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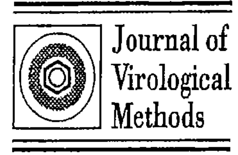
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Performance of a quantitative human immunodeficiency virus type 1 p24 antigen assay on various HIV-1 subtypes for the follow-up of human immunodeficiency type 1 seropositive individuals

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Abstract

The heat-denatured signal-amplified p24 antigen assay is a low-cost test allowing the determination of plasma levels of HIV-1 p24 antigen in infected patients. This assay may be appropriate for monitoring disease progression in HIV seropositive patients in developing countries. Only a few data on the clinical validation of the test are available for HIV-1 non-subtypes B viruses that represent the vast majority of virus circulating in Africa.

The present study was undertaken to evaluate and compare the performance of a heat-denatured signal-amplified p24 assay for the determination of p24 viral load in the plasma of individuals infected with different subtypes of HIV-1 and using the RT-PCR-based RNA viral load test as the gold standard. A total of 120 plasma samples from individuals infected with HIV-1 strains belonging to group M (subtypes A → H) and group O, as well as recombinant strains, were tested in parallel with the heat-denatured signal-amplified p24 assay and the RNA viral load. Plasma p24 levels appeared to be correlated significantly with the plasma RNA viral loads ($R = 0.751$, $P < 0.0001$). The heat-denatured p24 antigen assay was capable of measuring the plasma level of p24 derived from all the HIV-1 subtypes and recombinants selected for this study, in contrast to the RNA viral load test which lacked sensitivity towards HIV-1 group O. The heat-denatured signal-amplified p24 assay is a reliable, sensitive and a more affordable tool that can be used for the follow-up of patients infected with B and non-B subtypes as well as recombinant forms of HIV-1 in developing countries.
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1. Introduction

Anti-retroviral therapy is becoming available more readily throughout the developing world as a result of increases in health budgets as well as drastic price reductions negotiated with pharmaceutical companies. Unfortunately, the cost of serological tests required for the follow up of the treatment remains highly prohibitive for the majority of the patients in resource-limited countries. This situation may contribute to render anti-retroviral therapy ineffective as well as participating to the emergence of drug-resistant HIV strains (Mayers, 1998). The development and implementation of reliable low-cost tools to monitor the effectiveness of treatment is therefore highly foreseen.

Changes in plasma levels of virus replication in combination with the $CD4^+$ lymphocyte count, are considered to be the endpoint assays for monitoring HIV disease progression. In general, these tests are too expensive for routine use by laboratories in developing countries. A single PCR-based determination of the RNA viral load can cost US$ 100 or more per test, representing a month of life-saving treatment for a HIV patient receiving anti-retroviral therapy (Stephenson, 2002). Alternative techniques such as the heat-denatured signal-amplified p24 antigen assay are thus being examined for the determination of viral replication levels. This assay is based on the detection of the viral p24 *gag* protein in the

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plasma by enzyme-linked immunosorbent assay (ELISA) instead of the amplification of the viral genomic material by PCR techniques. Although the p24 ELISA has been available for several years, it has regained new interest, since an increase in its sensitivity could be achieved by a combination of immune complex disruption by heat treatment of the plasma (Schüpbach and Böni, 1993) and the use of a powerful tyramide-based amplification system (Bobrow et al., 1989). Several evaluations have shown that the sensitivity of the p24 antigen and the viral RNA test for the determination of plasma virus levels are comparable and that of the p24 viral load may even correlate better with disease progression (Bürgisser et al., 2000) as compared to RNA viral load. These evaluations were conducted using mainly plasma from HIV-1 subtype B-infected patients. Considering the importance of HIV-1 variability in developing countries of Africa, a thorough validation of the p24 assay on a large panel of different genetic variants of HIV-1 needs to be done before the test can be implemented in the field (Janssens et al., 1997).

The first objective of the present study was to evaluate and compare the levels of viral load determined by the HIV-1 heat-denatured p24 assay with the one determined by the Roche HIV-1 RNA viral load assay in follow-up plasma samples from patients infected with diverse subtypes and circulating recombinant forms of HIV-1 and receiving anti-retroviral therapy. A second objective was to examine whether the heat-denatured signal-amplified p24 antigen assay was equally sensitive across the entire range of HIV-1 isolates selected for this study.

2. Material and methods

2.1. Patients and samples

Plasma samples were selected retrospectively from 40 anti-retroviral therapy treated patients attending the clinic of the Institute of Tropical Medicine in Antwerp. The treated patients were selected on the basis of the HIV-1 subtype and/or a declining or increasing RNA viral load trend throughout the follow-up period. Three different time-point samples per patient, from different HIV-1-infected individuals receiving anti-retroviral therapy, were tested.

The isolates from 98% of the patients were classified by *gag/env* heteroduplex mobility assay (HMA) (Heyndrickx et al., 1998, 2000): A/A ($n = 4$), B/B ($n = 5$), C/C ($n = 2$), D/D ($n = 3$), F/F ($n = 2$), G/G ($n = 3$), H/H ($n = 2$), and the recombinant strains CRF02_AG/A ($n = 1$), CRF01_AE ($n = 6$), C/A ($n = 1$), C/B ($n = 1$), D/F ($n = 3$), F/D ($n = 1$), G/A ($n = 1$), H/A ($n = 1$) and H/B ($n = 1$), H/? ($n = 1$). One HIV-1 strain was subtyped as G ($n = 1$) by sequencing the *pol* region. One HIV-1 strain belonged to group O (Ant-70) ($n = 1$).

The plasma was collected in EDTA tubes and stored at −80 °C until further use.

2.2. PCR-based RNA viral load

All the plasma samples infected with group M viruses were tested for HIV-1 RNA viral load with the Amplicor HIV-1 Monitor test V1.5 (Roche Diagnostic Systems, Branchburg, USA) according to the instructions of the manufacturer. The lower limits of this test are 400 ($\log_{10} 2.60$; standard procedure) or 50 ($\log_{10} 1.70$; ultrasensitive procedure) RNA copies/ml of plasma depending on the protocol used. For the samples infected with a group O virus, the viral load was measured with an "in-house" semi-quantitative method described earlier (Fransen et al., 1994). The estimated limit of this test is 227 RNA copies/ml of plasma (Van Kerckhoven et al., 1994). All the negative values were set equal to the detection limit of the test.

2.3. The heat-denatured p24 signal-amplified antigen assay

All the plasma samples were tested for concentration of p24 antigen using the Alliance HIV-1 p24 ELISA kit in combination with the ELAST ELISA amplification system (Perkin-Elmer Life Science, Boston, USA; Bobrow et al., 1989) according to the instructions of the manufacturer and as described elsewhere (Böni et al., 1997). Briefly, 100 μl of plasma was mixed with 50 μl of an in-house virus lysis buffer and incubated for ten minutes at room temperature (Schüpbach et al., 2003). The mixture was further diluted with 450 μl of 0.5% Triton X-100 and the tubes transferred for 5 min to a heat block, pre-heated at 100 °C. The samples were removed and left to cool to room temperature. Two hundred and fifty microlitres of the sample was tested in duplicate. Four plasma samples from HIV seronegative blood donors were included in each test. A standard curve generated with dilutions of p24 antigen of known concentration was incorporated into each run. The cut-off level for positivity was determined for each plate individually by calculating the average absorbance of the four HIV-1 negative controls plus three times their standard deviation (Schüpbach et al., 2000). The absorbance values were read at 450 nm with the reference at 630 nm with a Bio-Rad 550 micro plate reader. The optical density (OD) values were used to calculate the concentration of the p24 antigen against the standard curve. The data were analysed with the Microplate Manager 4.0 software (Bio-Rad Laboratories, Hercules, USA). All the negative values were set equal to the detection limit of the test.

The specificity of the assay was determined previously as 99.2% (Nadal et al., 1999).

2.4. Statistical analysis

The normality of data distribution was examined by the Smirnov–Kolmogorov statistical test. Correlations between two sets of data were measured with the two-tailed Spearman rank correlation test. A two-tailed decision query was used with a total α-error set at 0.05. All the analyses were

done with the SPSS V.10 programme (Chicago Inc.). The group O samples were excluded from the statistical analysis as they were tested for their viral load with an "in-house" semi-quantitative method.

3. Results

3.1. Follow-up plasma samples

The origin and number of samples of the treated patients were as follows: Europe ($n = 75$), Africa ($n = 42$) and Asia ($n = 3$). The median interval between the first and second time point was 5.7 months (0.9–52.7 months). The median for time points two and three was 9.0 months (0.9–39.4 months).

3.2. Correlation between heat-denatured p24 antigen concentration and viral RNA copy number in plasma

The Smirnov–Kolmogorov test for data distribution indicated that p24 antigen concentration but not RNA copy number were distributed normally. The heat-denatured p24 antigen assay was positive in 110 out of the 120 (92%) samples with a mean value of $\log_{10}$ 3.59 fg/ml. The RNA viral load was detectable in 86 out of 120 (72%) samples with a mean $\log_{10}$ 4.40 copies/ml. There was a significant positive correlation between p24 concentration and RNA copy numbers ($R = 0.751$, $P < 0.0001$, two-tailed Spearman) (Fig. 1). Both tests were plotted in one graph as below the 10,000 RNA copies/ml viral loads obtained by the ultrasensitive and the standard method are highly correlated ($R = 0.936$, intercept = 0.044) (Sun et al., 1998).

From the 120 selected samples, 34 tested negative for viral RNA (27 samples were lower than $\log_{10}$ 2.60 copies/ml and seven lower than $\log_{10}$ 1.70 copies/ml). From the 27 samples negative for the standard protocol, only four samples were negative for p24. Out of the seven samples negative with the ultrasensitive protocol, only one had undetectable p24 levels. Ten of the 120 samples were negative by the heat-denatured p24 antigen assay. Of these ten samples, belonging to three different patients, five had detectable RNA levels.

3.3. Correlation between longitudinal changes in p24 antigen concentration and viral RNA copy number in the plasma

In 33 patients, the changes in p24 correlated well with those of viral RNA while seven others showed a discrepancy (Fig. 2a–j). The patients under treatment were divided into three groups. Those where the RNA viral load and the p24 both increased between the first two time points ($n = 26$), those in whom both concentrations decreased ($n = 7$) and those with a discrepancy between the p24 and the RNA viral load changes ($n = 7$). The seven patients with discrepancies between p24 concentration and RNA viral load over time

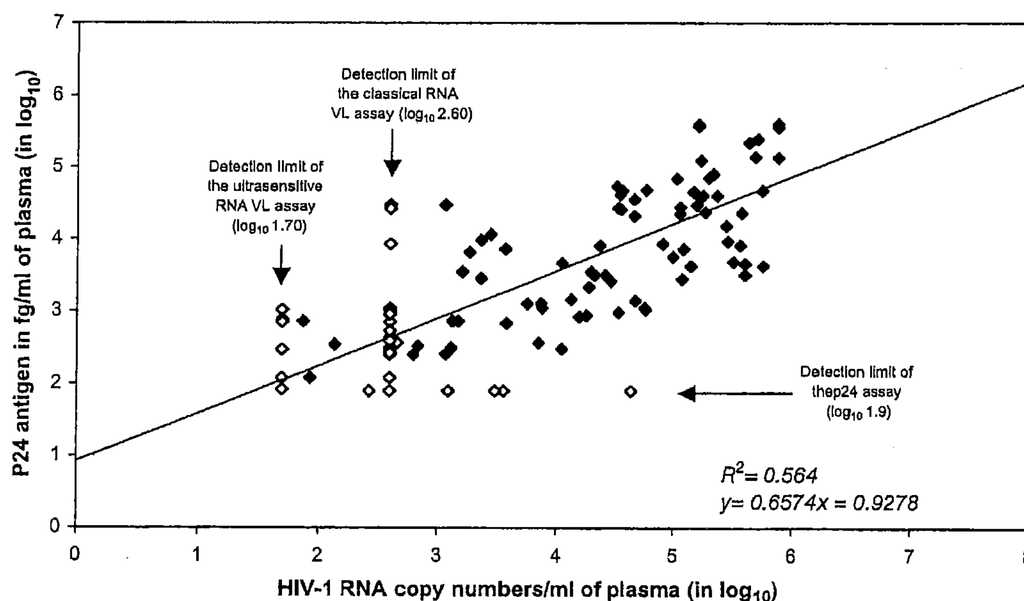

Fig. 1. Correlation between plasma HIV-1 viral loads and plasma HIV-1 p24 concentration in a group of HIV-1-infected patients. P24 and RNA viral load have been determined in parallel in a group of HIV-1-infected persons ($n = 40$) with the Alliance HIV-1 p24 ELISA kit and the Amplicor HIV-1 Monitor test V1.5, respectively. Log_{10} values of p24 antigen concentration and HIV-1 RNA viral load have been plotted against each other. In a scatter plot, the arrows indicate cut-off values for the Amplicor (standard and ultrasensitive version) and the Alliance test. The correlation coefficient between the two sets of data was calculated by the Spearman ρ-test ($R = 0.751$, $P < 0.0001$). The solid line (—) represents the linear correlation between the RNA viral load and the p24 antigen concentration. The equation was calculated after the outlier points (i.e. corresponding to values equal to the cut-off points) were excluded from the analysis. These outlier points are represented by the transparent diamonds (◇).

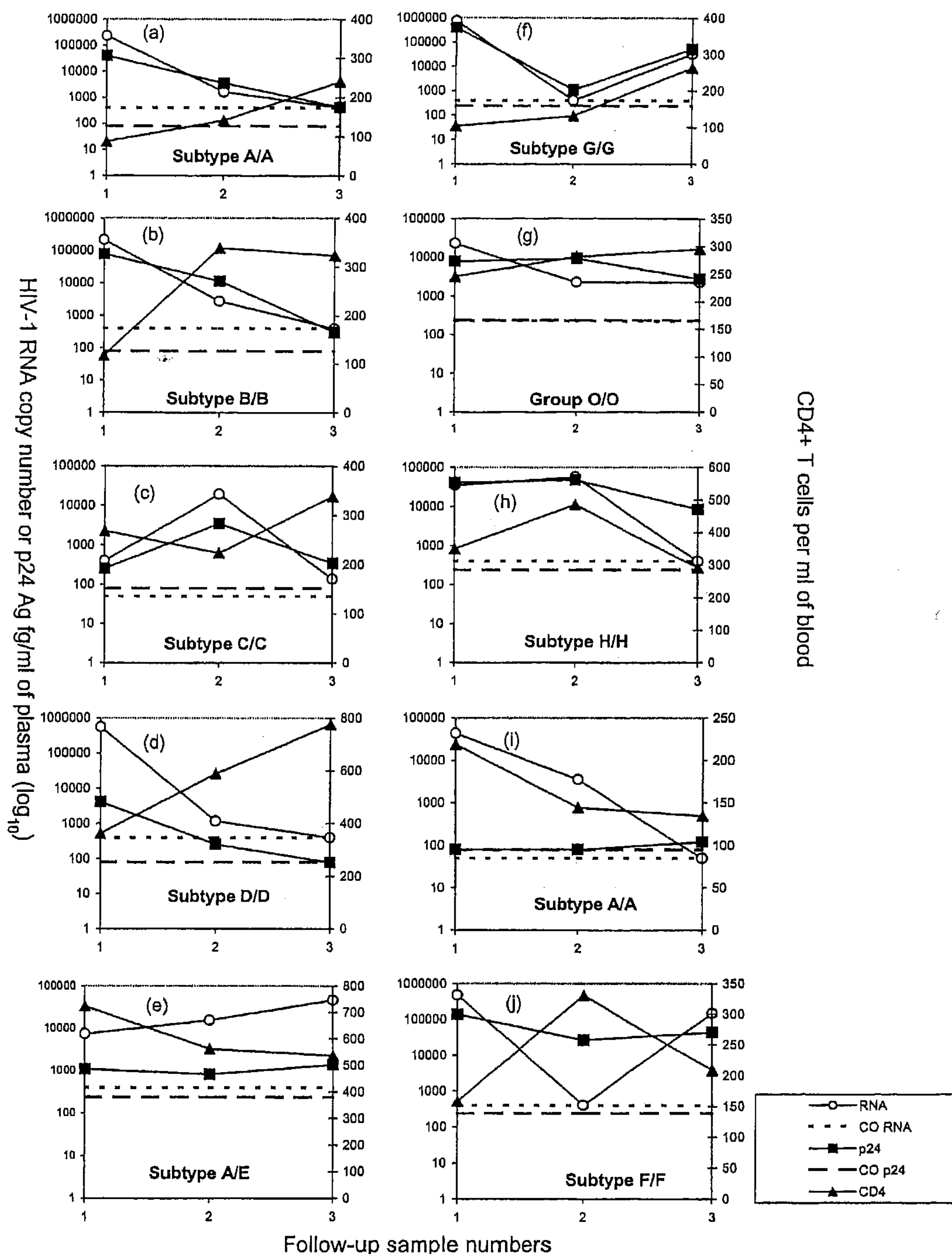

Fig. 2. Representative curves of the evolution of plasma HIV-1 RNA viral load, plasma HIV-1 p24 antigen and $CD4^+$ cell levels over time in patients infected with diverse subtypes of HIV-1. Each graph represents the data generated from the virologic and immunologic follow up of one patient over three time points. RNA viral load (○), p24 antigen concentration (■) and $CD4^+$ T cell absolute count (▲) have been determined for each time point. The broken lines represent the cut-off values for the RNA viral load assay (- - -) and the p24 antigen assay (— — —). The two letters of the genetic subtype indicate the genetic subtype for *gag* and *env*, respectively. P24 concentration, as measured by the Alliance HIV-1 p24 ELISA kit, could be detected for the whole range of HIV-1 subtypes. The RNA viral load of in the plasma infected with the group O virus (graph g) was determined by an in-house semi-quantitative assay. RNA viral load for the group M viruses was determined by the Amplicor HIV-1 Monitor test V1.5. Changes in p24 antigen concentration were in general positively correlated with changes in RNA viral load for each individual patient. In some instances, an atypical lack of inverse correlation between $CD4^+$ cell absolute count and p24 and/or RNA viral load was observed (graphs f, h and i).

were infected with virus of subtype A/A, F/F, G (*pol*) and group O/O and the recombinant subtypes A/E, D/F and F/D.

3.4. Correlation between heat-denatured p24 antigen and CD4$^+$

From six samples, no CD4$^+$ data were available. The correlation between heat-denatured p24 antigen and CD4$^+$ was $R = -0.309$ ($P = 0.001$). The correlation between the CD4$^+$ count and RNA was $R = -0.330$ ($P < 0.0001$).

3.5. Sensitivity of the heat-denatured p24 assay towards HIV-1 subtypes

All the group M subtypes used in this evaluation could be detected by the heat-denatured p24 antigen assay. P24 could also be detected in all three samples taken from a group O-infected patient (Fig. 2g). P24 concentration adjusted for viral load ($\log_{10}$ of p24 fg/RNA copy number) for the group of subtype B/B-infected samples was expressed as quartiles ($q1 = 0.180$, $q2 = 0.646$ and $q3 = 2.00$) and used as reference for the other groups of subtypes. Medians (q2) of p24 adjusted for viral load of the majority of subtypes were comparable to that of the reference, i.e. comprised between q1 and q3 of subtype B/B. Conversely, median of p24 adjusted for viral load of subtypes A/A ($q2 = 0.186$), A/E ($q2 = 0.115$), F/D ($q2 = 0.015$) and H/B ($q2 = 0.178$) appeared to be lower than that of subtype B/B.

One patient infected with a virus belonging to subtype A/A, consistently showed a null ratio of p24 concentration over RNA viral load (Fig. 2i). These three samples were re-tested with the same protocol with the not yet optimised INNOTEST® HIV antigen mAb test (Innogenetics, Ghent, Belgium). The samples reacted positive, indicating that the sensitivity of the Alliance HIV-1 p24 ELISA test can be improved towards this subtype. All the viruses from the seven patients with discrepancy between p24 antigen and RNA load belonged to different subtypes.

4. Discussion

The present set of data indicates that levels of plasma HIV-1 p24 measured by the heat-denatured p24 ELISA test were correlated significantly with plasma RNA viral load as measured by the PCR-based reference assay, regardless of the viral subtype. This report confirms previous findings that the improved version of the p24 ELISA is as sensitive as PCR-based virus detection for the measure of viral load (Schüpbach et al., 2001).

All the subtypes and recombinant strains of HIV-1 selected for this study were detected by the heat-denatured p24 assay, including the HIV-1 group O (Ant 70) subtype. This indicates that the heat-denatured p24 assay may detect a broader spectrum of HIV viruses as compared to the RNA viral load Roche test (Swanson et al., 2001). So far, the Roche test has never been described to be able to detect any group O viruses. Considering that the group O consist of a pool of highly divergent strains with no defined clades (Gürtler et al., 1994), we recommend that more HIV-1 strains from group O should be tested in the p24 assay in order to evaluate further its sensitivity toward this group of outlier viruses.

Some of the groups of subtypes evaluated in the present study had a very small size. For this reason, we have not been able to demonstrate statistically significant differences in p24 detection per RNA copy number between different subtypes. Nonetheless, our data suggests an inferiority of the Alliance HIV-1 p24 ELISA for detecting p24 from virus strains clustering with subtype A within their *gag* region. A successful detection of p24 levels by the INNOTEST® HIV antigen mAb test, in the samples previously negative or with a low ratio of p24 concentration over RNA viral load with the Alliance kit, indicates that this assay can be improved further in terms of sensitivity towards the different HIV subtypes. For comparison, the INNOTEST® HIV antigen mAb test is an assay, which not only detects heat-denatured p24 antigen from HIV-1 group M and O, but also from HIV-2 (Fransen et al., 1997). Incorporation of monoclonal antibodies with specificities covering a broader range of HIV variants in the present assay should be considered.

The Alliance kit clearly showed a higher sensitivity than the Roche RNA assay toward samples with a lower range of RNA viral load. Ultracentrifugation experiments have demonstrated that in chronically infected patients only a small fraction of the total p24 antigen can be pelletable, i.e. enclosed within viral particles as a structural protein (Schüpbach, 2002). While the p24 protein inside the viral particle becomes undetectable if the RNA copy numbers falls below 6000–12000 copies/ml (Coffin, 1996), the p24 antigen outside the particles is usually present in a large excess with a half-life of 42 ± 16 days. It may remain detectable for some time even if the viral RNA has fallen below the detection limit under effective anti-retroviral therapy, thus explaining the higher diagnostic sensitivity of p24 as compared to viral RNA in these patients. We did not have evidence that the discrepancy between p24 concentration and RNA viral load changes over time, observed in some patients, was associated with a particular subtype of HIV-1. A lower sensitivity of the p24 assay towards some strains of HIV-1 may partly explain this discordance. Whether some early changes in viral replication patterns due to the effect of the therapy or to opportunistic infections also contribute to this discrepancy is still unclear. Indeed, the short window of observation in the present study may not be representative of the overall long-term relationship between p24 production and RNA viral load in the plasma. The significant correlation between heat-denatured p24 concentrations and levels of CD4$^+$ T cells further confirms the value of the heat-denatured p24 assay to monitor the efficacy of anti-retroviral therapy. In contrast, RNA viral loads were not significantly associated with CD4$^+$ absolute counts. This is in accordance with other reports indicating the better association of p24 concentration

with CD4+ levels during anti-retroviral therapy as compared to HIV RNA viral loads (Schüpbach et al., 2003). Because of the small size of the population studied ($n = 40$) and the few time points included in the observation period, we recommend that further investigations on the correlation of heat-denatured p24 levels and CD4+ T cell count should be undertaken in cohorts of both naive and treated HIV-infected individuals.

In conclusion, our data demonstrate that overall p24 viral load is highly correlated with HIV-1 RNA in the plasma regardless of the subtype of the virus. The heat-denatured p24 assay may be superior to the HIV RNA viral load in terms of sensitivity towards a broader range of HIV subtypes as well as the correlation with the immunological status of the patient, as measured by the CD4+ absolute count. A slightly lower detection of p24 antigen in some *gag* subtype A virus remains to be confirmed but could potentially be improved by further modifications of the assay.

Taking into account both financial and assay performance, we confirm that the heat-denatured p24 antigen assay can be used as an affordable alternative to the quantitative HIV-1 RNA tests available today. Further testing in laboratories on the field should be done, in order to assess the reliability and reproducibility of the heat-denatured p24 assay in settings where only limited logistic is available. Similarly, we recommend that the capacity of the heat-denatured p24 assay to predict the outcome of anti-retroviral therapy should be further assessed. Field data are ultimately needed in order to evaluate whether the heat-denatured p24 antigen could be as reliable as the RNA viral load, in combination with the CD4+ count, for the follow-up of HIV-infected patients.

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