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Comparison of techniques for extracting viral RNA in plasma for dengue diagnosis by reverse transcriptase-PCR

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Abstract

This study aims to determine if the rate of RT-PCR detection of Dengue virus from clinical plasma would vary between the RNA extracts of QIAamp® Viral RNA and TRIzol® LS in the first week of Dengue infection. Plasma samples from 31 individuals clinically suspected of being infected with dengue virus in day 1 to 7 of fever onset were extracted for RNA using the protocol of QIAamp® Viral RNA and TRIzol® LS. The paired RNA extracts of plasma samples were analyzed for the presence of Dengue virus RNA using RT-PCR. Out of 31 samples, a significantly higher rate of RT-PCR detection was obtained with QIAamp® than TRIzol® (74% vs. 48%, p=0.039). In comparison to Dengue NS1 antigen positivity, a significantly lower rates of RT-PCR detection was obtained with TRIzol® (77% vs. 48%, p=0.035), while there was no significant difference with QIAamp® (77% vs. 74%, p=1.000). In comparison to Dengue IgM/IgG antibody positivity, the rates of RT-PCR detection with QIAamp® was significantly higher (42% vs. 74%, p=0.031) while there was no significant difference with TRIzol® (42% vs. 48%, p=0.832).These results suggest that RNA extraction using QIAamp® Viral RNA provides more sufficiently pure RNA template for a conventional RT-PCR than TRIzol®LS.

Key words : Dengue virus; Diagnosis; NS1 antigen; IgM/IgG antibody; RNA extraction; RT-PCR

INTRODUCTION

Dengue is a mosquito borne virus that causes the most significant illness in terms of human morbidity and mortality.[1]In the past decades, *Dengue virus* through its vectors (*Aedesaegypti*and *Aedesalbopictus)* has spread in tropical and subtropical regions worldwide, placing risk of infection to more than 2.5 billion people. *Dengue virus* causes a wide range of diseases from acute febrile dengue fever (50100 million cases), to dengue hemorrhagic fever (>250,000 cases) leading to approximately 24,000 dengue related deaths each year. [2,3]

To date, there are no specific antiviral drugs available, so in cases with dengue hemorrhagic fever, a sensitive diagnostic test followed by an immediate supportive management is of utmost importance.[4] For the early and specific dengue diagnosis, currently applied methods were: virological detection of *Dengue virus* RNA and serological determination of Dengue NS1 antigen and IgM/IgGantibodyin serum or plasma.[5,6]Different methods have been applied for the diagnosis of *Dengue virus* infection. Consequently, comparing results from various authors especially in the performance of dengue RT-PCR has not produced reproducible results. Even among diagnostic laboratories with similar test systems, primer design and amplification conditions for denguediagnosis, a great variation in detection sensitivity corresponding to 33100% of the expected resultswas demonstrated.[7]These might be accounted to differences in the methods of RNA extraction, performance of enzymes andreagents, and the procedural variations administered by different institutions. Thus, leading to a huge discrepancy in results which can be seen if a formal external quality assessment (EQA) program will look over into these laboratories

Thereby, to lessen the gaps of sensitivity and to increase the total sensitivity of dengue RT-PCR, we compared the QIAamp®Viral RNA and TRIzol® LSby focusing on their ability to provide enough sufficiently pure RNA templates fromplasma samples for amplification by a conventional RT-PCRprotocol.[8]In addition, we also compared theperformance of these methodsin the presence of acute (NS1 antigen) and the convalescent phase markers (dengue IgM/IgG antibodies). Finally, this study was patterned to an approach that is useful in a clinical setting by acquiring the specimen from patient prior to their admission in a hospital.

MATERIALAND METHODS

Clinical Samples

We identified 31 patients with suspected dengueinfection (based on clinical aspects) in day 1-7 of fever onset presenting atSan Lazaro Hospital (Manila, Philippines). From the EDTA whole blood specimens collected, aliquots of plasma were prepared and stored at -20°C before testing for dengue infection by RT-PCRwithin each weekday of collection.In addition a dengue serologic test wasalso used to screen the samples. Negative controls of plasma werealso obtained from 3 patients negative for Dengueserology and RT-PCR.This work was approved by the San Lazaro Hospital Research and Ethics Review Board.

Specimen Preparation

Four milliliters of EDTA whole blood was drawn from eachpatient by venipuncture. The plasma samples were isolated from the whole blood bycentrifugation at 1,000×*g*. A 250and 140 µl aliquots of plasma were transferred to 1.5-ml microcentrifuge tubesand were sent to the laboratory for refrigeration where they were stored at −20 °C until use.

Serological Screening

Plasma aliquots were initially screened using the SD Bioline® Dengue Duo according to manufacturer's instruction. Briefly,the

test devices were placed on a flat surface. For 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 15-20 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 plasma samples, infected culture fluid (ICF) was also used as positive controls. Strains used were: D1, Hawaiian; D2, New Guinea B; D3, H83; and D4, No 17. 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 of the infected culture fluid (ICF), viral antigen was detected by sandwich-ELISA following the micro-sandwich method. An ICF with the titer of ≥ 9.6 units was used.

RNA extraction

The 250 µl and 140 µl of plasma aliquots were subjected to RNA extraction by the two different methods, the TRIzol® LS (GIBCO BRL, USA), and the QIAamp® Viral RNA (QIAGEN Inc, USA), respectively. Briefly, the TRIzol® LS method consisted of addition of 750 µl of the TRIzol® LS reagent to a microcentrifuge tube followed by 250 µl of each clinical sample. 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 ×*g* for 15 min. The aqueous phase was transferred to a new tube, precipitated with 500 µl isopropanol, and centrifuged at 12,000 ×*g* for 10 min. The resulting pellet was washed in absolute ethanol and precipitated at 7500×*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. The QIAamp® Viral RNA kit was used according to the manufacturer's instructions. A total of 140 µl of each clinical sample was incubated with buffer AVL for 10 min. After viral particle lysis, the samples were mixed with ethanol and applied to the spin column, centrifuged, and washed twice with buffers AW1 and AW2. RNA was then eluted from the columns using 20 µl of AVE buffer. All RNA extracts in micro centrifuge tubes were stored temporarily in crushed ice.

cDNA synthesis

The cDNA strand was synthesized immediately from the RNA extracts of both methods. cDNA synthesis was carried out on a 10 µl final volume containing 5 µl of RNA template and 5µl of RT mixture consisting of 0.5 µl random primers (50µM), 0.5 µl dNTP mix (10 mM), 0.5 µl DEPC treated H_2O, 2 µl 5× RT buffer, 0.5 µl DTT (0.1M), 0.5 µl RNAse OUT™ RNAse Inhibitor (40 U/µL) and 0.5 µLSuper Script III® Reverse Transcriptase (200 U/µl). The sample was incubated at 37°C for 1 hr, and was stored at -4°C until further use.

RT-Polymerase chain reaction

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(5'TCAATATGCTGAAACGCGCGAGAAACCG-3') and DC2 (5'TTGCACCAACAGTCAATTCTGGTTC-3'), which anneal to a conserved region located on the C/prM genes. RT-PCR was performed according to the following cycles: 3 min denaturation step at 94 °C at 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 (infected culture fluid and dengue positive plasma) and Negative (dengue negative plasma and triple distilled H_2O) controls were included for each step of the process (RNA extraction, cDNA synthesis and PCR).

Statistical analysis

The comparison of success rates between Dengue RNA isolation methods and serological screening was analyzed by the McNemar test for paired samples, with a 5% level used to establish a statistically significant difference. Statistical

Table 1. Serological positivity and the RT-PCR detection rates of Dengue RNA from TRIzol® and QIAamp® extracts

Results	Number of Dengue positive samples (%)				
	SD Bioline®			TRIzol® LS	QIAamp® Viral RNA
	NS1	IgM/IgG	NS/IgM/IgG		
Positive	24 (77)	13 (42)	30 (97)	15 (48)	23 (74)
Negative	7 (23)	18 (58)	1 (3)	16 (52)	8 (26)

p value for NS1 vs.		*p* value for IgM/IgG vs.		*p* value for NS1/IgG/IgM vs.	
TRIzol	QIAamp	TRIzol	QIAamp	TRIzol	QIAamp
0.035	NS[a]	NS	0.031	0.001	0.016

[a]NS: not significant

comparisons were made by using SPSS (version 16) software.

RESULTS

Positivity rates of serologic markers and the success rates of RNA extraction

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 four tests within all phases. Among the serological tests, 24 (77%) and 13 (42%) were positive for Dengue NS1 antigen and IgM/IgG, antibody respectively. Among the virological tests, 15 (48%) and 23 (74%) were positive for the RT-PCR of TRIzol LS® and QIAamp® Viral RNA extracts, respectively (Table 1).

Comparison of serologic markers to the success rates of RNA extraction

In comparison to Dengue NS1 antigen positivity, a significantly lower success rate was obtained by TRIzol® LS (48% vs. 77%, p=0.035), while for QIAamp® there is no significant difference (74% vs. 77%, p=1.000). In comparison to Dengue IgM/IgG antibody positivity, the success rate of QIAamp® was significantly higher (42% vs. 74%, p=0.031) while for TRIzol LS®, there was no significant difference (42% vs. 48%, p=0.832). The success rates of TRIzol LS® and QIAamp® Viral RNA were both significantly lower than the combined NS1/IgM/IgG in plasma (97% vs. 48%, p=0.001; 97% vs. 74%, p=0.016, respectively) (Table 1).

Positivity rates of serologic markers and the success rates of RNA extraction in phases of infection

When the dengue NS1 antigen, dengue IgM/IgG antibody, and the success rates of TRIzol® LS and QIAamp® Viral RNA extraction were examined among the 21 acute phase samples: 19 (91%), 7 (34%), 12 (57%) and 18 (86%) were positive, respectively. Among the 10 convalescent phase samples: 5 (50%), 6 (60%), 3 (30%) and 5 (50%) were positive, respectively (Table 2).

Comparison of serologic markers to the success rates of RNA extraction in phases of infection.

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

Results	Number of Dengue positive samples (%)				
	SD Bioline®			TRIzol® LS	QIAamp®Viral RNA
	NS1	IgM/IgG	NS/IgM/IgG		
Acute	19 (91)	7 (34)	21 (100)	12 (57)	18 (86)
Convalescent	5 (50)	6 (60)	9 (90)	3 (30)	5 (50)

Results	p value for NS1 vs.		p value for IgM/IgG vs.		p value for NS1/IgG/IgM vs.	
	TRIzol	QIAamp	TRIzol	QIAamp	TRIzol	QIAamp
Acute	0.039	NS	NS	0.003	0.003	NS
Convalescent	NS[a]	NS	NS	NS	0.022	NS

[a]NS: not significant

Table 3. Comparison of Dengue RT-PCR Positivity of TRIzol® LS and QIAamp® Viral RNA Extracts

Phase of samples	No. of samples	No. of samples Dengue positive by RT-PCR		p value for TRIzol® vs.QIAamp®
		TRIzol®	QIAamp®	
Acute	21	12 (57%)	18 (86%)	NS[a]
Convalescent	10	3 (30%)	5 (50%)	NS
Total	31	15 (48%)	23 (74%)	0.039

[a]NS: not significant

In comparison to Dengue NS1 antigen positivity among acute phase samples, a significantly lower success rate was obtained by TRIzol® (91% vs. 57%, p=0.039), while for QIAamp® there was no significant difference (91% vs. 86%, p=1.000). In comparison to IgM/IgG antibody positivity among acute phase samples, a significantly higher success rate was obtained by QIAamp® (34% vs. 86%, p=0.003) while for TRIzol LS®, there was no significant difference (34% 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 by TRIzol®(100% vs. 57%, p=0.003) while for QIAamp® Viral RNA, there is no significant difference (100% vs. 86%, p=0.242). No significant differences among TRIzol®, QIAamp®, NS1, IgM/IgG and the combined NS1/IgM/IgG were found from convalescent samples as shown in Table 2. In comparison to the combined serological positivity among convalescent phase samples, a significantly lower rates of RT-PCR detection was also obtained with TRIzol® (90% vs.30%, p=0.022) while with QIAamp®, there was no significant difference (90% vs. 50%, p=0.143) (Table 2).

Comparison of RNA extraction rates between TRIzol LS® and QIAamp® Viral RNA

A significantly lower success rate of Dengue RNA extraction was obtained by TRIzol® when compared directly to QIAamp® (15 vs. 23, p=0.039). No significant differences were found in the acute and convalescent phases of infection (Table 3).

DISCUSSION

The WHO still considers PCR as an experimental technique and still needs to be better standardized for dengue diagnosis. A number of RT-PCR procedures that detect *Dengue virus* RNA in clinical specimens have been reported. These RT-PCR methods vary in terms of the amplified gene regions of the genome and in ways RT-PCR products were detected. Yet, RT-PCR of similar test system in different laboratories can still demonstrate a great variation in terms of their sensitivity.[7]

One probable reason is that the RNA extraction reagents or kits used in diagnostic laboratories worldwide were labeled as "for research use only". Just like TRIzol® and QIAamp®, these reagents for RNA extraction were not given any claims of test performance for diagnostic purposes, so therefore these reagents or kit should at least be subjected to stringent regulations and evidence must be shown to prove its effectiveness particularly for dengue diagnosis. Aiming at the improvement of molecular diagnosis of dengue, the researchers compared the commonly used reagents for extraction of RNA in liquid samples, the QIAamp® Viral RNA and the TRIzol® LS. Plasma from patients identified in the emergency room of San Lazaro Hospital was processed utilizing these two methods while all other steps of RT-PCR were kept constant.

As expected, the rate of Dengue NS1 antigen positivity and the success rates of RNA extraction by two methods were higher among acute phase samples.[9] For the antibodies (IgM/IgG), the rate of positivity as expected was higher among convalescent phase samples.[10,11]

For the performance of RNA extraction methods, QIAamp® Viral RNA likely provides more sufficient RNA template for a conventional RT-PCR than TRIzol® LS. Combining these findings to the comparisons mentioned earlier with the presence of detectable NS1 antigen and IgM/IgG antibodies, it could be inferred that QIAamp® Viral RNA method of extraction proved better than TRIzol® LS.

For this study, the differences between the precipitation and wash procedures might have effects in the yield of TRIzol® LS and QIAamp® Viral RNA. For TRIzol® LS, succeeding steps were done in several microcentrifuge tubes, while for the QIAamp® Viral RNA, all extraction and wash procedures were done on a single silica based membrane. Another explanation could be related to the concentration of the starting material used in the different protocols used in this study. The starting volumes for QIAamp® and TRIzol® LS were 140 µl and 250 µl, respectively and higher volumes of sample might also introduce higher amounts of inhibitory substances present in the sample.[12]

Regardless of these findings, and even if RT-PCR was claimed as the most accurate single assay for dengue diagnosis its cost effectiveness might still be argued in a developing country like the Philippines where limiting factors such as the economy and a lack of technical support gives the RT-PCR for dengue diagnosis a disadvantage. [13,14]

Despite the cost, nonetheless, there are compelling reasons why this current study, still aims to increase Dengue RT-PCR sensitivity. First, PCR has the ability to identify dengue serotype. The typing of the respective dengue virus provides important information for the clinician and the patient due to the risk of a severe secondary infection by another serotype.[15] Second, RT-PCR based assays are applied in the studies of Dengue pathogenesis and phylogenetic studies and as a virological endpoint in clinical trials of anti-viral therapies.[5,16]Third, the diagnostic sensitivity of in-house and commercial dengue RT-PCR assays vary greatly in laboratories worldwide and it might be due to differences in the yield of viral RNA template. Lastly, Dengue is expanding globally to include more developed countries where the capacity for molecular diagnostics is higher and where validated assays could be deployed.[13,17]

CONCLUSION

Overall, RT-PCR is an efficient method in the detection of dengue virus infections, and it will be a helpful tool for clinicians for the detection of an acute dengue infection, if sampling and diagnostic PCR is optimized in all phases of its procedure.

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