

# Infant HIV and Nanotechnology

N.Goel\*, R.Sharma\*\*

\*Department of Microbiology, Pt.BDS Post Graduate Institute of Medical Sciences, Rohtak, Haryana, India 124001 Email: [ngoel\\_2003@yahoo.com](mailto:ngoel_2003@yahoo.com)

\*\*Center of Nanomagnetism Biotechnology, TCC & Florida State University, Tallahassee FL 32304

## ABSTRACT

Background: New methods have emerged in detection and monitoring HIV using nanotechnology techniques but HIV in infants got less attention. Purpose: We report here the Human Immunodeficiency virus (HIV) and Acquired Immune Deficiency Syndrome (AIDS) of under 5 years old children, especially in developing countries. Study Design: Different from adult HIV, in infants it is a challenge of accuracy in diagnosing HIV infection. The success depends on accuracy and sensitivity of HIV infection diagnostics. Materials and Methods: Different HIV-1 DNA and RNA Assays, P24 antigen assay, CD4 counting and DBS methods were compared for adult and infant HIV diagnosis and feasibility in tertiary care hospital set up. Results: The HIV DNA and RNA assays were qualitative and semiquantitative methods. Other methods p24 antigen biobarcode amplification, nanoarray and FITC (Fluorescein-5-isothiocyanate) doped silica nanoparticle for CD4/CD8 counting were upcoming possible nanotechnology based methods but these are in infancy. Conclusion: HIV DNA and RNA techniques offer good HIV detection in infants. P24 antigen and CD4/CD8 counting offer better quantitative results and further precision by nanoparticle based miniature bar code analysis.

**Key words:** infant HIV, DNA, RNA, CD4/CD8 counting, nanobarcode

## 1 INTRODUCTION

case of HIV was detected in 1981. According to World Health Organization (WHO 2005), 40 million people got infected with HIV worldwide and over 25.8 million in sub-Saharan Africa South and Southeast Asia and Latin America [1]. It is estimated that untreated 3 million people die every year of HIV/AIDS. Drugs restraining HIV successful treatment depends upon early diagnosis of the Disease by highly sensitive laboratory diagnosis and monitoring techniques to diagnose the HIV infection accurately in the early phase of disease. Currently viral nucleic acid (DNA or RNA) detection tests are the most sensitive assay for the diagnosis and monitoring of HIV infection, but these tests are expensive, technologically complex, require expert technical staff and well equipped laboratory. There is a need to develop sensitive, technically simple, relatively inexpensive point of care type tests. It can be useful for the remote settings and less equipped laboratories. Nanotechnology is a significant advancement in molecular diagnostics. Recently several nanotechnology based techniques have been evaluated in medical testing and monitoring of HIV infection that could provide new tools for clinical diagnosis due to their potential for high degree of sensitivity, high specificity, multiplexing

capabilities and ability to operate without enzymes. In the recent past the various technologies that have shown the high potential to diagnose and monitor HIV infection include ultrasensitive nanoparticle (NP)-based assay, fluorescent dye doped nanoparticle assay, nanotechnology based biosensors and protein array. This paper will focus on currently available tests to diagnose HIV infection and recent developments in the field of diagnosis using Nano technology.

## 2 HIV ANTIBODY DETECTION

### 2.1 Current approach

HIV antibody detection is the most common screening method to detect HIV worldwide. Antibodies to HIV generally appear in circulation 2-12 weeks after the infection. Antibodies can be detected in blood, serum, plasma, saliva or urine. Enzyme immuno assay or enzyme linked immunosorbent assay are the easiest and most widely used tests. For the detection of antibodies many assays are available commercially. These assays differ in format, in the nature of antigens (viral lysate, recombinant antigens and synthetic peptides) used for capture, and the conjugates used for detection of antibodies. HIV antigen or antibody (depending upon the tests ) is attached to a solid phase (matrix or support). Matrix can be wells, strips of a microtitre plate (polystyrene), plastic beads, nitrocellulose paper etc. Conjugates are most often antibodies (IgM, IgG, IgA) or antigens coupled to enzymes (alkaline phosphatase, horse reddish peroxidase etc) fluorochromes, or other reagents that bring about a reaction that can be measured or visualized. Enzymes generate a color signal on interaction with the substrate and fluorochromes generate fluorescence. The color produced at the end of reaction is measured on Elisa reader as OD value. The sensitivity and specificity of different kits used is between 98% - 99.99%.

### 2.2 Rapid Antibody Detection Tests

FDA has approved several rapid antibody detection kits for the testing of HIV antibodies. List of approved assay is available on ([www.fda.gov/cber/products/testkits.htm](http://www.fda.gov/cber/products/testkits.htm))[.2] The assays used are based on principle of particle agglutination, immunodot (dipstick), immunofiltration, or immune chromatography. Rapid antibody tests are sensitive (99.3-100%) and specific (98.6-100%). The results of rapid tests kits are available within 20 minutes. These tests are technically simple, require no specific instrument and can be performed in remote settings.

Detection of oral antibody in saliva has many advantages over serum. It can be collected noninvasively, safe, easy to collect, store, ship and sufficient quantity can be taken repeatedly for analysis. The disadvantage of salivary sample

is that the antibodies may be present in lower amounts so a highly sensitive test is required to detect the antibodies in saliva. Recently FDA has approved Orasure Kit for detection of salivary anti-HIV antibodies. Future application of Nano technology based bio sensors for Salivary Anti HIV antibody detection is obvious. Recently nano technology based nano electrical-mechanical system (NEMS) biosensors have been developed that exhibit remarkable sensitivity and specificity for oral fluid analyte up to a single marker [3]. Although

antibody detection seems to be an easy and cheap diagnostic test yet there are certain limitations of using this test. The major limitation is that antibodies generally appear in circulation 2- 12 weeks after the infection so the test cannot be used for the persons who are in window phase (time from entry of virus in body to development of antibodies) of disease. Next important limitation of using the test is in the infants and children younger than 18 months because of maternal antibodies in blood.

**Table 1** Tests currently available for diagnosis of HIV infection

<ul style="list-style-type: none"> <li>• Antibody Blood tests</li> <li>HIV antibody enzyme immunoassay/ Enzyme linked immunosorbant assay(EIA/ELISA), Rapid HIV tests</li> <li>Immunofluorescent assays(IFA), Western blot, Immunoblot and recombinant immunoblot assay</li> <li>• Antibody tests on other fluids</li> <li>Oral (saliva) HIV antibody EIA/ELISA</li> <li>Urine HIV antibody/ELISA</li> </ul>	<ul style="list-style-type: none"> <li>• Other Blood Tests</li> <li>P24 antigen assay</li> <li>Plasma/serum viral load (HIV-1 RNA) such as: RT PCR, b DNA, NASBA</li> <li>Culture of peripheral blood mononuclear cells (PBMC)</li> <li>PBMC HIV DNA detection for HIV.</li> <li>• Tests available to monitor the disease</li> <li>CD4 / CD8 cell count</li> <li>Quantitative Viral RNA assay.</li> </ul>
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### 2.3 Detection of p24 Antigen

HIV p24 is a protein product of HIV replication that is present in varying amount in the blood of HIV infected individual. HIV p24 is an immunogenic protein so antibodies to p24 are also produced. Thus, it is present in the blood in the form of free p24 and antibody bound p24 (immune complex). The simplest of direct detection test of HIV virus component is p24 antigen capture assay. This is an enzyme immuno assay type test in which solid phase consists of antibodies to p24 antigen of the HIV. Specimens containing p24 antigen is incubated in the presence of antibodies to p24. An enzyme reaction resulting in the development of color in the subsequent steps of the test indicate a reactive test. The intensity of color developed is proportional to the amount of antigen present. Many commercial EIAs for detection and quantification of p24 antigen in serum, plasma, cerebrospinal fluid and or cell culture supernatant are available. p24 antigenaemia is transient during primary HIV infection. Throughout the course of HIV infection equilibrium exist between p24 and p24 antibodies. During the first few weeks of infection level of p24 antigen are high later after the development of p24 antibodies level of p24 antigen declines. The greatest use of p24 antigen detection assay is in the screening of patients suspected of having acute HIV viral syndrome since, viral antigen is present prior to the development of antibodies, diagnosing infection in infants and neonates, resolving discordant and equivocal serological results, monitoring response to antiviral therapy and detecting window period infections in transfusion services. Disadvantage of antigen detection assays include poor sensitivity (only 69% in patients of AIDS and low in neonates less than one month old), failure to detect antigen in patients having high titer of p24 antibody, failure to detect HIV-2 infection. The regular p24 antigen detection test is relatively insensitive and there FDA approved commercial kits [4] Lower limit of detection of the antigen is 10 pg /ml by this test. Such low level of antigen may not be detectable in serum even when virus is actively replicating. In fact only about 50-60% of AIDS patients, 30-40% of AIDs related complex and only 10% of asymptomatic patients have p24 antigenemia.

Several modifications have been made in p24 antigen detection assay to increase the sensitivity of test. To increase the sensitivity of p24 antigen assay it is necessary to dissociate anti- p24 antibody from p24 (immune complex dissociated p24 antigen detection test). One simpler method that is gaining support for the detection of HIV infection in infants is the heat-denatured, signal-amplified p24 (ultrasensitive p24 [Up24]) antigen assay[5]. Several p24 antigen detection assays have been developed by using immune complex disruption method, using more effective lysis buffer and incorporating a tyramide-mediated booster assay. It has been reported that the boosted ELISA can decrease the lower limit of detection of p24 antigen to about 1 pg/ml.

#### 2.3.1 Novel approach of detecting p24 antigen using Nano particles

Recently newer methods of detecting p24 antigen quantitatively using nano particle were developed. These methods are more sensitive (lower limit of detection is 0.1 pg/ ml). Nanoparticle based biobarcode amplification technique is an ultra sensitive nanoparticle based assay, called the biobarcode amplification (BCA) assay. It uses oligonucleotide (the barcodes) as surrogate for the indirect amplification of the disease markers and a micro array based chip detection method, which utilizes NP probes and a silver amplification process for signal enhancement and optical readout. In this technique anti p24 antibodies are coated on the surface of a microtitre plate. Targeted p24 antigen is captured on it. Second biotinylated p24 antibody is added, this immune complex is detected by streptavidin- coated gold nano particle. This whole complex is bound to the biotinylated barcode DNA LT68. Released biobarcode are hybridizes to chips (slide coated with LT68 complementary oligonucleotides). The released barcodes are identified with NP based scanometric detection. This assay can detect 0.1pg/ ml of HIV p24 antigen as compared to 10pg /ml by regular p24 antigen assay[6]. This assay may provide a simple and sensitive tool for HIV p-24 antigen detection in settings where HIV-RNA testing is not routinely performed.

Biobarcode amplification assay can be used to detect specific HIV 1 p24 Gag protein from plasma.

### 2.3.2 Application of nanoarray for detection of p 24 antigen

Dip-pen nanolithography is used to generate nano scale pattern of antibodies against the HIV-1 p24 antigen on gold surface. HIV -1 p24 antigen in plasma obtained directly from HIV-1 infected patients is hybridized to the antibody array in situ, and bound protein is hybridized to a gold functionalized nanoparticle probe for signal enhancement. The nanoarray features in the three component sandwich assay is confirmed by atomic force microscopy (AFM). It can detect plasma concentration of p24 antigen up to the .025 pg/ml. This level corresponds to the less than 50 copies of RNA /ml of plasma[7]

**2.4 Detection of HIV-1 DNA** assay allows for a small amount of DNA to be amplified exponentially. Amplification of proviral DNA allows detection of cells that harbor quiescent provirus as well as cells with actively replicating

virus. In HIV-1 DNA PCR assays double-stranded DNA located in PBMCs is separated into 2 single strands by heating. On cooling, the HIV-1 DNA strands re-anneal with complementary nucleotide sequences of HIV-1-specific primers in the reagent mixture, which allows synthesis of new complementary DNA strands. After 1 heating and cooling cycle, the number of DNA strands that contain the HIV-1 proviral sequence that originated in the test sample has doubled, and through repetition of these steps numerous times, amplification of the HIV-1 proviral DNA from the test sample proceeds in a logarithmic manner. After a set number of amplification steps, HIV-1 proviral sequences are detected by hybridizing amplified DNA to a synthetic, enzyme-labeled HIV-1 DNA probe. A positive result is indicated by a color change in a chromogenic substrate. It is the most sensitive test to diagnose HIV infection in infant and children. Novel approach of detecting HIV -1 virus DNA using Hyper-rayleigh Scattering Spectroscopy was reported using gold-nanorod-based sensing of sequence specific HIV-1 virus DNA[9]

Table 2: Different CD4/CD8 detection methods are shown using CD antibodies by enzymes or immunostaining methods.

Method	Manufacturer	Detection System
FACS Count	Beckton& Dickinson	Fluochrome labeled anti-CD3 Cd4 and CD8 monoclonal antibodies (MAb)
Coulter manual Cd4+ count Kit	Coulter Corp.	Beads conjugated to anti-CD4 MAb
Zymune Kit	Zynaxis Inc.	Anti-CD4 & CD8 MAb
Dynabeads	Dynal A/S	Magnetic beads coated with antiCD4 and cD8 mAb
Capecellia Immunoalkaline phosphatase assay	Sanofi Diagnostic Pasteur	Anti-CD4 and CD8 MAbSlide based staining with anti CD3,Cd4 and CD8 MAb

## 3 MONITORING IMMUNE FUNCTIONS: NANOTECHNOLOGY

CD4 + T-lymphocytes cells help to organize the immune system's response to various microorganisms including viruses. HIV infects these cells and uses them for replication. Even in an asymptomatic individual millions of CD4+ cells are infected by HIV and destroyed every day. With time, counts gradually decline and fall below the critical level of 500 cells/mm<sup>3</sup> and continue to decrease till death due to AIDS. The rate of decline of CD4+ lymphocytes is related to early viral set point. CD4 cell count is one of the important parameter in addition to plasma HIV -RNA for determining the rate of progression of HIV disease and AIDS death. This parameter is used routinely in management of HIV infected patients. Centre for Disease Control and Prevention (CDC) ,USA recommends prophylaxis in an HIV infected person whose CD4+ count is less than 200 cells/μL or CD4 + T lymphocyte percentage is less than 14%. In infants and young children lymphocyte subsets (including CD4 counts) are higher than those of adults. This fact shows the percentage of CD4+ T-cells of blood subsets, i.e., CD4/CD45%, CD4/CD8% or CD4/CD3% means a more reliable indicator of HIV infection than absolute counts in children. Various methods are used for quantification of CD4 + T-cells. Flowcytometry is the reference method but the technique is expensive, requires highly trained personnel and has high maintenance cost. A number of methods are

available (Table-2) and WHO recommends that anyone of these can be used depending upon the resources and expertise available locally. To know the percentage of CD4+ T-cell by using two fluorescent dyes of different emission wavelength, at least, one laser and two PMT detectors are in general needed. However, the above methods require antibodies with fluorescence dye of at least two different emission wavelengths or two individual detectors (PMT), which prevent the device from being portable. Also, the complexity of sample preparation process cannot fulfill criteria of WHO on a diagnostics for Third World. By these reasons, some diagnostic methods cheap and easy to use are proposed nowadays. However, still those proposed utilize two detectors or one detector with low resolution. Use of FITC (Fluorescein-5-isothiocyanate) doped silica Nanoparticle for CD4 counting FITC doped silica nanoparticles are 2,500 times brighter than FITC by theoretical calculation with assumptions of molecular density of FITC as 1kDa/nm<sup>3</sup> and nanoparticles tightly doped with FITC. Therefore, one detector is able to detect CD4% with FITC conjugated CD45 antibody and FITC-doped silica nanoparticles conjugated CD4 antibody. Fluorescence intensity between FITC conjugated CD4 antibody and FITC-SiNPs conjugated CD4 antibody is compared, two parameters can be measured using only single fluorescent dye doped SiNPs brighter [10]

### 3.1 Diagnosing HIV infection in Infants and children

Part of the controversy surrounding the various diagnostic assays and the difficulty in establishing HIV diagnosis in

new born relates to the timing of HIV infection. Infants who are presumed to have been infected with HIV in utero may have detectable virus at birth, whereas infants presumed to have been infected around the time of delivery may not have detectable virus until days or weeks after birth. Thus in non breast feeding infants an accurate diagnosis can be made during the first few weeks of life with virologic assays. Virological tests for the diagnosis of HIV infection include culture of virus, Nucleic acid amplification tests (NAAT) [i.e. tests that detects HIV -1 DNA or HIV-1 RNA] and assay for a viral capsid protein (p24 antigen).

### 3.2 HIV-1 DNA Assay

DNA PCR is positive in about 40% of infected infants at 48 hours after the birth and in 93% infected neonates by age 14 days. By far it is the most experienced test to diagnose HIV infection in infant and children. In several studies sensitivity was found to be as high as 90%-100% by 1 month of age. Similarly high specificity has been observed by 1 month of age in non breast fed infant. In a study done in India using in housed developed technique sensitivity of HIV DNA PCR was 100% whereas specificity was only 53.9% in infants of 1.5 months to 7 months. The DNA PCR kits should be validated by the WHO before they can be used widely for field implementation. Once validated, virological tests have proven effective in the field as shown in South Africa, where regulators approved Roche Amplicor HIV DNA PCR. The drawback of this technique is that it is expensive, complex laboratory equipments and skilled technician are required. Moreover there are chances of false positivity if laboratory contamination occurs, lack of sensitivity in diagnosing HIV infection at birth and inability to assess viral load but it is still widely used because it is cheaper than other virological assays.

### 3.3 HIV RNA Assay

HIV 1 RNA assays detect plasma (cell free) viral RNA using different techniques. Method of amplification of HIV-1 RNA include target (nucleic acid sequence based amplification and reverse transcriptase PCR) and signal (branched chain DNA) amplification techniques. Various studies reported HIV-1 RNA assay sensitivity ranging from 25-50% within first few days of life to 100 % by 6-12 weeks of age. Early studies in HIV exposed infants using HIV RNA PCR assay found that the method matched or exceeded the sensitivity and specificity of HIV DNA PCR and viral culture methods. Unlike HIV DNA PCR which is a qualitative test, HIV RNA is quantitative test which can measure the viral load in a patient. Thus it can be used to guide the initiation of ART and monitor treatment response. When HIV-1 RNA quantitative assay are used for the diagnosis of infants a plasma level of more than 10,000 copies/ ml is generally required before the assay result is interpreted as being positive. HIV RNA assay result of less than 10,000 copies/ml should not be interpreted as definitely positive and test should be repeated. HIV RNA PCR is done routinely to monitor HIV in infected patients in high income countries but owing to their high cost, automation, power requirement and technical complexities implementation of expanded viral

load testing technique has been delayed in resource limited countries. This method does have several drawbacks like false positive results for the patients with low viremia (<10,000 copies/ml) and decreased sensitivity of these assays in recipients of antiretroviral prophylaxis but more research is needed to answer the question whether ART exposure history of the mother or child affects the result of HIV-1 testing with NAAT.

### 3.4 Use of Dry Blood Spot(DBS) for infant diagnosis

Early infant diagnosis using virological assays leads to initiation of ART and thus reduces mortality and morbidity in infants, but owing to high cost of these technologies only few central laboratories can be set up with all sophisticated instruments and skilled technicians. Blood spotted on the filter paper is seen as an attractive alternative for collection and transportation. DBS does not require drawing blood and can be done using a single drop of blood from heel, toe or finger stick. HIV -1 DNA PCR assay using DBS is sensitive and specific test for the diagnosis of HIV -1 infection in infant. The difficulties that hinder the effective implementation of this programme are mainly labeling errors, transportation of filter paper, lack of quality control, cross contamination and delay in report. Moreover about a quarter of the children lost to follow up.

### 3.5 National AIDS control organization (NACO) guidelines for Diagnosis of Infant HIV

For HIV testing in infants: first HIV DNA PCR at age of 6 weeks, if positive the test is repeated immediately or as early as possible, if the first PCR test negative second PCR at 6 months is done. If the child was breast fed or was taking mixed feed and initial PCR test shows negative at 6 weeks, PCR test is done again after 6-8 weeks to exclude any infection. If symptoms develop at any time child should be tested at that age by PCR or Antibody detection. Report of HIV positive is only after 2 PCR tests show positive similarly HIV negative report was given after 2 Negative PCR tests. In India a National pediatric HIV /AIDS Initiative program was launched in Nov. 2006 with the collaboration of Clinton Foundation HIV/AIDS initiative (CHAI), NACO, various technical agencies and NGOs.

## 4 CONCLUSION

There is no doubt that nanotechnology constitute the solution of many important problems encountered in conventional diagnostic techniques and may open new areas of modern analysis. But even if a real progress is achieved in this field, further optimization research studies are required in order to fully explore their real potentials.

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