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Comparison of Matrix-Based and Filter Paper-Based Systems for Transport of Plasma for HIV-1 RNA Quantification and Amplicon Preparation for Genotyping

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Two ambient-temperature, dry plasma transport systems, ViveST tubes and RNA*Sound* RNA sampling cards, and two extraction methods were compared to frozen plasma for HIV-1 RNA recovery. Significant RNA loss occurred: ViveST+MiniMag > ViveST+QIAamp > RNA*Sound*+QIAamp. RNA loss and low specimen volumes may affect the sensitivity of genotyping specimens with HIV-1 RNA of <4.70 $\log_{10}$ copies/ml.

Plasma samples from HIV-1-infected individuals are monitored for drug resistance and HIV-1 RNA viral load (VL) before and during antiretroviral therapy (ART) in many treatment programs, generally at central laboratories (1). Quantification of HIV-1 RNA is generally performed on plasma samples frozen shortly after collection. However, the expense and logistics of maintaining this cold chain for transport from remote to central laboratories can be prohibitively expensive for low-resource communities. To more economically transport plasma, dried blood and plasma spots and matrix-based tubes have been developed to ship plasma at ambient temperatures. Dried blood spots prepared with whole-blood samples on Whatman 903 filter paper produce HIV drug resistance genotypes comparable to those with frozen plasma (2–6) and quantify plasma HIV-1 RNA loads with a mean loss of 0.23 $\log_{10}$ copy (c)/ml (2). Given that Whatman 903 filter paper holds only 50 μl of plasma or whole blood per circle, methods able to transport larger specimen volumes at ambient temperatures and reliably preserve HIV-1 RNA might reduce costs and increase access to recommended monitoring of HIV-1 VL and genotypes.

The ViveST system (ViveBio, Alpharetta, GA) includes matrix-based tubes for ambient temperature storage and shipment of plasma specimens. These tubes can hold up to 1 ml of plasma, which is slowly loaded by dripping the plasma from a pipet onto the matrix. Once the plasma has completely dried (≥8 h), the tube containing the matrix and a desiccant is shipped at ambient temperatures. Storage of the plasma for 2 to 56 days in the ViveST tubes at ambient temperatures has been reported to decrease plasma HIV-1 RNA values by 0.23 to 0.53 $\log_{10}$ c/ml compared to those for frozen plasma (7). In another study, plasma samples in ViveST tubes shipped to three U.S. sites at ambient temperatures lost HIV-1 RNA (mean, 1.07 $\log_{10}$ c/ml) compared to frozen plasma samples (A. M. McClernon, G. Cloherty, and D. R. McClernon, presented at the Association for Molecular Pathology Annual Meeting, Washington, DC, 12 to 14 November 2014). Reproducible quantification of replicate aliquots from four plasma specimens led the latter authors to suggest that the loss in HIV-1 RNA is sufficiently consistent that a correction factor might provide reliable VL quantification. Variable preservation of HIV-1 RNA for resistance genotyping has been reported for plasma samples stored in ViveST tubes (7, 8). A study of 20 plasma samples, with a broad range of VL (3.49 to 5.41 $\log_{10}$ c/ml), found 99.9% concordance of drug resistance mutations between frozen aliquots and corresponding aliquots dried in ViveST tubes (7). Another study of 50 plasma samples with relatively lower VL (median, 3.54 $\log_{10}$ c/ml; interquartile range −IQR], 3.32 to 4.11) yielded genotypes from 36% of the specimens dried in ViveST tubes compared to 96% from frozen plasma, with 98.9% concordance of resistance genotypes between specimen storage conditions (8).

The RNA*Sound* blood RNA card system (FortiusBio, San Diego, CA) represent a filter paper-based system for storage and transportation of biological specimens. The cards hold 200 μl of whole blood or plasma, which after drying can be stored with a desiccant and shipped at ambient temperatures. To our knowledge, no studies of plasma stability in RNA*Sound* have been published. The manufacturer reports that viral RNA in dried plasma is stable for at least 7 days. In this study, we assessed HIV-1 RNA recovery by quantifying VL and amplifying HIV-1 *pol* from plasma aliquots of specimens stored in ViveST tubes (RNA extracted by two methods), stored on RNA*Sound* cards, and frozen.

To compare the ViveST and RNA*Sound* systems to frozen plasma, previously genotyped residual deidentified plasma samples from HIV-1-infected individuals were obtained from the University of Washington Clinical Virology Laboratory, Seattle, WA. Each frozen plasma sample was thawed and divided into three aliquots: (i) 140 μl underwent immediate extraction of RNA (QIAamp viral RNA minikit; Qiagen, Valencia, CA); (ii) a median of 950 μl (range, 400 to 1,000) was loaded onto a ViveST matrix tube and dried overnight in a biosafety hood; and (iii) 200 μl was loaded onto an RNA*Sound* card and dried for ≥2 h at ambient temperature.

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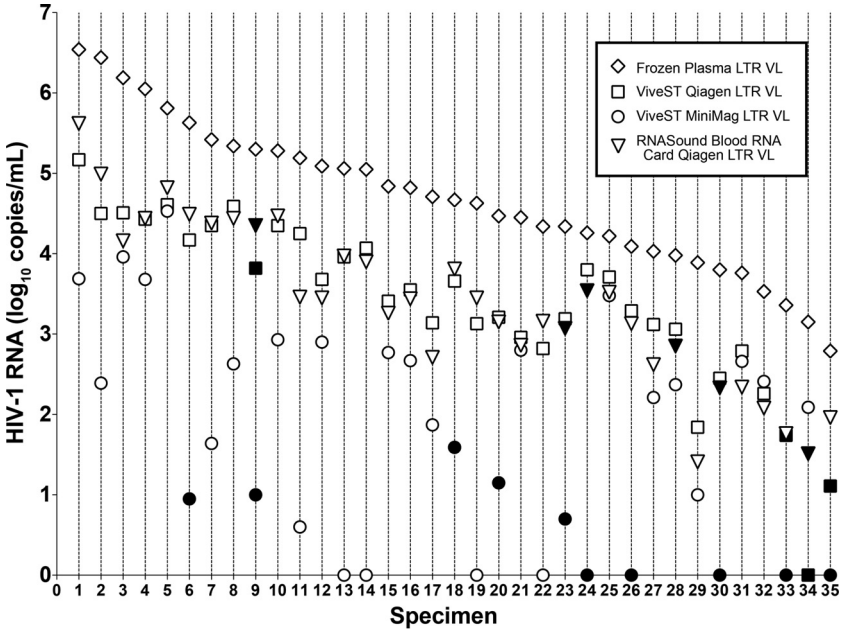

FIG 1 Comparison of HIV-1 RNA quantification and drug resistance genotyping following preservation of plasma samples in ViveST tubes (extracted by QIAamp or MiniMag) versus on RNA*Sound* cards (extracted by QIAamp) versus frozen (extracted by QIAamp). Aliquots of 35 clinical plasma specimens had RNA extracted from frozen plasma by QIAamp or were loaded into ViveST tubes, which following storage overnight at ambient temperatures had RNA extracted by QIAamp and MiniMag, or onto an RNA*Sound* card, which once dried had RNA extracted by QIAamp. The recovery/quantification of HIV-1 RNA with each storage and extraction method by a real-time PCR that amplified 114 bp of 5′ long terminal repeat (LTR)-*gag* is shown. The HIV-1 RNA yield from each condition and volumes of plasma extracted is normalized to $\log_{10}$ copies per milliliter of plasma. Amplification of 1,257 bp of HIV-1 *pol* for genotyping was performed using 10 μl of extracted RNA, which included RNA from the following plasma volumes: frozen, 23 μl; ViveST+QIAamp, 22 μl; ViveST+MiniMag, 152 μl; and RNA*Sound*+QIAamp, 33 μl. Successful amplification of *pol* is indicated by open shapes and failed amplification by closed shapes.

To extract the RNA from the ViveST tube, each dried matrix was rehydrated with 1 ml of RNase-free water, and the plasma was recovered according to the manufacturer's instruction. The recovered plasma totaled ~940 μl and was extracted immediately by two silica-based kits: (i) 140 μl using a QIAamp kit and (ii) ~800 μl using the NucliSENS MiniMag system (bioMérieux, Durham, NC), following the manufacturers' instructions. Each RNA*Sound* card was rehydrated with 720 μl of Qiagen buffer AVL that includes carrier RNA plus 180 μl of RNase-free water and extracted immediately following the instructions for the QIAamp kit. The RNA extracts (10 μl) from each of the four methods were reverse transcribed separately (Thermo Fisher SuperScript III; Thermo Fisher Scientific, Waltham, MA) and the HIV-1 cDNA (20 μl) was quantified in duplicate (10 μl/reaction) by quantitative real-time PCR (qPCR) (9). The HIV-1 RNA yields from each condition and volumes of plasma extracted were normalized to $\log_{10}$ copies per milliliter of plasma. Based on the volumes of plasma extracted, the lower limit of quantification of the real-time assay was 2.94 $\log_{10}$ c/ml for ViveST+QIAamp and fresh extractions, 2.09 $\log_{10}$ c/mL for ViveST+MiniMag, and 2.78 $\log_{10}$ c/ml for RNA*Sound*+QIAamp.

The recovery of nucleic acids for HIV-1 drug resistance genotyping was compared separately by amplifying 10 μl of each extracted RNA (derived from 23 μl frozen plasma, a median of 22 μl plasma from ViveST+QIAamp, a median of 152 μl plasma from ViveST+MiniMag, and 33 μl plasma from RNA*Sound*+QIAamp) in a nested PCR of HIV-1 *pol* (1,257 bp; 1st round forward primer CCTAGGAAAAAGGGCTGTTGGAAATGTGG and reverse primer AATCCCTGSATAAATYTGACTTGCCCARTT; 2nd round forward primer GArAGACAGGCTAATTTTTTAGGGA and reverse primer AAyTTCTGTATATCATTGACAGTCCA) encoding amino acids in protease (PR) 1 through reverse transcriptase (RT) 251 using a TaKaRa PrimeScript One Step RT-PCR kit (version 2; TaKaRa, Shiga, Japan) and MyTaq DNA Polymerase (Bioline, London, United Kingdom). Amplification was assessed by electrophoresis in an agarose gel stained with ethidium bromide, and band intensity was assessed visually to determine if it appeared adequate for genotyping. HIV-1 genotyping was not attempted on these amplicons.

The 35 frozen plasma samples (HIV-1 subtypes B [33 samples], AG/G [1 sample], and A/AE [1 sample]) had a median HIV-1 RNA of 4.67 $\log_{10}$ c/ml (range, 2.79 to 6.54). Drying the plasma diminished the HIV-1 RNA values compared to those for the frozen plasma, with median reductions of 1.3 $\log_{10}$ c/ml (range, 0.5 to 3.2), 2.7 $\log_{10}$ c/ml (range, 0.7 to 4.7), and 1.3 $\log_{10}$ c/ml (range, 0.7 to 2.5) for ViveST+QIAamp, ViveST+MiniMag, and RNA*Sound*+QIAamp, respectively ($P < 0.001$ for each comparisons by F tests for overall significance) (Fig. 1). The loss of HIV-1 RNA observed from plasma samples stored in ViveST tubes was greater than previously reported (7, 10). The correlation of ViveST+QIAamp, ViveST+MiniMag, or RNA*Sound*+QIAamp to frozen plasma found R^2 values of 0.707, 0.063, and 0.703, respectively, which indicates that a conversion factor would provide moderate to poor confidence in providing an accurate VL. The ViveST+MiniMag extraction was particularly variable, and 9 (26%) samples failed to amplify by qPCR, with median plasma HIV-1 RNA of 4.64 $\log_{10}$ c/ml (range, 3.91 to 6.09). As only one sample extracted by ViveST+QIAamp (HIV-1 RNA of 5.08 $\log_{10}$ c/ml) and none of

the RNA*Sound*+QIAamp samples were below the limit of HIV-1 RNA qPCR detection, the MiniMag extraction appears inefficient for either capturing RNA or removing inhibitors of PCR.

Amplification of HIV-1 *pol* for genotyping was successful from 100% of the frozen plasma specimens and for 89% of ViveST+QIAamp, 71% of ViveST+MiniMag, and 83% of RNA*Sound*+QIAamp specimens (Fig. 1). This higher rate of amplification failure than that for qPCR is likely due to the reverse transcription/amplification of a longer RNA fragment (114 versus 1,257 bp) and/or reduced efficiency of the one-step RT-PCR used for genotyping compared to that of the two-step RT-PCR used for qPCR. The plasma samples failing amplification of *pol* correspond to those with lower VL ($P < 0.0001$ by Mann-Whitney U test), except specimen 9, in which, given the successful amplification of frozen plasma for genotyping, we suspect that inhibitors of PCR may have persisted following storage in ViveST and RNA*Sound*. Our results are consistent with those of previous studies that successfully genotyped 36% of samples after storage in ViveST tubes with RNA extracted using the NucliSENS EasyMag system, which has the same chemistry as the MiniMag system (8). ViveST tubes have the advantage of holding up to 1 ml of plasma, while RNA*Sound* cards hold 200 μl. The larger volume appears advantageous for genotyping of samples with lower HIV-1 RNA levels by increasing the total number of HIV-1 RNA templates available for extraction, reverse transcription, and amplification. Given our HIV-1 RNA recovery, only a subset of the specimens would theoretically provide ≥50 HIV-1 cDNA templates/10 μl of RNA extract (the minimum number of templates needed to detect minority mutant variants comprising ≤20% of the viral population by consensus HIV genotyping): 54% of ViveST-Qiagen, 37% of ViveST-MiniMag, and 57% of RNA*Sound* card-Qiagen samples. Indeed, the VL was <4.70 $\log_{10}$ c/ml in all of the RNA*Sound*+QIAamp and ViveST+QIAamp specimens, providing <50 viral templates/10 μL. In contrast, HIV-1 RNA recoveries from several specimens with >6 $\log_{10}$ c/ml extracted from ViveST tubes by MiniMag were below the threshold, consistent with potential inhibitors of PCR.

Limitations of the study include the fact that the stability of the HIV-1 plasma RNA in the ViveST and RNA*Sound* systems was tested over a short duration of storage and only at ~23°C and ~15% humidity. Also, to save resources, amplified HIV-1 *pol* was not sequenced, so the detection of minority variants could not be compared.

In conclusion, both the ViveST tubes and RNA*Sound* cards preserved the majority of plasma HIV-1 RNA at ambient laboratory temperatures over a relatively short period of time. The proportion of HIV-1 RNA lost was moderately predictable with ViveST+QIAamp and RNA*Sound*+QIAamp and unpredictable with ViveST+MiniMag. The variable loss of HIV-1 RNA reduced the lower limit and reproducibility of HIV-1 RNA quantification. Furthermore, it diminished the input of viral templates for HIV-1 drug resistance genotyping, which reduces the likelihood that minority mutants would be detected by consensus sequencing when the plasma HIV-1 RNA is <4.70 $\log_{10}$ c/ml. The variable loss of HIV-1 RNA using these storage and transport systems emphasizes the need for an improved inexpensive modality to transport plasma samples for virus quantification and genotyping.

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