

# P24 ANTIGEN (DETECTED BY HYPERSENSITIVE ASSAYS), A TECHNICAL SOLUTION IN HIV INFECTION MONITORING: REVIEW

*Antigenul P24 (detectat prin tehnici hipersensibile), soluție tehnică în supravegherea infecției HIV: trecere în revistă*

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## ABSTRACT

In resource-constraint settings, aggravated by the actual global recession, but where the overwhelming majority of HIV/AIDS infections worldwide occur, the viral load (HIV-1 RNA) testing is limited. To date, in specialized and well founded centers, the treated or naive patients are monitored by testing the HIV-1 RNA using PCR and real-time PCR amplification kits. A potential alternative is HIV-1 p24 boosted ELISA assay (p24), a technology accessible for every hospital laboratory, which costs ten times less, at least, as compared to molecular assays.

Although p24 is of demonstrated predictive value for the CD4 decline and disease progression, it was not immediately accepted as appropriate alternative to the viral load.

At present, p24 is used and recognized as diagnostic test in children born to positive mothers, having chance to become alternate monitoring test in chronic HIV/AIDS patients.

**Key words:** viral load testing, molecular assay

## REZUMAT

În condițiile unor resurse limitate, agravate de actuala recesiune globală, dar în cadrul căreia au loc majoritatea infecțiilor cu virusul HIV/SIDA pe întregul glob, testarea încărcăturii virale (HIV-1 RNA) este și ea limitată. În prezent, în centre specializate construite în acest scop, pacienții suspecți sau tratați sunt monitorizați testându-li-se HIV-1 RNA folosind PCR și kituri PCR de amplificare în timp real. O alternativă posibilă este tehnica hipersensibilă ELISA (p24), o tehnologie accesibilă oricărui laborator de spital, care costă de zece ori mai puțin, cel puțin, în comparație cu tehnicile moleculare. Deși p24 are o valoare predictivă demonstrată pentru scăderea CD4 și pentru progresia bolii, nu a fost imediat acceptată ca o alternativă potrivită la aflarea încărcăturii virale.

În prezent, p24 este folosit și recunoscut ca test de diagnostic la copiii născuți din mame pozitive, având șansa să devină o alternativă viabilă de test de monitorizare la pacienții infectați cronic cu HIV/SIDA.

**Cuvinte cheie:** test al încărcăturii virale, tehnică moleculară

In resource-constraint settings, aggravated by the actual global recession, but where the overwhelming majority of HIV/AIDS infections worldwide occur, the viral load (HIV-1 RNA) testing is limited. To date, in specialized and well founded centers, the treated or naive patients are monitored by mainly testing the HIV-1 RNA using PCR and real-time PCR amplification methodology.

Nucleic acid-based amplification assays are the mainstay of viral load monitoring in high-income countries. Assay design is complicated by the high level of genetic heterogeneity characteristic of HIV-1 and the emergence of recombinant strains. (1,2) This diversity remains an ongoing challenge for assay development and necessitates constant molecular surveillance.

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Increasingly, the trend in the field and in resource-poor settings is to move toward real-time technology options that are faster and have higher throughputs, larger dynamic ranges, and fully automated extraction steps. Examples include the Roche TaqMan and Abbott RealTime assays which are to date the only real-time assays with Food and Drug Administration approval. (3) These validated assays undergo quality control by the manufacturers, recognize most HIV-1 subtypes, and are familiar to many clinicians. However, these assays are still technologically complex and require physical resources, such as uninterrupted electricity, air conditioning, and access to clean water that may not be available in all less-developed settings. The cost of capital equipment to conduct nucleic acid testing, the high costs of reagents and consumables, together with lack of funds for public health programs, prohibits implementation on a large scale in many resource-poor environments.

Plasma viral load (HIV RNA) quantification was always considered the most representative and sensitive laboratory test for monitoring progression of HIV infection and response to antiretroviral therapy. (4,5)

Active replication of virus occurs in all clinical stages of infection. It is possible to detect and quantify virus throughout the course of HIV infection. Measurement of levels of HIV RNA over time have been of great value in delineating the relationship between levels of virus and rates of disease progression, viral turnover, the relationship between immune system activation and viral replication, and the time to development of antiviral drug resistance. Also the HIV RNA assays allow an evaluation of the changes in viral burden that occur over a matter of hours, whilst p24 antigen capture assays reflect changes in total viral burden over an extended period. Measurements of HIV RNA levels should be made approximately every six months and more frequently in a setting where changes are made in antiretroviral therapy (ART, HAART).

However, testing the viral load shows some limits yet, in terms of: specificity (false positives in the absence of contamination/carryover, or false negatives still appear); (6) the sensitivity is not absolute (approximate 20 copies/ml at best); the circadian variability; the interference of cortisol levels; (7) nevertheless, the technology, the personnel skills, and the cost.

Since the beginning of the HIV natural history, testing for p24 was found being of value in detecting early HIV infection, screening blood, diagnosing infection in the newborn, and monitoring antiviral therapy. (8)

P24 is a diagnostically important HIV-1 protein that has been used for years in diagnostic assays such as the fourth generation HIV-1/2 (combo) screening assays. (9,10) The production and release of p24 and of particle-associated HIV-1 RNA from infected cells are tightly linked, as they both derive from unspliced HIV-1 messenger RNA. P24 is a component of the HIV-1 precursor proteins Pr160 (*gag-pol*) and Pr55 (*gag*), thus being stoichiometrically linked with the nucleocapsid p9, which is directly involved in the encapsidation of HIV-1 RNA. P24 is estimated to be present at 1,200 molecules in each virion. (11) Ultracentrifugation experiments demonstrated that, in contrast to HIV-1 RNA, p24 antigen may also be found outside infected cells or particles, either as a soluble protein in plasma, as a protein bound in immune complexes, or as a component of a subviral particle. Consequently, HIV-1 RNA and p24 antigen, despite being closely interrelated, may originate from sources, which are common in part, but not identical. (12)

Standard ELISA assays can provide indirect measures of viral load, such as p24 levels, that require less equipment and skills, but these detection systems are frequently faced with several problems, as follows. A major limitation is that the p24 test is insensitive when testing blood, both because low levels of antigen are difficult to detect, and because antigenemia occurs only transiently during different stages of infection. (13)

In normal conditions, p24Ag is bound with the corresponding antibodies in immune complexes that can mask its presence and cause under-detection or false negative results. A second problem is the presence of immunoglobulin-specific, rheumatoid factor-like antibodies, which may cause over-detection or false positives by bridging the capture and the tracer antibodies of the ELISA antigen test. A third problem is the low sensitivity of the test compared to nucleic acid-based methods. Another limitation was the shortage of information regarding the capability of p24 detection of various HIV subtypes. Also, it has been reported that the degree of antigenemia seems to vary depending on the population tested. For example, several studies have shown that African patients with AIDS have higher levels of antigen than a similar group of individuals in the United States. (14) Conversely, African patients infected with HIV but who are asymptomatic have been shown to have lower levels of p24 antigenemia than a similar group in the United States.

To improve sensitivity of the p24 antigen assay, manufacturers introduced an Immune Complex

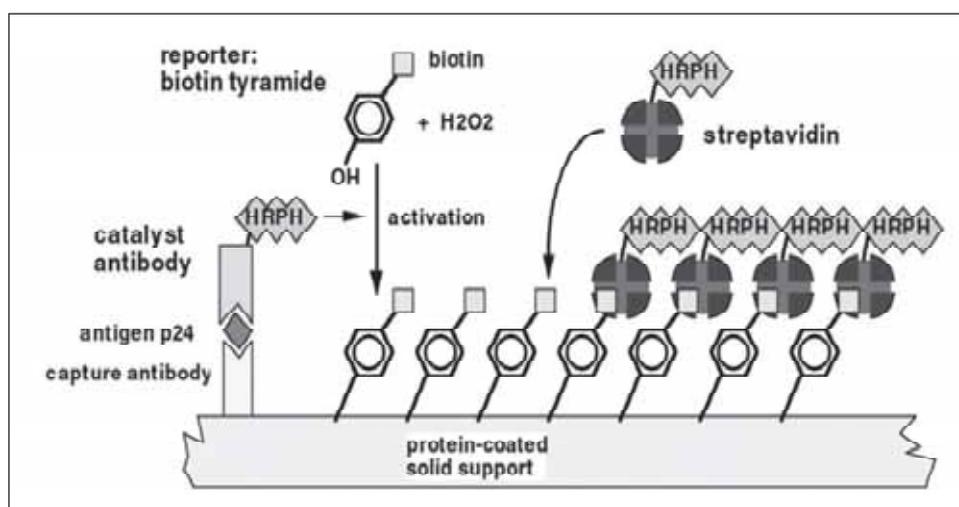
Dissociation (ICD) procedure using low pH or/and thermal treatments to dissociate p24 antigen/anti-p24 antibody complexes before performing the antigen assay. Using this procedure, an increased sensitivity of the assay was demonstrated, particularly for asymptomatic HIV-infected individuals. This dissociation procedure allows for detection of both free p24 antigen and complexed p24 antigen/antibody. The method not only increases the number of antigen positive individuals (epidemiologic sensitivity), but also can detect lower amounts of p24 antigen (analytical sensitivity). (15) Although ICD may result in an overall increase in sensitivity of the antigen test, detection of all HIV-infected individuals remained only about 50%.

Soon, reports have indicated that the p24 antigen assay can be modified to increase its sensitivity for detecting early HIV infection to a level approaching that of the PCR (viral load) test, at a much reduced cost and with faster turnaround times. During the past decade the p24 test has been significantly further improved. Elimination of antibodies that interfere with the test by heat denaturation permitted unobstructed measurement of the p24. (16) Moreover, boosting the p24 ELISA by tyramide signal amplification significantly decreased the detection limit to about 0.5-1 pg/ml. (17) The schematic principle of the improved reaction is shown in Figure 1.

The high diagnostic sensitivity achieved by these modifications have been published since. (20-

26) In the early 1990s, studies in patients soon after seroconversion found that the presence of p24 was a stronger predictor of progression to AIDS than was HIV-1 RNA concentration. (27,28) All those studies showed, however, that the p24 tests then available were considerably less sensitive than RT-PCR for HIV-1 RNA. (27-29)

However, p24 may be more effective in monitoring and predicting disease outcome, (28) and several studies have demonstrated the predictive value of p24 for CD4 decline, disease monitoring and ending. (30,31) It was shown that during primary HIV infection subjects with higher concentrations of p24 antigen tend to have higher plasma HIV titers, and a greater likelihood of progression to AIDS for HIV infected subjects who are positive for serum p24 antigen. (32-34) Though precise significance of p24 antigenemia is unclear, it has been found that for a group of asymptomatic HIV infected patients having similar CD4+ T cell counts, those with detectable levels of p24 antigen, are three times more likely on an average to show progression to AIDS, over a period of 3 years, than those in whom p24 antigen levels cannot be detected. Among HIV antigen positive, asymptomatic, antibody positive homosexual males, 23.9% progressed to AIDS over a 21 month period, compared with 1.3% of HIV antigen negative, asymptomatic, antibody positive homosexual males. (35) In the San Francisco cohort study, (36) p24 antigen levels correlated with disease progression,



**FIGURE 1.** Principle of tyramide-mediated signal amplification of ELISA. (18) The tracer antibody which is labeled with horseradish peroxidase (HRPH), also known as conjugate, is used as a catalyst antibody for the activation of the biotin tyramide reporter molecule. The activated reporter binds to tyrosine residues of any immobilized protein. Added HRP-labeled streptavidin thus provides a highly increased number of enzyme molecules which in turn will transform more substrate molecules (H<sub>2</sub>O<sub>2</sub>) hereby generating an enhanced colored signal. Picture reproduced from (19).

with 59% of p24 antigen positive patients progressing to AIDS over three years compared to 15% of patients who were p24 antigen negative. In this study, rising level of circulating p24 antigen preceded a fall in the percentage of CD4 cells. In addition, multiple clinical trials have demonstrated that patients receiving anti-retroviral therapy show a decline in their circulating levels of p24 antigen. (37)

P24 proved its suitability over time for anti-retroviral treatment monitoring. Numerous reports in the literature describe the performance of the quantitative p24 antigen assay (Perkin Elmer/NEN HIV-1 ELISA p24 antigen kit) with a heat denaturation step for dissociating immune complexes for monitoring viral response under ART. (38-43) Some describe less successful outcomes, (44) and the assay is no longer being developed for viral load monitoring. P24 antigen testing is used less often, however, for monitoring the effectiveness of anti-HIV drug regimens now that RNA levels can be determined. (21,23,24,45,46)

The assay is now used widely as an alternative to nucleic acid testing for diagnosis of HIV infection during early infancy; this use appears to be the focus of the supplier. (26,38,47)

Nevertheless, p24 has not yet been totally accepted as an adequate alternative to HIV-1 RNA, mainly for two reasons. First, the p24 changes observed after initiation of HAART, treatment stop, or occurrence of viral failure are frequently less pronounced when compared to the changes of HIV-1 RNA. (48,49) Second, the test is also less sensitive than the meanwhile further improved tests for HIV-1 RNA which now detect less than 50 copies/ml. (24) Prompted by occasional observations that p24 was undetectable in samples from patients whose high HIV-1 RNA concentration should easily have permitted detection of the particle-associated p24, 50 an inefficient virus lysis by kit buffer (Triton-based) was suspected as a possible cause for the failure. This possibility was addressed and corrected by the introduction of a new buffer, based on an easy formulation (containing both denaturing and non-denaturing detergents) at hand in every laboratory. The SNCR buffer increased the measured p24 concentration about 1.5- to 3-fold in HIV-negative plasma reconstituted with purified HIV-1 particles, not affecting the background. Among 127 HIV-1-positive samples with moderate to high concentrations of HIV-1 RNA the increase was about 3-fold across the entire concentration range ( $P < 0.0001$ ). The specificity before neutralization of negative samples was 98.0% for the SNCR buffer

and 96.9% for kit buffer. Specificity after confirmatory neutralization of reactive samples or a follow-up test was 100% with either buffer. (51)

The possibility of using p24 assay method with ICD was carefully considered in our country since the early years of management of HIV infected patients. The first results were obtained by Dr. Duiculescu D., and defended in his MD thesis (52), followed nine years later by the works of Dr. Ionescu P. (52, 53) The third thesis yet to be finished belongs to Comanici M., BSc which focused on the p24Ag detected by hypersensitive methods in the diagnostic, evolution and surveillance of anti-retroviral therapy from HIV infection. Prompted by the acute need for adequate solutions for HIV/AIDS patient monitoring, we started by reproducing the boosted ELISA method of Schupbach and collaborators (54) for the quantitative detection of p24Ag in detergent/heat denaturated plasma samples. The kinetic reads were performed using the software Quanti-Kin CD Agp24 3-Plus. (55) The first results were communicated on the occasion of a local symposium, then at CEFOR conference and published in Romanian Journal of infectious Diseases. (56-58)

We concluded that this method which employs the thermal denaturation of detergent treated samples followed by tyramide catalyzed amplification of the enzymatic chromogenic reaction improved the ELISA test performances by 10 orders of magnitude to the limit (sensitivity) of detection, and at least one order of magnitude to the lower limit of quantification. These achievements encouraged the local opinion of infectionists about the value of the method with respect of reduced probability of false results, and a reliable improvement of analytical sensitivity, sufficient for p24 antigenemia detection in majority of HIV positive samples (17,59).

Although HIV RNA is a better prognostic marker for diseases progression to AIDS, p24Ag seems more adequate parameter for predicting the immune decline (CD4 loss) and the survival period. (30) The comparative results on our serial samples investigated support the assay efficacy in signalling the therapeutic failures, in a manner at least equivalent to Roche test used at the time (COBAS AMPLICOR HIV-1 Monitor version 1.5, Roche Diagnostic Systems, Inc. Branchburg, NJ). The complementary information brought by the content of therapeutic cocktails could help interpretation of results for p24 antigenemia, especially those so-called discrepant (undetectable p24 levels in HIV

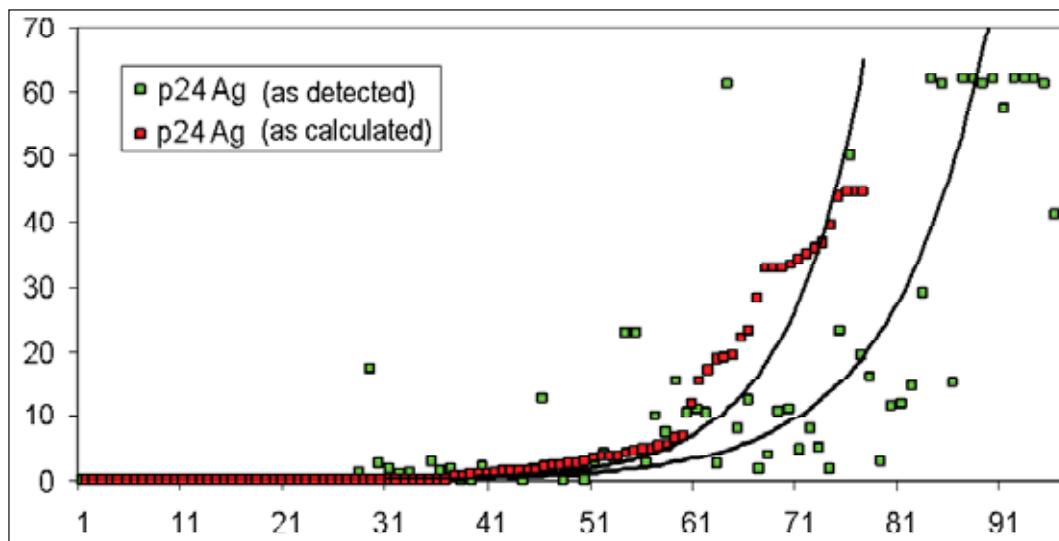


FIGURE 2

RNA positive patients, and undetectable viral loads in p24 positive samples).

In order to harmonize the quantitative values obtained for p24 and those of viral loads which are more familiar for clinicians, we compared the measured values to the calculated values for p24, considering 1,200 molecules for each HIV virion. The correlation was satisfactory as shown in figure 2.

The cost of the hypersensitive assay is about 10 times lower than PCR test, and facilities, instrumentation and technical skills required are common. The Quanti-Kin program operates under DOS platforms in any PC, and drives regular microplate readers; moreover, the use of this program is optional. Other commercial ELISA kits for the detection of p24 antigen could be used, proven their capability of recognizing denatured antigens, employing tracer antibodies conjugated to peroxidase/HRP, and OPD as chromogen (such as Genetic Systems HIV-1 Ag El A, Bio-Rad,

Hercules, CA HIV p24 Ag Assay, Coulter Inc., USA, etc.). All these arguments are pleadable for the introduction and scaling-up the ultrasensitive p24 testing in monitoring programmes of HIV/AIDS patients in Romania.

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