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Detection of HIV-1 p24 antigen in patients with varying degrees of viremia using an ELISA with a photochemical signal amplification system

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Abstract

Background—We describe a photochemical signal amplification method (PSAM) for increasing the sensitivity of enzyme-linked immunosorbent assays (ELISAs) for the detection of HIV-1 p24 antigen, and present a preliminary validation study on ELISA+PSAM technology for detection of HIV-1 p24 antigen in clinical samples.

Methods—ELISA+PSAM is compatible with commercially available microtiter plate readers, employs an inexpensive illumination device and the amplification takes around 10 min.

Results—The PSAM technology not only increases the analytical sensitivity for detection of HIV-1 p24 antigen by approximately 40 times, but also significantly increases the clinical sensitivity of the ELISA: in instances where viral RNA load is <3,000 copies/ml, conventional heat mediated immune complex disruption ELISA (HM-ELISA) cannot detect any HIV positive samples whereas HM-ELISA+PSAM can detect HIV infection in approximately half of the samples (clinical sensitivity is 52.63%). For viral RNA loads between 3,000 and 30,000 copies/ml, the clinical sensitivities of the HM-ELISA and HM-ELISA+PSAM are 32.6% and 91.3%, and for that >30,000 copies/ml, clinical sensitivities of HM-ELISA and HM-ELISA+PSAM are 52.3% and 100%, respectively.

Conclusions—The HM-ELISA+PSAM represents an advancement in monitoring HIV-1 disease progression and treatment in the global healthcare setting.

Keywords

HIV p24 antigen; Immunoassay; ELISA; Signal amplification; Increase of sensitivity; Clinical sensitivity; Specificity; Photochemistry; Illumination

1. Introduction

In the last 25 years, HIV1 infection and acquired immunodeficiency syndrome (AIDS) became global epidemics with 35 million people living with the disease. In 2013 alone, there

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were 2.1 million new infections, and 1.5 million deaths [1]. Starting antiretroviral therapy (ART) on time may improve the quality of life and prolong life considerably, while antiretroviral drugs, obstetrical interventions, and safer infant feeding practices can reduce mother-to-child transmission (MTCT) rate to 1–20%, depending on the interventions provided [2]. HIV-1 RNA, anti-HIV antibodies, and HIV-1 capsid protein (p24 antigen) are the main viral markers used to detect HIV infection, and to monitor disease progression. For early detection of HIV-1 infection, p24 antigen or anti-HIV antibody/p24 antigen combination assays are used which can reduce the seroconversion “window period” to an average of 17 days post-infection and aid in identification of acute infection [3].

The p24 antigen appears within 2 weeks of HIV infection as a result of an initial burst of viral replication that is associated with high levels of viremia during which the individual is highly infectious. Analysis of p24 antigen is complicated by the fact that when antibodies to HIV become detectable, p24 antigen becomes undetectable, most likely because of the formation of antigen-antibody complexes in the blood. However, when detected, p24 antigen is highly specific for infection. In MTCT of the disease, assays for HIV-1 p24 are also useful for diagnosing HIV infection in infants since HIV antibody tests can yield false positives due to maternal HIV antibodies [2, 3]. While nucleic acid testing (NAT) remains the gold standard for pediatric samples, currently commercially available NAT assays are expensive, require complex instrumentation for quantification of the viral load, require highly trained staff, and are sensitive to contamination. These factors ultimately limit the use of NAT assays in resource-limited settings. Thus, there is an ongoing need for less expensive, simple and accurate techniques for monitoring patients for HIV status in resource-poor settings.

Most commercial HIV-1 p24 assays employ a standard ELISA format for the capture and detection of p24 antigen. These methods are capable of detecting in the range of 5–25 pg/ml of HIV-1 p24 antigen in the absence of signal amplification [4]. At this level of sensitivity, it is difficult to consistently detect p24 antigen in HIV-positive individuals, especially in those cases where the infections are asymptomatic. Studies show that only about 50–60 % of AIDS patients, 30–40% of AIDS-related complex (ARC), and 10 % of asymptomatic patients will be detected as HIV positive via standard ELISA [5]. In recent decades, several HIV p24 detection methods using signal amplification systems have been developed in order to increase the analytical sensitivity of EIA/ELISA [1, 6]. While some of these methods allow for ultrasensitive detection of HIV p24 detection, these methods are generally characterized by complex protocols, expensive reagents, or a combination of both.

Recently, we presented a photochemical signal amplification method (PSAM), which is simple and straightforward, requiring minimal effort to achieve markedly improved results from commercially available ELISA reagent systems [7,8]. PSAM is based on an autocatalytic photochemical reaction of a commonly-used colorimetric substrate for ELISA assays [7]. Briefly, ELISA+PSAM consists of 2 steps: the first step being a conventional ELISA based on the use of one of the most sensitive chromogenic detection systems: the

¹Abbreviations: AIDS: Acquired Immuno Deficiency Syndrome; ART: Anti Retroviral Therapy; HM-ICD: Heat Mediated ICD; HM-ELISA: Heat Mediated (ICD) ELISA; ICD: Immune Complex Disruption; MTCT: Mother-To-Child Transmission; NAT: Nucleic Acid Test; PSAM: Photochemical Signal Amplification Method; VDB: Virus Disruption Buffer.

combination of horseradish peroxidase (HRP) enzyme and its substrate, orthophenylenediamine (OPD). Within this step, HRP catalyzes oxidation of OPD, yielding a yellow solution containing the product of this reaction, the dye 2, 3-diaminophenazine (DAP) (Fig. 1, reaction (1.1)). In the second step, the PSAM system takes advantage of the fact that DAP is a good photosensitizer, and the small amount of DAP produced during the enzymatic reaction acts as a catalyst for a subsequent autocatalytic photosensitized amplification reaction (Fig. 1, reaction (1.2)). The possible mechanism of the photosensitized reaction (1.2) is discussed in [7]. In our previous feasibility study, we also showed that ELISA+PSAM allows a nearly 40-fold increase in the analytical sensitivity and dynamic range of the Alliance HIV-1 p24 ELISA test from PerkinElmer, Inc (PE p24 ELISA) [8]. It should be emphasized, however, that the aforementioned calculations of the analytical sensitivity for conventional ELISA and ELISA+PSAM assays were based on the use of “ideal” negative (i.e. normal, uninfected) sera. In order to determine clinical sensitivity and specificity of the assay, and in general, in order to demonstrate that PSAM could be used in clinical practice, validation studies using hundreds clinical samples must be performed.

We present a pilot validation study of ELISA+PSAM technology (based on the use of the PE p24 ELISA) for detection of HIV-1 p24 antigen in human serum or plasma. In order to validate the ELISA+PSAM method for detection of HIV p24 antigen, we used over 200 negative and positive clinical samples. We showed that the ELISA+PSAM has overall better clinical sensitivity over the conventional ELISA for detecting HIV-1 infection in patients with low- as well as middle- viral RNA titers. Building on our previous work [8] on feasibility of this method, here we present pilot preliminary clinical studies involving plasma from HIV-1 positive patients and seroconversion panels, to demonstrate the potential utility of the ELISA+PSAM technology in the early detection of HIV-1 P24 antigen in patients.

2. Materials and Methods

2.1 Materials

Alliance HIV-1 p24 ELISA kits were from PerkinElmer, Inc. Pathogen-free human serum and OPD substrate tablets were obtained from Sigma and sodium ascorbate was from Alfa Aesar. Standard 96-well clear microplates were obtained from R&D Systems. A Labsystems Multiskan MS/352 microplate reader was used for optical density measurements. Blood plasma and serum samples (negatives) were obtained from Biological Specialty, seroconversion panels from Seracare and archived HIV-1 positive plasma samples were from Children's Memorial Hospital (CMH): these de-identified samples were in accordance with the Institutional Review Board guidelines and all subjects signed an informed consent agreement prior to participation in this study.

2.2 Conventional ELISA and ELISA+PSAM methods

2.2.1 Samples pre-treatment procedure—PE p24 ELISA was used for detection and quantification of the major structural core component of HIV-1 virus, p24 antigen. In PE p24 ELISA, either acid- or heat-mediated immune complex disruption (ICD) procedures can be used for disrupting antigen-antibody complexes, allowing detection of both free and

bound to antibodies p24 antigen. In this study, the heat-mediated (HM)-ICD procedure was used because it is considered to be more sensitive [9]. HM-ICD for conventional ELISA (HM-ELISA) and HM-ELISA+PSAM were carried out as described in our previous work [8], with some modifications. Briefly, 50 µl of samples (standards and clinical samples) were added to polypropylene screw-capped microcentrifuge tubes containing 25 µl of virus disruption buffer (VDB) (30 mmol/l Tris-HCl (pH 7.2), 450 mmol/l NaCl, 1.5% Triton X-100, 1.5% deoxycholic acid sodium salt, 0.3% sodium dodecyl sulfate, 10 mmol/l EDTA) initially described in [10]. The tubes were incubated for 10 min at room temperature. 225 µl of 0.5 % Triton X-100 in PBS was added to each tube and the tubes were boiled for 5 min. After cooling the tubes to room temperature for 15 min, the samples were centrifuged at 4000 rpm in a microcentrifuge from Eppendorf, Model 5415 for 5 min to remove any clotted material.

2.2.2 PE p24 ELISA—Two hundred fifty microliters of the pre-treated samples (as described above) were added to the wells in the provided microtiter plate coated with a monoclonal antibody to HIV-1 p24 for capturing HIV-1 p24 antigen. The wells were incubated for 2 h at room temperature with shaking followed by 10 washes with wash buffer (provided in the PE p24 kit). The wells were then incubated with a detector antibody (biotinylated rabbit polyclonal) for 2 h at 37 °C. The wells were washed 10 times and then incubated with a 1:100 dilution of Streptavidin-HRP conjugate (provided in the kit) for 30 min at room temperature. After washing, 100 µl of OPD substrate solution was added and incubated for 30 min at room temperature. Then, 100 µl of stop solution (0.1 mol/l sulfuric acid) was added to the wells and absorbance (optical density, OD) was read at 492 nm and 620 nm. A calibration curve for determination of p24 antigen concentrations was prepared by plotting the difference between the signals (OD) obtained at 492 nm and 620 nm vs. p24 concentrations prepared as double dilutions of p24 antigen in negative control (normal human serum)[11].

2.2.3 PE p24 ELISA+PSAM procedure—As discussed in our previous work [8], minor modifications to the conventional ELISA were made to reach the maximum signal-to-noise ratio of the assay. Briefly, in the HM-ELISA+PSAM assays, the conventional HM-ELISA steps were the same as described above, except 1: 6,000 dilutions of streptavidin HRP conjugate (High sensitivity) and Sigma OPD tablets were used instead of the PE kit's streptavidin-HRP conjugate and OPD tablets, respectively. The OPD substrate solution contained OPD and H₂O₂ in concentrations of 6.0×10^{-3} mol/l and 4×10^{-3} mol/l, respectively in 0.05 mol/l phosphate-citrate buffer, pH 5.0 (PCB). After 30 min incubation with the OPD substrate solution, 100 µl OPD substrate solution was transferred to the clean microtiter plate (R&D Systems). Thirty microliters of the PSAM reagent solution (300 µM of sodium ascorbate in PCB [8]) was then added to 100 µl OPD substrate solution. Samples were irradiated in 2 min time increments for the first 4 min and in 1 min increments after that - for a total of 12 min using an in-house made illumination device. The illumination device, configured for even illumination of 96-well microtiter plates, and parameters of light irradiation were described in [8] (Supplemental Digital Content 1). Optical density of the samples was measured at 450 nm using the Labsystems Multiskan MS/352 microplate reader after each period of illumination. Calibration curves for determination of p24 antigen

were prepared by plotting the signals (OD 450 nm) vs. p24 concentrations prepared as double dilutions of p24 antigen in negative control (normal human serum).

2.3 Preliminary validation studies

In order to validate the PSAM method for detection of HIV p24 antigen in human serum or plasma, we used 106 archived positive plasma samples characterized by the reference test (Roche Amplicor HIV-1 Monitor RNA viral load assay), 76 negative samples and 3 seroconversion panels containing serial bleed plasma specimens. Analytical sensitivity or limit of detection (LOD) (these terms are used interchangeably here) of the conventional HM-ELISA test was determined via the least squares fit to the standard (calibration) curve at an absorbance (optical density, OD) equal to the cut-off defined by the manufacturer (i.e., mean negative control OD + 0.050) [11]. For the HM-ELISA+PSAM assay, LOD was estimated as the p24 concentration corresponding to the absorbance equal to the twice the background value using the least squares fit to the standard curve. The real cut-off of the HM-ELISA+PSAM assay for clinical analysis was determined by receiver operating characteristic (ROC) analysis. The clinical specificity and clinical sensitivity were determined based on the use of this cut-off value. For evaluation of confidence intervals and statistical significance, we used standard statistical methods including FREQ procedure and Fisher's exact test [12].

3. Results

3.1 Overview

Early detection of HIV infection can not only help diminish the spread of the disease within a population, but can also provide a basis for health-care opportunities and preventive options within the population of infected individuals [2]. p24 antigen is found in serum or plasma in either free form or bound by anti-p24 antibody. Free p24, which characteristically appears only early and late during infection, can be measured by ELISA without sample pre-treatment. In contrast, the detection of bound p24 antigen requires an ICD procedure, i.e. sample pre-treatment with an acid or heat to dissociate the antibody-antigen immune complex. The total (free and bound) p24 antigen can be detected by ELISA using samples pre-treated with the ICD procedure. Since heat-mediated ELISA (HM-ELISA) provides higher sensitivity of p24 antigen detection than the acid-mediated ELISA [9], in this study we mainly used HM-ELISA. We performed acid-mediated ELISA only for direct comparison with data provided in the seroconversion panels.

In order to compare the analytical and clinical sensitivities of conventional HM-ELISA versus HM-ELISA+PSAM for the determination of p24 antigen, an LOD for each method must be determined. For this purpose, the cut-off value, a signal corresponding to the lowest analyte concentration that can be measured, is defined and the corresponding LOD is calculated using the regression analysis. In general terms, the limit of detection of an analytical system is the lowest concentration of analyte which produces a signal statistically-significant different from a 'blank' or 'background' signal [13]. There are several approaches for the determination of the cut-off values of an analytical assay. For sake of simplicity we consider only 3 of them. In method 1, the cut-off is calculated according to the formula:

mean (negatives) + $n \times SD$, where mean (negatives) is the mean of signals obtained for several (3–6) negative controls (background), n is the whole number, and SD is the standard deviation between negative controls. In method 2, the cut-off value can be estimated according to the formula: cut-off = mean (negatives) $\times n$. In method 3, the cut-off = mean (reference standard) $\times/+ A$, where mean (reference standard) is the mean of signals obtained for replicates of the standard sample (could be negative or any standard positive containing a certain concentration of the analyte), $\times/+$ is either multiplication or addition, and A is a certain factor defined based on what is acceptable for the desired level of clinical sensitivity and specificity. In the latter method, dozens or hundreds clinical samples are used, and the optimal cut-off point is determined by plotting the clinical sensitivity and specificity for various cut-off points by means of ROC analysis to achieve the best relationship between the clinical sensitivity and specificity.

The first and second methods for the cut-off assessment are used, mainly, for estimation of the analytical sensitivity and, unlike the method 3, do not take into account that signals for real world negative samples may lie in a rather wide range. Thus, method 3 provides a more accurate assessment of the cut-off value as compared to that used in methods 1 and 2. Therefore, we used method 2 for the initial estimation of the analytical sensitivity of the HM-ELISA+PSAM assay and method 3 for the assessment of the real cut-off value, and the value determined by method 3 is used in the subsequent statistical analysis.

3.2 Analytical Performance Characteristics

3.2.1 Analytical sensitivity of the conventional HM-ELISA and HM-ELISA +PSAM for HIV-1 p24 antigen detection—Calibration curves for HM-ELISA and HM-ELISA+PSAM for detection of p24 antigen are shown in Fig. 2A and 2B, respectively. The analytical sensitivity or limit of detection (LOD) for conventional HM-ELISA without signal amplification is equal to 7.1 pg/ml, as calculated according to the manufacturer's instruction using the regression analysis and the cut-off OD (i.e., cut-off = mean negative control OD + 0.050) [11].

Fig. 2B shows several HM-ELISA+PSAM calibration curves prepared for different times of illumination. The HM-ELISA+PSAM assay was most sensitive after 8 min of illumination (Fig. 2B, big squares) with a lower limit of detection of approximately 0.2 pg/ml estimated as twice the background signal according to the described above method 2. On the other hand, when method 1 for calculation of LOD (as described above) is used, HM-ELISA +PSAM assay has a cut-off equal to Mean (negatives) + $n \times SD$, with $n=3$ [13] and the corresponding LOD is approximately 0.15 pg/ml. This value is close to the LOD obtained using method 2 (0.2 pg/ml). Thus, the HM-ELISA+PSAM assay has approximately 40-fold higher analytical sensitivity as compared with that of the conventional HM-ELISA. Below, we will also use method 3 for determination of LOD which corresponds to the real cut-off of the method.

It should be noted that in our earlier work [8], we achieved lower limit of detection of 4 pg/ml and 0.1 pg/ml for the conventional HM-ELISA and HM-ELISA+PSAM, respectively. Possibly due to variations in the commercially available PE HIV-1 p24 antigen detection kits, in the present study we achieved LODs approximately 2-fold higher than we reported

previously. This may be attributed to the lower activity of HRP in the streptavidin-HRP conjugate currently supplied with the kit: experiments on the activity of HRP comparing streptavidin-HRP of earlier and current kits show higher activity in the former (data not shown). Other possible reasons for the decreased analytical sensitivity of the current version of the PE p24 antigen kit could be lower affinity of antibodies or streptavidin-HRP conjugates than those of the corresponding reagents used previously.

3.2.2 Reproducibility and accuracy—In order to evaluate the reproducibility and accuracy of the HM-ELISA+PSAM assay for the quantitative determination of p24 antigen in human serum or plasma, the panel of the reference quality control (QC) samples was prepared. Panel members were prepared by diluting positive control samples in recalcified nonreactive human plasma (Sigma). For estimation of the intra-assay precision of the HM-ELISA+PSAM, the panel members selected for testing were those with p24 concentrations closest to the target LOD values of 0, 0.2, 0.4 and 0.8 pg/ml. We calculated the coefficient of variance (%CV) for signals obtained for replicates ($n = 4$) of these 4 QC samples and are in the range from approximately 3% to approximately 15%, which is typical for quantitative immunoassays [14].

For the estimation of the inter-assay (within-laboratory) precision and accuracy, 5 QC samples containing p24 antigen in a wide range of concentrations were prepared. Then, the HM-ELISA+PSAM assay was run 4 times, and the p24 antigen concentration in replicates of QC samples was calculated using the HM-ELISA+PSAM calibration curve and regression analysis. The inter-assay precision of the HM-ELISA+PSAM was estimated by calculating %CV for these samples.

The accuracy of the HM-ELISA+PSAM can be expressed in terms of average bias or relative error (RE) of measurement, $\text{Mean RE (\%)} = (\text{mean calculated concentration} - \text{theoretical concentration}) / \text{theoretical concentration} \times 100$. The accuracy of HM-ELISA+PSAM was determined by calculating the concentrations of HIV p24 antigen in the QC samples as described above and comparing the mean of calculated concentrations with the corresponding theoretical concentrations.

The results for the inter-assay precision and accuracy of the HM-ELISA+PSAM are summarized in Table 1. The average bias (mean relative error) is <10 % for all 5 analyzed samples, which shows that the HM-ELISA+PSAM is a high accuracy method. Table 1 also shows that the inter-assay precision for 4 out of 5 QC samples is below 20%, which is considered to be an acceptable range for these parameters [14]. Only one QC sample with low p24 concentration (0.312 pg/ml) showed the inter-assay precision >20%. It should be emphasized, however, that the data can only be considered preliminary since, according to guidance from Clinical and Laboratory Standards Institute (CLSI) document EP5-A2, a much higher number of tests than used in the current pilot study must be performed for full characterization of the method [15].

3.2.3 Preliminary validation of HM-ELISA+PSAM

3.2.3.1 Determination of the real cut-off for HM-ELISA+PSAM: In order to determine and compare analytical and clinical sensitivities of conventional HM-ELISA and HM-

ELISA+PSAM more precisely, the real cut-off values and corresponding limits of detection for these methods must be determined. As we mentioned above, Perkin Elmer uses the mean absorbance value for negative control + 0.05 as the cut-off value for its HIV-1 p24 test. Most likely, PE used method 3, as described above, for the determination of the real cut-off value.

In order to determine the actual or real cut-off value for the HM-ELISA+PSAM assay, we also used method 3. In more detail, we compared the clinical sensitivity and specificity for various cut-off values by means of ROC analysis, which is a way of displaying clinical sensitivity (true positives fraction by test) versus specificity (true negatives fraction by test) across a range of cut-offs [16]. Then, we selected the cut-off value which provides the acceptable clinical sensitivity and specificity.

The examined cut-off points were based on the absorbance, OD at 450 nm, obtained for a reference standard with the concentration of 0.31 pg/ml. The cut-off points being validated were: cut-offs 1 – 5, $(1 + k) \times \text{OD } 450 \text{ nm } (0.31 \text{ pg/ml})$, where $k = 0$ (0%), 0.05 (5%), 0.1 (10%), 0.2 (20%) and 0.3 (30%), respectively.

It has been shown that a p24 antigen level of $>5 \text{ pg/ml}$ (approximate PE ELISA LOD) is predictive of disease progression with an accuracy comparable to that of CD4 lymphocyte count ($<350 \text{ lymphocytes/mm}^3$) and HIV-1 RNA viral load $>30,000 \text{ copies/ml}$, which are cut-offs used in guidelines for the initiation of antiretroviral therapy [17]. Therefore, in order to perform ROC analysis and compare the sensitivities of the conventional HM-ELISA and HM-ELISA+PSAM in the ranges of p24 concentrations around and below PE ELISA LOD of 5 pg/ml , we segregated the positive samples based on RNA viral loads in 3 ranges: Range 1: RNA load $<3,000 \text{ copies/ml}$, Range 2: RNA load between $3,000 \text{ copies/ml}$ and $30,000 \text{ copies/ml}$ and Range 3: RNA load $>30,000 \text{ copies/ml}$. Our categorization of HIV-positive samples in these ranges was similar to the manner in which clinical samples were segregated as containing $<$ or $>30,000 \text{ copies}$ of viral RNA/ml for the evaluation of the ultrasensitive p24 antigen ELISA involving tyramide signal amplification [18].

For ROC analysis, clinical sensitivity of the HM-ELISA+PSAM was calculated using 87 positive samples with the RNA load $>3,000 \text{ copies/ml}$ (ranges 2 and 3 combined). The clinical specificity of the HM-ELISA+PSAM assay was evaluated from healthy non-infected individuals. The negative samples included 50 plasma and 26 serum samples.

Clinical sensitivities and clinical specificities obtained for HM-ELISA+PSAM using cut-offs 1 through 5 are collected in Table 2. The clinical specificity calculated using the 26 negative serum samples is 100% for all 5 examined cut-off values. This means that for the HM-ELISA+PSAM assay, the false positive rate for serum samples is zero for all cut-offs, including cut-off 1 ($\text{OD } 450 \text{ nm } (0.31 \text{ pg/ml})$). If serum samples had been used as negative samples, cut-off 1 could have been selected which would have resulted in a higher analytical sensitivity of the method. In this study, however, the only positive samples were plasma samples, and for accurate evaluation of the method, it is preferable to base the calculations on positive and negative samples of the same type (i.e., plasma). Based on the HM-ELISA+PSAM results obtained for plasma samples only (Table 2) an increase in the specificity is

accompanied by a decrease in the clinical sensitivity. This is a common phenomenon characteristic for many tests [16].

We selected cut-off 4 ($1.2 \times \text{OD } 450 \text{ nm}$ (0.31 pg/ml) with $k = 0.2$ (20%)) for plasma samples because this cut-off point provides an acceptable value of specificity of 98% (false positive rate of 2%) along with high clinical sensitivity of 95.4 % within the defined range of RNA viral load (Table 2). Thus, in HM-ELISA+PSAM screening of clinical samples, the sample reactivity is determined by the following rule [5]: if the ratio of sample OD at 450 nm to cut-off < 1 , the sample is considered negative for HIV-1 p24 antigen by this test; if the ratio of sample OD at 450 nm to cut-off ≥ 1 , the sample is considered positive for HIV-1 p24 antigen by this test.

3.2.4 Clinical specificity—Based on the cut-off 4 value defined above, the clinical specificities of the HM-ELISA+PSAM for 50 plasma and 26 serum samples are presented in Table 3. Out of 76 samples 1 plasma sample gave a false positive result, and, as mentioned above, clinical specificity for plasma and serum samples is 98% and 100%, respectively. Overall, taking into account both plasma and serum samples, HM-ELISA+PSAM specificity has a value of 98.68%. According to FDA and CDC guidelines, this value of the clinical specificity is acceptable [19,20], and is comparable to specificities for other p24 tests [21].

3.2.5 Clinical sensitivity—The clinical sensitivity of the HM-ELISA+PSAM assay for detecting infection with HIV in samples over different ranges of RNA viral load was evaluated using plasma samples from 106 infected individuals undergoing HAART therapy who were exhibiting different degrees of viremia. Based on the cut-off 4 value described above, the clinical sensitivities of HM-ELISA+PSAM along with those for the conventional ELISA are summarized in the Table 4. The lower and upper values of the confidence interval (CI) were calculated based on the FREQ Procedure [12] and the formula: $P \pm 1.96 (P(1-P)/N)^{1/2}$, where P is the observed sensitivity, and N is the number of samples analyzed (the sample size) [22]. Two procedures are used for the calculation of the bounds of the confidence interval within which the true value of the specificity will be with 95% probability. Since the results obtained using the 2 procedures are practically the same, the results obtained using only the latter formula are shown in the Table 4.

The clinical sensitivity of the HM-ELISA+PSAM is significantly higher than that for the conventional HM-ELISA in all ranges of HIV RNA viral load. This means that the HM-ELISA+PSAM allows one to detect more positive samples than the conventional HM-ELISA in these concentration ranges. Indeed, as can be seen from Table 4, in Range 1 (viral load $< 3,000$ copies/ml), the conventional HM-ELISA cannot detect p24 antigen in any of the 14 studied positive samples, while HM-ELISA+PSAM can detect p24 antigen in 10 samples out of 19 positive samples (i.e. a clinical sensitivity of 52.63%). In the Range 2 (viral load between 3,000 and 30,000 copies per ml), p24 antigen was detected in 15 (32.6 %) and 42 (91.3 %) samples by HM-ELISA and HM-ELISA+PSAM, respectively out of total 46 positive samples studied. Similarly, in the range 3 (i.e. RNA viral load above 30,000 copies per ml), among 41 positive samples HM-ELISA+PSAM can identify 100 % as positives while the conventional HM-ELISA can identify only 52.3 % as positives. It is also worth noting that in the combined ranges of HIV concentrations 1 and 2 (RNA viral load below

30,000 copies per ml); 2 and 3 (RNA viral load above 3,000 copies per ml); and 1, 2 and 3 (all positives), the calculated sensitivities of the HM-ELISA+PSAM assays are significantly higher than HM-ELISA (Table 4).

The statistical significance of the aforementioned differences between the clinical sensitivities and specificities of the conventional HM-ELISA and HM-ELISA+PSAM was calculated using several statistical tests: Pearson Chi-Square Test, Likelihood Ratio Chi-Square Test, Mantel-Haenszel Chi-Square Test, and Fisher's Exact Test. All tests show that ELISA+PSAM has a significantly better clinical sensitivity than conventional ELISA for all 6 studied RNA viral loads ranges, and there is no significant difference in specificity between conventional HM-ELISA and HM-ELISA+PSAM.

3.3 Seroconversion detectability

Several factors, including testing method, individual host responses and viral characteristics, determine the time between virus exposure and when HIV RNA, antigen, and antibodies can be reliably detected. Viral RNA is generally detectable 11 days after exposure with nucleic acid amplification methods. The current serologic window period, i.e., when HIV infection is not detectable with antibody tests, is approximately 3–4 weeks. While circulating HIV antigens are theoretically detectable from as early as 2 weeks after exposure to 3–5 months, it is only practically detectable between 16–22 days after infection. Thus, conventional methods that detect the p24 antigen can shorten serologic window period by approximately 1 week (2–3 weeks after infection) [23].

We performed a preliminary study to compare the ability of the conventional HM-ELISA and HM-ELISA+PSAM to detect HIV p24 antigen when used to test serial bleed specimens from HIV-infected individuals. Three seroconversion panels PRB956, PRB958, and PRB973 (a total of 15 serial bleed specimens from 3 HIV-infected individuals) were tested. Every member of these seroconversion panels was screened by conventional HM-ELISA as well as HM-ELISA+PSAM. The data for the tests other than PE p24 HM-ELISA and HM-ELISA +PSAM were extracted from inserts provided with the seroconversion panels [24].

Table 5 shows the signal-to-cut-off ratios obtained for samples from the seroconversion panel PRB956. Samples with signal-to-cut-off ratios >1 , and consequently considered positives by the test, are marked with the bold font. One can see from Table 5 that in the panel PRB956, HM-ELISA+PSAM was able to detect p24 antigen in the 3rd, 4th and 5th members of the panel while all conventional ELISAs including PE HM-ELISA could only detect p24 antigen in the 4th and 5th members of the panel. It should also be noted that the RNA viral load for the 3rd sample in this panel is in the range between 3,200 and 9,100 copies/ml (as estimated by various NATs [24], which is lower than the estimated LOD for the conventional commercially available assays including PE p24 ELISA tests based on either acid- or heat-mediated ICD.

Table 6 shows a summation of the data obtained from 3 seroconversion panels studied. In the panel, PRB958, the HM-ELISA+PSAM assay detected the 3rd member onwards as positives while PE acid-mediated and HM-ELISA could detect only after the 4th member onwards of the panel PRB958 as positives. For this panel, the results obtained with HM-ELISA+PSAM

were coincident with some commercially available ELISAs while the PE ELISAs were coincident with others [24]. The HIV RNA load in panel member 2 is between 1,000 – 3,100 copies/ml, which is likely just below the limit of detection of HM-ELISA+PSAM. The 3rd member's samples contained between 130,000 and 290,000 HIV RNA copies/ml (as detected by different NATs [24]) which is above the LOD of both our HM-ELISA+PSAM and most sensitive, commercially-available p24 ELISA methods including PE ELISAs. Given the wide gap between RNA loads for the 2nd and 3rd members, seroconversion panel PRB958 is likely not an ideal panel for studying the early limit for detection of HIV antigen. It should be mentioned, however, that it is not clear why the conventional PE ELISAs were not able to detect p24 antigen in the 3rd panel member. We speculate that this has been caused by deterioration of one or more samples in the PRB958 panel (see below).

For the panel PRB973, HM-ELISA+PSAM detected from the 2nd member onwards as positives, while the conventional HM-ELISA and other commercially available ELISAs only could detect from the 3rd member onwards PRB973 as positives [24]. The HIV RNA load in panel member 2 is between 6,400 – 28,000 copies/ml which is within the limit of detection of HM-ELISA+PSAM.

Thus, for seroconversion panels PRB956 and PRB973, HM-ELISA+PSAM detects HIV p24 antigen earlier than other conventional assays by 5 days, for both panels. For the panel PRB958, HM-ELISA+PSAM detected HIV p24 antigen earlier than some commercially available assays and at the same time for others. In the latter case, however, the gap between RNA loads for the 2nd and 3rd members was too wide which makes the panel PRB958 is not suitable for estimation of the window period for detection of HIV p24 antigen. The data obtained for the panels PRB956 and PRB973 implies that the window period for detection of HIV p24 antigen can likely be reduced by 5 days through the use of HM-ELISA+PSAM. While these preliminary results are indeed promising, future large-scale clinical trials with many seroconversion panels are needed to substantiate the claim that this method will significantly impact the early detection of HIV infection.

Additionally, the signal-to-cut off ratio results presented in Table 6 suggest that HM-ELISA +PSAM can detect the HIV p24 antigen in Fiebig Stage I [3] at which point only nucleic acid (RNA) tests are generally sensitive.

Overall, number of samples identified as positives by different ELISAs for 3 seroconversion panels are summarized in Table 7. In aggregate, HM-ELISA+PSAM identified 10 samples as HIV-positives while other conventional HIV-1 p24 tests were able to identify just 7. In addition, in panels PRB956 and PRB973, HM-ELISA+PSAM identified 3 samples as positives while all other conventional ELISAs were able to identify only 2 (Table 7). Thus, the preliminary data demonstrate improved HIV p24 antigen delectability in seroconversion panels by the HM-ELISA+ PSAM.

4. Discussion

The results obtained in this study confirm that HM-ELISA+PSAM dramatically increases the analytical and clinical sensitivity of a commercially available assay, the conventional

HM-PE p24 antigen ELISA. Given the clinical challenges involved in detection and treatment of AIDS, PSAM technology holds the potential to significantly improve the diagnostic utility of p24 immunoassays. Indeed, our previous [8] and current experiments show that the analytical sensitivity of the p24 antigen assays and, consequently, their dynamic range, is increased approximately 40-fold as compared to the conventional tests. The documented lower detection limit of the PerkinElmer Ultrasensitive HIV-1 p24 ELISA, which uses a rather complex tyramide amplification system (ELAST), is around 0.5 – 1 pg/ml [11,25]. Therefore, the analytical sensitivity of the HM-ELISA+PSAM, 0.1 – 0.2 pg/ml, is approximately 5-fold higher than that of the conventional Ultrasensitive assay, which uses a comparatively labor intensive and expensive ELAST amplification system.

It should be noted that in the above calculations, the HM-ELISA+PSAM analytical sensitivity was, mainly, estimated based on the use of the cut-off value equal to the twice the background signal obtained for “ideal” negative (normal) serum. In order to determine clinical sensitivity and specificity of the assay, the real cut-off value needed to be determined. For this purpose, we used ROC analysis, in which clinical sensitivity and specificity were determined for several cut-off values, and the test cut-off was selected based on the acceptability of clinical sensitivity and specificity. In this study, we selected cut-offs calculated based on the formula, $\text{cut-off} = (1 + k) \times \text{OD}_{450 \text{ nm}} (0.31 \text{ pg/ml})$. Such an approach for selecting cut-offs is quite common. For example, similar strategies for obtaining cut-offs based on percentages of absorbances of a positive control signal were utilized in detection of Schmallenberg antibodies in ruminants using whole blood antigen. A range of 8–15 % for ratio of absorbance of sample to absorbance of positive control gave acceptable sensitivity and specificity of 99.19 % and 100 % respectively [26].

In this study, the cut-off, selected based on the ROC analysis, is equal to $1.2 \times \text{OD}_{450 \text{ nm}} (0.31 \text{ pg/ml})$. This cut-off corresponds to the LOD for detection of p24 antigen of approximately 0.4 pg/ml. This LOD is 2-fold greater than the LOD estimated based on using the twice the background (negative control) value. An increase in the assay cut-off, and, consequently, the corresponding LOD, is a common phenomenon when real clinical samples are used for LOD determination as compared to those estimated using just negative control.

As an example of how real LODs differ from estimated LODs, the analytical sensitivity of the PE Ultrasensitive assay using HM-ICD ELISA with ELAST amplification [11] was determined as follows: a sample was considered positive if the absorbance of that sample was greater than or equal to the cut-off, which was defined as the sum of mean of 6 negative controls and 5 times the standard deviations between these 6 negative controls [27], or as the sum of mean of 4 negative controls and 3 times the SD between these 4 negative controls [28]. As mentioned above, the estimated analytical sensitivity of this test for p24 determination is 0.5 – 1 pg/ml. However, the cut-offs for p24 positivity and for p24 negativity for the Ultrasensitive PE HIV-1 p24 assay for real Tanzanian plasma samples were calculated as corresponding to 3.5 pg/ml and 2.3 pg/ml, respectively [29]. These real LODs are 2.5 - 7-fold higher than those for the estimated LODs of the same assay (0.5 – 1 pg/ml). The effect of increasing of the assay cut-off, and hence LOD, when real clinical samples are used for its determination may be caused by a number of factors, including sample type and sample preparation procedure, the presence of interfering substances in the real clinical

samples and others [30]. For example, in this study we showed that the non-specific binding for serum negative samples is less significant than that for plasma samples. Although serum samples are generally favored for use in most ELISAs, there are many instances where plasma samples may contain higher quantities of the analyte of interest than serum [31], and thus plasma samples may be preferable for analysis by ELISA in some situations.

In order to estimate the clinical sensitivity of the HM-ELISA+PSAM for patients at different stages of the HIV infection, we used samples divided into 3 primary groups with various HIV viral load. A similar approach was followed in a study where samples were divided into 3 groups depending on whether their cell associated HIV DNA load was 100, 500 or 2,500 DNA copies/ 10^6 peripheral blood mononuclear cells [32]. Our approach permitted us to evaluate the conventional HM-ELISA and HM-ELISA+PSAM sensitivities in each of the studied concentration ranges and compare the results with a gold standard HIV molecular assay. In this pilot study, we showed that the HM-ELISA+PSAM is more sensitive than the conventional HM-ELISA assay over all primary 3 RNA viral load concentration ranges. The difference is particularly significant in the ranges of RNA viral load below 30,000 copies/ml. Indeed, when RNA viral load is under 3,000 copies/ml, conventional HM-ELISA cannot detect any HIV positive samples whereas HM-ELISA+PSAM can detect approximately half of the samples, with HM-ELISA+PSAM having a clinical sensitivity of 52.63%. When RNA viral load is between 3,000 and 30,000 copies per ml, the clinical sensitivities of the conventional HM-ELISA and HM-ELISA+PSAM are 32.6% and 91.3%, respectively. When viral RNA load is >30,000 copies/ml, HM-ELISA+PSAM was able to detect 100 % of the HIV- positive samples while clinical sensitivity was only 52.3% for the conventional HM-ELISA.

In other studies, it has been shown that for ultrasensitive HM-ICD p24 antigen ELISA with tyramide signal amplification, clinical sensitivity for HIV positive samples with viral load >30,000 copies of RNA per ml was 99% [33] and 100 % [18]. While at this range (i.e., with viral RNA load >30,000 copies/ml) clinical sensitivity is comparable to that of HM-ELISA +PSAM, it is at the lower ranges (viral RNA load <30,000 copies/ml), that HM-ELISA +PSAM has significantly higher (80%) clinical sensitivity (Table 4) as compared to that achieved by ultrasensitive HM-ICD p24 ELISA (46.4%) [18]. A direct comparison between the ultrasensitive p24 antigen assay and HM-ELISA+PSAM assays to detect HIV-positive samples with viral RNA load under 30,000 copies/ml will, however, be required to fully comparatively assess the capabilities of the 2 systems for detecting earlier stages of HIV disease progression.

An additional confirmation of the fact that HM-ELISA+PSAM is more sensitive than HM-ELISA comes from the analysis of the results obtained using seroconversion panels. These results showed that HM-ELISA+PSAM allows one to detect HIV infection approximately 5 days earlier than conventional HM-ELISA, and more seroconversion panel members were identified as positives by HM-ELISA+PSAM than by conventional HM-ELISA and commercially available ELISA tests (see Table 7).

The results obtained by the HM-ELISA+PSAM for seroconversion panels showed that HM-ELISA+PSAM is more sensitive than conventional HM-ELISA, but these results could have

been even more impressive. From Table 7, it is evident that conventional PE p24 acid-mediated (kit insert information) and heat-mediated ELISA (our data) identify the same panel members, and hence the same number of panel members, as positives. In each case, though, the signal-to-cut-off ratio is higher for acid-mediated ELISA than for HM-ELISA (Table 5 for panel PRB956 and data not shown for panels PRB958 and PRB973), which is inconsistent with the fact that the heat-mediated ELISA is much more sensitive than the acid-mediated ELISA [[9], and our own results]. For a direct comparison, we performed acid-mediated ELISA and compared the obtained results with those provided in the inserts of all 3 seroconversion panels studied. As can be seen from Table 5, for the seroconversion panel PRB956 the signal-to-cut-off ratios obtained by PE acid-mediated ELISA as per the information provided in the seroconversion panel insert are higher as compared to those obtained by us using both acid-mediated ELISA and HM-ELISA. The similar results were also obtained for other 2 seroconversion panels studied. This fact could be explained by the deterioration of the samples since their initial testing. Such deterioration of the samples over time is possible (as per personal conversation with Scientific and Technical Sales Manager, Seracare, Dr. Rachel Gao), and could be caused by multiple freeze/thaw cycles or occur during aliquoting, storage and transportation. If not for the anomalous results in seroconversion panels, the results obtained by HM-ELISA+PSAM would have been even more impressive. While this preliminary study included only 3 seroconversion panels, more vigorous validation studies of the HM-ELISA+PSAM are required before conclusively claiming that this method will shorten the window between virus exposure and HIV p24 antigen detection in human serum or plasma. Regardless, the results of the present study clearly show superiority of the HM-ELISA+PSAM over the conventional PE acid- or heat-mediated ELISA in terms of the clinical sensitivity.

As mentioned above, several signal amplification strategies have been applied to p24 ELISAs in order to address the need for more sensitive detection of HIV-1 p24 antigen. The advantages of the HM-ELISA+PSAM assay over other signal amplification methods include ultrasensitive detection capabilities and extended dynamic range of the assay without the use of the complex and expensive equipment. PSAM technology increases analytical and clinical sensitivity of conventional HM-ELISA and requires only 1 additional short step of illumination of the microtiter plate using an inexpensive illumination device. Unlike other ultrasensitive techniques, the PSAM is simple and straightforward, achieving markedly improved results from commercially available ELISA reagent systems.

5. Conclusions

Application of the photochemical signal amplification method (PSAM) to heat-mediated immune complex disruption ELISA has enhanced the analytical sensitivity approximately 40 times over the conventional Perkin Elmer HM-ELISA for the detection of HIV-1 p24 antigen. This provides a much cheaper and less labor-intensive procedure as compared to PE Ultrasensitive Assay with tyramide signal amplification. As part of our preliminary validation studies for the PE HM-ELISA+PSAM, we have demonstrated that this technology has much higher clinical sensitivity in detection of HIV-1 infection in patients with RNA viral loads >3,000 copies/ml. Moreover, this technology has more success in detecting p24 antigen in samples with the RNA viral load less than 3000 copies/ml as compared to the

conventional ELISA. While our current study of >200 clinical samples serves to establish our method with a high analytical sensitivity and acceptable clinical performance, it will be necessary to undertake a more comprehensive study with a much higher number of patient samples to further demonstrate its clinical significance.

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Highlights

- HIV p24 ELISA with photochemical amplification (ELISA+PSAM) was validated.
- Over 200 clinical samples were used for preliminary validation of ELISA+PSAM.
- ELISA+PSAM's clinical sensitivity is higher than that for ELISA at low RNA load.
- ELISA+PSAM presents significant advancement in monitoring HIV disease progression.
- ELISA+PSAM shows potential to detect HIV p24 earlier than other immunoassays.

Figure 1.

A scheme of oxidation of OPD catalyzed by horseradish peroxidase (HRP) (1.1) and autosensitized by 2, 3-diaminophenazine (DAP) (1.2).

Figure 2.

Calibration curves for determination of HIV-1 p24 antigen prepared for the conventional HM-ELISA (A) and the HM-ELISA+PSAM at different times of illumination (B). The calibration curve obtained after 8 min of illumination (B, closed large squares) provides the highest sensitivity of the assay, with a lower limit of detection of 0.2 pg/ml.

Table 1

Accuracy and inter-assay (within-laboratory) precision of HM-ELISA+PSAM.

Sample	Theoretical Concentration of p24 antigen (pg/ml)	Accuracy		Inter-assay precision
		Mean Calculated Concentration of p24 antigen (pg/ml)	Mean Relative error (%)	
1	0.312	0.322	3.24	31.00
2	0.625	0.680	8.79	9.08
3	1.25	1.221	2.28	14.45
4	2.5	2.667	6.66	18.12
5	5.0	5.361	7.22	14.32

Table 2

ROC analysis of HM-ELISA+PSAM using HIV-1 positive and negative samples.

No. of samples	Sample type	RNA Range No.	RNA load (copies/ml)	cut-off 1	cut-off 2	cut-off 3	cut-off 4	cut-off 5
				Sensitivity (True positive (TP) fraction, %)				
87	Plasma positives	2&3	3,000	98.85	97.70	96.55	95.40	95.40
				Specificity (True negative (TN) fraction, %)				
50	Plasma negatives	N/A *	0	84	86	90	98	100
26	Serum negatives	N/A *	0	100	100	100	100	100

* N/A - not applicable.

Table 3

Clinical specificity of HM-ELISA+PSAM with cut-off 4 for plasma and serum samples.

Sample type	Method used	Total samples	Negative by test	False positives	Clinical Specificity, %
Plasma	HM-ELISA+PSAM	50	49	1	98
Serum	HM-ELISA+PSAM	26	26	0	100
Total	HM-ELISA+PSAM	76	75	1	98.68

Table 4

Clinical sensitivities of HM-ELISA and HM-ELISA+PSAM for plasma samples in various ranges of HIV concentrations.

Range No.	RNA viral load (copies/ml)	Method	Total No. positives	No. Positives by test	Clinical sensitivity, %	Lower - Upper confidence interval
1	3,000	HM-ELISA	14	0	0	0 – 0
		HM-ELISA+PSAM	19	10	52.63	30.2 – 75.1
2	3,000 – 30,000	HM-ELISA	46	15	32.61	19.1 – 46.2
		HM-ELISA+PSAM	46	42	91.30	83.2 – 99.4
3	30,000	HM-ELISA	42	22	52.3	37.2 – 67.4
		HM-ELISA+PSAM	41	41	100	100 – 100
1 & 2	30,000	HM-ELISA	60	15	25.0	14.0–35.96
		HM-ELISA+PSAM	65	52	80.0	70.3–89.7
2 & 3	3,000	HM-ELISA	88	37	42.04	31.7 – 52.4
		HM-ELISA+PSAM	87	83	95.40	91.0 – 99.8
1, 2&3	All positive	HM-ELISA	102	37	36.27	26.9 – 45.6
		HM-ELISA+PSAM	106	93	87.74	81.5 – 94.0

Table 5

The data for ELISA-based HIV p24 tests for samples from Seroconversion panel PRB956.

Panel members	HIV RNA * (x10 ³ , copies/ml)	Signal-to-cut-off ratio						
		Insert information				Present study		
		Abbott HIV Ag-1 Monoclonal EIA	Coulter HIV-1 p24 Antigen	Innogenetics HIV-1 p24 ELISA	PE HIV p24 Acid mediated ELISA	PE HIV p24 Acid mediated ELISA	PE HIV p24 HM- ELISA	PE HIV p24 HM- ELISA +PSAM
PRB956-01	BLD **	0.3	0.3	0.3	0.3	0.37	0.26	0.52
PRB956-02	0.71–0.84	0.4	0.3	0.4	0.3	0.36	0.25	0.59
PRB956-03	3.2 – 9.1	0.3	0.3	0.4	0.4	0.36	0.28	1.05
PRB956-04	63–230	1.5	3.2	7.5	5.9	0.89	1.15	2.41
PRB956-05	60–570	3.5	7.2	20.2	17	1.75	2.84	3.04

* As determined recently by various methods (insert information);

** BLD = below the limit of detection

Table 6

The data for HIV virus detection tests for samples from Seroconversion panels PRB956, PRB958 and PRB 973

Panel no.	Member no.	Days since first bleed	Fiebig stage*** [3]	HIV RNA* (x10 ³ , copies/ml)	Signal-to-cut-off ratio		
					Other commercial tests*	Present study	
						PE HIV p24 HM-ELISA	PE HIV p24 HM-ELISA +PSAM
PRB956	PRB956-01	0	I	BLD**	<1 (0.3)	0.26	0.52
	PRB956-02	40	I	0.71–0.84	<1 (0.3–0.4)	0.25	0.59
	PRB956-03	42	I	3.2–9.1	<1 (0.3–0.4)	0.28	1.05
	PRB956-04	47	II	63–230	<1 (0.3–0.4)	1.15	2.41
	PRB956-05	50	III	60–570	>1 (3.5–20.2)	2.84	3.04
PRB958	PRB958-01	0	I	BLD** -0.43	<1 (0.3–0.6)	0.25	0.38
	PRB958-02	2	I	1–3.1	<1 (0.3–0.4)	0.24	0.29
	PRB958-03	7	I	100–290	(0.8–5.4)	0.86	2.62
	PRB958-04	9	II	300–700	>1 (2.1–17.8)	1.86	3.27
	PRB958-05	15	III	40–1600	>1 (2.9–19.8)	3.01	3.52
	PRB958-06	17	III	470–1200	>1 (3.3–21.5)	2.93	3.47
PRB973	PRB973-01	0	I	0.68–2.7	<1	0.28	0.73
	PRB973-02	2	I	6.4–28	<1	0.33	1.05
	PRB973-03	7	II	91–220	>1	1.20	3.98
	PRB973-04	11	III/IV	500–2200	>1	4.64	5.42

* As determined recently by various methods (insert information);

** BLD = below the limit of detection;

*** Fiebig stages for HIV detection in blood: stage I: only RNA assay positive; stage II: RNA and HIV-1 p24 antigen tests positive, antibody EIA non-reactive; stage III: RNA, HIV-1 antigen and HIV IgM-sensitive EIA reactive, but Western blot without HIV-1-specific bands; stage IV: as stage III, but indeterminate Western blot pattern; stage V: as stage IV, but reactive Western blot pattern, without p31 (pol) reactivity; stage VI: as stage V, but full Western blot reactivity.

Table 7

Number of samples identified as positives by various tests in 3 seroconversion panels.

Panel	No. of samples	Insert information				Present study		
		Abbott HIV EIA	Coulter p24 Antigen	Innogenetics HIV-1 p24 ELISA	PE HIV p24 Acid mediated ELISA	PE HIV p24 Acid mediated ELISA	PE HIV p24 HM-ELISA	PE HIV p24 HM-ELISA +PSAM
PRB9 56	5	2	2	2	2	1	2	3
PRB9 58	6	3	4	4	3	3	3	4
PRB9 73	4	2 **	N/A	2 *	2	1	2	3
Total	15	7			7	5	7	10

* - Abbott 4th generation Antigen/antibody Combo test;

** - bioMérieux VIDAS p24 II.