Stabilized viral nucleic acids in plasma as an alternative shipping method for NAT

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BACKGROUND: Preservation of the integrity of viral nucleic acids in blood specimens during shipping and handling is crucial for NAT and viral load monitoring. An economical and convenient method is described for nucleic acid stabilization by using an RNA stabilizing solution (RNAlater, Ambion) in plasma that is designed for the shipment of samples to tropical countries.

STUDY DESIGN AND METHODS: HCV, HIV, and HBV FFP were compared with RNAlater-treated plasma and dried plasma spots (DPSs) after incubation at 37°C, which was chosen as an upper limit of ambient shipping temperature, for up to 28 days. HCV-infected chimpanzee plasma was shipped at either room temperature after RNAlater treatment or as frozen plasma in liquid nitrogen from Liberia to New York City. They were then compared for HCV RNA levels. The nucleic acid stabilities were determined by quantitative PCR by using a molecular beacon assay on a sequence detection system (ABI 7700, PE-Biosystems) and by visualizing the PCR components on an acrylamide gel.

RESULTS: Quantitative PCR data showed that a 60:40 or greater ratio of RNAlater:plasma volume successfully stabilized HCV RNA and HIV RNA in plasma for up to 28 days at 37°C. HBV DNA in plasma was stable for up to 14 days at 37°C without any stabilizing solution. DPSs on filter paper stabilized viral nucleic acids, but the recoveries were 3 to 10 times less than those with frozen plasma. The integrity of the 5′ UTR region of HCV RNA in RNA later-treated chimpanzee plasma was intact when its PCR component was viewed on an acrylamide gel.

CONCLUSION: The DPS method stabilized nucleic acids, at least with the extraction method used, was less sensitive than use of RNAlater, and required tedious manual handling. RNAlater provides a convenient way of stabilizing viral nucleic acid in plasma at ambient temperature during sample transportation.

NAT will become a standard screening method for blood donation in the near future. Furthermore, quantitative PCR is becoming a valuable tool for the assessment of plasma viral load during antiviral therapy. Plasma sample integrity is a key factor for successful PCR assays for the previously mentioned applications. Shipping, handling, and storage conditions of clinical specimens are known to influence the stability of viral nucleic acids. The degradation of viral RNA during routine specimen processing, storage, and shipping could result in failure of its detection. The stability of viral nucleic acid in blood samples for PCR assay was addressed during collection, processing, storage,[1-5] and multiple freeze–thaw cycles.[6] Blood specimens are routinely transported to NAT sites in EDTA or in plasma preparation tubes (Becton Dickinson, Franklin Lakes, NJ) at 2°C to 8°C within 24 hours.[4,7] However, samples for viral load testing that require long-distance transportation are usually shipped as plasma in dry ice to preserve the integrity of viral nucleic acid, which is costly, and in some developing countries, this option is not readily available. India is currently embarking on a national program to confirm ELISA-positive samples resulting from blood bank screening nationwide by NAT in a central location. The use of an overnight blood shipment of plasma in dry ice shipments would not be practical because of their relatively inefficient transportation systems. Thus, a simplified and economical

ABBREVIATION: DPS(s) = dried plasma spot(s).

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method for preserving the integrity of viral nucleic acid in plasma is needed in such regions.

In this study, we report an economical method for preserving the integrity of viral nucleic acids in plasma, suitable for shipment at ambient temperature, by using an RNA stabilization solution.

MATERIALS AND METHODS

Virus stocks

A stock of HCV was obtained from a chronically HCV-infected blood donor (NYBC #292). The HBV-infected plasma (CPDA-1) was taken from a chronically infected chimpanzee (NYBC #75-546). HIV cell culture supernatant (HIV/III-CEM, NYBC #30b) was diluted in human seronegative CPDA-1 plasma for use in this study.

Stabilization solution

RNAlater (Ambion, Austin, TX) is a tissue RNA stabilization solution; the manufacturer does not recommend its use with plasma samples. Nevertheless, we tested this solution for viral RNA stabilization by mixing it at various ratios with plasma and by incubating the mixture at 37°C. Ten HCV-infected chimpanzee plasmas were collected in Liberia, West Africa. One set of plasma was mixed with RNAlater (60 parts RNAlater and 40 parts plasma, [vol/vol]) and was transported at room temperature, and a duplicate set of plasma was flash frozen and transported in liquid nitrogen via air to New York City.

Dried plasma spots

Fifty μL of HCV-, HIV-, and HBV-containing plasma was spotted onto filter paper (no. 903; Schleicher & Schuell, Keene, NH). The plasma spot (approx., 1.5 cm in diameter) was dried for 3 hours in a laminar flow hood[8] and was then incubated at 37°C.

Sample extraction

For extraction from frozen plasma and RNAlater and/or plasma mixtures, HCV and HIV RNA were extracted with a viral extraction kit (Qiagen, Chatsworth, CA), and HBV DNA was extracted with blood kits (Qiagen). The extracted HIV samples were treated with 10 U DNAase 1 (Ambion) for 60 minutes at 37°C to remove HIV DNA. For extraction from DPSs, the plasma-containing filter paper circles were cut into three pieces to ensure submersion of all the materials from the 1.5-cm disk under lysis buffer. HCV and HIV disks were suspended in 140 μL of sterile water and 560 μL of lysis buffer (Qiagen AVL) to meet the required volume for the Qiagen procedure, and they were incubated at room temperature for 1 hour with occasional vortexing. HBV disks were suspended in 200 μL of sterile water and 200 μL of lysis buffer (Qiagen AL), incubated at 56°C for 1 hour and then further extracted as recommended by the manufacturer.

Quantitative PCR assay

Mixtures for cDNA synthesis and PCR were set up with a pipetting station (Biomek 2000, Beckman, Fullerton, CA) in a laminar flow hood in a room dedicated to PCR set up.

Molecular beacons were single-stranded oligonucleotide probes that form a stem and loop structure.[9] The loop contained a probe sequence that was complementary to the target amplicon, and the stem was formed by the annealing of complementary arm sequences that are located on either side of the probe sequence. A fluorophore was linked covalently to the end of one arm, and a quencher was linked to the end of the other arm. Molecular beacons did not fluoresce when free in solution. They fluoresced when hybridized to a target strand.

Reverse transcription. The reaction was carried out in a 20-μL volume containing 10 μL of purified nucleic acid, 20 units of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Rockville, MD), 12 U of RNAse inhibitor (RNasin, Promega, Madison, WI), 50 m*M* of Tris-HCl (pH 8.9), 75 m*M* of KCl, 10 m*M* of DTT, 1.5 m*M* of $MgCl_2$, 0.1 m*M* of dNTP, and 1 μ*M* of reverse primer (HCV 5′ UTR, 5′-gtactcaccggttccgcaga-3′; HIV GAG, 5′-atttctcctactgggataggt-3′). The reaction was run at 42°C for 45 minutes and at 90°C for 2 minutes.

PCR. The PCR master mix (30 μL) was added to the previously mentioned cDNA reaction tube. The PCR master mix contained 2.5 units of a DNA polymerase (AmpliTaq Gold, Applied Biosystems, Foster City, CA), 4 m*M* of $MgCl_2$, 50 m*M* of KCl, 1 μ*M* of forward primer (HCV 5′ UTR, 5′-acgcagaaagcgtctagcc-3′; HIV GAG, 5′-aaccaagggaagtgacata-3′; no additional reverse primers were added), 0.5 μ*M* of molecular beacon probe (HCV 5′ UTR, 5′-FAM-ccgagc*cttagtatgagtgtcgtgcagcct*gctcgg-DABCYL-3′; HIV GAG, 5′-FAM-catggc*cagtacccttcaggaacaaatagga*gccatg-DABCYL-3′; probe sequence italicized), and 10 m*M* of Tris-HCl, pH 8.3. The reaction was run at 95°C for 10 minutes to activate AmpliTaq gold and was subsequently subjected to 40 cycles of 95°C for 30 seconds, at 55°C for 60 seconds, and at 72°C for 30 seconds on a spectrofluorometric thermal cycler (ABI PRISM 7700, PE-Biosystems, Foster City, CA). HBV PCR was carried out as described previously here by using primer sequences encoding surface antigens: a forward primer (5′-aaattcgcagtccccaacc-3′), a reverse primer (5′-atgaggcatagcagcaggatg-3′), and a probe (5′-FAM-cgacgg*ctggatgtgtctgcggcgttttat*ccgtcg-DABCYL-3′, probe sequence italicized) in a 50-μL final volume.

Detection and quantitation. Fluorescence was monitored during every thermal cycle at the annealing step. A software program (Sequence Detection version 1.6.3, PE-Biosystems) determined the copy number of the target template by analyzing cycle-to-cycle change in

fluorescence signal as a result of the amplification of template during PCR and by comparing unknowns to a curve generated from serially diluted, known synthetic RNA or plasmid DNA standard samples. Synthetic HCV RNA transcribed from the linearized plasmid (full-length HCV-H strain, subtype 1b), HIV-1 RNA from DNAse 1-treated HIV cell supernatant (NYBC #30), and pJW-So plasmid encoding the HBV S antigen (subtype ayw) were used as quantitation standards for HCV, HIV-1, and HBV, respectively. All standards were calibrated with EUROHEP panels (CLB, The Netherlands) for a determination of the copy numbers. Aliquots of the quantitative PCR products from chimpanzee specimens were also run on a 5-percent polyacrylamide gel, stained with ethidium bromide, and visualized under UV light.

Quality control. Four to 10 HCV-, HIV-, or HBV-positive plasma samples were coassayed in each PCR as external positive controls, containing an average of log 4.55 copies per mL for HCV, log 5.90 copies per mL for HBV, and log 6.45 copies per mL for HIV, to monitor extraction and amplification efficiencies. Only assays with the average of positive controls within two SDs of accumulative run averages were accepted as valid. Four to 10 negative human plasma samples were also included as negative controls.

Statistical analysis

HCV, HIV, and HBV plasma specimens treated with RNAlater or prepared as DPSs were incubated at 37°C and were compared with frozen plasma by using the data from quantitative PCR by a pair-wise *t*-test.

RESULTS

Stabilization solution

HCV, HIV, and HBV plasma samples without the addition of stabilizing solution were incubated at 37°C for 1 to 28 days and were then subjected to nucleic acid extraction and quantitative PCR assay simultaneously when all samples had been collected. The quantitative PCR data showed that HCV RNA and HIV RNA held without stabilizing solution started to degrade within 1 and 3 days at 37°C, respectively, although HBV DNA was stable up to 14 days, after which there was a minor drop in viral load (Table 1).

To determine optimal amount of RNAlater for stabilization, HCV plasma samples were mixed with RNAlater at various ratios and were then incubated at 37°C for 7, 14, and 28 days before nucleic acid extraction and quan-

TABLE 1. Sample degradation of unstabilized viral nucleic acids in plasma at 37°C shown by quantitative PCR

	Quantity (log molecules mL)		
Days	HCV	HIV	HBV
0 (FFP)	4.88 ± 0.15*	7.72 ± 0.21	6.84 ± 0.11
1	4.63 ± 0.08†	7.61 ± 0.21	6.81 ± 0.14
3	4.10 ± 0.17†	7.39 ± 0.19†	6.77 ± 0.12
7	3.77 ± 0.20†	6.77 ± 0.11†	6.77 ± 0.16
14			6.76 ± 0.20
21			6.72 ± 0.05†
28			6.56 ± 0.17†

* Mean ± SD quantity of six replicates determined by quantitative PCR.
† Significantly different from Day 0 sample ($p < 0.05$).

TABLE 2. Optimal ratios of RNAlater and plasma for stabilization of HCV RNA in plasma at 37°C

Ratio		Quantity (log molecules/mL)		
RNAlater:plasma	FFP*	7 Days	14 Days	28 Days
0:100	5.03 ± 0.27†	3.25 ± 0.29‡	3.29 ± 0.17‡	3.24 ± 0.22‡
20:80	4.71 ± 0.10	4.25 ± 0.25‡	4.21 ± 0.19‡	3.44 ± 0.20‡
40:60	4.64 ± 0.10	4.79 ± 0.14	4.38 ± 0.21‡	3.62 ± 0.15‡
60:40	4.40 ± 0.11	4.47 ± 0.36	4.35 ± 0.34	4.28 ± 0.18
80:20	4.11 ± 0.11	3.89 ± 0.35	4.31 ± 0.28	4.17 ± 0.19
90:10	3.76 ± 0.23	3.56 ± 0.21	3.61 ± 0.27	3.72 ± 0.14

* No RNAlater was added; sample volumes were substituted with normal human plasma.
† Mean ± SD quantity of six replicates determined by quantitative PCR.
‡ Significantly different from FFP ($p < 0.05$).

TABLE 3. HCV, HIV, and HBV nucleic acid stabilization by RNAlater in plasma at 37°C

		Quantity (log molecules/mL)					
	FFP	1 Day	3 Days	7 Days	14 Days	21 Days	28 Days
HCV	5.59 ± 0.19*	5.63 ± 0.16	5.62 ± 0.17	5.68 ± 0.11	5.76 ± 0.14	5.69 ± 0.13	5.64 ± 0.14
HIV	7.42 ± 0.19	7.39 ± 0.34	7.46 ± 0.23	7.38 ± 0.25	7.59 ± 0.20	7.55 ± 0.19	7.40 ± 0.17
HBV	6.84 ± 0.11	6.37 ± 0.21†	6.23 ± 0.11†	6.26 ± 0.19†	6.30 ± 0.10†	6.39 ± 0.12†	6.16 ± 0.24†

* Mean ± SD quantity of six replicates determined by quantitative PCR.
† Significantly different from FFP ($p < 0.05$).

TABLE 4. Comparison of HCV RNA quantities from HCV-infected chimpanzee plasma shipped as frozen and RNAlater-treated plasma

Chimpanzee number	RNA recovery (log RNA mol/mL)	
	FFP	RNAlater*
147	3.74†	3.87
167	5.11	5.09
194	7.03	7.17
213	3.82	3.68
214	4.86	4.80
275	5.36	5.02
280	5.21	5.29
325	5.77	5.48
328	6.74	6.59
353	5.44	5.44
Mean ± SD	5.31 ± 1.06	5.24 ± 1.07‡

* RNAlater-treated plasma (60 RNAlater:40 plasma, vol/vol).
† Quantity determined by quantitative PCR.
‡ p = 0.45 vs. FFP.

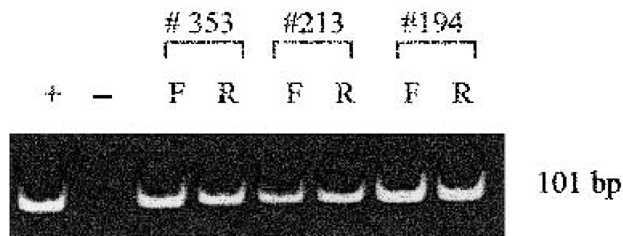


Fig. 1. PCR products obtained from three HCV-infected chimpanzees were compared for RNA integrity: duplicate aliquots of frozen (F) and RNAlater-treated (R) plasma. A 101-bp product from HCV 5′ UTR was amplified in quantitative RT-PCR. Quantities ranged from log 3.68 to 7.18 per mL. Lane 1, positive control; Lane 2, negative control; Lanes 3 and 4, chimpanzee #353; Lanes 5 and 6, chimpanzee #213; Lanes 7 and 8, chimpanzee #194.

TABLE 5. Stability and sensitivity of detection of HCV, RNA, HIV RNA, and HBV DNA stored at 37°C as DPSs

		Quantity (log molecules/mL)					
	FFP	0 Day	3 Days	7 Days	14 Days	21 Days	28 Days
HCV	5.15 ± 0.05*	3.93 ± 0.41	3.99 ± 0.09	4.13 ± 0.12	4.24 ± 0.35	4.26 ± 0.19	4.19 ± 0.17
HIV	7.53 ± 0.06	6.70 ± 0.11	6.88 ± 0.05	6.98 ± 0.01	6.92 ± 0.12	7.04 ± 0.04	6.94 ± 0.04
HBV	6.84 ± 0.10	5.79 ± 0.15	5.95 ± 0.16	5.88 ± 0.15	5.81 ± 0.19	5.75 ± 0.19	5.63 ± 0.10

* Mean ± SD quantity of three replicates determined by quantitative PCR.

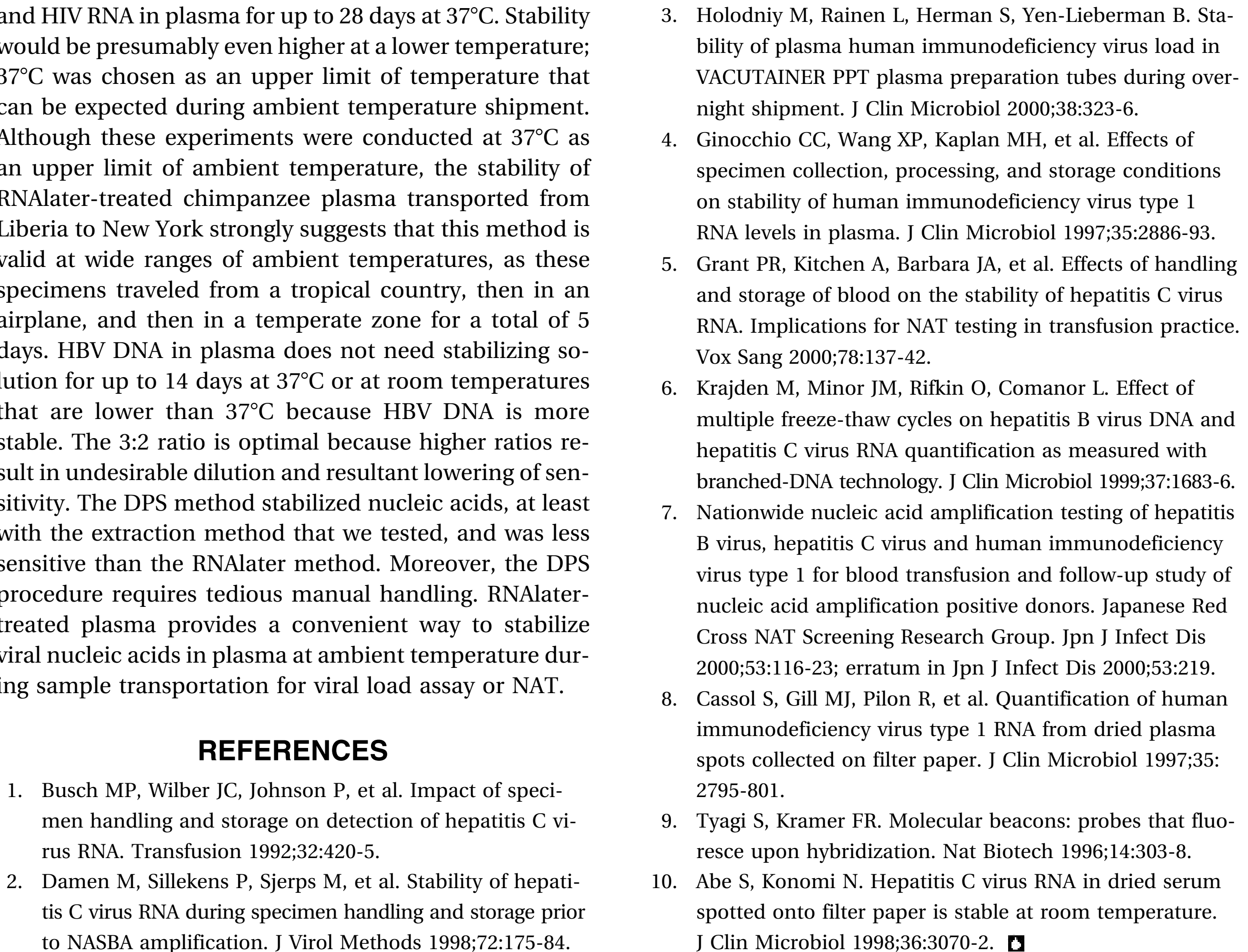
titative PCR assay. The quantitative PCR assay showed that plasma at a ratio of 3:2 (60 μL RNAlater:40 μL plasma) or higher ratios of RNAlater to plasma successfully stabilized HCV RNA in plasma at 37°C for up to 28 days (Table 2). Table 3 shows successful stabilization of HCV and HIV RNA in plasma for up to 28 days at 37°C at a ratio of 50 μL of plasma mixed with 90 μL of RNAlater. Plasma and/or RNAlater volumes were determined based on the data in Table 2 and to meet the total sample volume of 140 μL in the Qiagen extraction procedure. HBV DNA in RNAlater solution seemed to lose approximately log 0.5 copies per mL during SDS-based buffer extraction procedures used in the Qiagen blood kit when compared with fresh frozen samples. The difference was significant ($p < 0.05$), although the RNAlater-treated samples taken at different times were not different from each other. Considering the fact that untreated HBV DNA plasma was stable up to 14 days at 37°C (Table 1), the difference may be due to interference between RNAlater and the SDS buffer system. We did not see this reduction with HCV RNA or HIV RNA where guanidine–thiocyanate-based buffer was used for extraction. To determine whether our experimental temperature, 37°C, provided a reasonable guide for shipment at ambient temperature, HCV-infected plasma was collected from 10 chimpanzees in Liberia and was shipped to New York City via air either in RNAlater at room temperature or frozen in liquid nitrogen. The chimpanzee plasma transported from Liberia to New York City with either method showed no significant differences in their viral loads (Table 4). An acrylamide gel showed that PCR bands from RNAlater-treated and frozen specimens were not different from each other, indicating the integrity of the 5′ UTR region of HCV RNA in RNAlater solution at ambient temperature (Fig. 1).

DPSs

In an attempt to compare virus stability in RNAlater with that in DPSs, 50 μL of HCV, HIV, and HBV plasma was spotted on a filter paper and dried in a laminar flow hood and then incubated at 37°C for 0, 3, 7, 14, 21, and 28 days before extraction and quantitative PCR. Despite the claims that DPSs could be used to stabilize viral RNA in plasma,[8] the PCR sensitivity was approximately 3 to 10 times lower in our hands than in previous reports, probably because of the relative ineffectiveness of the extraction method for DPSs (Table 5). Even though a prolonged lysis time was allowed for DPS extraction, the recovery of viral nucleic acids was significantly less than that for FFP. Similar results were also observed by other investigators.[10] Furthermore, the procedures for DPS preparation and extraction were far more tedious than those used for RNAlater treatment.

DISCUSSION

Based on the quantitative PCR assay, a 3 to 2 or greater ratio of RNAlater to plasma volume stabilized HCV RNA



and HIV RNA in plasma for up to 28 days at 37°C. Stability would be presumably even higher at a lower temperature; 37°C was chosen as an upper limit of temperature that can be expected during ambient temperature shipment. Although these experiments were conducted at 37°C as an upper limit of ambient temperature, the stability of RNAlater-treated chimpanzee plasma transported from Liberia to New York strongly suggests that this method is valid at wide ranges of ambient temperatures, as these specimens traveled from a tropical country, then in an airplane, and then in a temperate zone for a total of 5 days. HBV DNA in plasma does not need stabilizing solution for up to 14 days at 37°C or at room temperatures that are lower than 37°C because HBV DNA is more stable. The 3:2 ratio is optimal because higher ratios result in undesirable dilution and resultant lowering of sensitivity. The DPS method stabilized nucleic acids, at least with the extraction method that we tested, and was less sensitive than the RNAlater method. Moreover, the DPS procedure requires tedious manual handling. RNAlater-treated plasma provides a convenient way to stabilize viral nucleic acids in plasma at ambient temperature during sample transportation for viral load assay or NAT.

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