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Assessment of LyseNow® Perforated Card on Preservation of Dengue Virus RNA in Whole Blood

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ABSTRACT

Degradation of RNA in clinical specimens during storage and transportation is a major problem in accurate diagnosis of RNA virus infection. Concerns on degradation of RNA made cold-chain a necessity during transportation/storage of specimens for downstream molecular applications. However, cold-chain is not only costly but also can be scarce or compromised by insufficient supporting infrastructure or staff in resource-constrained settings. In this study, we employed dengue virus serotype-1 (DENV-1), an RNA virus, to test the performance of LyseNow® Perforated Card with commonly used Whatman FTA™ Micro Card in RNA preservation of the DENV-1 spiked in human whole blood, at different time points up to 109 days, stored at both room temperature and 37°C. There were a 35-fold and 800-fold reduction of DENV-1 RNA from the LyseNow® Perforated Card and Whatman FTA™ Micro Card, as compared to the un-loaded spiked whole blood. Once dried on the cards, the DENV-1 RNA remained stable on the LyseNow® Perforated Card stored at both room temperature and 37°C up to the end of the study of Day 109. On the other hand, the DENV-1 RNA recovered from spiked whole blood spotted on the FTA™ Micro Card showed additional 8-fold and 20-fold reduction in RNA after stored at room temperature and 37°C for 109 days, respectively. Our results showed LyseNow® Perforated Card had superior ability in preserving DENV-1 RNA during prolonged storage even an elevated temperature of 37°C.

Keywords: *paper-based card, RNA preservation, Dengue virus, whole blood*

INTRODUCTION

Efficient and accurate diagnosis of dengue fever is of primary importance for clinical care (i.e. early detection of severe cases, case confirmation, differential diagnosis with other infectious diseases, and proper care management), surveillance activities, outbreak control, pathogenesis, vaccine development, and clinical trials. Molecular testing to confirm dengue virus infection during the acute phase has become a routine in many laboratories due to the same day "time to results" and the confirmative nature of the tests [1].

Although molecular testing is considered highly sensitive and specific [2], the accuracy of test results is relying on the preservation of target analytes in the specimens (Labcorp "Introduction to Specimen Collection") which oftentimes were remotely collected and required transportation to core testing facilities [3]. Concerns on degradation of RNA made cold-chain a necessity during transportation/storage of specimens for downstream molecular applications [4-6]. However, cold-chain is not only costly but also can be scarce or compromised by insufficient supporting infrastructure or staff in resource-constrained settings [7]. It is, therefore, highly desirable to explore an alternative method to transport/store dengue virus samples at ambient temperature. Samples preserved on dried blood spot (DBS) cards (Whatman 903 filter paper, GE Health care) were used in large scale routine field evaluation of human immunodeficiency virus 1 (HIV-1) viral load and drug resistance monitoring by molecular testing in Africa and Asia [8] and were tested in another study for *hepatitis C* virus **(**HCV) RNA levels intended to provide a viable low-cost method for field samples to ensure broad access to HCV screening, diagnosis and treatment monitoring [9]. The DBS method was considered by Fourati et al. [10] to be included in their approach for simplified HCV diagnostic algorithms. Paper products treated with proprietary chemicals have been used in studies to preserve RNA of HIV-1 [11] and influenza viruses, both type A and B [12]. LyseNow® Perforated Card (Fortius Bio, San Diego, CA), is a thick filter paper-based card treated with proprietary chemicals intended

to preserve the nucleic acid in samples spotted and dried on the card stored at room temperature.

There is no publication on the effectiveness of the card in preserving viral RNA. In this study, we employed dengue virus serotype-1 (DENV-1), an RNA virus, to test the performance of LyseNow® Perforated Card with commonly used Whatman FTA™ Micro Card in RNA preservation of the DENV-1 spiked in human whole blood, at different time points up to 109 days, stored at both room temperature and 37°C.

Materials and Methods

Preparation of DENV-1 Spiked Human Whole Blood Sample

Fresh normal human whole blood with CPDA-1 was purchased from Valley Biomedical (Winchester, VA) and was used for the study the day it was received. DENV-1 strain WP74 was grown and maintained in Vero cells with a titer of 1.7 x 10^7 plaque forming unit (PFU)/mL. The DENV-1 was spiked into the fresh blood at a final concentration of 1 x 10^4 PFU/mL. Three 120 µL aliquots each of the un-spiked (normal) and DENV-1 spiked fresh whole blood were stored at -80°C to serve as the un-loaded liquid baseline negative and positive controls.

Cards Spotting

LyseNow® Perforated card (LyseNow card), provided by the manufacturer FortiusBio, are filter paper-based cards which have been treated with proprietary chemicals for viral/cell lysis and preservation of the RNAs thus released (Figure 1A). Each of the LyseNow card has seven perforated discs, 3 mm in diameter, to ensure easy sample collection from the cards for downstream sample processing/application. Whatman FTA™ Micro Card (FTA card) (GE Healthcare Life Science, Chicago, IL), also filter-based and treated cards, were purchased for comparison (Figure 1B).

(A)

(B)

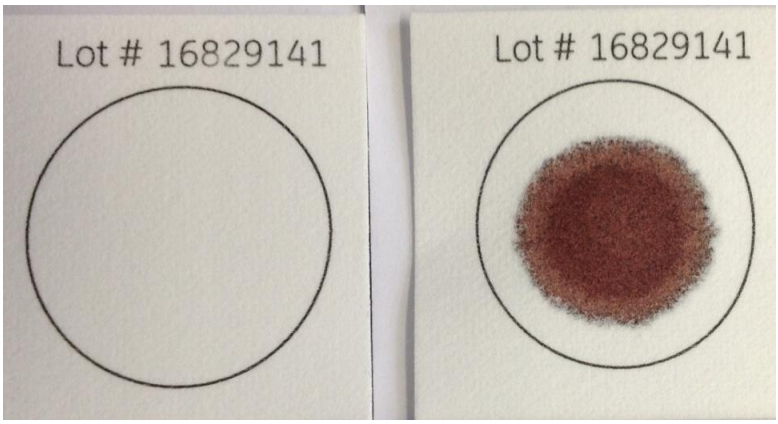

Figure1. *LyseNow card (A) and FTA card (B) before and after whole blood sample application. One hundred µL normal whole blood or DENV-1 spiked whole blood was spotted on the center of the card.*

One hundred µL of sample was spotted on the center of each of the LyseNow card and FTA card. Spotted cards were left to dry in the biosafety cabinet overnight. Cards collected the next morning (designated as Day 0) were sorted, packaged in moisture-barrier plastic pouches with desiccants, and stored either at room temperature or 37°C. At 0, 9, 16, 30, 58, and

109 days, spotted cards were taken to harvest the discs for RNA elution and extraction. Each treatment was run in triplicates.

Elution and Extraction of RNA from the Spotted Cards

For spotted LyseNow card, the seven perforated discs were removed from the card using clean pipet tips. For FTA card, seven discs of 3 mm in diameter were punched out using 3 mm hole puncher. The seven discs from one single card were collected in a 1.6mL graduated Micro centrifuge tube. To each tube with seven discs, 450 µL of ATL Buffer (Qiagen, Stockach, Germany) was added followed by 2 minutes incubation at 60°C with shaking at 1400 rpm on an Eppendorf Thermo mixer 5350. Up on completion of the RNA elution, all liquid from each tube was used for RNA extraction. RNA extractions were performed using the Qiagen EZ1 Advanced XL machine following the manufacturer's instruction. The load and elute volume was set at 400 µL and 60 µL, respectively. Each 60 µL eluate was divided into 3 X 18µL aliquots and immediately stored at -80°C. Un-loaded samples at 100 µL were mixed with 300 µL ATL buffer for RNA extraction.

Real Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Modified McAvin et al. [13] RT-PCR reactions were performed with QuantumStudio 5 thermo cycler (Applied Biosystems, Foster City, CA). Forward and reversed primers were both at 0.7 µM and probe was at 0.27 µM (Integrated DNA Technologies, Inc., Coralville, IA) in the master mix using the Super Scrip III Platinum One Step RT-PCR Kit (Fisher Scientific, Hampton, NH). An initial step of 30 minutes at 50°C and two minutes at 95°C was followed by 40 cycles at 95°C for 15 seconds, 61°C for 45 seconds.

RESULTS

The unloaded normal whole blood was negative for DENV RNA (undetected) by RT-PCR. The normal whole blood spotted on the cards was tested at Day 0, 30, and 109 and none had any signal. Un-loaded whole blood spikedwith DENV-1 at 1x104 PFU/ml gave a mean Ct value of 22.753 (Table 1). After the spiked whole blood was spotted on the cards and dried overnight (Day 0), samples from the LyseNow card and FTA card had a mean Ct value of 27.859 and 32.395, respectively (Table 1).

Table1. *Comparison of real time RT-PCR results for unloaded and Day 0 normal whole blood and DENV-1 spiked whole blood samples.*

	Normal whole blood			DENV-1 spiked whole blood[a]		
	Unload	LyseNow[b]	FTA[c]	Unload	LyseNow[b]	FTA[c]
Ct[d]	Und[e]	Und	Und	22.753	27.859	32.395

[a] Using DENV-1 strain WP74 virus stock with a titer of 1.7 x 10^7 PFU/mL to reach a final concentration of 1 x 10^4 PFU/mL.

[b] One hundred µL of normal or spiked whole blood was spotted on the each of the LyseNow card. Seven perforated discs were removed for RNA elution and extraction on Day 0.

[c] One hundred µL of normal or spiked whole blood was spotted on each of the FTA cards. Seven discs of 3 mm in diameter were punched out for RNA elution and extraction on Day 0.

[d] The mean Ct of three independent samples.

[e] Und: Undetected.

The increase of 5.106 and 9.642 in mean Ct of DENV-1 RNA from the LyseNow card and the FTA card reflected a 35-fold and 800-fold reduction in RNA recovery, respectively, as compared to the un-loaded spiked whole blood stored at -80°C, in (Table1). And the disparity in DENV-1 RNA preservation between the two cards widened with increased time in storage (Figure 2).

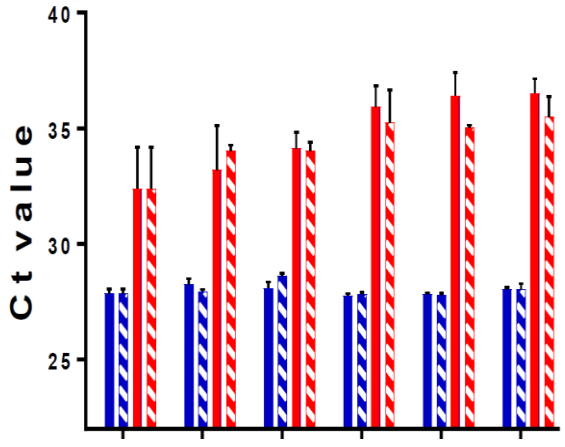

Figure2. *Time course study of the DNEV-1 RNA stability in spiked whole blood at 1x10^4 pfu/ml spotted on LyseNow card and FTA card.*

The RNA of DENV-1 from spiked whole blood spotted on the LyseNow card (blue) were

recovered at indicated days after stored at 37°C (filled) or room temperature (striped).

RNA of DENV-1 from spiked whole blood spotted on the FTA card (red) were also recovered at indicated days after stored at 37°C (filled) or room temperature (striped).

Each bar represents the averages of three samples, and the error bars represent standard deviation. Ct, cycle threshold.

Once dried on the cards, the DENV-1 RNA remained stable on the LyseNow card stored at both room temperature and 37°C up to the end of the study at Day 109 (mean Ct at 27.859, 28.023, and 28.048 for Day 0, Day 109 at room temperature, and Day 109 at 37°C) (Figure 2). On the other hand, the DENV-1 RNA recovered from spiked whole blood spotted on the FTA card showed an increase in Ct associated with both the duration of storage and the temperature of storage. The mean Ct was 32.395, 35.513, and 36.714 for Day 0, Day 109 at room temperature, and Day 109 at 37°C, respectively (Figure 2). The changes in the Ct value of samples stored at room temperature and 37°C reflected about an 8-fold and 20-fold reduction in RNA recovery between Day 0 and 109 on the FTA card.

Discussions

Preliminary study (data not shown) on LyseNow card was conducted using whole blood spiked with DENV-1 at two concentrations (10^3 and 10^6 PFU/mL). The initial percentage loss of RNA on Day 0 was similar regardless of the concentration of virus spiked in the whole blood. After the initial loss on Day 0, the RNA stability was maintained through Day 28 (the end point of the study).

Based on the preliminary study and findings by Wu et al [14] that highest % of clinical samples tested by plaque assay using Vero cells, had a DENV titer near 10^4 PFU/mL, we decided to spike whole blood with DENV-1 at 10^4 PFU/mL for this study. The results from this study showed a calculated 800-fold reduction on Day 0 on FTA card and a 5800- and 9200-fold reduction in recoverable RNA on Day 30 stored at room temperature and 37°C respectively (Figure 2). In the RNA stabilization work by Dauner et al. [15] using DENV-1 WP-74, FTA card had a calculated 65-fold loss on Day 0 and a 230-fold and 370-fold loss on Day 28 at room temperature and 37°C, respectively. The differences on RNA recovered from FTA card by Dauner et al. and us could be due to the different ways that (1) the sample on the cards were harvested and (2) the RNAs were eluted in the two studies. We used a 3 mm hole puncher to harvest seven discs from the cards, in order to match the way samples were harvested from the spotted LyseNow card, while Dauner et al. took a quarter of the spot of each FTA card and minced this quarter of a spot into small pieces with scissors. However, the minced method using a pair of scissors is both labor intensive and time consuming and thus making it impractical for routine samples' collection. For RNA elution, Dauner et al. shacked the FTA card pieces at 1,000 to 1,200 rpm in AVL buffer (Qiagen, Valencia, CA) for one hour at room temperature while shacking at 1,400 rpm in ATL buffer for two minutes at 60°C was the method used in this study. Stability of the RNA of DENV-2 and DENV-3 spiked in normal human whole blood and spotted/dried on filter paper (Blood Sampling Paper, NOBUTO, Toyo Roshi Kaisha Ltd., Japan, Type I) was performed by Prado et al [16]. They found dengue virus RNA stable on the filter paper for nine weeks at room temperature.

The calculated 35-fold DENV-1 RNA loss with the LyseNow card on Day 0 is definitely superior to an 800 fold loss with the FTA card in our study. However, the results may vary with different elution protocol. Also, the LyseNow card being a thicker filter-paper based card than FTA card would likely retained larger volume of samples than FTA card at the same total area harvested. The DENV-1 RNA in whole blood samples stored on FTA card appeared to be much less stable than those stored on LyseNow card. Both duration of storage (up to 109 days) and elevated temperature (37°C) contributed to increased degradation of DENV RNA in whole blood on FTA card but not on LyseNow card (Figure 2). In addition, greater variation in RNA recovery within the same treatment was observed with the FTA card in our study.

We believe this was caused in part by the way samples were collected from the FTA card. Seven discs of 3 mm in diameter were punched out using a 3 mm hole puncher from each FTA card. Although special attention was made to punch-out the 7 discs in a pattern as close to the LyseNow card design as possible, our manually punched out discs' locations on each FTA card just cannot be exact. The perforated card design combined with sample application on the exact same marked location on the card not only makes it easier to collect samples from the Lyse

Now card for downstream sample processing but also greatly improved the consistency of discs' harvesting with a higher degree of repeatability of test results. In summary, the LyseNow card has several advantages over the FTA card including easy sample application, easy collection of applied samples for RNA elution, higher rate of recovery of DENV-1 RNA, superior ability in preservation of DENV-1 RNA during prolonged storage even at an elevated temperature of 37°C, and the card produced much lower variability in results from the replica cards of the same treatment in this investigation. This product is an ideal tool to keep and preserve RNA from blood samples in resource-limited setting where cold chain is not available.

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