Measurement of HIV-1 Viral Burden Utilizing Dried Spots of Whole Blood, Plasma and Mother’s Milk Collected on Filter Paper

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ABSTRACT

We studied the use of dried spots of bodily fluids (plasma, whole blood and mother’s milk) on filter paper as a way of sample collection and storage for HIV-1 viral load testing under stringent field conditions. Plasma placed directly in lysis buffer, customarily used for viral load assays, was used as the comparison in all our experiments. Utilizing reconstruction experiments we demonstrate no statistical differences in determining viral loads in plasma and mother’s milk spotted on filter paper or placed directly in lysis buffer. We identify that the addition of whole blood directly in lysis buffer was unreliable and could not be considered a feasible option. On the contrary, viral load measurements in whole blood spotted onto filter paper correlated with both plasma viral load values on filter spots or in lysis buffer (Pearson correlation coefficient 0.7706 and 0.8155 respectively). In conclusion, dried spots from plasma, whole blood or mother’s milk provide a feasible way for collection, storage and shipment of samples for subsequent viral load measurement and monitoring. Virus material spotted and dried on filter paper is an inexpensive good alternative way of collecting patient material to monitor HIV-1 viral load. Measuring HIV-1 burden from whole blood dried on filter paper provides a suitable alternative for low technology settings with limited access to refrigeration as can be identified in Sub-Saharan Africa.
INTRODUCTION

In recent years there has been an increased access to antiretroviral therapy for HIV-infected patients in resource limited settings (7), which has led to a growing need to have monitoring systems in place that are capable of assessing the effects of these treatments. Monitoring of therapy in the context of many developing nations poses unique challenges. Over ninety percent of new HIV infections occur in developing countries where there is limited access to equipment for processing of specimens and often also inadequate storage facilities for preserving sample integrity until testing. In order to overcome the logistical obstacles, innovative approaches to simplify methods of sample collection from far-to-reach areas have been applied with good results. Spotting and drying of blood samples on filter paper for clinical testing has proven to be a highly effective method for sample collection and storage (5,11). Various types of paper were used when screening for various metabolic disorders in neonates (4,38). Latterly on it has been applied more widely to the diagnosis of other infectious disease as well, since this approach is feasible for collection of large numbers of field specimens (19,22,26,31,36,37).

To provide alternative ways for access to viral load measurement, we developed and evaluated the use of filter papers as a collection and storage medium for HIV-1 infected blood, plasma and breast milk samples. The analyses were performed in combination with the Primagen Retina™ Rainbow assay for viral load measurements that is able to detect all known subtypes and circulating recombinant forms (CRFs) of HIV-1. Dried plasma spot technology is the least expensive way of shipping samples spotted on the filter paper and dried. Once samples are dried on filter paper they stay stable for HIV-1 RNA detections for long periods of time (9,11), whereas the virus that is spotted is no longer infectious (website

In addition to dried plasma and mothers-milk spots we also tested dried whole blood spots as this would be the utmost simple formulation for sample collection. In addition, we compared the Rainbow assay (13) to the Roche Amplicor HIV-1 Monitor assay v1.5, the most widely used technology in the field.
MATERIALS AND METHODS

**Reconstruction experiments for viral load measurements:** We evaluated filter paper as a carrier and storage medium for HIV nucleic acids in reconstructed experimental conditions where virus was spiked into a body fluid. The human plasma used was collected as a byproduct of the lymphocyte purification protocol and human milk, surplus, donated by a breast-feeding mother.

We used a panel of virus isolates, comprising all subtypes and the circulating recombinant forms one and two (CRF 01/ CRF 02). These isolates were quantified using several methods including electron microscopy (17). Each virus was diluted in the body fluid in use and at the desired concentrations spanning from one hundred to one million virus particles per ml. Subsequently 200 µl of the fluid spiked with the virus was dissolved in lysis buffer (L6) as required for the RNA isolation by the Boom method (8) or laid on Schleicher and Schuell 903 filter paper (Keene, NH, USA) in four spots of 50 µl each. All samples spotted on paper were air dried for 30 minutes and then stored for 24 hours to several weeks, sealed in a bag with desiccant, at room temperature.

**Subjects and samples:** The validation of the method for using air-dried filter paper in clinical samples was performed in two sets of blood-derived material collected from HIV-1 infected individuals.

A set of 103 plasma samples were collected, between January 2000 and December 2002 from 103 HIV-1 infected patients visiting the outpatient clinic of the University Medical Center of Utrecht University in the Netherlands. The samples were analyzed immediately upon arrival for HIV-1 viral RNA levels using the Amplicor v1.5 assay, whereas the remainder of the sample was stored at –80 °C until further use. These samples encompassed a range of viral
loads from >6 log_{10} copies RNA per ml to below detection level of the Amplicor assay, which means <50 copies RNA per ml. From the 103 plasma samples, 19 were proven to be of a non-B subtype.

A set of 35 samples collected in Ethiopia in 2003 were from HIV-1 seropositive individuals enrolled in the cohort of factory workers. The Ethio-Netherlands AIDS Research Project (ENARP) cohort was intended to study the natural history of HIV-1 infection in Ethiopia and has been described elsewhere (34,35). Blood was collected using EDTA anti-coagulant as required for the routine viral load determination using the NucliSens HIV-1 QT assay (bioMérieux, Boxtel, The Netherlands). Blood samples from 3 healthy sero-negative cohort participants were included as controls. For the purpose of the study each of these samples was prepared in four different ways, each compared in our analysis. Whole-blood or plasma (200 µl) was aliquoted in lysis buffer and stored immediately at −80 °C until processing. Additionally, a same volume-amount of whole-blood and plasma were spotted on filter paper in 4 spots of 50 µl each.

**Viral load determination:** The viral RNA from all samples was assayed by the Primagene Retina Rainbow assay, performed according to instructions of the manufacturer. In short, 5 µl of purified nucleic acid from a total elution volume of 50 µl was assayed by mixing it with the amplification reagents. After addition of the enzymes, the samples were placed for 90 minutes in a fluorimeter with a thermostat. After the run, the results were analyzed and reported as the number of copies per ml, or “<500 copies per ml” if the sample was positive but not quantifiable, or “not detectable” if no signal was detected.
The method for isolating virus RNA from HIV-1 containing fluids that were dissolved in lysis buffer was previously described (8). For the filter paper spots there was an additional elution process after punching the plasma spots of the paper. All 4 spots were incubated for at least 2 hr or over night at room temperature in 3 ml lysis buffer following removal of the papers from the lysis buffer and further processing according to standard protocols for extraction of nucleic acids using a silica-based isolation method by Boom and colleagues (8). The purified RNA was dissolved in a total elution volume of 50 µl.

**Statistical Analysis:** Statistical analyses were performed using GraphPad Prism version 4 (GraphPad Software Inc., San Diego, California, USA) and SPSS for Windows v11.5 (SPSS Inc., Chicago, Il.). Correlations were substantiated with the Pearson statistical test and the group values were compared with the Wilcoxon Matched-Pairs Signed-Ranks Test unless otherwise stated. Significance level was set at p<0.05 in all cases. For purposes of analysis, samples which were negative or positive but below the limit of detection of the assay were reported at the cut-off value (2.7 log_{10}/ml).
RESULTS

Reconstruction experiments: By adding cultured virus particles to uninfected human plasma or mother’s milk, we reconstructed samples with a known RNA-copy content (17) prior to quantification by the Rainbow assay. We used viral isolates that had previously been quantified by electron microscopy to reconstruct samples with virus concentrations spanning a range of 2-6 log₁₀. The virus-spiked samples were subsequently processed, in two different ways, either directly dissolved in lysis buffer or laid as spots on blotting paper. Fig. 1 shows the correlation of the viral RNA measurements generated by the two formulations (virus in plasma or in milk subsequently dissolved in lysis buffer or spot dried on paper). Regardless of whether human plasma or mother’s milk was used as carrier prior to the RNA isolation there was a high correlation between the two formulations: the Pearson correlation coefficients were 0.91 for the plasma-set of directly in lysis buffer or via dried spot and 0.93 for the mothers milk-set (p <0.001 for both). A good linear correlation was found between the values we generated with the Rainbow-Retina assay and the theoretical values according to the Electron microscopy quantification (Pearson correlation coefficient = 0.82, p<0.001, data not shown). Furthermore, when we compared the results from the paper blot formulation between plasma and milk (Pearson correlation coefficient =0.99, p = 0.001) with a good linearity across the 5-log range (r² = 0.98) (data not shown).

These results provide evidence that isolation and quantification of viral RNA from samples collected on blotting paper would be a viable alternative to the traditional freezing for transportation of clinical samples. This is an important incentive for limited-resources settings, especially under the consideration that monitoring will be a key factor for the curtailing of the HIV-1 pandemic.
Evaluation of the viral RNA measurements with subtype B viruses: 84 patient samples from the Netherlands, all infected with a subtype B virus, were selected for the comparison of the two formulations, plasma in lysis buffer or plasma on paper. These samples had been previously analysed with the Amplicor v1.5 assay and the viral load values were spanning from undetectable to \(10^{6.5}\). The RNA copy-number values generated by the Retina Rainbow assay for both the plasma in lysis buffer and plasma on paper were compared (Fig. 2A). The correlation was high with a Pearson correlation coefficient of 0.96 (p<0.001).

Since the viral load values measured by the Roche Amplicor HIV RNA v1.5 assay were available to us, we compared them with the results we generated with the Retina Rainbow assay (Fig. 2B). The Amplicor assay has a sensitivity of approximately 50 copies per ml and the Retina Rainbow assay in its standard format has a sensitivity of 500 copies per ml. This indicates that samples between 50 and 500 copies per ml are unlikely to be detected with the Retina Rainbow assay. The samples that were part of the comparison studies were selected based on the Amplicor results. By using this method, there was a one-sided bias in the lower range towards the favor of the Amplicor assay since all samples were positive with the Amplicor assay and likely negative with the Rainbow assay.

Evaluation of the viral RNA measurements with non subtype B viruses: Since the vast majority of HIV-1 isolates world–wide encompasses subtypes other-than-B with subtype C being the most prevalent, we wanted to verify that non-B HIV-1 isolates could be detected as equally as subtype B plasma isolates spotted on paper. For this purpose we analyzed a set of 19 plasma samples from the Netherlands collected from individuals infected with non B HIV-1 viruses (CRF01 AE, CRF AG, D, and C including one CRF06 cpx and one K) together with a
set of 19 plasma samples collected from HIV-1 subtype C infected individuals in Ethiopia. The subtype C isolates were detected with a similar correlation as the subtype B isolates (Fig. 3).

These samples were analyzed by the Rainbow assay dissolved directly in lysis buffer or laid as four 50 µl spots on blotting paper. The correlation between the two formulations was similar as for the subtype B isolates, with a Pearson correlation coefficient of 0.9164 and a linearity $r^2$ value of 0.8398.

**Viral load measurements on whole blood dried spots:** The purpose of this study was to show there are simpler ways of low cost for monitoring HIV-1 viral load measurements. We therefore wanted to study whether whole blood collected from a finger-prick for example would be feasible for viral load measurements since it is less expensive and laborious than separating plasma from whole blood specimens. When viral RNA was isolated from whole blood that was dissolved directly in lysis buffer the viral load values generated by the Rainbow assay were not reproducible and correlated poorly with values obtained from the plasma in lysis buffer from the same donor. Also, the whole blood values were systematically lower than the plasma samples (data not shown). This indicates that there are factors in whole blood that inhibit efficient isolation and/or amplification of the viral genetic material. Collecting whole blood in lysis buffer therefore would not be a valid method for sample collection for subsequent viral load measurement. Nevertheless, we found that measurements from whole blood after it had been spotted and dried on filter paper correlated well with both the plasma in lysis buffer or plasma spotted on paper (Fig. 4A). The Pearson correlation coefficient is 0.7706 and 0.8155 respectively. Despite the fact we only analyzed a set of 17 samples we found no significant difference when compared to the values generated from plasma directly in lysis buffer.
buffer (Fig. 4B). The values generated from the whole blood spots tended to be slightly lower than the plasma samples but we did not take into account the relative volume differences due to the high red blood cell content of whole blood, which adjustments could be made for.
DISCUSSION

The increased access to life saving antiretroviral drugs has raised hope for many AIDS patients in resource limited countries but also raises challenges for the appropriate monitoring. The determination of viral endpoints remains the gold standard by which current clinical trials assess HIV treatment efficacy (21,28). Besides, viral load measurements have an established value in predicting the clinical progression to disease (14,24,25,33), in monitoring response to antiretroviral therapy (20,33) as well as in assessing the risk of vertical transmission to newborns by HIV sero-positive mothers (16,27). However the prohibitive costs of carrying out viral RNA measurements for many areas in the developing world is limiting, and has mandated the search for suitable alternatives validated against this standard (18,23). The costs associated with carrying out HIV viral load testing in developing nations can be partially decreased by the development of suitable technologies allowing for simpler methods of sample collection and preservation. Samples collected in this way may be subsequently transported to centralized testing facilities without risk of specimen deterioration and without the need for expensive shipping facilities including dry-ice shipping.

Detection of HIV-1 DNA (11,30) or RNA (3,10,36) using dried blood specimens collected on filter paper has previously been reported. Apart from its use for viral load testing the DBS/DPS technique of sample collection has been utilized for other purposes including as a means for conducting studies on perinatal HIV transmission due to the small volumes required (6,16). In addition molecular epidemiology studies for genotyping of HIV-1 have also exploited the technique (12,32).

Recently the dried blood spot (DBS) technology was extended further to provide a simple method for CD4+ cell enumeration feasible to undertake in the field (29). Using a
combined viral load and CD4 measurement on one sample of dried whole blood, such as from a heel or finger prick, would be optimal for monitoring patients on antiretroviral programs in resource-poor settings like Ethiopia. The current investigation suggests that whole blood on filter spot could be a way of monitoring high viral loads or dramatic therapy failures. Plasma spotted onto filter paper provided a better estimation of HIV-1 viral load in a patient compared to plasma in lysis buffer as a reference. Logistically this presents an added step of requiring blood collection equipment for separation of plasma from whole blood, which may not always be readily available in remote areas. If the feasibility of using dried whole blood spots for CD4 cell enumeration is demonstrated to be satisfactorily in Ethiopia, whole blood spotting onto filter paper immediately after collection, could be followed by spotting of plasma for the viral load testing aspect.

Previously it was shown that the porphyrin moiety of heme from contaminating erythrocytes inhibits nucleic acid amplification by PCR (1) but the blotting of the blood on the paper could lead to irreversible absorption of inhibiting factors. In our study, while whole blood directly in lysis buffer remains unreliable, when spotted on paper a substantial amount of inhibiting factors seemed to be retained on the filter paper during the process of RNA extraction providing a good readout.

The dried blood and plasma spots used in this study were maintained at room temperature (22-24 °C) at the site of sample processing with no perceptible loss of viral RNA compared to the standard approach of sample collection, processing of plasma, and storing at –80°C in lysis buffer until analysis. The data were in agreement with findings by other investigators who report remarkable stability of RNA under extreme climate or prolonged storage conditions (9,15). We found that the correlation was highest with the plasma
spot/plasma in lysis buffer comparison, consistent also with a high correlation reported by other investigators (10).

We used the LTR-based Retina Rainbow viral load assay for our study since this assay was previously shown to consistently detect all subtypes of HIV-1, even those that were under-detected using other commercially available tests (13). In addition it has a format adapted for use with either dried blood or dried plasma specimens. Parallel testing of plasma samples in our study using the more established Amplicor assay together with the Rainbow test, showed that the results of the two assays correlated well. In addition the comparison of the Retina Rainbow assay with the NucliSens assay was similarly good (data not shown) with both been reported to detect efficiently HIV-1 subtype C (2,13), the dominant subtype both world-wide and in our Ethiopian cohort.

Providing an easy way of collecting, storing and shipping of samples can aide in the monitoring of circulating strains of virus in different parts of the globe, usefully contributing to the monitoring of vaccine efforts and therapy distribution to curtail the pandemic. From the present study it can be concluded that this technology may be used meaningfully in resource-limited settings for collection of specimens from populations not readily served by laboratories. It is feasible to carry out under field conditions without access to refrigeration or electrical power supply, although the results presented here suggest that HIV viral load testing from samples of whole blood as dried spots in combination with the Rainbow assay requires somewhat further optimization. DBS/DPS technology offers the advantages of a stable environment for the analysis, as well as the ease of sample collection and shipment with minimal biohazard risks thereby providing a highly suitable and affordable alternative to common practice and could be exploited for multiple purposes.
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LEGENDS TO THE FIGURES

**Figure 1:** Correlation between filter spot and fluid placed in lysis buffer. $\log_{10}$ measurement of the HIV-1 RNA content in plasma or milk as assessed by the LTR-based NASBA (Retina-rainbow) assay. The virus-containing fluid was dissolved directly in lysis buffer or was initially spotted on filter paper (four spots of 50 $\mu$l each) prior to RNA isolation and quantification. The values from the spotted samples are plotted against the values obtained from the corresponding sample dissolved directly in lysis buffer. The linear correlation between the two formulations together with the 95% confidence interval is shown. The broken line with the open circles is human plasma containing virus from a panel of cultured isolates, encompassing subtypes A, B and CRF01. The solid line with the closed circles is human milk containing virus from the same panel of cultured isolates.

**Figure 2:** HIV-1 Subtype B infected patient sera. **A:** assessed by the Retina-rainbow (LTR-based NASBA) assay. 200 ml of the sera were dissolved in lysis buffer or dried as spots (four times 50 $\mu$l) on filter paper prior to RNA isolation and quantification. The $\log_{10}$ RNA level values from the spotted sample were plotted against the corresponding value from the sample dissolved in lysis buffer. The solid lines express the linear correlation between the two formulations and the dotted lines the 95% confidence interval. **B:** $\log_{10}$ RNA level values in this panel of patient samples as measured by the Retina-rainbow assay (X axis) plotted against the $\log_{10}$ RNA values as determined by the Amplicor v1.5 assay.

**Figure 3:** HIV-1 non B subtype infected-sera assessed by the Retina-rainbow assay as described in figure 2A The bold symbols are representing 19 subjects sampled in the
Netherlands infected with HIV-1 A, C, D, K, CRF01, CRF02 and CRF06. The open symbols are representing 19 subjects sampled in Ethiopia infected with HIV-1 C.

**Figure 4:** Assessment of whole blood collected as dried spots on filter paper for the monitoring of HIV-1 viral burden. In panel A the $\log_{10}$ RNA values derived from 200 µl blood spotted on filter paper were plotted against the $\log_{10}$ RNA values derived from 200 µl plasma dissolved in lysis buffer (solid line/closed circles) or against the $\log_{10}$ RNA values derived from 200 µl plasma spotted on filter paper (broken line/open circles). The panel B is a pair-wise comparison of the $\log_{10}$ RNA values from whole blood spotted on paper to the $\log_{10}$ RNA values from plasma dissolved in lysis buffer that is the standard used formulation.