalescent	5 (50)	6 (60)	9 (90)	7 (70)	3 (30)

Results	*p* value for NS1 vs.		*p* value for IgM/IgG vs.		*p* value for NS1/IgG/IgM vs.	
	WB	Plasma	WB	Plasma	WB	Plasma
Acute	NS	0.039	0.002	NS	NS	0.008
Convalescent	NS	NS	NS	NS	NS	NS

*a*NS: not significant.

Table 3: Comparison of Dengue RT-PCR Positivity of Whole Blood and Plasma RNA Extracts.

Phase of samples	No. of samples	No. of samples Dengue positive by RT-PCR		*p* value for TRIzol® vs. QIAamp®
		WB	Plasma	
Acute	21	19 (91%)	12 (57%)	0.016
Convalescent	10	7 (70%)	3 (30%)	NS
Total	31	26 (84%)	15 (48%)	<0.001

*a*NS: not significant

DISCUSSION

The WHO still considers PCR as an experimental technique. Therefore, it still needs continuous optimization to increase its diagnostic sensitivity in cases of dengue infections. A number of RT-PCR procedures that identifies dengue virus RNA in clinical specimens have been reported. These RT-PCR methods vary in terms of the gene regions amplified and in ways RT-PCR products were detected.[7] Yet, RT-PCR of similar test system can still demonstrate a variation in sensitivity just by varying the blood component used in the RNA extraction phase.

Aiming at the improvement of molecular diagnosis of dengue, the researchers used liquid based methods with optimized concentrations of Guanidine isothiocyanate for extraction of dengue RNA in whole blood and plasma. This is by using TRIzol® and TRIzol® LS reagent, respectively. Whole blood and its corresponding plasma from patients identified in the emergency room of San Lazaro Hospital (Manila, Philippines) was processed by utilizing either of this reagents in the RNA extraction phase while all other steps of RT-PCR were kept constant.

As expected, the positivity rates of dengue NS1 and dengue RT-PCR in whole blood and/or plasma were higher among acute phase samples.[9] For the dengue antibodies (IgM/IgG), the positivity rates as expected was higher among convalescent phase samples.[10,11]

For the performance of the samples, whole blood likely provides more dengue RNA templates for a conventional RT-PCR than its corresponding plasma. Combining these findings to the comparisons mentioned earlier with the presence of detectable NS1 antigen and IgM/IgG antibodies, it could be inferred that RNA extraction in whole blood has a better yield of dengue RNA than its corresponding plasma.

For this study, the result is still in agreement with Klungthong *et al.* despite some differences in the reagent used in the RNA extraction phase. Klungthong *et al.*[12] used TRIzol® only for both whole blood and plasma, whereas in our study, we used TRIzol® and TRIzol® LS for whole blood and plasma, respectively. According to the TRIzol® Reagent Technical inserts, TRIzol® reagent was designed to isolate RNA from cellular and tissue samples whereas TRIzol® LS was designed to isolate RNA from liquid samples such as plasma. The difference between TRIzol® and TRIzol® LS Reagent is that, the later has more concentrated monophasic solution of phenol, Guanidine isothiocyanate and other components.

Thereby, this study still compared whole blood and its corresponding plasma for the isolation of dengue RNA using the more appropriate reagent suggested by the manufacturer (Life Technologies Corp.). This study was also patterned to an approach that is useful in a clinical setting, since a diagnostic test will only help in the management of the patient if it can diagnose during the acute phase of illness and before a probable plasma leakage occurs at the convalescent phase. Looking back at the results, the increased positivity rates of RT-PCR with whole blood is likely due to presence of virus-infected cells found in the buffy coat, despite the potential presence of RT-PCR inhibitors such the hemoglobin in whole blood.[12] This may have increased the amount of dengue virus RNA extracted from the patient's blood sample, increasing the RT-PCR positivity rates that would be otherwise significantly lower if plasma was used.

Despite these findings, and even if RT-PCR was considered as one of the most accurate assay for dengue diagnosis, its cost effectiveness might still be argued in developing countries where limiting factors such as insufficient technical support and being expensive gives RT-PCR a disadvantage.[13,14] Nonetheless, there are still reasons why we aim to increase dengue RT-PCR

sensitivity. First, PCR can identify dengue serotypes. This information is important for the physician due to the risk of a severe secondary infection by other serotypes.[15] Second, RT-PCR based assays are applied in the pathogenesis and phylogenetic studies of dengue.[16] Third, the diagnostic sensitivity of dengue RT-PCR may significantly decrease due to differences in the yield of dengue RNA template. Lastly, dengue is expanding globally to include more developed countries with increased capacity in utilizing molecular diagnostics. [17]

CONCLUSION

Overall, RT-PCR is an efficient method in the diagnosis of dengue virus infections, and it will be more helpful for clinicians and researchers if sufficient dengue RNA templates can be isolated in clinical samples for research and diagnosis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS USED

DOST-PCHRD: Department of Science and Technology - Philippines Council for Health Research and Development.

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