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Detection and Quantification of Human Immunodeficiency Virus Type 1 p24 Antigen in Dried Whole Blood and Plasma on Filter Paper Stored under Various Conditions

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The quantification of human immunodeficiency virus type 1 (HIV-1) by an assay measuring heat-dissociated (HD) p24 antigen (Ag) in specimens of whole blood and plasma stored on filter paper, and of plasma stored in tubes, was compared to HIV-1 RNA plasma levels determined by real-time reverse transcription (RT)-PCR. The stability of p24 Ag on filter paper under conditions simulating specimen transport was also evaluated. The HD p24 Ag in both plasma and whole-blood specimens stored on filter paper correlated with plasma HIV-1 RNA levels (Spearman rank $\rho = 0.74$ [$P < 0.0001$] and $\rho = 0.56$ [$P = 0.0001$], respectively). The sensitivity of the HD p24 Ag assay was similar when plasma and whole blood on filter paper were contrasted to the real-time RT-PCR assay (80% versus 82.5% and 78.6% versus 83.3%, respectively). However, while the specificity of the HD p24 Ag assay of plasma on filter paper was 100%, the specificity was diminished in whole-blood specimens. The storage of specimens on filter paper for 2 weeks at 37°C, 24°C, or 0°C did not alter the detection or quantification of HD p24 Ag. These results suggest that transport and storage of plasma on filter paper and quantification of HD p24 Ag may be a reliable method for HIV-1 load monitoring.

While antiretroviral therapy is reaching resource-limited countries, the laboratory tests critical to monitoring their use are costly and often not available. Specifically, measurements of viral load and CD4 lymphocytes in the blood are central to optimal management of antiretroviral treatment (31). In resource-limited settings, convenient, low-cost assays are urgently needed (31). Validation of less expensive assays may enable their adoption and lead to improvements in the management of human immunodeficiency virus type 1 (HIV-1) treatment.

The quantification of p24 antigen (Ag) has been considered for viral load monitoring (3, 4, 13, 15, 19, 21–23, 25–28). The p24 Ag assay measures the virus directly and has been shown to correlate with levels of plasma HIV-1 RNA in untreated subjects (21, 23, 30) and persons receiving antiretroviral therapy (3, 23, 27, 28). In addition, detection of p24 Ag has been explored for early diagnosis of pediatric HIV-1 infection (19, 20, 24). Assays that quantify p24 Ag are less expensive, time consuming, labor intensive, and costly than the RNA assay (21, 23, 27–29) and may be a practical alternative for viral load monitoring.

Dried specimens collected on filter paper have the potential to further reduce the expense of viral load testing by simplifying sample collection, storage, and shipment (14, 17, 18). Transport of specimens on filter paper has proved effective for detection of HIV-1 DNA (1, 7, 8, 9), quantification of HIV-1 RNA (4, 6, 12, 18), and monitoring the emergence of drug resistance mutations (8). Here we report the evaluation of filter paper for transport and storage of human whole blood and plasma for subsequent quantification of viral core Ag (p24 Ag). Our goal was to examine a simple, inexpensive, practical method for specimen processing and storage that could be integrated with an existing inexpensive validated kit for HIV-1 load quantification.

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MATERIALS AND METHODS

Specimens. Excess whole blood and/or plasma from HIV-1-infected patients submitted for genetic sequencing to the virology laboratory was placed in 2-ml polypropylene tubes (Sarstedt, Newton, NC) and transported to our laboratory for assay of heat-dissociated (HD) p24 Ag. Aliquots of 50 μl whole blood or plasma anticoagulated by collection into EDTA-containing Vacutainer tubes were spotted onto filter paper (903 Paper; Schleicher & Schuell, Inc., Keene, NH). After drying, the specimens were placed in common plastic Ziploc bags. Aliquots of each specimen were stored at −80°C, and to test the effects of various ambient temperatures that could occur during specimen transport, at 37°C and low humidity (5 to 10%), at 24°C and ~30% humidity, or at 0°C for 2 weeks prior to storage at −80°C.

HD p24 Ag assay. Specimens were eluted from filter paper by incubation in 0.5% Triton X-100 buffer for 60 min in a 1.5-ml polypropylene tube (USA Scientific, Ocala, FL). The eluted whole blood or plasma in 0.5% Triton X-100 was separated from the filter paper by centrifugation at 13,600 × *g* for 3 min in an Ultrafree-MC tube (Millipore, Billerica, MA). The eluate was then transferred to a 2-ml screw-cap polypropylene tube and heated at 100°C for 5 min in a dry heat block and assayed using the HIV-1 p24 enzyme-linked immunosorbent assay (ELISA) kit (Perkin-Elmer Life Sciences, Wellesley, MA) boosted by the ELAST ELISA amplification system (Perkin-Elmer Life Sciences) (26). Briefly, treated whole blood or plasma (0.25 ml) was transferred to wells of the HIV-1 p24 Ag ELISA kit, and the wells were covered and incubated for 2 h at room temperature on a microtiter plate shaker. Wells were washed with 1× wash buffer, and then 0.1 ml of biotinylated detector antibody was added and the mixture was incubated for 1 h at 37°C. After a wash, 0.1 ml of streptavidin-horseradish peroxidase solution was added to the wells and the plate was incubated for 15 min at 37°C. After a wash, 0.1 ml of biotinyl-tyramide solution was added to the wells and the plate was incubated for 15 min at room temperature. Following a wash, 0.1 ml of streptavidin-horseradish peroxidase diluted in buffer solution was added to the wells and the plate was incubated for 15 min at room temperature. *O*-Phenylenediamine substrate solution (0.1 ml) was added to the

wells after a wash. At the end of 30 min, the colorimetric reaction was stopped by the addition of 0.1 ml of stop solution and the optical density (OD) read at 490 nm using an ELISA reader. Kit standards were included in each assay run. The assay lower limit of detection was established as the mean of the negative controls plus 3 standard deviations. All specimens were tested in duplicate, and the mean of the two values was used in statistical analysis.

Quantification of plasma HIV-1 RNA by real-time PCR. Viral particles were pelleted from blood plasma (500 μl) by centrifugation at 23,000 × *g* for 1 h at 4°C, lysed (5.75 M guanidinium isothiocyanate, 190 mM dithiothreitol, 25 mM Tris-HCl, 30 μl of glycogen [10 mg/ml]), and then extracted with isopropanol (600 μl) and ethanol (1 ml of a 70%, vol/vol, solution). The overall specimen preparation schema followed the manufacturer's procedure for the Roche Monitor HIV-1 RNA US-RT-PCR assay, with the exception that the pellet was resuspended in 50 μl rather than 100 μl of lysis buffer. Real-time PCR was done on each specimen using a master mixture (40 μl) composed of manganese acetate at 25 mM (6 μl; Applied Biosystems, Foster City, CA), 5× EZ buffer with ROX (10 μl; SYNTHEGEN, Houston, TX), deoxynucleoside triphosphates at 10 mM (6 μl; Amersham Pharmacia Biotech, Piscataway, NJ), forward primer HXB2-Gag-F (CAA GCA GCC ATG CAA ATG TT) at 20 μM in AE buffer (1 μl), reverse primer SK431-B (TGC TAT GTC ACT TCC CCT TGG TTC TCT) at 20 μM in AE buffer (1 μl), probe HXB2-*gag* (6-carboxyfluorescein–AAA GAG ACC ATC AAT GAG GAA GCT GCA GAA–6-carboxytetramethylrhodamine) at 10 μM (0.5 μl), 12.75 μl diethyl pyrocarbonate H_2O, RNase inhibitor at 26.4 U/μl (0.75 μl; Amersham Pharmacia Biotech), and r*Tth* at 2.5 U/μl (2 μl; Applied Biosystems), and sample (10 μl) was added to each reaction tube. The tube was sealed and placed in the reaction plate and then into the TaqMan PCR machine (ABI Prism 7700). The PCR program thermocycler conditions were set at 60°C for 30 min for reverse transcription, 95°C for 5 min for denaturation, and 42 cycles of amplification at 95°C for 20 s, 52°C for 20 s, and 60°C for 1 min. All primers were high-performance liquid chromatography purified and purchased from Invitrogen (Frederick, MD), and all probes were high-performance liquid chromatography purified and purchased from Applied Biosystems. The standard curve for the assay was prepared from Armored RNA HIV-1 of subtype B (Ambion, Inc., Austin, TX) in TSM buffer (10 mM Tris, pH 7.0; 100 mM NaCl; 1 mM $MgCl_2$; 0.1% gelatin; diethyl pyrocarbonate H_2O; carrier *Escherichia coli* tRNA at 10 μg/μl). The working stock contained 30,000 RNA copies/μl, as verified by measuring the A_{260} and confirmed using the Roche Monitor HIV-1 RNA assay.

HIV-1 genotyping. HIV-1 subtypes of *pol* were assessed as described previously (10) using the Stanford database HIVseq Sequence Analysis Program (www.hivdb.stanford.edu).

Statistical analysis. HD p24 Ag and HIV-1 RNA concentrations were $\log_{10}$ transformed for all analyses. Sensitivity statistics were calculated for the samples from HIV-1-infected individuals. Specificity statistics were calculated using samples from 20 uninfected controls. Spearman rank correlation coefficients were used to evaluate the strength of the relationship between HIV-1 RNA concentrations and HD p24 Ag levels. Spearman's rho (ρ) statistic was calculated from the rankings of observations within the population sampled. Two features of the Spearman statistic support its use for this analysis: (i) that it is not limited to detecting linear relationships and (ii) that it requires no arbitrary assumptions to allow inclusion of measurements that fall below the detection limit. The Spearman correlation coefficients were calculated for both plasma and whole-blood samples.

Linear regression was chosen to analyze the effects of different short-term storage temperatures on measurements of HD p24 Ag, as the fit of the linear model and the degree by which the slope differs from 1.0 is critical to determining whether temperature affects the assay results. Slope estimates and r^2 statistics (i.e., the proportion of the total variance explained by the linear model) are reported. Similarly, for HD p24 Ag measurements in samples on filter paper and samples in polypropylene tubes, linear regression slopes and r^2 values are reported. For the linear regression modeling, observations that fell in the undetectable range were assigned a value equal to the detection limit of the assay, 10^2 fg/ml.

RESULTS

HIV-1 specimens studied. The p24 Ag concentrations were determined for a total of 42 whole-blood and 40 plasma specimens from 49 HIV-1-infected individuals. The HIV-1 polymerase gene subtypes of 43 plasma specimens included 40 subtype B specimens, 2 subtype CRF01_AE specimens, and 1 subtype C specimen. The sequence and subtype were not determined for the remaining six specimens (virus from these subjects was never PCR amplified in our laboratory). Plasma HIV-1 RNA levels were available from all of the 49 subjects. Values for all four tests (whole blood on filter paper HD p24 Ag, plasma on filter paper HD p24 Ag, plasma in tube HD p24 Ag, and plasma HIV-1 RNA) were available from 33 HIV-1-infected patients. Whole-blood and plasma specimens from 20 healthy laboratory workers were also evaluated by the p24 Ag assay to serve as negative control specimens.

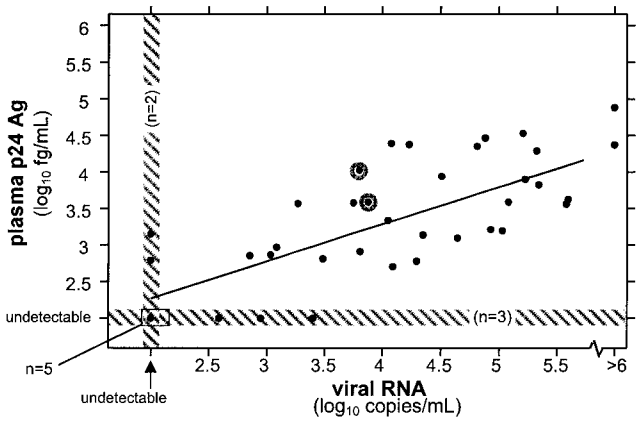

FIG. 1. Plasma HIV-1 loads determined by detection of HD p24 Ag from filter paper and HIV-1 RNA concentrations determined by real-time PCR in 40 specimens from HIV-1-infected patients. The subtype CRF01_AE specimens are circled, and the single subtype C specimen tested negative for both RNA and p24 Ag (however, a previous specimen from the subject with subtype C had been sequenced).

Concordance of HIV-1 detected by HD p24 Ag and by real-time PCR. The concordance of qualitative detection of HIV-1 in plasma specimens stored on filter paper and tested in the HDp24 Ag assay to plasma specimens stored in tubes and evaluated by the real-time PCR of HIV-1 RNA was 87.5% (35 of 40), with 75% (30 of 40) of the specimens being positive in both assays and 12.5% (5 of 40) being negative in both assays. The nonconcordant specimens had low viral loads (<3.5 $\log_{10}$) (Fig. 1). The sensitivities of the two assays were similar, with a virus detected in 33/40 (82.5%) specimens by the HIV-1 RNA assay and in 32/40 (80%) by the HD p24 Ag assay.

The concordance of HIV-1 detection in whole blood stored on filter paper by the HD p24 Ag assay to the detection of HIV-1 RNA by real-time PCR of plasma stored in tubes was 85.7% (36 of 42 specimens), with 73.8% (31 of 42) positive in both assays (including two subtype CRF01_AE specimens) and 11.9% (5 of 42) negative in both assays (including the single subtype C virus). The HD p24 Ag assay yielded 33 positive results (78.6%), while the RNA assay had 35 positive results (83.3%). The viral loads in the nonconcordant specimens were 3.75 $\log_{10}$ and 3.689 $\log_{10}$ in the HD p24 Ag assay ($n = 2$) and ranged from 3.48 $\log_{10}$ to 4.35 $\log_{10}$ in the RNA assay ($n = 4$).

HD p24 Ag was not detected in any of the 20 plasma specimens from laboratory workers without HIV-1 infection when stored on filter paper or in tubes (100% specificity). Testing of whole blood on filter paper yielded a higher mean OD compared to plasma in the specimens from both HIV-1-infected

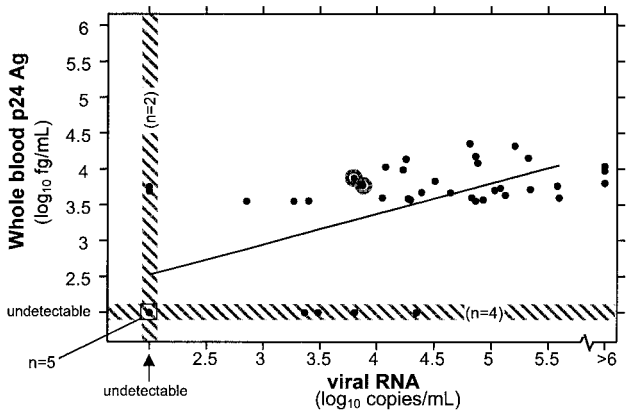

FIG. 2. HIV-1 loads determined by detection of HD p24 Ag in whole blood on filter paper and HIV-1 RNA concentrations determined by real-time PCR in plasma in specimens from 42 HIV-1-infected patients. The subtype CRF01_AE specimens are circled, and the single subtype C specimen tested negative for both RNA and p24 Ag (however, a previous specimen from the subject with subtype C had been sequenced).

and uninfected individuals. The mean difference in OD (± standard deviation) between whole blood and plasma in non-HIV-1-infected persons was 0.12 ± 0.05, and in HIV-1-seropositive persons it was 0.08 ± 0.02. In fact, the OD for HD p24 Ag in the whole-blood specimens from all 20 non-HIV-1-infected individuals exceeded the negative cutoff and thus tested falsely positive.

Quantification of HIV-1 by HD p24 Ag compared to real-time PCR of plasma viral RNA. The quantification of HD p24 Ag in plasma on filter paper correlated with the plasma HIV-1 RNA level. The Spearman rank correlation of these two assays was $\rho = 0.74$ ($P < 0.0001$) (Fig. 1). The HD p24 Ag assay detected virus in 30% (3 of 10) of specimens with viral loads of <1,000 RNA copies/ml, 88.9% (8 of 9) of specimens with viral loads between 1,000 and 10,000 copies/ml, and 100% (21 of 21) of specimens with viral loads of >10,000 copies/ml. The quantification of plasma HD p24 Ag stored in polypropylene tubes yielded very similar results to that of plasma stored on filter paper. The concordance was 97.5% (39 of 40 specimens), with 77.5% (31 of 40) positive in both assays and 20% (8 of 40) negative in both assays. The slope of the linear regression on the $\log_{10}$-transformed values was 1.037, with 95% confidence bounds of 0.96 and 1.11. The proportion of total variation explained by the linear model, r^2, was 0.95.

The relationship between quantity of HD p24 Ag measured in whole blood on filter paper and plasma HIV-1 RNA levels was statistically significant but not as strong as for plasma. The Spearman rank correlation for testing of whole blood on filter paper compared to plasma in tubes was $\rho = 0.56$ ($P = 0.0001$) (Fig. 2). Detection of p24 Ag increased with the plasma HIV-1 RNA. The HD p24 Ag assay detected viral Ag in 37.5% (3 of 8) of the specimens with viral loads of <1,000 RNA copies/ml, 57.1% (4 of 7) of the specimens with viral loads between 1,000 and 10,000 copies/ml, 93.8% (15 of 16) of the specimens with viral loads between 10,000 and 100,000 copies/ml, and 100% (11 of 11) of the specimens with viral loads of >100,000 copies/ml.

Effect of storage temperature on the quantification of HD p24 Ag. A total of 36 whole-blood and 28 plasma specimens on filter paper were stored for 2 weeks at various temperatures to simulate specimen transport prior to freezing at −80°C. The HD p24 Ag concentration in these filter papers was determined within one assay, along with another aliquot of plasma from the same specimens stored in tubes at −80°C from the time the specimen was initially processed, <24 h from the blood draw, as the reference standard. As indicated in Fig. 3, storage of specimens for up to 2 weeks at 37°C, 4°C, or 0°C did not systematically alter the detection or quantification of p24 Ag compared to plasma specimen storage at −80°C. The linear regression analysis slopes were 0.99, 0.980, and 0.982 for specimens stored at 37°C, 24°C, and 0°C, respectively. The proportions of total variation explained (r^2values) were greater than 0.99 in all three regressions. Whole blood also performed favorably, with slopes estimates of 1.01, 1.00, and 1.01 and r^2

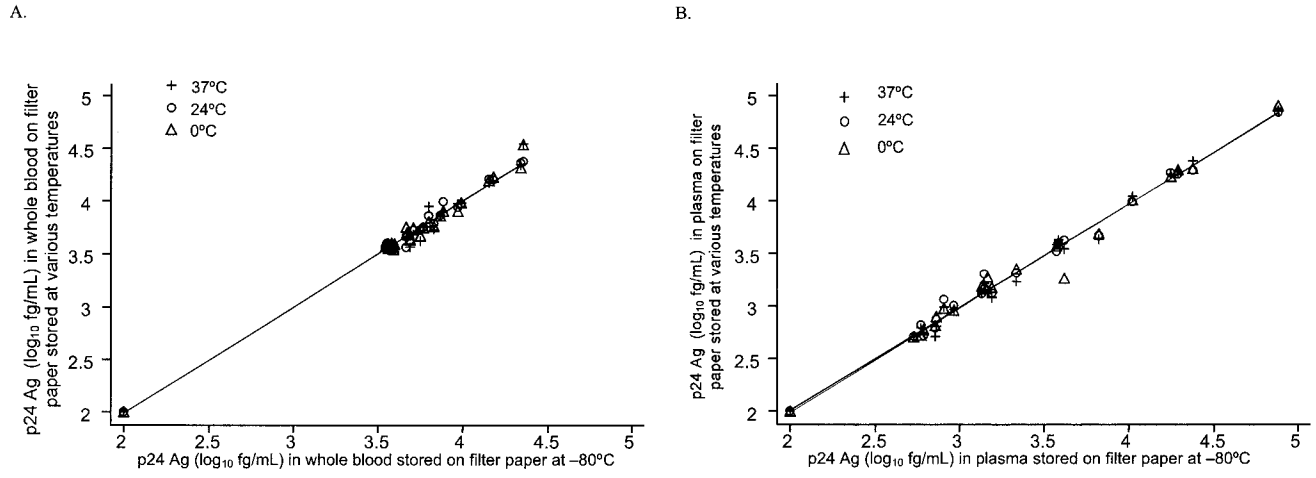

FIG. 3. Stability of p24 Ag in whole blood (A) and plasma (B) on filter paper at different temperatures for 2 weeks prior to freezing at −80°C is shown compared to an aliquot of plasma from the same specimen frozen in a polypropylene tube at −80°C within 6 h of collection. The samples stored at 37°C are represented by plus signs, the samples stored at 24°C by circles, and the samples stored at 0°C by triangles.

values of >0.99 for the linear regressions relating specimens stored on filter paper at 37°C, 24°C, and 0°C, respectively, to those stored at −80°C. In all six of the regression analyses, the 95% confidence intervals for the slope estimates were quite narrow (maximal width, ±0.037) and comfortably included the value 1.00, indicating no systematic effect of storage temperature on HD p24 Ag measurement.

DISCUSSION

In this study, the detection and quantification of HIV-1 in plasma were similar when samples were stored on filter paper and submitted to an HD p24 Ag assay and when they were stored frozen in polypropylene tubes and evaluated by real-time PCR of HIV-1 *gag* RNA. The quantification of plasma viral loads by the HD p24 Ag assay correlated closely to real-time PCR of HIV-1 RNA, except in specimens with low viral loads. Only 10 specimens with <1,000 RNA copies/ml were evaluated, which was too few to comprehensively compare these methods near the cutoff points of these assays. In contrast, virus was successfully quantified by the HD p24 Ag assay in 96% of the 36 specimens with a viral load of >1,000 RNA copies/ml, with a strong correlative values. Furthermore, detection and quantification of HD p24 Ag in plasma were very similar for samples stored on filter paper to those of samples stored in a polypropylene tube. These data suggest that the p24 Ag assay of plasma on filter paper could be useful for monitoring viral loads, especially when they are >1,000 RNA copies/ml, and that storage and transport on filter paper have little or no effect on HD p24 Ag detection and quantification.

The correlation of HD p24 Ag in whole blood stored on filter paper to the plasma HIV-1 RNA load was relatively weak, for reasons not fully elucidated in our study. The OD readings from whole blood were increased compared to the concordant plasma specimens. Opsonization of pathogens by complement and antibodies can lead to their binding to complement receptor 1 (CD35), also called the immune adherence receptor, on primate erythrocytes (16). Erythrocyte-associated HIV-1 RNA has been detected in persons with or without detectable virus in plasma, providing evidence that HIV-1 immune complexes bind to erythrocytes (13). Since the p24 Ag is largely bound in immune complexes (11), quantification of this Ag in whole-blood samples would be expected to detect a higher level of this viral protein compared to testing of plasma. However, the higher OD readings were also evident in the whole blood of non-HIV-1-infected laboratory workers (20 of 20 of whom tested in the low positive range for HD p24 Ag), suggesting that a component of whole blood increases the OD. One possibility is that hemoglobin, released from erythrocytes lysed at 100°C, may coat the bottom of the microtiter well and remain there even after several washes. The hemoglobin could increase the optical absorbance and the OD. Importantly, if the relatively high OD of whole blood was derived from a nonspecific component, this could lessen the quantitative correlation between HD p24 Ag and HIV-1 RNA, especially at low viral loads. Such a nonspecific effect could explain the flattening of the curve we observed with testing of HD p24 Ag in whole blood (Fig. 2). These results stand in contrast to studies showing comparable quantification of HIV-1 RNA in dried whole-blood and plasma spots (5, 18) and to plasma stored in tubes (18). Importantly, the false-positive reactions of whole blood on filter paper indicate that this method should not be used for early diagnosis of infants without further evaluation of negative specimens to determine the cutoff for a positive reaction, as others have done (19).

A significant correlation between HD p24 Ag and HIV-1 RNA has been reported (3, 19, 23, 28), primarily in studies evaluating HIV-1 subtype B. However, in several other studies the correlation was weak or absent (2, 5, 22). Bonard and colleagues showed a weak correlation ($r = 0.33$) for quantification except for HIV-1 RNA levels of >5 $\log_{10}$ copies/ml ($r = 0.62$; $P < 0.001$) in African adults infected primarily with HIV-1 CRF02_AG strains (2). Bürgisser and colleagues found a poor correlation ($r = 0.39$) in a study of mostly non-B subtypes (5), and plasma p24 Ag levels did not parallel HIV-1 RNA levels following structured treatment interruptions (22). However, Pascual and colleagues reported a correlation between plasma p24 Ag and HIV-1 RNA when assessing a panel of subtypes A to F and 18 specimens from Malawi (all subtype C) (21). Our study evaluated mostly subtype B variants. The paucity of data on non-B subtype virus suggests that further validation of the assay, especially with non-B subtypes, is warranted.

An important finding of our study was that whole blood and plasma on filter paper could be stored for 2 weeks at ambient temperatures without adversely affecting the measurement of p24 Ag. The conditions evaluated, 2 weeks at 37°C or 24°C, should encompass the room temperatures of most laboratories. Importantly, the use of desiccant and moisture-tight bags is advisable in environments with high humidity to minimize the risk of fungal growth on the blood spots.

The procedures used to perform the HD p24 Ag assay are relatively simple and do not require costly equipment. Also, the assay is also generally less expensive than commercial HIV-1 RNA assays. The cost of the HD p24 Ag assay has been estimated to amount to 5 to 20% of a HIV-1 RNA assay (21, 23, 29).

The reliable preservation and transport of plasma p24 Ag on filter paper have far-reaching epidemiological and public health implications. The close correlation we observed between HD p24 Ag in plasma stored on filter paper and HIV-1 RNA levels in plasma stored in tubes and the stability of HD p24 Ag over a range of environmental temperatures suggest that the assay could provide a convenient option for viral load monitoring in regions where transport of specimens to laboratories for testing is problematic. Testing of whole blood on filter paper would provide even greater convenience; however, in our evaluation, quantification of HD p24 Ag in whole blood was inferior to testing of plasma because a component of whole blood appeared to nonspecifically increase the OD readout of the assay. In conclusion, the attributes of filter paper for specimen transport and laboratory safety, combined with the close correlation of HD p24 Ag with plasma viral RNA and the stability of p24 Ag over a large range of temperatures, provides impetus for continued refinement of the assay as a practical and economical tool for monitoring viral loads in HIV-1-infected patients.

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