Quantification of HIV-1 p24 by a highly improved ELISA: An alternative to HIV-1 RNA based treatment monitoring in patients from Abidjan, Côte d’Ivoire

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Abstract

Background: Quantification of HIV-1 RNA remains difficult to implement in Africa. Simple and inexpensive tests for antiretroviral treatment (ART) monitoring are needed.

Objective: To evaluate an HIV-1 p24 ELISA, which combines efficient virus disruption, heat-denaturation and signal amplification, in a West African setting.

Study design: Eighty-six HIV-1 infected patients from Abidjan, Côte d’Ivoire, were tested for p24, HIV-1 RNA, and CD4+ count at baseline, and twice within 8 months after ART initiation.

Results: All patients responded to ART with a minimal HIV-1 RNA drop of 0.5 log_{10} at first follow-up. Forty-one (47.7%) then rebounded >0.5 log_{10} or persisted above 1000 copies/mL by week 24. The predicted baseline concentration of p24 corresponding to 100,000 copies/mL of HIV-1 RNA, above which ART is recommended, was 4546 fg/mL (95% confidence interval 3148–6566). A prediction model of virologic failure, occurring after an initial response to ART, correctly classified 84% of patients using baseline p24, p24 change on therapy, and achievement of undetectable p24 as explanatory variables. The model and further bootstrap evaluation suggested a good ability to discriminate between sustained or failing virologic response to ART.

Conclusion: HIV-1 p24 and RNA based-ART monitoring in a low-resource country dominated by HIV-1 CRF02 AG appeared comparable.

**Abbreviations:** ART, antiretroviral therapy; CRF, circulating recombinant form; DAI, drug access initiative; ELISA, enzyme linked immunosorbent assay; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; OD, optical density; RETRO-CI, retrovirus Côte d’Ivoire; RNA, ribonucleic acid; SNCR, Swiss National Center for Retroviruses

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1. Introduction

In industrialized countries, use of effective antiretroviral therapy (ART) has significantly reduced morbidity and mortality among HIV-infected persons. However, partly due to high costs and complexity associated with laboratory monitoring of patients, very few patients have benefited from ART in developing countries, particularly in Africa. The major laboratory parameters involved in monitoring patients on ART include CD4+ cell counts and plasma viral load. Currently commercial assays for quantification of the viral load...
are expensive and require highly trained staff and sophisticated equipment. Therefore, there is need for less expensive, simple and accurate techniques for monitoring patients on ART in resource-poor settings. HIV-1 p24 antigen, which can be quantified by ELISA, is a potential alternative to RNA-based viral load tests (Schupbach, 2003a). During the past decade, both elimination of antibody interference by heat-denaturation and signal amplification have greatly improved p24 detection (Lymuya et al., 1996; Schupbach and Boni, 1993). Studies in developed countries have demonstrated the high sensitivity of the test (Steindl et al., 1998; Sutthent et al., 2003), established its predictive value for disease progression in early and late disease (Ledergerber et al., 2000; Sterling et al., 2002), and demonstrated its potential for antiretroviral treatment monitoring in both adult and pediatric HIV-1 infection (Ribas et al., 2003; Nadal et al., 1999; Steindl et al., 1998). The p24 antigen may still be measurable in patients with undetectable RNA (Schupbach 2005; Schupbach et al., 2003b). These studies provide evidence that the concentration of the p24 antigen, which is representative of other viral proteins, is associated with short-term and long-term disease outcome (Ledergerber et al., 2000; Sterling et al., 2002).

Few studies have been published in Africa on the performance of this assay. Differences in antibody levels in HIV-infected Africans (Brown et al., 1995) due to endemic disease burden, and the occurrence of different HIV-1 subtypes (Janssens et al., 1997; Carr et al., 1999; Peeters et al., 2003), may influence the performance of the p24 antigen test in Africa compared with regions of the world where HIV-1 subtype B are predominant.

In this study, using a modified p24 antigen test and HIV-1 RNA as gold standard, we estimated the correlation between the two tests, the level of p24 corresponding to a viral load of 100,000 copies/mL (the level at which initiation of therapy is currently recommended if CD4+ cell counts are >350 cells/µL), the correlation between p24 change and HIV-1 RNA viral load change on therapy, and we developed and validated a prediction model of virologic failure for patients receiving ART.

2. Materials and methods

2.1. Study population

In collaboration with UNAIDS, the Ministry of Health of Côte d’Ivoire started a pilot Drug Access Initiative (DAI) in 1998 aimed at providing ART to HIV-infected patients in the country (Djomand et al., 2003). We selected all HIV-1 infected patients participating in the DAI for whom data were available on RNA viral load at baseline and at two follow-up visits within 8 months after initiation of ART treatment, and who had a favorable virologic response to therapy observed at their first follow-up visit after initiation of therapy, defined by a minimum 0.5 log10 decline in HIV-1 RNA from baseline.

2.2. Laboratory testing

Whole blood was drawn from participants into EDTA tubes (Becton Dickinson, San Jose, CA, USA). Plasma was separated from cells within 4 h by centrifugation at 200 × g, and then aliquoted and stored at −70 °C. HIV-1 antibody status was determined using an enzyme-linked immunosorbent assay (ELISA)-based parallel testing algorithm combining three ELISA assays and Western blot (Nkengasong et al., 1999a). HIV-1 RNA plasma viral load was quantified by the Amplicor HIV-1 Monitor assay, version 1.5 (Roche Diagnostics Systems, Branchburg, NJ). The limit of detection of the assay was 200 copies/mL. This assay has been shown to efficiently quantify CRF02-AG viruses that predominate in the Côte d’Ivoire epidemic (Nkengasong et al., 1998, 1999b).

CD4+ and CD8+ counts were determined by three-color flow cytometry using FACScan (Becton Dickinson, San Jose, CA, USA). The TriTEST kit and MultiSET software (Becton Dickinson) were used for labeling and analysis.

2.3. P24 antigen test

The p24 assay used an efficient virus lysis buffer to improve detection of virion-associated p24 and possibly other forms of aggregated p24 (Schupbach et al., 2003b). Briefly, 100 µL of plasma were mixed into 50 µL of a solution containing 30 mM Tris/HCl pH 7.2, 450 mM NaCl, 1.5% Triton X-100, 1.5% deoxycholic acid (sodium salt), 0.3% sodium dodecylsulfate, and 10 mM EDTA and left for 10 min at room temperature in a 1.5 mL Eppendorf tube. After further dilution with 450 µL of the antigen test kit’s pre-diluted dissociation solution (0.5% Triton X-100 in PBS), the mixture was boiled for 5 min on a Techne (Cambridge, UK) dry heat block pre-heated to 100 °C.

Duplicate 250 µL aliquots of each specimen were tested using the HIV-1 Core Profile ELISA in combination with the ELAST ELISA Amplification System (Perkin-Elmer Life Science Products, Geneva, Switzerland). The reaction product was quantified on a Dynex MRX TCII Microplate Reader (Microtech Produkte, Embrach, Switzerland) using a combination of kinetic and endpoint reading. Kinetic reading, performed immediately after addition of the ortho-phenylene diamine (OPD) substrate from the HIV-1 Core Profile ELISA Amplification System. The bivariate plot function of the StatView® Version 5.0 program for Macintosh (SAS Institute, Cary, NC) calculated a log–log power fit regression curve with tails of log10(concentration) = A + B log10(OD) + C [log10(OD)]2 + D [log10(OD)]3. The concentration of p24 antigen in a sample was calculated with this equation by inserting the
sample’s OD (OD/time for kinetic readings) value and the constants A–D, established by the standard regression curve. At levels less than 20 pg/mL, the concentration was based on endpoint reading. At levels of ≥20 pg/mL the concentration was based on the kinetic reading if this yielded a higher concentration than endpoint reading. To guarantee the reproducibility of the results generated at SNCR and RETRO-CI, the first 46 plasma specimens were tested in both laboratories. No significant differences were observed. Based on the satisfactory reproducibility, further p24 antigen testing has been performed at RETRO-CI.

2.4. HIV-1 subtype identification

HIV-1 subtype was identified by amplifying cDNA comprising the C2–V5 region of gp120 env reverse transcribed from virus contained in plasma and sequencing the first approximately 330 bases corresponding to C2/V3/C3, as described previously (Bürgisser et al., 2000).

2.5. Data analysis

For variance stabilization and normalization of error terms in regression models, both HIV-1 RNA and HIV-1 p24 antigen were log10-transformed prior to analyses. Values below the limit of detection were fixed at 200 copies/mL for HIV-1 RNA and at 100 pg/mL for p24 antigen. Using data at baseline, prior to initiation of antiretroviral therapy, the degree of linear relationship between p24, HIV-1 RNA, and CD4+ cell count was estimated using Pearson’s correlation coefficient. Maximum likelihood linear regression models, both HIV-1 RNA and HIV-1 p24 antigen were log10-transformed prior to analyses. Values below the limit of detection were fixed at 200 copies/mL for HIV-1 RNA and at 100 pg/mL for p24 antigen.

To determine whether p24 antigen and CD4+ cell count measurements could be utilized for monitoring patients receiving therapy, we developed and evaluated a prediction model for therapy failure using follow-up viral load measurements, defined as >0.5 log10 increase or persistence above 1000 copies/mL after 24 weeks of therapy. Viral load failure was modeled using logistic regression (Hosmer and Lemeshow, 1982) with potential explanatory variables including p24 antigen change, baseline p24 antigen, follow-up p24 antigen below detectable limit, CD4+ cell count percent change from baseline, CD4+ cell count increase >25 cells/μL from baseline, baseline CD4+ cell counts, recent CD4+ cell count below 50 cells/μL during therapy, age, and sex.

Model performance was assessed using 1000 bootstrap samples from the study population (Efron and Tibshirani, 1986). To assess prediction accuracy of our model, we constructed and estimated the area under a receiver operating characteristic (ROC) curve by comparing patients’ known outcomes with the predicted outcome using varying probability scores, ranging from zero to one (Hanley and McNeil, 1982). In addition, the Kappa statistic was calculated to measure agreement in classification of therapy failure based on observed and predicted values (Fleiss, 1981). The median, 2.5th, and 97.5th percentiles of the area under the ROC curve, prediction model sensitivity and specificity, and the Kappa coefficient were computed from 1000 bootstrap replicates. The Hosmer–Lemeshow goodness-of-fit test was used to evaluate model fit (Hosmer and Lemeshow, 1982).

3. Results

We monitored the effect of ART during a median 133 days (range: 61–239 days) in 86 patients from Abidjan, using HIV-1 RNA, p24 antigen and CD4+ cell counts as markers for their success or failure. The median age of the patients (48 males and 38 females) was 36 years (range 2–63). Median concentrations of HIV-1 RNA, p24 and CD4+ cells count at baseline and follow-up are shown in Table 1.

Thirty-eight (44%) of the 86 patients started a triple combination of antiretroviral drugs consisting of two nucleoside reverse transcriptase inhibitors (NRTI) selected among zidovudine, lamivudine, stavudine, zalcitabine or didanosine plus either a protease inhibitor (nelfinavir, indinavir, saquinavir) or the non-nucleoside reverse transcriptase inhibitor (NNRTI) nevirapine or efavirenz. Forty-eight patients (56%) initiated dual combination therapy consisting of two NRTIs.

3.1. Relationships between p24, HIV-1 RNA, and CD4+ cell count

Of the 86 baseline specimens that were tested, 83 (97%) were detected by the p24 antigen test. Sequencing of the C2–V2 region of HIV-1 RNA amplified from plasma of the three patient with undetectable p24 identified viruses of HIV-1 subtype A in two patients and of subtype D in the third. Scatterplots of baseline measurements of log10-transformed viral load, log10-transformed p24 antigen, and CD4+ cell counts are depicted in Fig. 1. These measurements were performed on specimens collected prior to initiation of HIV

<table>
<thead>
<tr>
<th>Parameter: median (min, max)</th>
<th>Start therapy “baseline”</th>
<th>1st follow-up visit</th>
<th>2nd follow-up visit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days therapy</td>
<td>0</td>
<td>35 (15.92)</td>
<td>133 (61, 239)</td>
</tr>
<tr>
<td>HIV-1 RNA (log10 copies/mL)</td>
<td>5.5 (3.0, 7.3)</td>
<td>2.9 (2.3, 6.2)</td>
<td>2.9 (2.3, 7.2)</td>
</tr>
<tr>
<td>p24 Antigen (log10/g/mL)</td>
<td>3.9 (2.0, 5.6)</td>
<td>2.4 (2.0, 4.9)</td>
<td>2.5 (2.0, 4.5)</td>
</tr>
<tr>
<td>CD4+ T-lymphocytes (cells/μL)</td>
<td>115 (4, 684)</td>
<td>217 (8, 960)</td>
<td>241 (9, 1251)</td>
</tr>
</tbody>
</table>
antiretroviral treatment. The Pearson correlation between p24 and HIV-1 RNA was 0.49, \( p < 0.001 \) (Fig. 1). From the regression model, the expected value for p24 antigen (untransformed) when the viral load is 100,000 copies/mL was 4546 fg/mL (95% confidence interval, 3148–6566). CD4 counts at baseline were inversely correlated with p24 concentration \( (r = -0.25; \ p = 0.02) \) and showed a trend for correlation with HIV-1 RNA \( (r = -0.20; \ p = 0.07) \).

Scatterplots of changes in concentration of viral load, CD4+ cell count, and p24 antigen during the first and second follow-up visit are shown in Fig. 2. These data include both patients with a continuously effective antiretroviral therapy and patients experiencing a viral rebound under ongoing treatment. The Pearson correlation between change in log_{10}-transformed p24 antigen and change in log_{10}-transformed viral load was 0.79, \( p < 0.001 \). The predicted p24 antigen increase when viral load increased 0.5 log_{10} was 0.17 log_{10}; 95% CI = 0.08, 0.27. CD4 change between the first and second follow-up visit correlated inversely with changes in p24 \( (r = -0.29; \ p = 0.006) \) and HIV-1 RNA \( (r = -0.21; \ p = 0.05) \).
Table 2
Binary and continuous prognostic factors for failure to antiretroviral therapy

<table>
<thead>
<tr>
<th>Prognostic factor: binary</th>
<th># Virologic failures (%)</th>
<th>Adjusted odds ratio (95% CL)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &lt; 25</td>
<td>7 (53.9)</td>
<td>1.8 (0.3, 13.1)</td>
<td>0.56</td>
</tr>
<tr>
<td>Age &gt; 25</td>
<td>34 (46.6)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>18 (47.4)</td>
<td>1.2 (0.3, 4.8)</td>
<td>0.80</td>
</tr>
<tr>
<td>Male</td>
<td>23 (47.9)</td>
<td>Referent</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Undetectable p24 on therapy</th>
<th>Virologic failures (%)</th>
<th>Adjusted odds ratio (95% CL)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>12 (37.5)</td>
<td>0.2 (0.1, 0.8)</td>
<td>0.02</td>
</tr>
<tr>
<td>No</td>
<td>29 (53.7)</td>
<td>Referent</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Prognostic factor: continuous</th>
<th>Median (min, max)</th>
<th>Adjusted odds ratio (95% CI)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change p24 Antigen on therapy</td>
<td>–</td>
<td>27.6 (5.7, 133.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Failing</td>
<td>0.3 (−1.2, 2.4)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sustained</td>
<td>0.0 (−1.7, 0.6)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Baseline p24 antigen (log 10)</td>
<td>–</td>
<td>5.5 (1.8, 16.1)</td>
<td>0.003</td>
</tr>
<tr>
<td>Failing</td>
<td>4.0 (2.0, 5.6)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sustained</td>
<td>3.8 (2.0, 4.8)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>% Change from baseline CD4</td>
<td>–</td>
<td>1.0 (1.0, 1.0)</td>
<td>0.22</td>
</tr>
<tr>
<td>Failing</td>
<td>86.7 (−23.8, 4106)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sustained</td>
<td>57.6 (−59.7, 2039)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

3.2. Prediction of treatment failure

Increases in log10-transformed p24 antigen while receiving therapy, higher baseline levels of p24 antigen at therapy initiation, and not achieving undetectable p24 antigenemia in response to therapy were significantly associated with treatment failure (Table 2). There were 41 patients (47.7%) with >0.5 log10 increase or sustained viral load above 1000 copies/mL following a minimal initial 0.5 log10 decline in viral load in response to therapy. A prediction model based on these covariates correctly classified 84% of patients. Upon further evaluation using bootstrap replicates, the area under the ROC curve was 90.7, 95% CI 82.2–96.7 (Fig. 3), suggesting a good ability to discriminate between sustained and failing virologic response to treatment. Furthermore, the Kappa statistic for agreement between observed and predicted therapy failure, based on a predicted value >0.5 was 0.71, 95% CI 0.51–0.86.

Prediction models for treatment failure based only on CD4 performed poorly.

4. Discussion

A good test to monitor virologic response to antiretroviral treatment in HIV-infected patients should (i) optimally detect and quantify all viruses present in the patient population, (ii) correlate significantly with the CD4+ cell count, and (iii) correlate with clinical progression to AIDS and survival. In the present study, we showed that the performance of a p24 antigen assay involving improved virus lysis buffer, efficient p24 antigen and antibody immune complex disruption, heat-denaturation and signal amplification, was comparable to the RNA viral load test in monitoring patients in a resource-limited country where HIV-1 CRF02_AG is the predominant virus, but where subtypes A and D, CRF06_AG and CRF06_cpx have also been found (Bonard et al., 2003; Ellenberger et al., 1999; Montavon et al., 2002).

Among a total of 86 patients starting ART, the signal amplification boosted ELISA for HIV-1 p24 in heat-denatured plasma pre-treated with an improved virus lysis buffer was capable of quantifying the virus at baseline in 83 patients (97%). This is comparable to the sensitivity of the same test reported in studies from Switzerland (Ledergerber et al., 2000; Schupbach et al., 1996), and Abidjan (Bonard et al., 2003). The observed high rate of positivity implies that...
the locally dominating CRF02_AG viruses are well detected by the assay.

There was significant correlation between p24 antigen and HIV-1 RNA and an inverse correlation between the concentrations of p24 antigen and CD4+ cell at baseline, and in the changes in concentrations of these markers following treatment initiation (Figs. 1 and 2). Despite their correlation, there are important differences between HIV-1 RNA and p24 measured in plasma. HIV-1 RNA is present only inside virus particles while the p24 antigen, at least in chronically infected patients, is found largely outside virus particles. This is demonstrated by ultracentrifugation experiments which show that most p24 antigen remains in the supernatant while virtually all HIV-1 RNA, together with the reverse transcriptase, is found in the pellet (Schupbach, 2003a). It should be noted that the rapid reduction of virus in plasma upon initiation of therapy is not necessarily accompanied by a similarly rapid disappearance of the p24 outside the particles, which is mostly immune complexed. We recently showed that detectable p24 antigen was found in 34% of ART-treated patients whose viral RNA was below 50 copies/mL during a median preceding interval of 24.7 months (Schupbach et al., 2005). The correlation of p24 and RNA viral load observed is consistent with those of other studies in Europe (Schupbach, 2003a; Schüpbach et al., 2001) and the United States among naïve and treated-experience patients when considered separately (Respess et al., 2005), but contrast sharply with that of another study with patients from Abidjan, that found p24 antigen measurement unreliable (Bonard et al., 2003). Bonard et al. did not use the improved virus lysis buffer, which significantly improves the correlation of p24 with HIV-1 RNA. The superior sensitivity conferred by the improved virus lysis buffer was also demonstrated in other studies (Jennings et al., 2003, 2005; Schupbach et al., 2006).

We used the statistical relationship between viral load and p24 to develop a prediction model for virologic failure in our study patients receiving ART. Significant explanatory covariates included in the prediction model were p24 antigen at therapy initiation, achieving undetectable p24 in response to therapy, and changes in p24 antigen while receiving therapy. An ROC curve was generated by varying the failure probability across the prediction range and observing the corresponding classification performances. ROC analysis results suggest an ability of this prediction model to effectively discriminate between patients with sustained or failed virologic response to treatment.

Since virologic failure may precede immunologic or clinical failure by months or years, early change in medication after virologic failure, rather than waiting for a CD4 decline or clinical progression is considered both cost-effective and clinically beneficial (Haubrich et al., 1998; Schupbach, 2003a). Although there is no consensus on when to switch therapy based on changes in viral load, the current DHHS guidelines recommend confirmation of detectable viremia with a second viral load test (DHHS Guidelines, 2006). Our results suggest that the modified p24 antigen test could be used for monitoring ART therapy in Africa, particularly in regions where the epidemic is dominated by subtypes known to be recognized well by the assay, for example subtype C (Pascual et al., 2002; Respess et al., 2005). ART is becoming increasingly available in African countries, but laboratory facilities with highly trained personnel capable of conducting the technically demanding HIV-1 RNA assays remain limited. p24 testing, which is simple and inexpensive, does not require cumbersome sample pretreatment and involves a protein which is very stable compared to HIV-1 RNA and also permits the use of serum instead of plasma (Boni et al., 1997; Schupbach, 2003a). These factors might contribute to improvements in the monitoring and treatment of HIV-infected persons in resource-poor settings.

In summary, we have shown that the performance of the p24 antigen assay based on efficient p24 antigen and antibody immune complex disruption, heat-denaturation, and signal amplification may be comparable to that of the RNA viral load test in monitoring patients in a resource-limited country where HIV-1 non-B subtypes predominates the epidemic. However, a number of open questions remain: because treatment recommendations are based on HIV-1 RNA concentrations and/or CD4+ cell counts, further studies are required to define the corresponding p24 antigen concentrations that indicate a need for ART therapy. Also, to permit large-scale use, there is a need for standardization of the procedure and production of a dedicated assay kit, which contains all necessary reagents.

**References**


