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MINIREVIEW

Determinations of Levels of Human Immunodeficiency Virus Type 1 RNA in Plasma: Reassessment of Parameters Affecting Assay Outcome

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INTRODUCTION

A growing number of adult and pediatric studies have demonstrated that human immunodeficiency virus (HIV) type 1 (HIV-1) RNA levels have importance in determining the risk of both disease progression and the transmission of infection from mother to infant (3, 4, 7, 9, 12, 15, 16, 19, 26–28, 34–36). However, there has been concern that the different assays for determination of HIV-1 RNA levels and the different techniques used for specimen handling and processing in those investigations may make interpretation of findings across studies difficult. Issues concerning specimen handling prior to testing of plasma for HIV-1 RNA levels are important since some reports suggest that variations in specimen handling may profoundly affect the detection of and quantification of plasma HIV-1 RNA (18, 19).

A workshop sponsored by the National Institute of Allergy and Infectious Diseases National Institutes of Health and entitled Technology Utilization for HIV-1 Blood Evaluation and Standardization in Pediatrics: A Special Emphasis on Plasma RNA Assays was held on 17 and 18 June 1996 in the Washington, D.C., area to address these issues. The primary purpose of that workshop was to convene a group of expert investigators from academia, government, and industry to develop recommendations on the most appropriate specimen collection, processing, storage, and shipping methods for blood collected in pediatric HIV-1 studies in both national and international settings, with an emphasis on what was required if quantitative plasma HIV-1 RNA assays were to be used in the investigations. Invited investigators presented data from studies with specimens that were acquired from adults and children and that addressed pertinent specimen-handling issues.

The highlights of the proceedings of the workshop are presented in this report. The information includes biological and specimen-handling factors that may affect the results of assays for plasma HIV-1 RNA levels and summaries of recommendations for the most appropriate specimen-handling methods for pediatric studies in both the national and the international settings.

EFFECT OF ASSAY AND BIOLOGIC VARIATION ON DETECTION

Assay variation. In order to interpret clinically significant changes in HIV-1 RNA levels, all sources of variability in the quantitation of the virus were considered. Assay variability data for plasma and patient blood samples spiked with HIV-1 were presented by industry and academic representatives. The intrinsic variability of each RNA quantitation method (intra- and interassay variability), independent of biological or other variables introduced by specimen processing, was determined over the dynamic range of the assays by using replicate samples with different operators and different kit lots. The standard deviation for all the kits assessed (Chiron Quantiplex [bDNA], Roche Amplicor HIV-1 Monitor Test, and Organon Teknika NASBA) appeared to be between 0.08 and 0.20 $\log_{10}$, with the variation observed among patient samples being greater than

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† Other attendees at the Technology Utilization for HIV-1 Blood Evaluation and Standardization in Pediatrics (TUBE) workshop were Paul Palumbo (University of Medicine and Dentistry-New Jersey Medical School, Newark), Indira Hewlett (Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, Bethesda, Md.), Richard Respess (Centers for Disease Control and Prevention, Decatur, Ga.), Kenneth Rich (University of Illinois, Chicago), Jane Pitt (Columbia Presbyterian Hospital, New York, N.Y.), and Fulvia Veronese (Office of AIDS Research, National Institutes of Health, Bethesda, Md.).

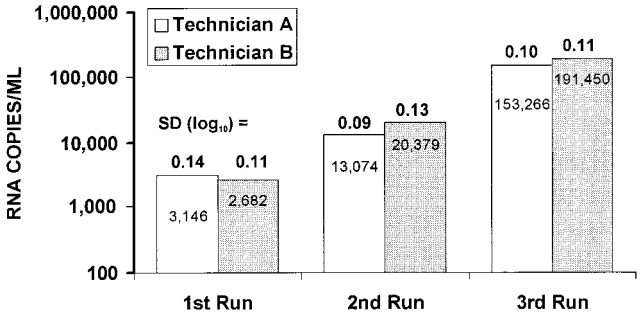

FIG. 1. Intraassay variability in plasma HIV-1 RNA levels for five replicates of three samples. The Roche Amplicor Monitor assay was used to quantitate plasma HIV-1 RNA levels (copies per milliliter) by two different experienced technicians in separate sample runs. The mean number of RNA copies per milliliter and the standard deviation (SD) ($\log_{10}$) for each sample run, by technician, are located within the columns and above the columns, respectively.

that observed among spiked samples (2, 39). Encouragingly, a recent study evaluating variation between the Chiron bDNA, Roche Amplicor, and Organon Teknika NASBA assays with patient specimens showed that the use of a common set of HIV-1 RNA standards could eliminate differences in the estimated absolute HIV-1 RNA copy number among these three commercial tests (2).

Variability due to operator differences was presented by T. Quinn (Fig. 1) for the Roche Amplicor assay. In this example, there was little variation due to the operators since both were experienced PCR technicians. However, depending upon the proficiency of the technician, variation could be significantly increased. This was confirmed in two recent publications which demonstrated that interlaboratory differences were greater than interkit differences for both spiked (39) and clinical (33) samples.

Biological variation. Normal biological fluctuations in HIV RNA levels can also complicate the interpretation of assay results. There have been a few longitudinal studies on variations in HIV-1 RNA levels (13, 30, 32) in patients with relatively high $CD4^+$ cell counts and other short-term studies involving either daily variations (17) or the use of baseline levels at initial clinical study visits (7). N. Michael presented data on plasma RNA levels for eight untreated individuals showing that variation was subject dependent (32). Five acutely and three chronically infected individuals were evaluated over 324 and 1,500 days, respectively. The variation in the estimated RNA copy number ranged from 0.25 to 1.30 $\log_{10}$ for the acutely infected patients and 0.32 to 0.93 $\log_{10}$ for the chronically infected patients. In a group analysis, biological variation was approximately 0.35 to 0.45 $\log_{10}$. However, variation within an individual may be even greater. Plasma HIV-1 RNA level data from the Women and Infants Transmission Study have also been examined, and preliminary analysis showed that variability in RNA levels in infants under 1 year of age was as high as 0.70 $\log_{10}$, with variability appearing to be higher in younger children than in older children.

Viral genotypic variation. Data presented suggested that the more specific and limited the genomic region used for RNA quantitation, the more likely it is that there will be differences in the quantitative measurements associated with differences in clade sequence. The Chiron bDNA assay, which assesses a larger portion of the HIV-1 genome with multiple probes, appeared to have fewer problems quantifying various HIV-1 subtypes than other, more target specific assays. In a study evaluating a limited number of RNA transcripts representing several different clades, the Chiron bDNA assay appeared to quantify the subtypes similarly (22). Modification of the procedures and/or the primers of other assays may improve detection of different clades of HIV-1 by these assays (20). A comprehensive set of reagents representing the various clades will be needed to provide standards for appropriate comparability studies with kits from various manufacturers.

Variation by compartment. Little is known about the variation in RNA levels in different body compartments such as semen and cervical-vaginal secretions. R. Coombs presented data on variations in the levels in semen over time and showed that the variations were 5- to 10-fold higher than those observed in plasma (8). Whereas reports assessing HIV-1 detection frequency in cervical-vaginal secretions have shown viral shedding to be intermittent, very few studies specifically addressed the issue of biological variation in this particular compartment (21, 29).

SPECIMEN HANDLING FACTORS THAT MAY AFFECT RNA STABILITY AND DETECTION

Blood fraction tested (plasma versus serum). The stability of HIV-1 RNA detection in plasma versus serum was presented by S. Herman, T. Yeghiazarian, R. Carroll, B. Jackson, and N. Michael (10, 18, 32, 37) (Table 1). Regardless of the specific assay used, when paired samples of plasma and serum were tested, there was a close correlation between the HIV-1 RNA levels detected in the plasma and serum specimens. However, consistently higher viral loads were detected in plasma samples, although the mean differences were not always statistically significant. The levels detected in plasma were typically 30 to 80% higher than those detected in serum. Thus, it appeared that viral load can reasonably be detected in both plasma and serum but that individual studies should consistently use either serum or plasma for RNA detection, with a preference for plasma if possible.

Anticoagulant and time of processing. The results of numerous studies on the effect of anticoagulants on viral RNA load determinations and HIV-1 RNA stability in whole blood or plasma are presented in Table 1. S. Herman and R. Dickover showed that for whole blood and stored plasma specimens collected and stored in tubes containing EDTA, about 10 to 15% and 20 to 25% more HIV-1 RNA tended to be detected at each time point at which they were examined than was detected in tubes containing ACD and heparin, respectively, as determined by the Roche Amplicor assay (10). (However, such differences are not necessarily found if a silica extraction procedure is used to prepare the specimens [see RNA Extraction Methods for RNA Detection].) The rate of loss of detectable RNA was greatest during the first 0 to 3 h and then 3 to 6 h postcollection; however, this loss was less with plasma than with whole blood. For whole-blood specimens, the amount of RNA lost relative to the level at 1 h was about 10% for tubes containing EDTA, 20% for tubes containing ACD, and 30% for tubes containing heparin by 6 h. After 6 h, the viral RNA levels in whole blood and plasma remained relatively stable at 4°C and room temperature (RT) for up to 24 to 48 h.

Data from A. Kovacs's laboratory were also presented. Kovacs and colleagues assessed (by the NASBA assay for RNA detection) variations in RNA levels due to collection in specimen tubes containing different anticoagulants, the use of different storage temperatures, and the timing of specimen processing. Analysis of variance showed no statistically significant difference in RNA levels by collection with an anticoagulant (EDTA or ACD) or without an anticoagulant (serum), by storage at RT or 4°C, or by processing at 0, 6, 24, or 48 h after

TABLE 2. Difference in HIV-1 RNA levels from baseline levels by temperature and type of anticoagulant[a]

Tube contents	Temp	No. of samples	Mean ± SD $\log_{10}$ difference in HIV-1 RNA levels from baseline levels at the following times:		
			6 h	24 h	48 h
EDTA	RT	7	+0.066 ± 0.200	+0.085 ± 0.237	+0.114 ± 0.259
	4°C	7	−0.001 ± 0.310	−0.077 ± 0.278	−0.060 ± 0.336
ACD	RT	7	−0.194 ± 0.309	−0.216 ± 0.083	−0.180 ± 0.286
	4°C	7	+0.057 ± 0.171	−0.108 ± 0.268	−0.119 ± 0.311
Serum	RT	5	−0.280 ± 0.419	−0.064 ± 0.606	−0.092 ± 0.388
	4°C	7	−0.007 ± 0.260	−0.076 ± 0.315	−0.061 ± 0.349
Total		40	−0.049 ± 0.278	−0.077 ± 0.313	−0.044 ± 0.320

[a] Analysis of variance showed no statistical significant difference by type of anticoagulant or temperature at each time point. The total number of samples was 160; the total number of patients was 7. The Organon Teknika NASBA assay was used for testing.

collection. In that study, the largest decrease in RNA levels also occurred within the first 6 h postcollection. Although not statistically significant, collection of specimens in tubes containing EDTA (versus tubes containing ACD or no anticoagulant [serum]) and storage of the tubes at 4°C produced consistently smaller differences in $\log_{10}$ RNA levels (23, 24) (Table 2).

It was postulated that the loss of RNA, seen most dramatically during the first 6 h after blood collection, might be due to the degradation of defective HIV-1 particles. Several of the participants also suggested that the 15% less plasma RNA signal for specimens in tubes containing ACD relative to that for specimens in tubes containing EDTA is probably due to the dilution factor (15%) of the ACD fluid volume in these tubes. In summary, those studies showed that both whole blood or plasma specimens tended to have slightly higher RNA levels when they were collected in tubes containing EDTA rather than tubes containing ACD or heparin and that over time there was a relatively small loss in the amount of RNA detected, with the greatest loss occurring during the first 6 h after collection.

RNA extraction methods for RNA detection. The effect of preextraction methods on RNA copy number was assessed by J. Kornegay, who used the Roche Amplicor assay. Eighteen paired plasma samples collected from HIV-infected women were placed in tubes containing heparin and were subjected to RNA extraction by either the silica extraction method (1) or by using a heparinase step prior to standard RNA extraction by the Roche Amplicor assay. The silica extraction method produced consistently higher HIV-1 RNA levels than the method in which the plasma samples were processed with heparinase. The yield by the heparinase method was 29% lower than that by the silica-binding method, on average (95% confidence interval = 20 to 37%) (Fig. 2). In a separate investigation of 99 patient samples collected and placed in tubes containing heparin and 11 samples collected and placed in tubes containing EDTA, no significant differences in RNA levels were seen when the silica extraction method was used for all extractions prior to testing by the Roche Amplicor assay (3). These observations suggest that the method of RNA extraction can have an important effect on the quantitation of RNA, and specifically, use of silica extraction prior to detection by the Roche Amplicor assay can circumvent the inhibitory action of heparin on reverse transcription-PCR.

TABLE 1. Results of studies on specimen-handling conditions with blood samples for HIV-1 RNA testing

Presenter (reference)	Assay	Sample no. and patient no.	RNA load in plasma vs that in serum	RNA load in different anticoagulants	RNA stability in whole blood	RNA stability in plasma	RNA stability with freezing and thawing of plasma	Stability at various temp	RNA stability during shipping
S. Herman (10)	Roche Amplicor	Samples from 18 to 38 patients	Greater in plasma	EDTA > ACD > heparin	Relatively stable to 24 to 48 h	Stable at RT and 4 to 8°C for 5 days	Stable up to four freeze (−80°C)-thaw cycles	Stable in plasma at −20°C for 10 yr	Plasma RNA stable at RT, 2 to 8°C, on dry ice overnight
S. Yeghiazarian (37)	Chiron bDNA	Samples from 19 patients	Greater in plasma	EDTA > ACD > heparin	Decline greater in whole blood vs plasma	Stable to 24 h at RT and 4°C	Stable up to three freeze (−80°C)-thaw cycles; 20% decline if freezing was at −20°C	Stable at −80°C; decline at −20°C by 20 wk	
R. Carroll and A. Kovacs (23, 24)[a]	Organon Teknika NASBA	160 samples from 7 patients	Greater in plasma	EDTA > ACD or serum	Relatively stable to 48 h at RT or 4°C				
B. Jackson (18)	Chiron and Roche assays	Samples from 40 patients	Greater in plasma	EDTA > ACD > heparin	Decline greater in whole blood vs plasma	Stable to 30 h at 4°C			
N. Michael (32)	Roche assay with silica extraction	33 matched samples from 9 patients	Greater in plasma, but not if adjusted for albumin						
R. Dickower (10)	Roche assay	Samples from 20 patients		EDTA > ACD > heparin		Stable to 24 to 48 h			
S. Fiscus	Organon Teknika NASBA	Samples from 35 patients					Stable up to four freeze (−80°C)-thaw cycles		Plasma RNA stable at RT overnight
B. Weiser	Organon Teknika NASBA	Samples from 2 patients					Stable up to three freeze (−80°C)-thaw cycles		

[a] R. Carroll presented work from the laboratory of A. Kovacs.

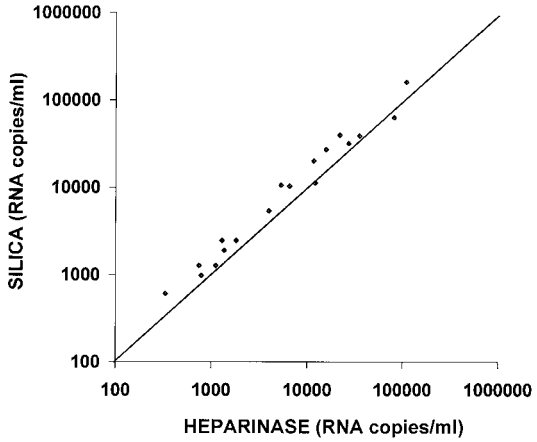

FIG. 2. Comparison of two RNA extraction methods, heparinase treatment and silica-binding extraction, on HIV-1 RNA copy number detected by the Roche Amplicor Monitor assay.

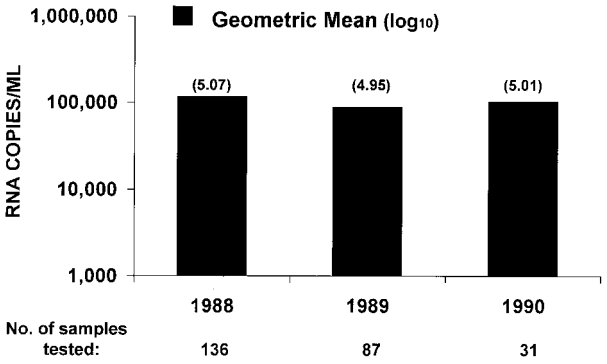

FIG. 3. Geometric mean baseline HIV-1 RNA level by year of study entry. The collected blood specimens were separated, and the serum was initially stored at −20 or −70°C prior to periodic shipment to a central repository. Serum specimens were stored centrally at −70°C for 4 to 7 years prior to batch testing by the Organon Teknika NASBA assay (28).

Long-term storage (temperature and time). RNA in plasma appeared to be stable for extended periods of time, particularly when it was stored at −80°C. Using the Chiron bDNA assay for detection, T. Yeghiazarian presented data from a study in which the differences between the RNA levels detected in specimens (samples with relatively high viral loads diluted in HIV-1-negative plasma) stored at −20 or −80°C for 80 weeks were evaluated (37). After 12 weeks of storage, a decline in RNA levels detected in samples stored at −20°C versus those stored at −80°C was noted, although the difference was not significant. However, by weeks 20 to 24, the difference in RNA levels in samples stored at −20°C versus the RNA levels in samples stored at −80°C became statistically significant, with specimens stored at −80°C having 30 to 80% more RNA than those stored at −20°C.

S. Herman presented data suggesting that storage of plasma for 10 years at −20°C and storage of serum for 4 to 7 years at −70°C appeared to have no significant effect on relative RNA quantitation by the Roche Amplicor assay. L. Mofenson reported on data from the National Institute of Child Health and Development IVIG Clinical Trial in which HIV RNA levels in 254 stored baseline serum samples from children enrolled in the study between 1988 and 1990 were assessed in 1996 by the NASBA assay (thus, the samples were stored for 6 to 9 years prior to testing). There was a similar distribution of values (over a 4-log range in HIV-1 RNA copy number) and no significant differences in geometric mean and median RNA levels, regardless of the year of study entry (28) (Fig. 3).

In another recent study with 10 specimens collected in tubes containing EDTA, ACD, or heparin and stored for 6 months prior to testing by the NASBA assay, the results indicated that the mean viral loss due to storage was within the expected variance of the assay (14). The findings of all these studies suggest that appropriate long-term storage does not have a significant effect on detectable HIV-1 RNA levels.

Freeze-thaw cycles. Repeated freeze-thaw cycles of plasma samples stored at −20 and/or −80°C were evaluated by S. Herman, T. Yeghiazarian, R. Carrol, B. Weiser, and S. Fiscus (10, 11, 37). There was an insignificant loss of RNA levels after one to four freeze-thaw cycles (Table 1). For example, S. Herman showed that the amount of HIV-1 RNA detected (by the Roche Amplicor assay) after two to four freeze (at −80°C)-thaw cycles as a percentage of the amount detected after the first freeze-thaw cycle for eight plasma specimens processed from tubes containing either ACD or EDTA averaged from about 120 to 150% (10). By the NASBA assay, Ginochio et al. (14) showed that the levels of HIV-1 RNA in 10 plasma specimens collected in tubes containing EDTA, ACD, or heparin after one freeze (at −70°C)-thaw cycle had a mean loss that was insignificant (14). In addition, although T. Yeghiazarian showed that three freeze (at −20°C)-thaw cycles produced a statistically significant decrease (about 20%) in HIV-1 RNA levels from the levels detected after only one freeze-thaw cycle, the decrease was within the expected variation of the Chiron bDNA assay used. No significant decrease was seen for specimens stored at −80°C; however, this could have been due to the lower baseline levels detected after one freeze (at −80°C)-thaw cycle (Fig. 4). Similar results were obtained regardless of the conditions of the freezing (at −80°C)-thawing, e.g., immediate sample processing with short thaw periods and quick freezing (presented by T. Yeghiazarian) to thawing at RT over 20 min or in a 37°C water bath for 10 min (presented by B. Weiser).

Shipping. Comparison of RNA copy numbers in samples from local and distantly located sites showed that there were no significant differences. S. Herman presented comparative data on 25 patient specimens collected in tubes containing EDTA or ACD, separated into plasma, and shipped overnight

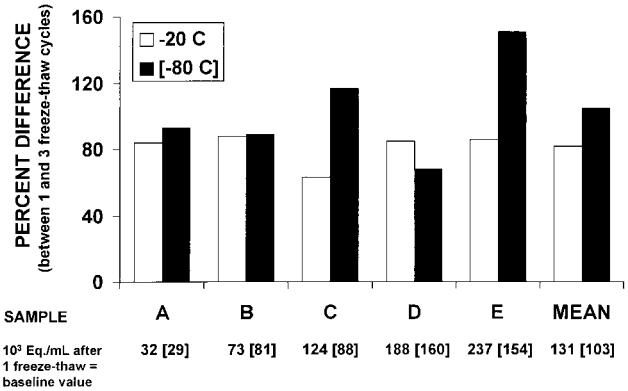

FIG. 4. Percent difference in HIV-1 RNA detection between one and three freeze-thaw cycles. The RNA equivalents per milliliter after the first freeze-thaw cycle, which is considered the baseline value, are given beneath the white columns (freezing at −20°C) and beneath the black columns (freezing at −80°C; numbers in brackets). HIV-1 RNA was detected by the Chiron bDNA assay (37).

TABLE 3. Difference in HIV-1 RNA levels between local and distant serum specimens[a]

Measurement	Mean $\log_{10}$ difference in HIV-1 RNA levels in the following specimens:		
	Total (n = 49)	Local (n = 20)	Distant (n = 29)
Mean	3.042	2.904	3.136
Median	3.298	3.158	3.428
Range	4.030	3.650	4.030
SD	1.193	1.043	1.296

[a] Local, specimens that were transported at room temperature prior to processing which generally took place within 6 h of collection; distant, specimens that were transported at RT via overnight delivery prior to processing. HIV-1 RNA levels were determined by the liquid hybridization PCR assay (38).

either on dry ice with cold packs (2 to 8°C) or at ambient temperature (10). Median RNA values (by the Roche Amplicor assay), expressed as a percentage of the median RNA level in samples shipped on dry ice, were 98% for specimens in tubes containing EDTA and 100% for specimens in tubes containing ACD for specimens shipped with cold packs and 90% for specimens in tubes containing EDTA and 80% for specimens in tubes containing ACD for specimens shipped at ambient temperature. Shipping of plasma specimens overnight at ambient temperature did not appear to decrease the detectable HIV RNA levels in comparison to the levels in local specimens that were quickly processed. S. Fiscus compared the RNA concentrations in the plasma of 25 perinatally HIV-1 infected infants using the NASBA assay; 11 infants were local to the study site and 14 infants were seen at clinics 75 to 300 miles away. The local plasma specimens were centrifuged and were frozen within 3 h of phlebotomy and had a median of 5.80 $\log_{10}$ RNA copies/ml (interquartile range, 5.56 to 6.40 $\log_{10}$ RNA copies/ml). The plasma samples which were shipped overnight at ambient temperature had a median of 5.63 $\log_{10}$ RNA copies/ml (interquartile range, 5.32 to 6.28 $\log_{10}$ RNA copies/ml).

M. Robb reported on the use of a laboratory-specific HIV-1 RNA assay (38) with serum specimens from adult and pediatric patients enrolled in different U.S. cohort studies that had been under long-term storage. These specimens were transported at room temperature prior to processing and arrived within 6 h of collection in most cases. Some samples were drawn at distant locations and were shipped overnight at room temperature prior to processing. Forty-nine maternal samples were analyzed, and 20 of these were drawn at locations requiring overnight shipment. The comparison of local and distantly acquired samples showed no differences in HIV-1 RNA levels in locally obtained samples and those whose processing was delayed (Table 3).

Effects of other factors on viral load detection. The effects of common antimicrobial agents in plasma samples, hemolysis, lipidemia, and elevated bilirubin levels on the HIV-1 RNA load detected by the Chiron bDNA assay were presented by T. Yeghiazarian (37). HIV-1-positive and HIV-1-negative plasma samples were spiked with (i) one of three pools of antimicrobials agents, one of two levels of hemoglobin (0.5 and 1.0 mg/ml), or (iii) 10 mg of bilirubin per dl. None of the substances interfered with the determination of the RNA copy number or gave false-positive results for the HIV-1-negative samples. Additionally, visible lipidemia was not found to alter the RNA levels in HIV-1-positive plasma samples.

ALTERNATE TESTING METHODS

In addition to the information presented on techniques for evaluation of plasma RNA levels, the workshop also reviewed data on some promising alternate testing methods. These included evaluation of HIV-1 RNA levels by using dried plasma spots (DPSs) on filter paper and methods for the detection of HIV-1 RNA levels in semen and cervical-vaginal secretions.

DPSs on filter paper. The feasibility of using DPSs for the quantitation of viral RNA and comparison of the results obtained with DPSs to those obtained by standard assays of plasma were described by S. Cassol (5). The effects of field conditions including humidity and temperature and the effects of short-term storage were assessed. Retrospective analysis of 51 paired specimens of cryopreserved plasma and DPS by a commercial assay (Roche Amplicor) indicated that the HIV-1 RNA levels from DPSs were equivalent to those obtained from fresh frozen plasma. The RNA levels obtained by assays with DPSs stored under different field conditions appeared to be stable. There was no evidence of a decline in RNA levels when the DPSs were stored at refrigerated or ambient temperature for up to 16 days or at 37°C for 3 days. Use of DPS specimens would be particularly useful for evaluating HIV-1 RNA levels in field trials in settings where the capacity for specific sample processing and refrigeration or freezing are not readily available.

S. Cassol also presented data for direct automated sequencing of viral DNA from dried blood spots (DBSs) for the detection of HIV-1 *env* subtypes A, B, C, and E (6). Results were presented for 51 DBSs from mostly asymptomatic HIV-1-infected individuals from five Asian countries. These specimens were shipped without refrigeration and were stored for more than 2 years. The subtypes identified were primarily subtype C with a few subtype A in India and subtypes B and E in Thailand, China, and Indonesia. Thus, the DBSs appeared to offer a practical approach for the genetic surveillance of the HIV-1 isolates from different populations, including recent seroconverters and vaccine trial participants.

Semen and cervical-vaginal specimens. A comparison of use of the Roche Amplicor and NASBA assays with seminal fluid was presented by S. Fiscus (11). Results from the two assays, expressed in terms of RNA copy number per milliliter of ejaculate, suggested that the detection of RNA in seminal fluid was complicated by the presence in semen of nonspecific inhibitors which interfere with the PCR step and that these inhibitors were removed by the use of the silica extraction method used in the NASBA assay. In addition, the HIV-1 RNA levels in seminal fluid samples were stable through at least four freeze-thaw cycles in that study.

One of the major problems associated with the quantification of HIV-1 RNA from cervical-vaginal secretions is that most such specimens are collected as a cervical-vaginal lavage and there is no standard method dealing with the dilution effect for a lavage specimen that may be only partially retrieved. R. Coombs presented data on the use of a "sno-strip" method (Akorn Inc., Abita Springs, La.) for the detection of HIV-1 RNA in cervical specimens. This method circumvents this obstacle by directly wicking up known quantities of cell-free cervical-vaginal fluid onto filter paper (8).

Data from Kovacs' laboratory was presented on the effect of time to processing and the best sample type (whole cervical-vaginal lavage specimen, supernatant or cell pellet) to detect and quantitate HIV-1 RNA by the NASBA assay. In that study, 27 cervical-vaginal lavage specimens from 9 HIV-positive women were refrigerated (4°C) after collection, processed as whole cervical-vaginal lavage specimens at 0, 3, 6, and 24 h

TABLE 4. Stability of HIV-1 RNA copies per milliliter detected in unseparated (whole) and separated (supernatant) cervical-vaginal lavage specimens processed at different times[a]

Specimen no.	Specimen	No. of HIV-1 RNA copies/ml at the following times:			
		0 h	3 h	6 h	24 h
1	Whole	42,000	60,000	15,000	31,000
2	Whole	3,600	1,700	1,700	1,500
3	Whole	14,000	8,500	6,000	5,400
4	Whole	3,800	100	100	100
5	Whole	25,000	33,000	21,000	9,600
1	SUP	5,600	11,000	4,600	4,100
2	SUP	1,800	2,000	1,200	940
3	SUP	3,200	3,500	2,400	1,600
4	SUP	100	100	100	100
5	SUP	100	100	100	100

[a] Specimens were stored at 4°C prior to processing (separation) and were tested by the Organon Teknika NASBA assay (23, 24). SUP, supernatant.

after collection, and centrifuged and separated into a supernatant and a cell pellet. Cervical-vaginal lavage samples stored at 4°C appeared to be stable for up to 24 h with no significant difference in $\log_{10}$ RNA levels in either whole cervical-vaginal lavage samples or the supernatant over time. Significantly more HIV-1 RNA was detected in whole cervical-vaginal lavage samples and cell pellets compared to the levels obtained in the supernatant (Table 4) (23, 24). Lastly, W. Meyer presented results for unfractionated frozen cervical-vaginal lavage fluids collected and processed in a routine manner by the NASBA technique for the detection of HIV-1 RNA. There appeared to be an inverse relationship between the detection of HIV-1 RNA in cervical-vaginal lavage specimens and the peripheral CD4 count, and the HIV-1 RNA levels detected in plasma generally exceeded the quantity detected in cervical-vaginal lavage specimens. Although the results of these limited studies with genital fluids are encouraging, understanding of the role of HIV-1 in these compartments will require further development of acceptable and standardized techniques for assay usage and specimen collection.

RECOMMENDATIONS FOR TESTING OF PERIPHERAL BLOOD FOR HIV RNA IN PEDIATRIC STUDIES

National studies. Workshop participants felt that although some studies have been published (22, 34), there remains an urgent need for further data to obtain a better understanding of the association between plasma HIV-1 RNA levels and disease progression in pediatric patients of different ages. Such studies were considered to be of the highest priority.

The potential of HIV-1 RNA detection as a diagnostic assay for HIV-1 infection in young infants was discussed. Although it was agreed that the "gold standards" for the detection of HIV-1 infection in children born to HIV-1-positive mothers are HIV-1 culture and/or positivity for HIV-1 DNA by PCR, theoretically, HIV-1 RNA can be detected before HIV-1 is detected by the other two tests. The level of HIV-1 RNA at the time of early detection might also be valuable in identifying the potential for disease progression. Thus, studies validating the detection of HIV-1 RNA as a diagnostic marker were considered an important priority, albeit secondary to that of validating HIV-1 RNA detection as a surrogate marker for disease progression.

On the basis of data presented at the workshop, the session participants also concluded that (i) of the various assays for the detection of HIV-1 RNA evaluated, one assay did not appear to be significantly superior to the others when considering specimen-handling techniques; (ii) further data are needed regarding the natural history of HIV-1 RNA in children and the correlation of the presence of HIV-1 RNA with clinical outcome and the response to therapy in pediatric patients; and (iii) data regarding HIV-1 RNA in adolescents (an important but distinct subset of pediatric patients) are needed to determine if pubertal changes modify the natural history of RNA in these patients.

The research priorities for the use of assays for the detection of HIV-1 RNA in pediatric HIV studies in the United States and the methods best suited to the collection, processing, shipping, and analysis of study specimens are summarized in Table 5. It was generally agreed by the participants that standardization of specimen handling and assay performance is important for the interpretation of the results. However, the information presented at the workshop suggested that a reasonable range of variability in these parameters could be acceptable, and this was reflected in the guidelines for specimen handling presented in Table 5.

International studies. The application of viral burden assays in developing countries poses unique challenges. Costly viral burden assays are not likely to play a major role in the medical management of HIV-1-infected patients in countries with limited resources. Rather, viral burden assays should be optimized to meet the research agenda of developing countries, which currently focuses on the more economically feasible goal of prevention. Differences among the clades of HIV-1 must be strongly considered in this setting; thus, such technical challenges to assays must be considered along with field conditions that depart significantly from the conditions found in clinics and laboratories in the developed world, and cultural sensibilities regarding phlebotomy volume and frequency must be respected.

In general, processing techniques should be simple and should be reliable in extremes of temperature and humidity. For these reasons, many experts at the meeting endorsed the use of DBSs or DPSs collected on filter papers. New collection tubes which are designed to separate plasma and cells and optimize the biological integrity of both, despite delayed processing after phlebotomy, are available. Alternatively, plasma, serum, and cells may be separated on-site by standard techniques and stored on liquid nitrogen, which is often more widely and reliably available than dry ice or high-quality freezers. However, all collection and storage devices may not perform as expected when they are deployed in less developed settings due to differences in the study population or the physical setting. For example, an endemic parasitic disease producing anemia may degrade the performance of cell separator collection devices. As a consequence, an element of the research designs in these settings must include revalidation of the techniques established in the developed world. Equipment requirements should be minimized to the extent possible, and equipment should be reliable in the setting of fluctuating availability of electrical power and environmental extremes. For example, the use of nonrefrigerated, fixed-angle centrifuges in assays deployed for use worldwide would be a significant advantage.

In view of the emphasis on HIV-1 transmission research in developing countries, the desire for viable peripheral blood mononuclear cells for functional studies, viral isolation, and flow cytometric studies may require the use of alternative anticoagulants such as heparin or ACD. Since the stability of cell-free RNA appears to be superior in tubes containing EDTA, it is likely that no single collection device will suffice for most

TABLE 5. Recommendations for handling of blood samples to be assayed for HIV-1 RNA with U.S. and international research cohorts

Area receiving recommendation	Research studies with U.S. cohorts	Research studies with international cohorts
I. Research priorities for HIV RNA testing	1. Validate use as a predictor of disease progression in pediatric patients 2. Evaluate as a diagnostic assay for HIV infection in young infants	1. Infant HIV diagnosis for assessment of early efficacy results from perinatal prevention trials 2. Measurement in plasma, cervical-vaginal secretions, and breast milk to evaluate mechanisms of perinatal HIV-1 transmission 3. Study of breakthrough in vaccine recipients 4. Pathogenesis studies of cofactors and clade differences related to disease progression
II. Component of blood used, specimen collection tube	1. Plasma is preferable to serum, but consistency is most important 2. Tubes containing EDTA were preferable to those containing ACD, followed by those containing heparin[a]	1. Both cells and plasma be collected and stored 2. Ideally, at a minimum samples would be collected in two tubes: (i) a tube containing EDTA would be used for plasma RNA and flow studies; (ii) tubes containing CPT, heparin, or ACD would be used depending on the research study and on-site laboratory capabilities.
III. Timing of specimen processing	1. Process (i.e., spin and separate) whole blood between 3 and 6 h after collection, particularly if the specimen is collected in a tube containing ACD 2. For specimens in tubes containing EDTA or other anticoagulants, processing could occur up to 24 h after collection	1. Ideally, the separation of plasma and cells was recommended to be done between 4 and 6 h but up to 24 to 48 h after specimen collection
IV. Stability and storage conditions	1. For whole blood, store the specimen at RT for up to 24 h if culture or lymphocyte function assays are not planned; otherwise, store at 4°C for up to 24 h until processing. 2. For plasma specimens, store at 4°C for up to 5 days, although storage at −70°C indefinitely is ideal, followed by storage at −20°C for up to 20 wk	1. Both cells and plasma should be frozen and stored in liquid nitrogen, given problems with electricity outages and −70°C freezers
V. Shipping conditions	1. Ship whole blood or plasma specimens within 24 h of sample collection; temperature extremes should be avoided by using an insulated container to pack and ship the specimens; freezing of whole blood should be avoided to preserve CD4 cells 2. Plasma specimens can be sent at RT or 4°C or frozen	1. Shipments should be on dry ice
VI. Future studies[b]	1. Future technical studies should focus on use of dried plasma spots for quantitative RT-PCR in plasma 2. Field deployable rapid diagnostic tests	1. Separation techniques applicable to developing country settings need to be validated 2. Future technical studies should focus on the use of dried plasma spots for quantitative RT-PCR in plasma 3. Development of assays that will detect multiple HIV clades well and a standardized panel of different clades of viruses for testing the assays

[a] If a silica extraction method for RNA is used prior to testing by the Roche Amplicor Monitor assay, then there may be less concern regarding which anticoagulant is used.

[b] Also see research priorities.

study needs. Future research should emphasize the development of collection tubes which provide biologically intact cells and stable cell-free RNA in plasma and which require only simple management efforts in the field. In addition, processing, storage, and transport paradigms need to be a focus of efforts in environments different from those present in the United States and Europe. A summary of the recommendations for studies with HIV-1-infected pediatric subjects in relationship to laboratory assay and specimen-handling issues in the international setting are presented in Table 5.

SUMMARY AND CONCLUSIONS OF WORKSHOP

Appropriate interpretation of HIV-1 RNA levels requires an understanding of differences in test results due to multiple factors, which include assay and biological variation as well as specimen-handling conditions. Multiple investigations with diverse patient populations and assays have suggested that the contributions of technical and biological variations to RNA levels were quite consistent and predictable and in the range of 0.3 to 0.6 $\log_{10}$ RNA copies/ml. To date, all of the studies that have assessed variations in the levels of HIV-1 RNA measured have been limited primarily to isolates of the B clade; thus, what is lacking is knowledge of the degree to which the clade subtype influences assay variation and whether the biological variation observed with the clade B subtype is consistent for other clades.

The major finding from the workshop was the unexpected stability of the HIV-1 RNA collected and stored under a variety of specimen handling conditions. HIV-1 RNA was shown to be relatively stable in whole blood, plasma, and serum, with the greatest stability being in plasma. Separated plasma was

found to have stable titers even after storage at room temperature for 24 to 48 h and repeated freeze-thaw cycles. Within the constraints of the studies described here, the potential differences in RNA levels due to various specimen-handling conditions were not large (10 to 20% due to the anticoagulant type used in the collection tube [30 to 80% if serum rather than plasma is used], 10 to 30% due to time at RT prior to processing within 24 h, 30 to 80% due to the use of a storage temperature of −20 or −80°C). Thus, the anticipated RNA levels for nonideally collected and processed plasma specimens may be only about 130% (0.11 $\log_{10}$) less than those for plasma specimens collected and processed ideally (assuming that these differences are additive). This 130% difference is relatively small compared to the potential total average standard deviation of up to about 400% or 0.6 $\log_{10}$ RNA copies/ml due to intra- and interassay (both 0.1 to 0.2 $\log_{10}$) and biological (0.1 to 0.2 $\log_{10}$) RNA copies/ml factors. On the basis of these findings, workshop participants concluded that retrospective studies, including those which have used sera or heparinized samples, should show biological comparability to studies performed under ideal conditions, and thus both retrospective and prospective studies are useful in providing an understanding of the role of HIV-1 RNA levels in blood in transmission and disease progression. However, for prospectively designed studies, workshop participants recommended that blood for quantitative HIV-1 RNA testing ideally be collected in tubes containing EDTA, processed within 6 h of collection (but up to 24 h is still acceptable), and then stored at −80°C until assayed.

Novel methodological approaches which could be useful in diagnosing and quantitating viral load in developing countries were also described, i.e., the use of DPSs, or in other body fluids such as cervical-vaginal secretions, i.e., sno-strip wicks. Finally, workshop participants determined what laboratory evaluations, including assays of HIV-1 RNA levels, with blood samples should be a priority in pediatric cohort studies while acknowledging that this ultimately depends on the study question being asked. Recommendations concerning specimen handling were then developed for international and domestic studies that use assays for detection of HIV-1 RNA.

The findings reported herein underscore the continued need for the exchange of information among investigators and industry with the aim of elucidating the technological parameters that influence the assays used to evaluate HIV-1 disease and therapeutic interventions. Only by understanding the factors that affect assay outcome can we appropriately discern their value and use in clinical studies and for patient management.

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