Human Immunodeficiency Virus Antibody Avidity Testing to identify recent infection among the newly diagnosed HIV-1 infected individuals and the effect of Antiretroviral Therapy on the Avidity Antibodies – A pilot study

Dissertation submitted as part of fulfilment for the M.D. (Branch-IV Microbiology) Degree examination of the Tamil Nadu Dr.M.G.R.Medical University, to be held in April-2017

CERTIFICATE

This is to certify that the dissertation entitled, "**Human Immunodeficiency Virus Antibody Avidity Testing to identify recent infection among the newly diagnosed HIV-1 infected individuals and the effect of Antiretroviral Therapy on the Avidity Antibodies – A pilot study**" is the bonafide work of Dr. Diviya Alex toward the M.D(Branch – IV Microbiology) Degree examination of the Tamil Nadu Dr. M. G. R. Medical University, to be conducted in April-2017.

Dr. Rajesh Kannangai

Guide

Professor and Head

Department of Clinical Virology

Christian Medical College

Vellore – 632004

Dr. V. Balaji

Professor and Head

Department of Clinical Microbiology

Christian Medical College

Vellore - 632004

Principal

Christian Medical College

Vellore - 632004

DECLARATION

I hereby declare that this M.D Dissertation entitled "Human Immunodeficiency Virus Antibody Avidity Testing to identify recent infection among the newly diagnosed HIV-1 infected individuals and the effect of Antiretroviral Therapy on the Avidity Antibodies – A pilot study" is the bonafide work done by me under the guidance of Dr. Rajesh Kannangai, Professor and Head, Department of Clinical Virology,Christian Medical College, Vellore. This work has not been submitted to any other university in part or full.

Dr. Diviya Alex

Department of Clinical Microbiology

Christian Medical College

Vellore-632004

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Human Immunodeficiency Virus Antibody Avidity Testing to identify recent

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1. Introduction

Human Immunodeficiency Virus (HIV) is the causative agent of Acquired Immunodeficiency Syndrome (AIDS) and was first isolated in 1983. It caused severe deterioration of the immune system. These are transforming viruses belonging [37] to the family *Retroviridae* and genus lentivirus. Human immunodeficiency virus is of two types – HIV-1 and HIV-2. Simian Immunodeficiency virus (SIV) is thought to have crossed species barrier from chimpanzees and sooty mangabeys to human beings resulting in [52] HIV-1 and HIV-2 respectively. The sequence homology between HIV-1 and HIV-2 is about 40-60%(1).

HIV is a major health issue worldwide. According to WHO, 36.7 million people are living with HIV worldwide. In 2015, 1.1 million people have died from HIV related causes worldwide(2). Among the people living with HIV about 0.8% are adults belonging to age group 15-49 years (3). The newly infected individuals with HIV were about 2.1 million globally. Sub-Saharan Africa ranked first with 25.6 million people living with HIV that is 70% of the global burden. Two thirds of the total new HIV infections in the world are in Sub-Saharan Africa (2). People living with HIV in Asia and the Pacific accounted for 5.1 million. There is an estimate of 300,000 new HIV infections in this region(4). In Southeast Asia, the prevalence of HIV infections in adults was 0.26% (3). New HIV infections have decreased by 35%. Death due to AIDS related causes have decreased by 28% between 2000 and 2015(2). Towards end of 2015, 17 million people living with HIV were receiving antiretroviral therapy (ART) worldwide(2).

1

India ranks third in the world with people living with HIV after South Africa and

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PAGE: 2 OF 86

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Contents

S.No	Content	Page no.
1	Introduction	1
2	Hypothesis and objectives	6
3	Review of literature	7
4	Materials and methods	32
5	Results	49
6	Discussion	70
7	Summary and conclusion	84
8	Bibliography	87
9	Annexure	96

1. Introduction

Human Immunodeficiency Virus (HIV) is the causative agent of Acquired Immunodeficiency Syndrome (AIDS) and was first isolated in 1983. It caused severe deterioration of the immune system. These are transforming viruses belonging to the family *Retroviridae* and genus lentivirus. Human immunodeficiency virus is of two types – HIV-1 and HIV-2. Simian Immunodeficiency virus (SIV) is thought to have crossed species barrier from chimpanzees and sooty mangabeys to human beings resulting in HIV-1 and HIV-2 respectively. The sequence homology between HIV-1 and HIV-2 is about 40-60%(1).

HIV is a major health issue worldwide. According to WHO, 36.7 million people are living with HIV worldwide. In 2015, 1.1 million people have died from HIV related causes worldwide(2). Among the people living with HIV about 0.8% are adults belonging to age group 15-49 years (3). The newly infected individuals with HIV were about 2.1 million globally. Sub-Saharan Africa ranked first with 25.6 million people living with HIV that is 70% of the global burden. Two thirds of the total new HIV infections in the world are in Sub-Saharan Africa (2). People living with HIV in Asia and the Pacific accounted for 5.1 million. There is an estimate of 300,000 new HIV infections in this region(4). In Southeast Asia, the prevalence of HIV infections in adults was 0.26% (3). New HIV infections have decreased by 35%. Death due to AIDS related causes have decreased by 28% between 2000 and 2015(2). Towards end of 2015, 17 million people living with HIV were receiving antiretroviral therapy (ART) worldwide(2).

India ranks third in the world with people living with HIV after South Africa and Nigeria(5). As per India HIV Estimation 2015 report, HIV adult (15-49 years) prevalence in India is 0.26%. Because of India's large population this equated to around 2.1 million people living with HIV in 2015. Among the states and union territories, Manipur had the highest adult HIV prevalence of 1.15%, Mizoram (0.80%) second in line and followed by Nagaland (0.78%), Andhra Pradesh & Telangana, Karnataka, and Gujarat (6,7). Andhra Pradesh &Telangana have the highest estimated number (0.3 million) of HIV infected individuals followed by Maharashtra, Karnataka, Gujarat, Bihar and Uttar Pradesh. These states account for two thirds (64.4%) of total estimated people living with HIV. According to HIV Sentinel Surveillance 2014-15, the prevalence of HIV in ANC clinic attendees continues to be low at 0.29%. The highest prevalence was seen in Nagaland (1.29%) followed by Manipur, Gujarat and Chhattisgarh(8).

According to the National Integrated Behavioural and Biological Surveillance (IBBS) 2014-2015 report, among the high risk groups – Intravenous drug abusers had the highest HIV prevalence of 9.9% followed by MSM 4.3% and Female Sex Workers 2.2% (9).

The various modes of transmission of HIV is sexual, parenteral by exposure to infected body fluids and from mother to child(2). HIV-1 targets a subset of T lymphocytes known as the CD4+T helper cells which serves as receptor for the virus. Entry of the virus into the T helper cells is followed by rapid and persistent replication. This results in dysfunction and depletion of CD4+T cell by various direct and indirect mechanisms. Most of the symptoms are due to decreased or weakened immunity(10). Once the

individual is infected the body starts producing antibodies against HIV-1. It is estimated that 50-70% of HIV infected individuals experience an acute syndrome approximately at 3-6 weeks of infection. Clinical latency is the period during which HIV infected individuals do not have any clinical symptoms. Clinical latency is defined as the time period from initial infection to the development of clinical disease and differs in different individuals. The median time for untreated HIV infected individual is 10 years. If an HIV infected individual has high levels of HIV RNA in plasma, they develop symptomatic disease faster than those individuals with low levels of HIV RNA. Thus comes the importance of an early detection(11).

HIV incidence is known as the rate at which a population acquires HIV infection and it can be used to measure the extent of current HIV transmission in the community(12). It is important for partner notification, to establish the incubation period, epidemiological surveillance. One of the methods of estimating the incidence is using laboratory tests for recent infection. Laboratory based are recommended to estimate HIV-1 incidence in cross sectional studies(13). This method provides direct measure of incidence and does not require repeated testing(14). Laboratory methods avoids the limitations of prospective studies such as bias, high cost and logistics.

Recent infection is the state that starts when an individual is infected with HIV for the first time and biological process of HIV infection is initiated. The duration of recent infection is usually defined as 6 months after initiation of the infection for first time.

Test for Recent HIV Infection (TRI) is defined as a laboratory test used to classify HIV infection as long term or recent HIV infection. Many different assays have been developed as Tests for Recent HIV Infections. Some of these assays are developed just

for identifying recent HIV infections while others are commercial assays used for HIV diagnosis which have been modified to identify recent infection. Avidity testing is based on fact that evolution and maturation of specific antibodies to HIV-1 occur within first two years of seroconversion. Avidity is defined as the strength of antigen-antibody binding when several epitopes on an antigen interact with several binding sites of an antibody and is known as the binding capacity of a maturing antibody to an antigen. The avidity of an antibody increases over time(15). The HIV-1 limiting antigen avidity enzyme immunoassay (LAg-Avidity EIA) is a quantitative avidity assay for differentiating recent HIV-1 infections from long-term HIV-1 infection. Individuals with recent HIV-1 infections have HIV-1 IgG with lower avidity compared to antibodies in HIV-1 infected individuals with long-term infections(16). This assay is based on the observation that when an individual is exposed to HIV-1 virus, the immune system initially produces HIV-1 antibodies with low avidity, and with progression of time, the immune system matures and produces HIV-1 antibodies with high avidity. More the amount of IgG antibodies with high avidity, the infection is more long term. The limiting antigen avidity assay is based on the principle that at high concentrations of antigen, antibodies with low avidity and antibodies with high avidity both can bind and because of the high density of antigen, the binding can occur with both binding sites of the antibodies. When the concentration of the antigen coated is lowered it permits the binding of only high avidity antibodies. Moreover, limiting the concentration of antigen prevents the binding with two sites simultaneously, so that the antibodies with low avidity will not be able to bind strongly to antigen. So based on this method we may be able to differentiate long term and recent infections(16). Studies show impaired

maturation and production of humoral immune response when HIV-1 infected individuals are treated early with antiretroviral therapy (ART)(17). When there is viral breakthrough that is the viral load is above 1000/ml in individuals who were on ART, the immune system reacts by increasing the proportion of HIV-1 specific antibody which may show changes in some of the tests for recent infection(18).

The main aim of this study was to determine the frequency of recent HIV-1 infections among the volunteers who attended the Integrated Counselling and Training Centre (ICTC) of a tertiary care centre by quantitative detection of matured HIV-1 IgG antibody levels using Limiting Antigen Avidity Enzyme Immunoassay (LAg Avidity EIA). We planned to compare the results obtained in LAg avidity assay with an in-house avidity assay and with the antibody pattern obtained in Western Blot. Also to study the avidity maturation of HIV-1 immunoglobulin among HIV-1 infected individuals on antiretroviral therapy (ART) using the LAg avidity assay.

2. Hypothesis and Objectives

Hypothesis

The Limiting antigen avidity assay is a useful technique to determine the quantity of matured immunoglobulin (IgG) to identify recent HIV-1 infection and ART leads to an impaired production and maturation of IgG in HIV-1 infected individuals.

Objectives

1. To determine the frequency of recent HIV-1 infections among the volunteers who attended the ICTC of CMC hospital by quantitative detection of matured HIV-1 IgG antibody levels using LAg avidity assay.
2. To compare the performance of LAg avidity assay with an in-house avidity HIV-1 antibody assay
3. To compare the normalized OD results obtained in the LAg avidity assay with HIV Western Blot antibody pattern to different antigens
4. To study the impact of antiretroviral therapy on avidity maturation of HIV-1 specific antibodies among HIV-1 infected individuals

3. Literature Review

3.1. History

Acquired immunodeficiency syndrome was recognized in US in 1981 when there were reports of pneumonia associated with rare organism like pneumocystis jirovecii among previously healthy homosexual men in United States. The term "acquired immunodeficiency syndrome," or AIDS was used first by public health officials in 1982. In 1983, Dr. Luc Montagnier in Pasteur Institute, Paris isolated the virus from an patient with generalized lymphadenopathy and named it as lymphadenopathy-associated virus (LAV)(19,20). Around the same time, Dr. Robert Gallo with his scientists in National Institute of health (NIH), Maryland isolated and named the virus as HTLV-III (human T-cell lymphotropic virus-type III), because it shared some features with HTLV I and HTLV II(21,22). Scientists under Dr. Jay Levy in University of California isolated the virus in 1984 and named it as AIDS associated Retroviruses (ARV)(23). In 1985, the name was changed to HIV (human immunodeficiency virus)(24). In 1986, the first case of AIDS in India was detected among commercial sex workers in Tamil Nadu(25).

3.2. HIV

3.2.1. Structure

Human Immunodeficiency Virus belongs to the family *Retroviridae* and genus lentivirus. The mature HIV particle is spherical and measures around 100nm. It has an outer host-derived lipid envelope. The major envelope proteins are glycoproteins gp120 and gp41 which forms the surface and transmembrane spikes respectively. It has a

cylindrical or conical inner core containing two identical copies of single stranded positive sense linear RNA. The core also contains enzymes that are required for functioning and replication of the virus – integrase, protease and reverse transcriptase(11,26,27).

3.2.2. Genome

The length of HIV-1 is 9.7kb. It has three structural genes that encode the structural proteins and six non-structural genes that encode various proteins that are involved in viral replication. The three main structural genes are the *gag, pol* and *env* genes. The *gag* gene encodes the core and matrix proteins – p24, p17, p6, p7. The *pol* gene encodes the enzymes – protease (p10), reverse transcriptase (p66/51) and integrase (p32). The *env* gene encodes the outer (gp120) and transmembrane (gp41) envelope proteins. The *gag-pol-env* genes are flanked by long terminal repeats which contain transcriptional regulatory proteins for gene expression. The non-structural genes are further divided into regulatory genes (*tat, rev* and *nef*) that encodes the regulatory proteins – transcriptional activator (p14), regulator protein (p19) and negative regulator protein (p27) respectively and the accessory genes (*vif, vpr* and *vpu*) that encodes the accessory proteins – viral infectivity factor (p23), viral protein R (p18) and viral protein U (p15-16) (11,27).

3.2.3. Molecular epidemiology

The phylogenetic clustering of HIV-1 viral isolates globally into clades or subtypes is based on 20-50% differences in envelope nucleotide sequences(28). They are classified into three groups. The group M (Main/Major), N (New, Non-M, Non-O) and O

(Outlier)(29). The *env* proteins of group M and O show as much as 30-50% variation. Group N is phylogenetically equidistant from group M and O(29). Group M has 9 different genetic subtypes A-D, F-H, J and K with E and I being circulating recombinants forms (CRFs)(28,30,31). The groups N and O are less frequent in human infections(32). Currently, 99.6% of all human infections globally is caused by group M of HIV-1 (32). The subtype C strains is prevalent in Africa, Latin America and Asia(28,31). India has a high prevalence of HIV-1 subtype C. The prevalence of subtype C varied in different parts of the country. In North India 78.4% and western India 96% of HIV-1 strains were subtype C(33,34). HIV-2 subtypes recognized in the world were A-H(35–37). When compared to HIV-1, HIV-2 strains were predominant in Africa. The HIV-2 strains identified in India till date are subtype A(38). The subtype A strain is the predominant in West African countries. Subtype A is estimated to cause 0.11% of all HIV infections in humans(32).

3.3. Transmission

HIV-1 is predominantly transmitted through sexual contact (both heterosexual and homosexual), by blood and blood products and from mother to child – intrapartum, perinatal and through breast milk. Transmission largely depends upon the viral load and the duration of exposure to that particular body fluid(10).

The common route of HIV transmission worldwide is through sexual contact and accounts for 80% of adult HIV infection. Increased risk of HIV transmission is seen in homosexuals and individuals with multiple sexual partners. The risk of HIV spread is increased by 300 times when there is concomitant sexually transmitted infections (STI) caused by *Herpes simplex-2, Hemophilus ducreyi, Treponema pallidum*(11).

The probability of acquiring HIV from infected blood is about 90%(11). Transmission of HIV through blood has reduced to a large extent after intense screening in transfusion and transplant settings by HIV antibody testing and molecular detection. Intravenous drug users acquire HIV infection through this route. The prevalence of HIV infection in our country is 9.9% in intravenous drug users(6). The relative proportions of mother to child transmission of HIV are 23-30% before delivery, 50-60% during delivery and 12-20% during breast feeding(11).

In India, the HIV epidemic is concentrated in two groups of people through whom it is transmitted to healthy population – one group the high risk which includes male and female sex workers, transgender and intravenous drug abusers. The second group is bridge population which comprises a group of people who bridge the high risk groups with general population, mostly they are clients or partners of male and female sex workers include long distance truck drivers and migrants(39).

3.4. Replication

The primary targets of the HIV virus are the immune cells namely CD4+ T lymphocytes and macrophages. Replication starts with the attachment of the gp120 protein of virus to the CD4 receptors and co-receptors present on the surface of target cells which results in conformational changes in gp120. The chemokine receptors, CCR5 and CXCR4 serves as the co-receptors for the macrophage trophic and non- macrophage trophic strains respectively. This results in exposure of the transmembrane envelope glycoprotein gp41 leading to fusion of the virion and target cells(40). Following fusion, matrix and capsid proteins in the virus are digested and releases viral RNA and enzymes into the cytoplasm of the cell. The viral genetic material gets incorporated into the cell

by endocytosis. The HIV enzyme reverse transcriptase uses the host nucleotides and converts the viral RNA into single-stranded DNA. During reverse transcription, random errors are made commonly due to the poor proofreading by reverse transcriptase. The double stranded DNA is synthesized from single stranded DNA by reverse transcriptase. The enzyme integrase integrates the double stranded DNA into the host DNA. Thus the virus replicates along with the DNA of the host cell. Following this, transcription occurs to produce viral mRNA which is translated into viral proteins in the cytoplasm. The final steps of HIV replication include assembly of the immature virion to the cell surface which leaves the host cell by budding. The mature virion that is released affects other immune cells thus continuing the process of replication(27,40).

3.5. Pathogenesis

The indicator of HIV disease is severe immunodeficiency due to progressive qualitative and quantitative deficiency of helper T cells by direct and indirect mechanisms(11). When the virus enters the body, there is massive multiplication of the virus in the target immune cells which results in CD4+ cell damage. Regardless of the host's active immune response, the virus escapes the immunological clearance and releases the virion from the destructed cells which are concealed within the regional lymph nodes, this results in viremia. This occurs usually within 2-6 weeks when a threshold of replication is reached(41). Primary viremia sets in, which is characterized by non-specific symptoms that resembles any viral infection such as fever, fatigue, lymphadenopathy, rash, sore throat, weight loss and muscle pain. Once the infection is established, it persists lifelong(42,43). During the primary viremia phase, there is high plasma viral load and a transient fall in the CD4 count(41). In proportion to the viral replication,

there is profound immune activation by the T cells which results in release of various proinflammatory cytokines like TNF-α and interleukins. This state along with the direct viral effects leads to dramatic depletion, impaired production and dysfunction of the CD4 helper cells(44). The period and the duration of clinical latency after the primary infection depends upon the individual's immune system. Once the CD4 count falls below 200 cells/µL, the infected individual is prone to get more opportunistic infections and progress to an advanced stage of HIV known as AIDS(41). The relationship between CD4 T lymphocyte count and viral load in an untreated HIV infected individual is depicted in **Fig 1.**

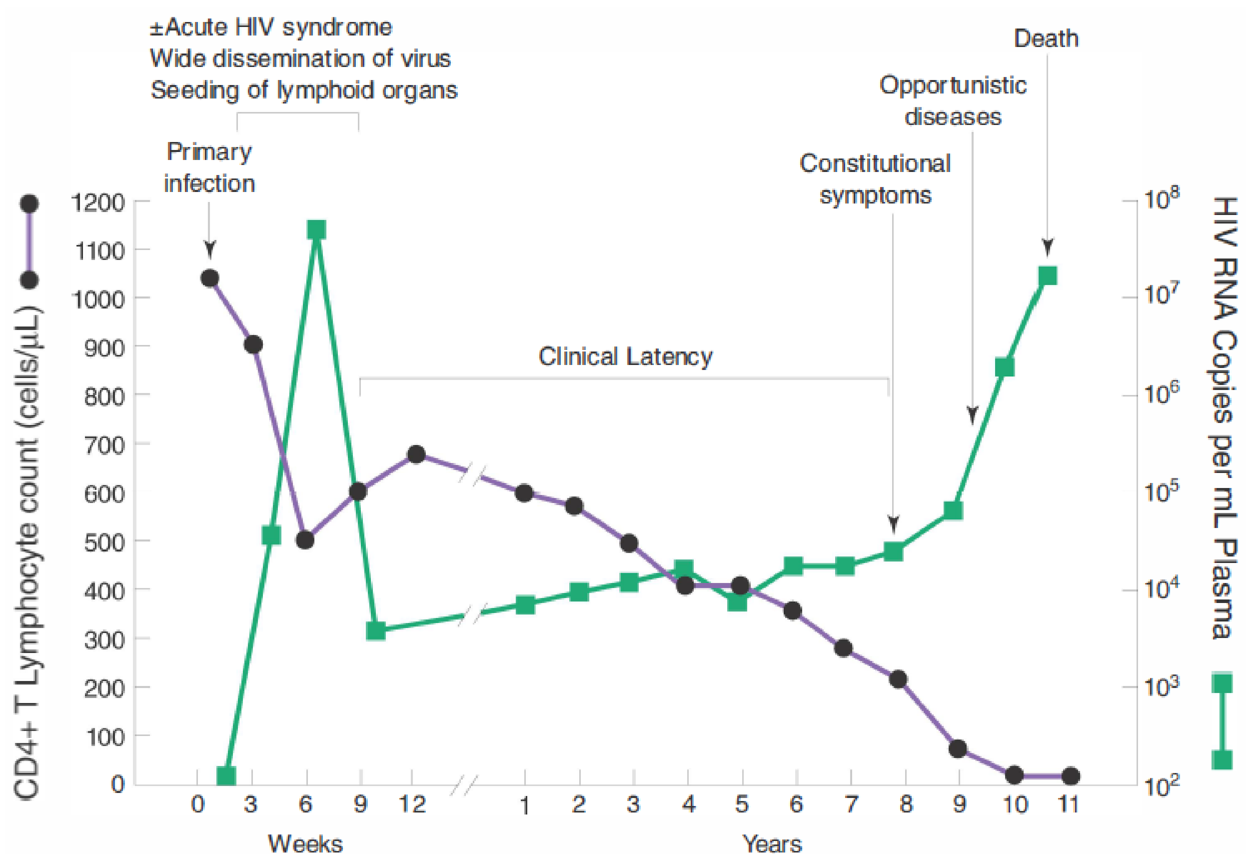


Figure 1: Graph depicting relationship between CD4 T lymphocyte count and viral load in an untreated HIV infected individual. Adapted from Harrison's Principles of Internal Medicine. Based on an original from Pantaleo et al (1993)

3.6. Immune Response

After primary viremia, the HIV infected individuals normally mount a good immune response that usually curtails the level of viremia and may delay the development of the disease for 10 years. The immune response of the body to HIV infection includes humoral and cell mediated immunity. The immune response is against antigenic determinants of virion and also against viral proteins which is usually expressed on the surface of infected cells(11).

3.6.1. Humoral immunity

HIV specific antibodies appear normally within 3-6 weeks of primary infection and without fail within 12 weeks; exceptions include inability to produce HIV specific antibodies in certain individuals(11). The detection of these HIV specific antibodies is the foundation of most of the screening tests used for detection of HIV infection.

The antibodies detected by ELISA and Western Blot are HIV binding antibodies and appear prior to the appearance of HIV neutralizing antibodies.

The neutralizing antibodies usually appear after the initial decrease in plasma viral load and are more closely associated with the arrival of HIV -specific CD8+ T lymphocytes(11). The first antibodies which appear are against the immunodominant region of the envelope protein gp41. This is followed by antibodies to gag protein p24 and the gag precursor p55(11). The antibodies to gp120 (envelope), p17 (gag) and p31 and p66 (pol protein) appear after antibodies to p24. Envelope proteins gp120 and gp41 are the only proteins which can elicit neutralizing antibodies. These neutralizing antibodies are considered as both protective and may also contribute to the pathogenesis

of HIV. The first neutralizing antibodies are against autologous infecting virus and usually appear after 12 weeks of infection. The virus is able escape the neutralizing antibodies because of the high rate of mutation. One important mechanism of immune escape is formation of glycan shield by linking of the glycosylation sites which interferes with envelope recognition(11).

There are 5 main sites in the HIV envelope that elicits the production of neutralizing antibodies(11). They are

i. CD4 binding site of gp120
ii. Glycan dependent epitopes in V1/V2 region of gp120
iii. Near the base of V3 region of gp120
iv. gp120/gp41 bridge
v. Membrane proximal region of gp41

Some protective antibodies engage in antibody dependent cellular cytotoxicity (ADCC) where natural killer cells have Fc receptors and Fc portion of anti HIV antibodies bind to these Fc receptors. These armed natural killer cells bind to the cells which express HIV antigens and destroys them. The antibodies against the envelope proteins which participate in ADCC are highest early in infection(11).

Avidity is the functional affinity and is the overall strength of the bonds between antibodies and their corresponding antigens. In HIV-1 infection, avidity increases over time following seroconversion. Maturation in antibodies against gp41 reflects the time since seroconversion and remains high in individuals with long term infection and in AIDS. But the same cannot be said of anti-p24 and anti-p17. Before the onset of AIDS,

the avidity of antibodies against p24 falls. The avidity of antibodies against gp41 is of value in identifying cases of recent HIV infection but not the avidity of antibodies against p24 or p17 (45). In early stages of primary immune response usually low avidity antibodies are produced. The hyper mutation in V region and the preferential selection of the high affinity B cells by antigen leads to the increase in the overall avidity over period of months. As this response matures usually when maximum antibody titers have been reached. The maturation of the antibody response is useful in serodiagnosis of viral infections for the timing of infection. Antibody maturation occurs in the presence of active viral replication(46). Using paired sera from cases of primary infection with a known date of onset of illness or seroconversion, an avidity maturation time course has been determined. Such avidity studies are now used in risk assessment in cases of rubella during pregnancy where it can confirm recent infections in the face of equivocal IgM results and for some time after maximum IgG antibody titers have been reached(45,47–49).

3.6.2. Cellular Immunity

T cell immunity can be mediated by inducer CD4+ T helper cells and immunoregulatory CD 8+ killer T cells. CD4+ T helper cells specific for HIV can be detected using flow cytometry to measure intracellular cytokine production or through lymphocyte proliferation assay using HIV antigen p24. These cells help HIV specific B cells and CD 8+ T cytotoxic cells in mounting an immune response. CD 4+ T cells may also directly kill HIV infected cells. During generation of an immune response to HIV, the CD4+ T cells may be the preferred targets of HIV infection by infected antigen

presenting cells. When there is high viral load, CD4+ T cells responds to HIV antigens by shifting from proliferation and IL-2 production to IFN-γ production(11).

HIV specific CD8+ T cells which are MHC class 1 restricted have been detected in the peripheral blood samples of HIV-1 infected individuals. These cells include cytotoxic T cells that produce perforins and T cells which can express an array of cytokines such as IFN-γ, IL-2, MIP- 1β and TNF α. Cytotoxic T lymphocytes (CTL) have been detected within weeks of HIV infection and before the appearance of virus in blood. They exert a selective pressure on the evolution of the population of circulating viruses. CD8+ T lymphocytes through their HIV specific antigen receptor bind to the target cells and results in the lytic destruction of target cells having autologous MHC class 1 molecules presenting HIV antigens. Two types of cytotoxic T lymphocyte activity are seen in HIV infected individuals. The first type is known as spontaneous CTL activity where the cytotoxic T lymphocytes directly lyses appropriate target cells in culture without prior in vitro stimulation. The second type of CTL activity indicates the precursor frequency of CTLs (CTLp); this CTL activity can be demonstrated by stimulation of CD8+ T cells in vitro using a mitogen such as phytohemagglutinin or anti-CD3 antibody(11).

The ability of CD8+ T lymphocytes from an HIV infected individual to inhibit the replication of HIV in tissue culture without killing infected targets is known as non cytolytic CD8+ T cell mediated suppression of HIV replication (11,43,50).

3.7. Classification of HIV infection

Two major systems of classification are used widely for HIV infection. The WHO classification system has clinical staging and is used widely in low resource setting where CD4 counts may not be available. The CDC staging system combines both CD4 counts and clinical staging together(51).

Table 1: WHO classification of HIV

Staging	Clinical Presentation
Primary HIV infection	Asymptomatic Acute retroviral syndrome
Stage 1	Asymptomatic Persistent generalized lymphadenopathy
Stage 2	Moderate unexplained weight loss (<10%) Recurrent respiratory tract infections Angular cheilitis Herpes zoster Recurrent oral ulcerations Seborrheic dermatitis Papular pruritic eruptions Fungal nail infections of fingers

Stage 3	Unexplained severe weight loss (>10%) Unexplained persistent fever for >1 month Unexplained chronic diarrhea for >1 month Persistent oral candidiasis Oral hairy leukoplakia Pulmonary tuberculosis Severe presumed bacterial infections (Pneumonia, meningitis, bacteremia, bone or joint infection) Acute necrotizing ulcerative gingivitis, stomatitis or periodontitis Anemia or Neutropenia or Chronic thrombocytopenia
Stage 4	**Conditions where a presumptive diagnosis can be made on the basis of clinical signs or simple investigations** HIV wasting syndrome *Pneumocystis* pneumonia Recurrent severe bacterial pneumonia Chronic herpes simplex infection (>1 month or visceral herpes at any site) Esophageal candidiasis Extra pulmonary tuberculosis Cytomegalovirus infection (infection of retina or other organs) Kaposi sarcoma Toxoplasmosis of the central nervous tissue HIV encephalopathy

	Conditions where confirmatory diagnostic testing is necessary: Extra pulmonary Cryptococcosis including meningitis Progressive multifocal leukoencephalopathy Disseminated non tuberculosis mycobacteria infection Disseminated mycosis Candida of the bronchi, trachea, or lungs Recurrent non typhoidal *Salmonella* bacteremia Chronic cryptosporidiosis or isosporiasis (with diarrhea) Invasive cervical carcinoma Atypical disseminated leishmaniasis Lymphoma Symptomatic HIV-associated cardiomyopathy or nephropathy Reactivation of American trypanosomiasis

Table 2: CDC classification of HIV

CD 4 cell count	Clinical Category		
	Category A	Category B	Category C
≥ 500 cells/µL	Asymptomatic, acute HIV or Persistent generalized lymphadenopathy (A1, A2, A3)	Symptomatic conditions except A and C (B1, B2, B3)	AIDS defining illnesses (C1, C2, C3)
200 – 499 cells/µL			
< 200 cells/µL			

3.8. HIV Testing Strategies

National AIDS Control Organisation (NACO) is a section of the Ministry of Health and Family Welfare (MOHFW) responsible for formulating policies and executing programmes for the prevention and control of the HIV in India. National AIDS control programme (NACP) was first established in 1992, which aimed at controlling the spread of HIV infection. NACP-IV (2012-2017) is the current programme. and it aims to reduce annual incidence of HIV infections by 50% (5,52).

Safety of blood and blood products is of prime importance because of the huge risk involved in the transmission of HIV through blood and blood products. The positive predictive value is low in populations with low HIV prevalence so the WHO/Government of India evolved strategies. There are three different strategies to detect HIV infection in the different population groups and different situations. The various strategies designated involve the use of categories of tests in many permutations and combinations(53).

1. ELISA or Rapid tests are used in strategy I, II, & III
2. Confirmatory tests with high specificity like western blot and line immunoassays, are used in cases of indeterminate/discordant result of ELISA or Rapid tests

Recommendation by NACO for HIV testing

ELISA kits with a sensitivity of ≥99.5% and a specificity of ≥98%.

Rapid kits with a sensitivity and specificity ≥99.5% and ≥98% respectively.

The different strategies for HIV testing used in different situations are shown in **Fig 2, 3, 4 and 5**

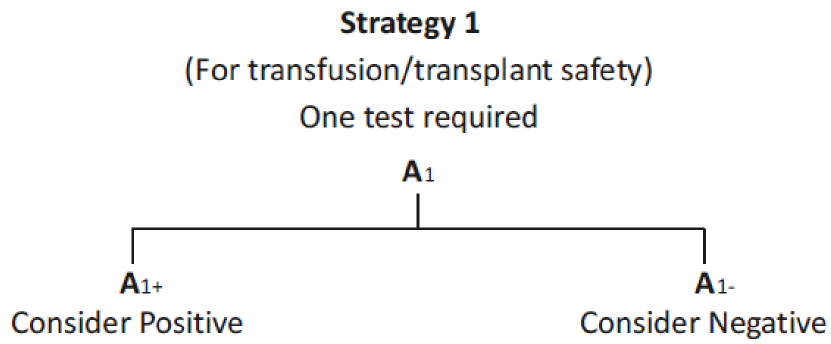


Figure 2: Strategy 1 – For blood transfusion/transplant safety

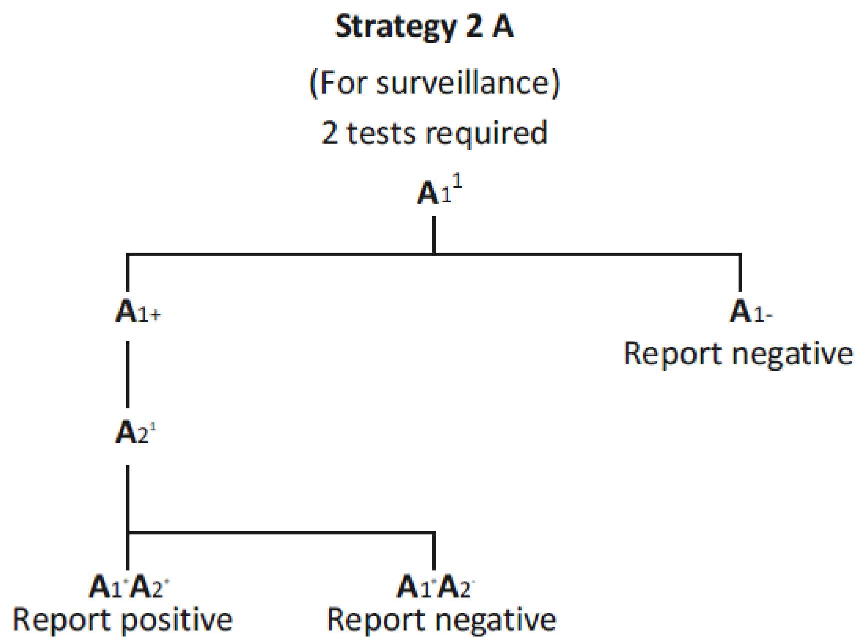


Figure 3: Strategy 2A is used for sentinel surveillance

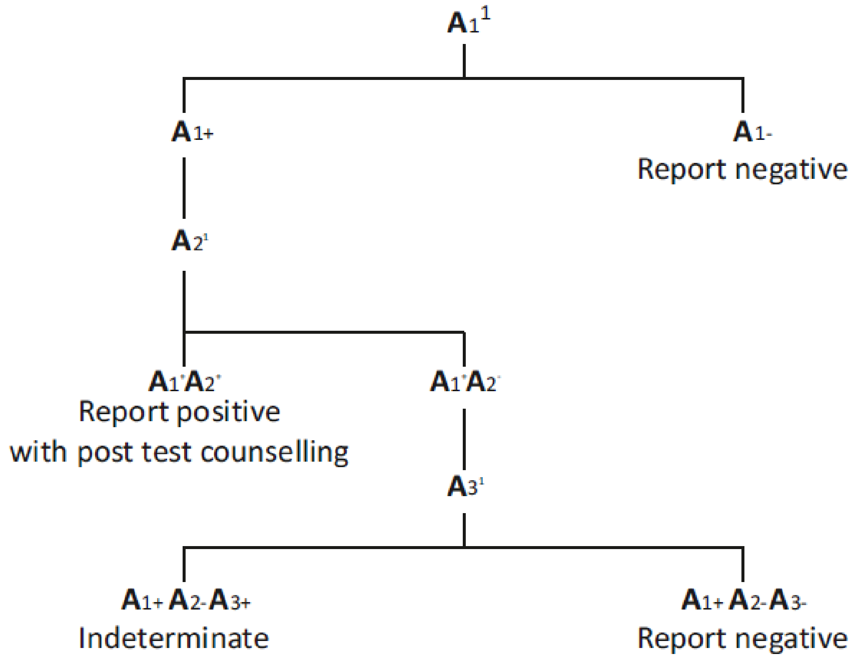


Figure4: Strategy 2 B – Used for diagnosis in symptomatic individuals

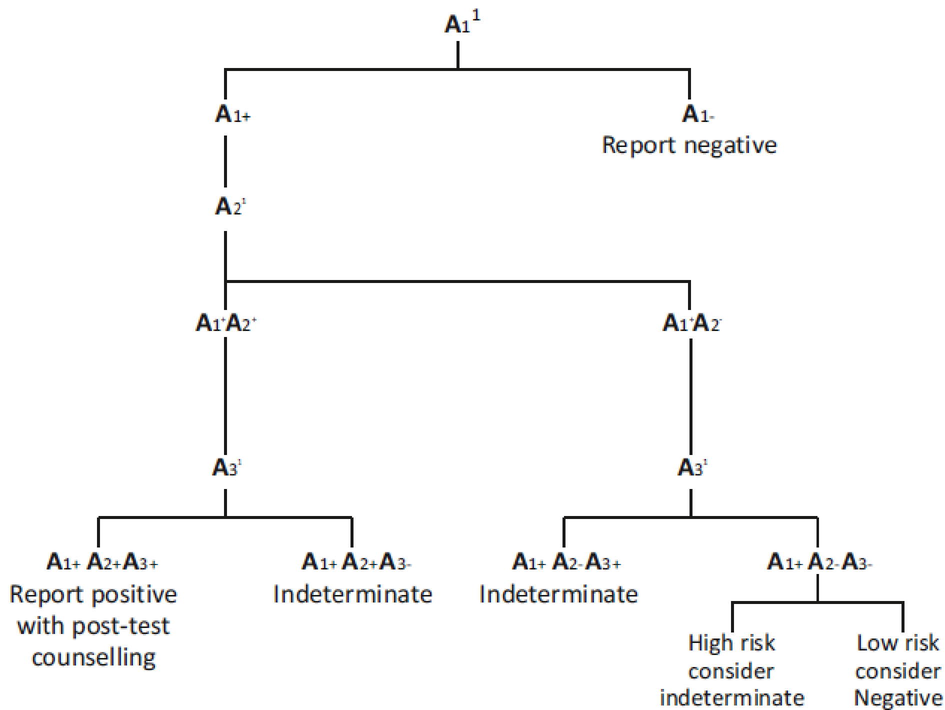


Figure 5: Strategy 3 - Used for diagnosis in asymptomatic individuals

The assays A_1, A_2, A_3 are 3 different assays which are based on different principles or different antigenic compositions. A_1 should be of high sensitivity. A_2 and A_3 should be of high specificity to minimize false positive reactions. A_2 and A_3 should also be able to differentiate between HIV 1 & 2 infections. In case of indeterminate, testing should be repeated on a second specimen taken after 14-28 days. If results continue to be indeterminate, then the specimen is to be subjected to Western Blot /PCR(53).

3.9. Recent infection

There are many developed laboratory tests which differentiates recently acquired HIV infections from long term infection based on the principle that the immunological response to HIV infection takes a few months to evolve after infection. The indicator of recent infection is usually a marker which is present during the initial period but later disappears. In such tests, "recent" usually means the duration of up to one year after an individual has acquired the infection (14).

HIV incidence is the rate at which a population acquires HIV infection. It is a quantitative indicator that measures the magnitude of current HIV transmission in a community.

Estimation the HIV incidence is done for population surveillance, for evaluating of the impact of preventive interventions and for evaluating the efficacy of early treatment or a new preventive intervention. Surveillance helps in identifying the incidence patterns between different population groups and over time. Surveillance may monitor HIV infection in the general population or in the high risk groups and advices governments regarding resource allocation.

Incidence ratios evaluates the outcome of an intervention by comparing the incidence between two populations or across two time periods. Only after approximating the absolute incidence with some accuracy are populations selected for early treatment or prevention cohort. The feasibility of population selection depends on detecting a specific number of new HIV infections in the population.

Estimation using laboratory tests for recent HIV infection includes estimating the number of individuals with newly acquired HIV-1 infection in a population using a test for recent HIV infection and using a mathematical formula to derive HIV incidence.

It does not require repeated test in individuals so can be used in samples collected in cross sectional studies. Its disadvantages are biases that arise in sample selection and chance of misclassifying long term infection as recent infection (false recent rate – FRR). Another limitation is the difference in assay performance across different population groups and different HIV subtypes. The incidence derived from multiple methods is more reliable than when it is based on a single method (12).

3.9.1. Tests available for recent infections

Majority of the Tests for Recent (HIV) Infections (TRI) are based on principle that maturation and evolution of HIV-1 specific antibodies occur within first two years of seroconversion(15).

Till date, there are 8 types of assays as tests for recent HIV infection. Among these assays , some have been specifically developed for identifying recent infection, while others are modifications of commercial HIV diagnostic tests(54–56).

1. **Less-sensitive enzyme immunoassay** – Majority of the antibody assays for HIV infection can be modified for use as a test for recent infection utilizing the principle that antibody titers rise for few months after acquisition of the infection Majority of the standard antibody assays for HIV infection can be altered for use as a test for recent infection utilizing the principle that antibody titers rise for several months after acquiring the infection. Janssen et al described this method first. It is based on the enzyme immunoassay (EIA) produced by Abbott laboratories and which was modified to create a less-sensitive HIV antibody test(57). The enzyme immunoassay was made less sensitive by diluting the plasma and reducing the incubation time. Confirmed HIV-1 positive samples were tested again using the less sensitive EIA. Individuals with recent HIV infection and an early immune response had lower level of HIV antibodies and therefore they tested negative in the less-sensitive EIA.

   Many other assays have also been modified in a similar manner way to estimate incidence. Two immunoassays which have been commercially modified as less-sensitive EIAs were the Avioq HIV-1 Microelisa and Abbott 3A11. For detection of recent HIV infection even rapid antibody tests have been modified. The limitation of these assays were that they used HIV-1 subtype B alone as antigen so it could not be used in other parts of the world where there is prevalence of other subtypes of HIV (12)

2. **Proportional assay** – This assay measures the proportion of all the immunoglobulin G in an individual's serum which is specifically against HIV. The principle is that the proportion IgG is lower in recent infection than in long

term infection. The commercial assay based on this principle is the IgG antibody capture BED-enzyme immunoassay (BED-CEIA). BED CEIA was developed particularly for the identification of recent HIV infection(58). BED capture EIA uses a synthetic antigen and this assay can be used against HIV subtypes B, E and D (12).

3. **Avidity assay** – Avidity is the strength of the bond between an antigen and an antibody. These assays are based on the principle that individuals with recent have antibodies of low avidity. After measuring the total anti-HIV response, a denaturation agent is added which separates the antibodies with weak bonds. Then avidity index is calculated as optical density (OD) of the well treated with denaturation agent divided by OD of well washed with phosphate buffered saline. Avidity index of ≤ 0.80 is considered as recent infection and avidity index > 0.80 is considered as long term infection(59,60).
4. **p24 antigen** – It is possible to detect p24 antigen within a few days after the presence of virus in the blood and before HIV antibodies can be detected. When the body initiates an immune response to the infection the level of p24 antigen falls. The presence of p24 antigen in absence of HIV specific antibodies is suggestive of recent infection. The use of this test is limited for detecting recent infection as the presence p24 antigen is brief (1-2 weeks) and unreliable (12).
5. **IDE-V3 assay** – The basis of this assay is the two conserved sequences in the envelope protein of HIV-1. They are

- Immunodominant epitope (i.e. IDE) of envelope protein gp41 comprising of two oligopeptides of 30 amino acids; and one of the oligopeptide is from group M and the other oligopeptide is from HIV-1 subtype D
- Conserved sequence from the V3 loop of glycoprotein120. It contains oligopeptides from HIV-1 subtypes A, B, C, D and E.

The assay uses a formula to calculate the combination of quantitative responses to the antigens from each region to differentiate recent infection from long term infection. The IDE-V3 assay has low sensitivity (12,61).

6. **HIV RNA** – The HIV RNA detection in the absence of HIV antibody is also utilized to identify recent HIV infection. Compared to p24 antigen, HIV RNA can be detected much earlier (62). If pooled HIV RNA is used, it can increase the accuracy of RNA amplification assay and lower the cost of testing. The limitation is, it requires very large sample population size for determining HIV incidence.
7. **IgG3 anti-p24** – During the course of an infection, the IgG isotypes formed in response to the infection varies. Isotype IgG3 is present transiently during the initial few months of HIV-1 infection. IgG3 response is most reliable against p24 antigen. An enzyme immunoassay was developed where IgG3 to p24 antigen was typically detectable only during the first four months of infection. The results from the initial studies of this assay have not been generalized to different groups with different subtypes of HIV infection (12).
8. **Line immunoassay –** It is an immunoblot that utilizes limited range of recombinant antigens and oligopeptides from HIV-1 and HIV-2. In routine

diagnostics it confirms the presence of HIV specific antibodies. Inno-Line immunoassay HIV 1/2 score is utilized to interpret results as either long term infection or recent infection (63). It is an expensive assay.

9. **HIV-1 LAg-Avidity EIA**

It is a quantitative limiting antigen (LAg) avidity enzyme immunoassay (EIA) for differentiating between long term and recent HIV-1 infections. Individuals with recent HIV-1 infections normally have HIV IgG with lower avidity than those with established HIV-1 infections. It measures avidity of the HIV-1 antibodies in both serum and plasma samples(16,64).

The earliest tests were desensitized or “less sensitive” commercially available HIV immunoassays (57,65–71) based on the principle that lower titers of HIV antibodies were typical of recent infections. All the less sensitive commercial immunoassays were a modification of HIV immunoassay that used HIV-1 subtype B as antigen so the assay was unreliable in populations in other parts of world (70,71). The subtype bias was overcome by the utilization of synthetic antigen which had sequences from different subtypes and a capture format helped to measure the proportion of HIV-1 antibodies which increased after seroconversion. The BED capture enzyme immunoassay utilizes a synthetic antigen with sequences from HIV-1 subtype B, E and D (58). But BED CEIA used in several studies (72–77) showed a high false recency rates which gave an overestimation of HIV-1 incidence being reported (78–81) and consequently adjustments were proposed to improve the accuracy of the incidence estimates after the test(82,83). HIV incidence utilizing the assays based on antibody avidity

have shown low false recency rates in studies in US populations (84,85). In order to overcome the biases of the previous assays, the wells were coated with a recombinant protein (rIDR-M) that had divergent sequences from immunodominant region of gp41 from all the major subtypes and recombinants of HIV-1 group M. Also limiting the concentration of the antigen in the wells detected only antibodies with high avidity. Studies done in Africa using this assay showed a false recency rate of less than 1%(86). As this assay is based on the binding strength of the antibodies or functional avidity, it is less likely to be affected by disease state compared to previous assays. Normalized optical density (ODn) cutoff of this assay is 1.5 to classify recent and long-term infections. In LAg avidity assay, ODn value = 1.5 denotes a mean duration of recent infection (MDRI) of 130 days (95% CI, 118-142)(87). The limitations of this assay is that the usefulness of this assay at individual level has not yet be assessed particularly when ODn values are close to the cutoff.

10. Limiting-Antigen Avidity Dot Immuno-Gold Filtration Assay

It is a rapid membrane based immunodiagnostic technique. Compared to the enzyme immunoassay, they are more suitable for onsite testing because it is rapid, economical and convenient and has visual characteristics. This is based on avidity of antibody between long-term and recent infection. This assay increases the sensitivity and accuracy by adding a silver staining technique. Its agreement with LAg avidity assay was 95.36% ($\kappa = 0.75$) and 92.10% with BED assay ($\kappa = 0.65$)(88).

3.10. Avidity and Antiretroviral therapy

HIV-1 infected individuals who were administered antiretroviral therapy (ART) showed impaired production and maturation of IgG compared to before administration of ART. ART seems to prevent the appearance of IgG antibodies(46). It can be due to suppression of viral replication by ART as HIV replication results in production of antibodies directly because of stimulating antigens or indirectly, due to release of cytokines like Interleukin-6 and interferon which stimulate the B lymphocytes. Western blot analysis has shown only limited number of recently HIV-1 infected individuals showing high reactivity to pol and gag proteins. Administration of ART prevents increase in avidity index and discontinuation of ART results increase in avidity index(17). ART in primary HIV infection partially prevents the emergence of HIV-1 IgG antibodies(89). Viral suppression naturally induced and due to ART have misclassified as recent infection those who have had long term HIV-1 infection when tested using the BED capture EIA. Also viral breakthrough that is when an individual on ART has an emergence of viral load >1000 copies/ml, such individuals showed an increased proportion of IgG measured using BED capture EIA(18).

3.11. HIV Drug resistance

In 2004, WHO and CDC, along with HIVResNet, developed a global strategy for the assessment and prevention of HIV drug resistance. And one of the components of the strategy was to check for transmitted drug resistance in recently infected HIV-1 individuals(90,91).

HIV drug resistance is of two type – transmitted drug resistance and acquired resistance associated with treatment failure.

Primary or Transmitted drug resistance

Transmitted drug resistance is defined as an occurring when individuals are newly infected with an already resistant virus(92,93). The increased use of antiretroviral drugs has resulted in an increase in the incidence of drug resistance and eventually a large pool of resistant virus strains becoming available to establish new infections. The transmission of drug resistant viruses does not depend on a particular route of infection (94).

Acquired Resistance occurs when HIV-1 infected individual on ART and was responding initially but later develops resistance to the antiretroviral drugs. That is, they acquired resistance following administration of ART.

Drug resistant HIV-1 can interfere with successful treatment and management of therapy in HIV-1 infected individuals. So when an individual has drug resistance, the treatment options available is limited and it affects our efforts to slow down the progression of the disease. Furthermore, this can lead to increased rates of transmitted drug resistance because of the pool of drug resistant strains available to infect more people (94).

4. Materials and Methods

The study was done in the Departments of Clinical Virology and Infectious Diseases, Christian Medical College and Hospital, Vellore. It was approved by the Institutional Review Board (Reference IRB Minutes No: 9527 dated 07.07.2015).

4.1. Materials

Study subjects:

The study participants were classified in 5 different groups

Group 1: Consecutive positive samples (using NACO strategy 3) of HIV-1 infected individuals from ICTC, CMCH. The study was explained to all the participating individuals and was recruited in the study only after getting a written informed consent. This cross sectional study was done during a period of 1 year (August 2015 – July 2016).

Inclusion criteria for samples from ICTC:

1. Adults >18yrs of age

2. HIV-1 infected individuals

3. Treatment naive

4. Individuals willing participate in the study by signing informed consent

Group 2: First negative sample from ICTC volunteers of each week who are negative for HIV infection to serve as controls.

Inclusion criteria for control from ICTC:

1. Adults >18yrs of age

2. Negative for HIV infection
3. Individuals willing participate in the study by signing informed consent

Archived samples – Paired sera

Group 3: Archived paired sera of HIV-1 infected individual prior to treatment and after 6 months to 1 year of treatment and showing response to treatment.

Inclusion criteria for archived samples

1. Adults >18yrs of age

2. HIV-1 infected individuals

3. Sufficient volume of sample- treatment naïve and following 6 to 12 months' treatment with CD4 counts and HIV-1 viral loads available

4. Individuals who have given informed consent for additional studies and wavier towards this study

Group 4: Archived paired sera of HIV-1 infected individual on ART for a minimum of one year, showing response to treatment and after developing drug resistance

Inclusion criteria for archived samples:

1. Adults >18yrs of age

2. HIV- 1 infected individuals on treatment for at least 1 year initially showing response to treatment and later showing treatment failure with CD4 counts and HIV-1 viral loads available

4. Individuals who have given informed consent for additional studies and wavier towards this study

Group 5: Archived samples (n = 3) positive by 4th generation assays were also used

These samples were tested using multiple 3rd and 4th generation assays, those samples which tested positive by 4th generation assays and negative by 3rd generation assays were assumed to be negative for IgG antibody and positive for HIV-1 antigen. All these were characterized samples positive only for antigen and negative for antibody. Hence we assumed that the sample will be negative for the avidity assay. So it was used to check the specificity of the assay.

Exclusion criteria for all groups

1. Individuals who haven't given consent for additional studies

2. Individuals < 18years of age

3. Pregnant women

4. HIV-2 infected individuals

5.1.1. Sample size:

Group 1 and 2

In our ICTC, 15-20 positives cases of HIV-1 come every month. Of these, based on history, 40-50% are recently infected. Based on this data, taking a prevalence (p) of 45% recently infected individuals among HIV-1 infected individuals with precision (d) of 10.

Here p (prevalence) = 45%, q (100-p) =55%

A precision (d) of 10, with 95% confidence interval (alpha error ($Z\alpha$) of 1.96)

$Sample\ size\ (n) = Z^2 \times p \times (100 - p) \div d2$

Sample size (n) = (1.96 x 1.96) x 45x 55/ 100 ≈ 96

Even though the calculated sample size was 96, blood samples were collected from 117 consecutive treatment naïve HIV-1 infected individuals who attended the ICTC, CMCH. The samples were collected from those who gave consent for the study.

For controls (Group 2), we collected the first sample of the week which tested negative for HIV in ICTC with written informed consent from the volunteer (n = 40)

For the group 3 the sample size was calculated in the following way,

Study by Re et al showed decrease in the avidity antibodies in approximately 80% of HIV-1 infected individuals(17).

With a precision d=15%. Prevalence (p1) = 82%, q1= (100-p) =18

$Sample\ size = Z^2 \times p \times (100 - p) \div d^2$

Sample size = 1.96x 1.96 x 82x 18/225 ≈ 26

In group 4 all the available pairs were taken (n=7)

Information collected from the study recruits included the individuals' age, sex, regional identification, marital status, occupation, history of exposure and any high risk behavior.

Samples tested

1. Serum samples from treatment naïve HIV-1 infected individuals (n = 117)
2. Serum samples from HIV negative individuals (n = 40)
3. Archived paired sera of HIV-1 infected individual prior to treatment and after 6 months to 1 year of treatment and showing response to treatment (n = 25 x 2 = 50)
4. Archived paired sera of HIV-1 infected individual on ART for 1year, showing response to treatment and after developing drug resistance (n = 7 x 2 = 14)
5. HIV-1 infected individuals who tested positive by 4th generation assays and negative by 3rd generation assays (n = 3)

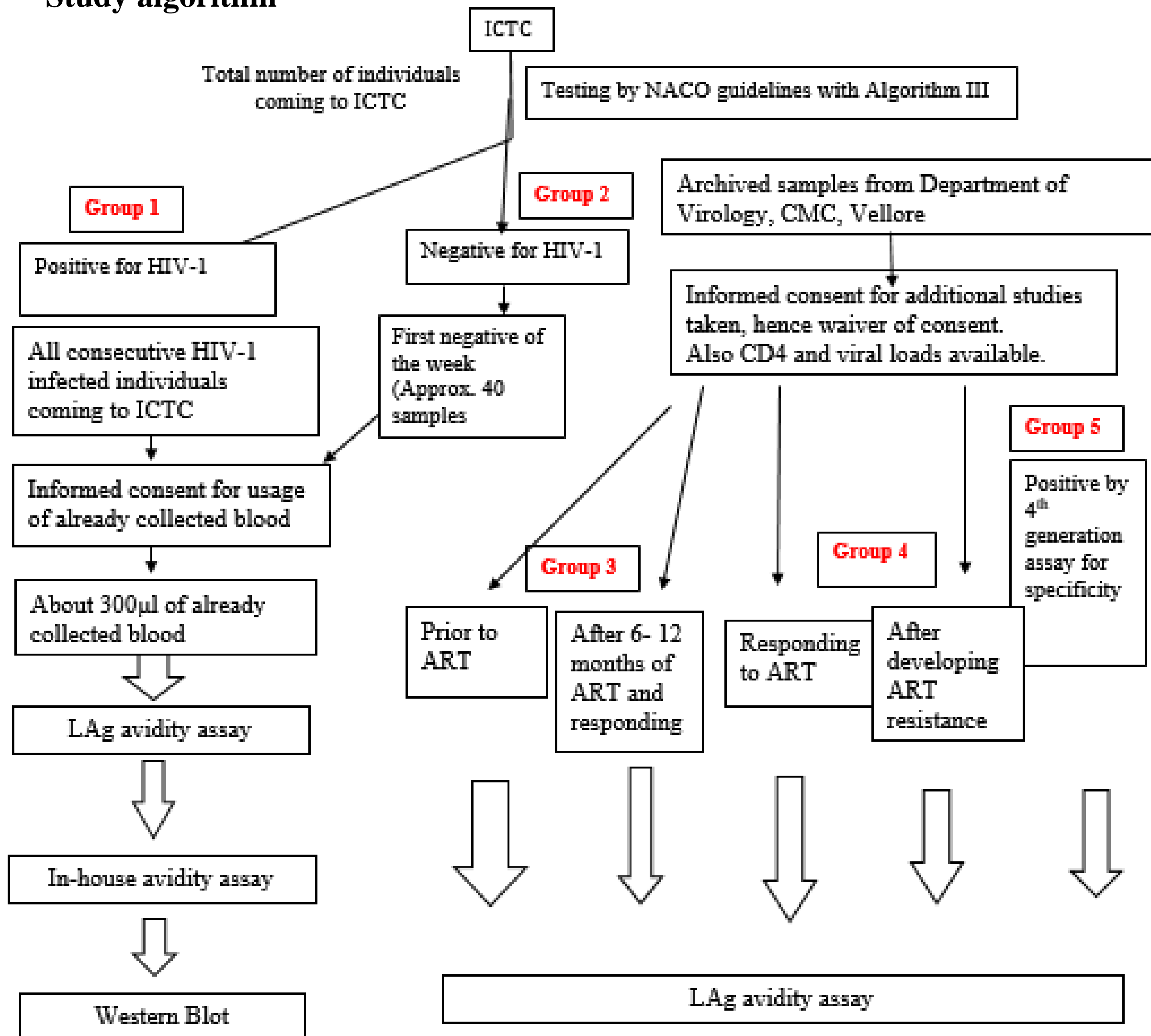
Specimen

A total of 2ml of blood sample was collected in a sterile red capped BD vacutainer (New Jersey, USA) in ICTC. After testing was done in ICTC, the remaining sample was obtained for the study with written informed consent. Serum was separated and stored in multiple aliquots of 200 µL at -60°C until testing.

For group 3, 4 and 5 the specimens were aliquots of plasma/ serum sample stored at

-60°C / -30°C until it was tested.

Study algorithm



5.2. Methods

5.2.1. HIV-1 limiting antigen avidity assay

The LAg Avidity enzyme immunoassay (Sedia Biosciences Corporation, Oregon, USA) is a commercially available single well assay based on avidity of the antibody.

The wells of this assay are coated with recombinant proteins containing the HIV-1 immune dominant region (IDR) of gp41.

1. The assay had four types of controls - the negative control, the calibrator, the low positive control and the high positive control. All the controls were tested in triplicates as per manufacturer's instruction and during the initial testing all specimens were tested singly.
2. Serum specimens was diluted 1:101 that is 500 μL of sample diluent and 5 μL of sample. The sample diluent contains phosphate buffered saline, blocking agents, detergent and preservatives. The Eppendorf tubes were marked with the sample ID and 500 μL sample diluent was added in each tube and 5 μL of each control, calibrator, and sample was transferred to respective Eppendorf tubes.
3. After mixing properly, 100 μL of diluted negative control was added to wells A1, B1 and C1 of the avidity plate; 100 μL of diluted calibrators was added to the wells D1, E1 and F1; 100 μL of diluted low positive controls was added to wells G1, H1 and A2 and 100 μL of diluted high positive control was added to wells B2, C2 and D2. After addition of controls, the samples were added in the respective wells. The plate was sealed and incubated for 1 hour at 37°C (±2°C).
4. After 1 hour, the avidity plate was washed 4 times with 1X wash buffer. To each well, 300 μL of wash buffer was added and allowed to soak for 10 seconds. After washing, residual buffer was removed by gently tapping the plate upside down on absorbent paper.
5. Then 200μL of dissociation buffer was added to each well. Plate was sealed and incubated for exactly 15 minutes at 37°C (±2°C).

6. During this time conjugate working solution was prepared, 12 ml of sample diluent was transferred to sterile clean petri dish and 12 µL of the goat anti-human IgG conjugated to horse radish peroxidase (HRP) concentrate was added to it and to prepare a 1:1001 dilution. The petri dish was closed and contents were mixed gently.
7. After the incubation, the plate was washed with 4 times with 1X wash buffer and residual buffer was removed by gently tapping the plate on an absorbent paper.
8. After this, 100 µL of the conjugate working solution was added to each well. The plate was sealed and incubated at 37°C (±2°C) for 30 minutes.
9. After the incubation period, the plate was washed 4 times with 1X wash buffer and residual buffer removed by gently tapping the plate on an absorbent paper
10. Then 100 µL of tetramethyl benzidine (TMB) substrate was added to each well. The plate was incubated for exactly 15 minutes at 25°C (±2° C) avoiding exposure to light.
11. After 15 minutes of TMB incubation, the reaction was stopped by addition of 100µL of stop solution (dilute acid) to each well.
12. The plate was read at 450 nm and 620-650 nm using a spectrophotometer (µQuant™, BioTek Instruments Inc. Vermont, USA) immediately after the addition of stop solution
13. Run validation and results were calculated according to the manufacturer's instruction. The median OD values of controls and calibrator must be within the ranges for the test to be valid. **Table 3.**

Table 3: Acceptable OD ranges for Controls and Calibrator in LAg avidity assay used in the study

	Negative control	Calibrator	Low positive control	High positive control
Minimum OD	0.000	0.400	0.190	0.830
Maximum OD	0.175	0.950	0.520	1.820

The normalized OD (ODn) was calculated for each control, calibrator and sample. Normalization of the OD value using an internal calibrator decreases the assay variability and increases the reproducibility(16,83). For the test to be valid the ODn of controls and calibrators must within range.

$$\text{ODn} = \frac{\text{Optical density of the sample, controls or calibrator}}{\text{Median optical density of the calibrator}}$$

ODn value of control and calibrator must fall within the acceptable range. **Table 4.**

Table 4: Acceptable ODn ranges for the Controls and Calibrator in LAg avidity assay used in the study

	Negative control	Calibrator	Low positive control	High positive control
Minimum ODn	0.000	1.000	0.370	1.500
Maximum ODn	0.240	1.000	0.700	2.400

14. During screening mode, if ODn was > 2.0, then long term infection and if ODn ≤ 2.0, then confirmatory testing was done.

15. During confirmatory testing, if the ODn was ≤1.5 then recent infection. If ODn was > 1.5, then long term infection. Confirmatory testing of the samples was done in triplicates. That is the controls, calibrators were tested as for the screening test but the difference is that all samples with ODn ≤ 2.0 were tested in triplicates. When samples are run in triplicate the mean OD is used to calculate the ODn. The algorithm and interpretation for LAg avidity assay is shown in **Fig 6.**

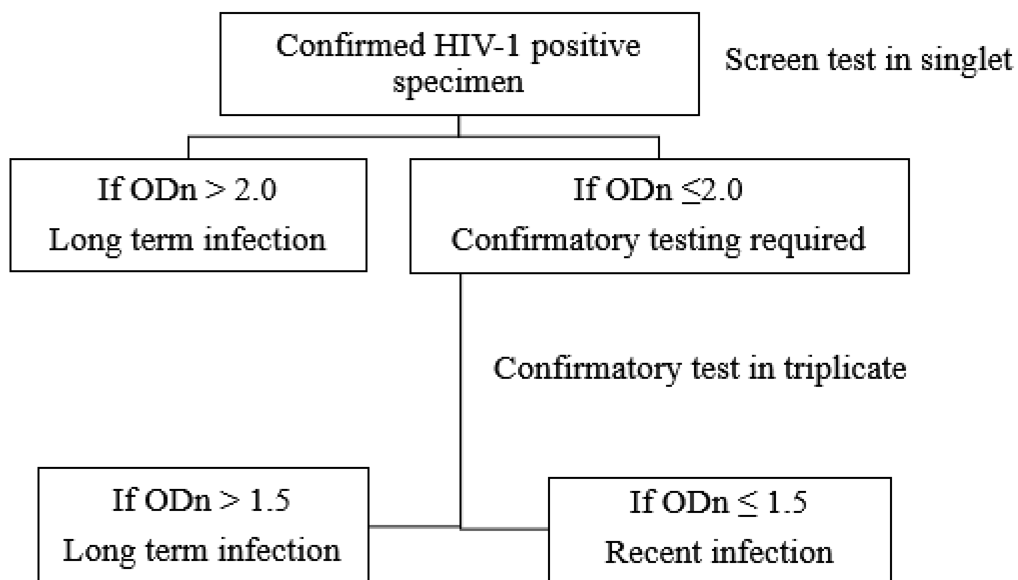


Figure 6: Algorithm for testing and interpretation of ODn values obtained in LAg avidity EIA

16. The avidity of the antibody was measured as normalized optical density value adjusted by calibrator and controls. Samples with ODn value below 1.5 was classified as ‘recent’ and the mean duration of recency (MDR) was 130 days.

5.2.2. In house avidity assay

The in house avidity assay was a modification of Microlisa HIV ELISA (Second generation ELISA using HIV envelope proteins gp41, gp120 for HIV-1 and gp36 for HIV-2) from J. Mitra (New Delhi, India) diagnostic products. The manufacturer's instruction was followed for addition of controls and sample to micro wells. The treatment of the duplicate wells with 7M urea solution (pH=3.0) is the modification used.

- **Preparation of 7M urea solution**
  - Urea was obtained from Fisher Scientific, Product No: 20885
  - The molecular weight of urea = 60.06 g/mol
  - 1M Urea solution = 1mole in 1L of distilled water (DW) = 60.06g in 1000ml of DW
  - 7M Urea solution = 7 x 60.06 g in 1000ml DW= 420.42 g in 1000 ml of DW
  - For preparation of 100 ml of urea solution 42.04 g of urea was added in 100ml of DW
  - The initial pH of the prepared solution was 6.4 and it set to 3.4 using pH meter CyberScan pH 1500 (Thermo Scientific).
  - The prepared urea solution was filtered and then stored at 4°C until use

Procedure

1. The controls were ready to use and required no dilution. 100µL of sample diluent was added to first well as blank, and 100µL of negative control was added in triplicate and positive controls were added 4 times as shown in **Table 5.**

Table 5: The plate configuration used for the in-house avidity assay

Washed with Wash Buffer						Treated with Urea solution					
BL	1	9	17	25	33	1	9	17	25	33	
NC	2	10	18	26	34	2	10	18	26	34	
NC	3	11	19	27	35	3	11	19	27	35	
NC	4	12	20	28	36	4	12	20	28	36	
PC	5	13	21	29	37	5	13	21	29	37	
PC	6	14	22	30	38	6	14	22	30	38	
PC	7	15	23	31	39	7	15	23	31	39	
PC	8	16	24	32	40	8	16	24	32	40	

*BL= Blank, NC = Negative control, PC = Positive control, the samples and their corresponding duplicates are depicting with the same number, the numbers in black depict the wells treated with wash buffer and the numbers in red depict the wells treated with 7M urea solution respective duplicate

2. The sample was diluted in sample diluent 1:11, that is 10µL of sample and 100µL of sample diluent. For a sample which was tested, 10 µL each was added in two different wells, that is, one well which was washed with wash buffer and second well which was washed with 7M urea solution as shown in **Table 5**. It was incubated at 37°C for 30 minutes.
3. After incubation, the entire plate was washed once with wash buffer (Phosphate buffered saline, included in the kit) using an automated strip washer (ELx50 strip washer, Bio-Tek Inc. USA). After wash, residual buffer was removed by gently tapping the plate over an absorbent paper.
4. Subsequently 300µL of wash buffer was added to the wells which had the controls and the samples. To the duplicate wells, 300µL of 7M urea solution with pH=3.0 was added and the plate was incubated for 15 minutes at room temperature. Urea solution was used as a dissociation agent and it has effective separation at pH=3.0(16).

5. After incubation, the plate was washed 5 times with wash buffer.
6. The concentrate of anti-human IgG conjugated with horse radish peroxidase was diluted with conjugate diluent in the ratio 1:100 and 100 µL of working conjugate was added to each well and the plate was incubated at 37°C for 30 minutes.
7. After incubation, the plate was washed 5 times with wash buffer.
8. Tetramethylbenzidine (TMB) substrate was diluted in TMB diluent in the ratio 1:1 and 100µL of working substrate was added to all the wells and plate was incubated at room temperature for 30 minutes avoiding exposure to light
9. After 30 minutes, the reaction was stopped using the stop solution (1N sulfuric acid).
10. The plate was read at 450nm using a spectrophotometer (µQuant™, BioTek Instruments Inc. Vermont, USA) after blanking the blank well within 30 minutes of adding the stop solution.

Test was valid when the controls were within limits as shown in **Table 6.**

Table 6: Acceptable limit for controls in house assay based on manufacturer's instruction for the commercial assay

	Blank	Negative Control	Positive Control
OD value	< 0.100	≤ 0.150	≥ 0.50

The Avidity Index (AI) is calculated by formula as previously described(45)

$$AI = \frac{\text{Optical density of the sample treated with urea}}{\text{(OD of the sample treated with wash buffer)}}$$

To be considered as recently infected the AI value cutoff was ≤ 0.80(60,59) and all samples above > 0.80 were considered as long term infection.

When the AI values were ≤ 0.80, the samples were tested in duplicates. In cases where there was discrepancy with AI and ODn. The samples were also tested in duplicates. And the median value was considered as the avidity index.

5.2.3. Western Blot

The assay used for the Western Blot was HIV BLOT 2.2 (MP Diagnostics, Singapore). It is a qualitative EIA for the detection of antibodies to HIV-1 and HIV-2 in human serum or plasma. It is a supplementary test used for specimens found repeatedly reactive using screening procedures like ELISA.

The nitrocellulose strips were blotted with separated antigenic proteins from inactivated HIV-1 by electrophoretic blotting procedures, combined with a specific HIV-2 synthetic protein on the same strip. Each strip was included with an internal sample control to reduce the risk of false negatives due to technical errors and to ensure that the sample has been added. The serum samples to be tested were inactivated at 56°C in water bath for 30 minutes.

Procedure

1. The individual nitrocellulose strips were removed from the tube and placed in trough with the numbered side of strip facing up
2. To each trough, 2ml of wash buffer (tris buffer with tween 20) was added and incubated at room temperature on a rocking platform for 2 minutes.
3. The buffer was removed by aspiration.

4. After aspiration, 2ml of blotting buffer (tris buffer with inactivated goat serum and non-fat milk powder) was added to each trough to prevent nonspecific binding of the proteins
5. Then 20µL of controls and sample were added to the respective trough. The tray was covered and incubated at room temperature on rocking platform and incubated overnight. The specific antibodies to HIV-1 if present will bind to the different antigenic proteins.
6. After an overnight incubation (16-20 hours), the tray was carefully uncovered to avoid mixing or splashing of samples. The contents of the tray were aspirated and the aspirator tips were changed between samples to avoid cross contamination.
7. The troughs were washed 3 times with wash buffer. The strips were allowed to soak for 5 minutes in the wash buffer between the washes (on the rocking platform)
8. After washing step, 2ml of working conjugate solution (goat anti human IgG conjugated with alkaline phosphatase and blotting buffer – 1:1000) was added to each trough and incubated for 30 minutes.
9. The conjugate was aspirated from the troughs and the strips were washed as in previous washing step
10. Then 2 ml of substrate BCIP (5-Bromo-4-chloro-3-indole-phosphate)/NBT (nitroblue tetrazolium) was added to each trough. It was covered and incubated for 15 minutes on rocking platform. The antigen-antibody reaction can be visualized by the presence of different bands.

11. The substrate was aspirated and the reaction was stopped using distilled water.
12. The strips were dried and graded based on the different bands.

For the run to be valid the bands on the controls must be satisfactory

- Non-reactive control
  - The band for serum control should be visible and there should be no HIV-1 or 2 specific bands
- Weak reactive control
  - It measures the sensitivity of the kit. The serum control band along with weak bands at p24 and or gp41 and gp120/gp160
- Strong reactive control
  - Serum control band along with bands at p17, p24, p31, gp41, p51, p55, p66, gp120/gp160 must be visualized. Also HIV-2 specific band should be visible.

Interpretation

The presence or absence of antibodies to HIV-1 is graded by comparing with the assay control strips tested with negative or non-reactive control, weak reactive and strong reactive controls. The different grades are negative, 1+, 2+, 3+ and 4+.

An American Red Cross criterion was used for the interpretation of the assay.

5.3. Statistical analysis

The data generated from study samples of controls and HIV-1 infected individuals were analyzed using independent sample ‘t’ test. The data obtained from western blot was analyzed using chi square test. The data generated from the archived paired sera of HIV-

1 infected individuals before treatment, after treatment and following virological failure were analyzed using paired 't' test. The data generated from these samples using these statistical methods were plotted using Box and Whisker plot. The comparison of the performance of the in-house assay with limiting antigen avidity assay was done by using degree of agreement kappa. The independent sample 't' test, the paired sample 't' test, degree of agreement was done using MedCalc Statistical Software v. 14.8.1 (MedCalc Software, Belgium; http://www.medcalc.org). The analysis of all demographic and laboratory data were performed using Microsoft Excel 2013. A p value of < 0.05 was considered significant.

5. Results

The findings on HIV-1 infected individuals (Group 1) and HIV-1 negative individuals (Group 2)

Demographic details

There were 896 volunteers who came to ICTC of a tertiary care centre during the study period - August 2015 to July 2016. All of them were tested according NACO strategy 3. Of these 132 individuals were found to be positive for HIV (14.73%). This data is shown in **Fig 7.**

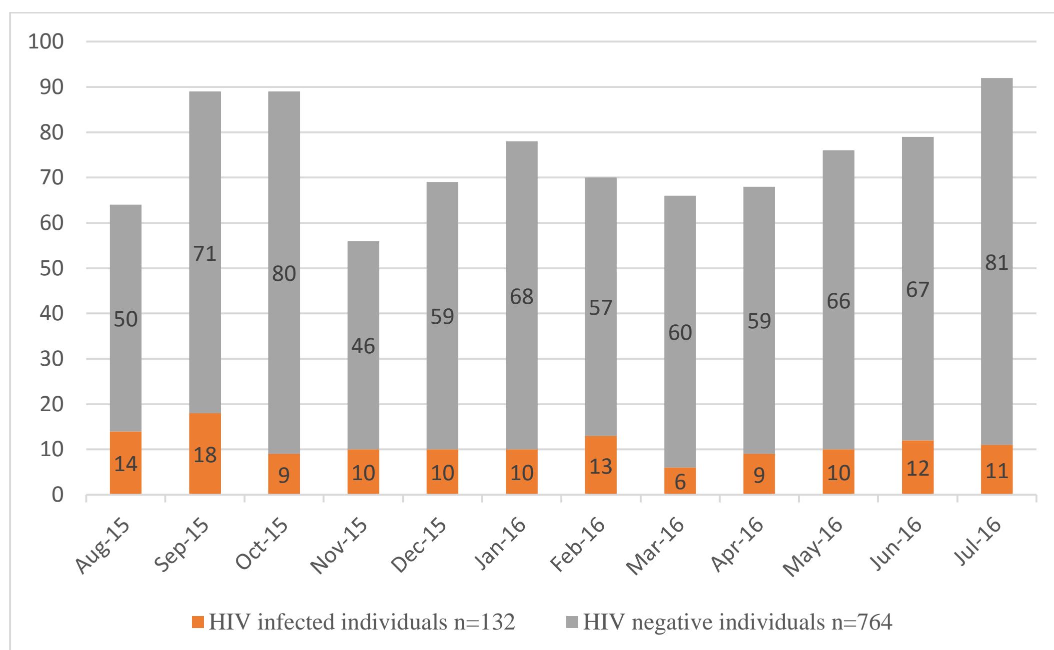


Figure 7: Distribution of volunteers (n = 896) to ICTC from August 2015 to July 2016, and showing the number of positive and negative samples month wise

Among these 117 treatment naïve HIV-1 infected individuals were recruited for the study. The remaining 15 were individuals who refused consent, or below the age of 18 years and one of the individuals had HIV-2 infection.

These HIV-1 infected individuals (n = 117) were from different states of the country, majority were from Tamil Nadu (n=47, 40.17%) followed by Andhra Pradesh (n=21, 17.95%) and West Bengal (n=18, 15.38%). There was also one individual from Bangladesh. The rest of the HIV-1 infected individuals were from other regions as shown in the **Table 7.**

Table 7: Geographic distribution of the HIV-1 infected individuals in the study (n= 117)

Geographic region	Number (%)
Tamil Nadu	47 (40.17%)
Andhra Pradesh	21 (17.95%)
West Bengal	18 (15.38%)
Jharkhand	15 (12.82%)
Bihar	4 (3.42%)
Chhattisgarh	3 (2.56%)
Uttar Pradesh	3 (2.56%)
Assam	2 (1.71%)
Manipur	2(1.71%)
Karnataka	1(0.85%)
Bangladesh	1 (0.85%)
Total	**117 (100)**

The gender distribution of the HIV-1 infected individuals (n = 117) recruited for the study is represented in **Fig 8.**

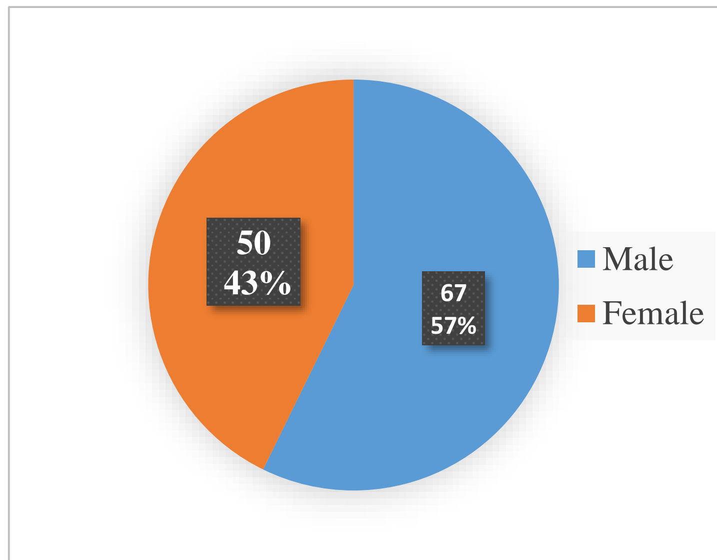


Figure 8: Distribution of HIV-1 infected individuals in the study population by gender (n =117)

The age group of the HIV-1 infected individuals (n = 117) ranged from 20-68 years with the mean age being 40 years (mean age in females = 38yrs; males = 41yrs). The age distribution of HIV-1 infected individuals in the study is shown in **Table 8.**

Table 8: Age distribution of HIV-1 infected individuals in the study (n = 117)

Age group in years	Number (%)
21-30	23 (19.66%)
31-40	41 (35.04%)
41-50	41 (35.04%)
51-60	10 (8.55%)
61-70	2 (1.71%)
Total	**117 (100.00%)**

In HIV-1 infected individuals of the study population, the transmission was predominantly through sexual contact as shown in **Fig 9.**

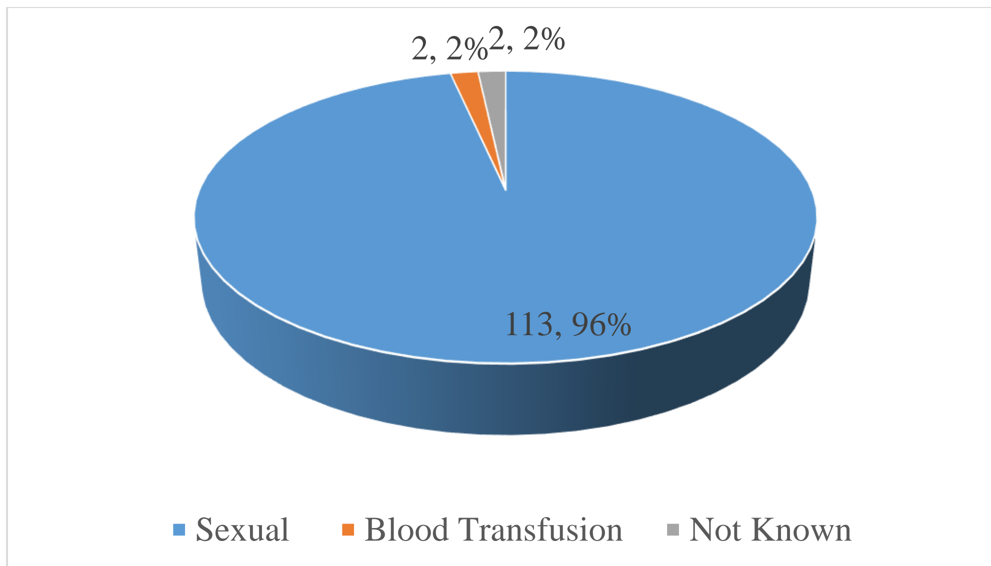


Figure 9: Mode of transmission among HIV-1 infected individuals in study population (n=117)

Of 113 who acquired HIV-1 infection by sexual transmission, the mode of acquisition is shown in **Fig 10.**

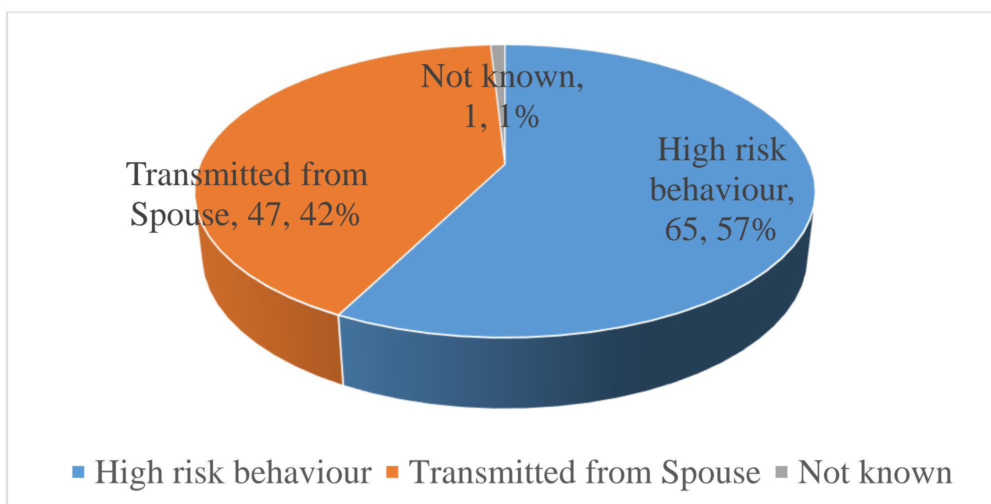


Figure 10: Pie chart showing the source of acquirement of infection in HIV-1 infected individuals who acquired the infection sexually (n=113)

The predominant mode of acquisition of infection in males and females were associated with high risk behavior and infection from spouses respectively as shown in **Fig 11.**

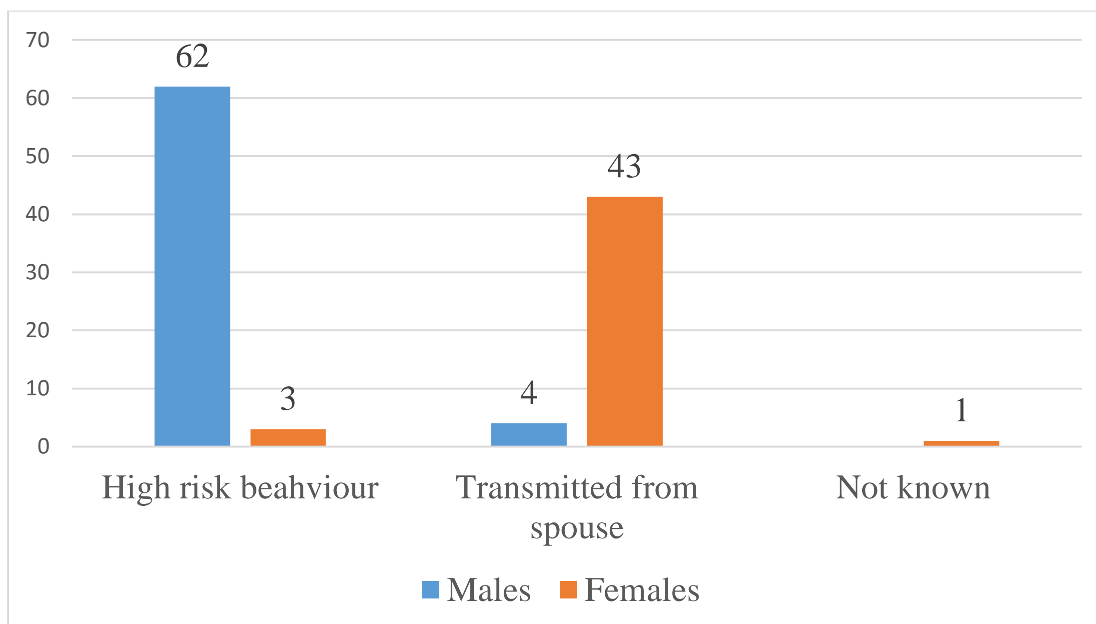


Figure 11: Mode of acquisition of HIV-1 infection in sexually transmitted HIV-1 infection, Males (n = 66), females (n = 47)

Group 2:

Among the volunteers who were recruited from ICTC as HIV **negative** controls, the male and female distribution is depicted in **Fig 12.**

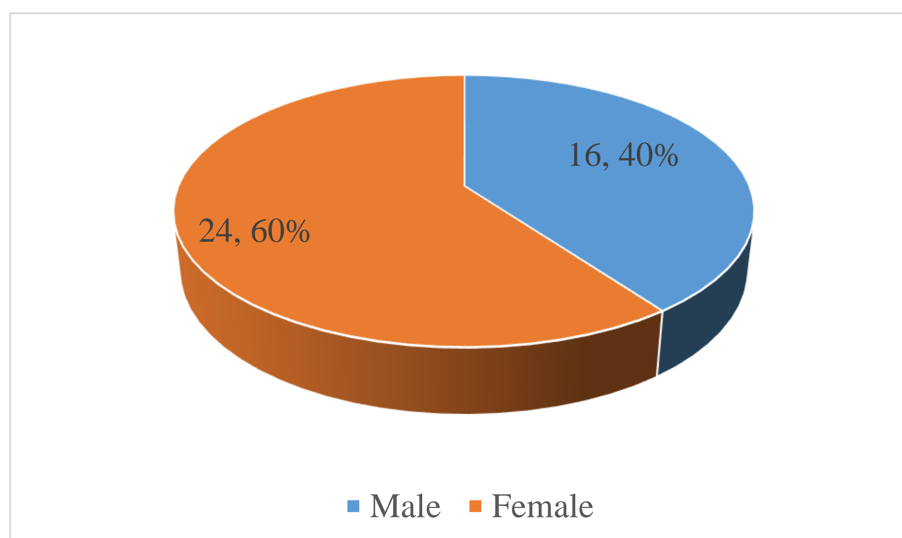


Figure 12: Distribution of HIV negative individuals recruited as controls for study by gender (n = 40)

The age of the HIV negative individuals recruited for the study ranged from 18-60 years with mean age being 36 years (mean age in males = 38 years and mean age of female =36 years). Majority of them were from Tamil Nadu (n = 28, 70%) like the HIV-1infected individuals. The state distribution of these HIV negative individuals are shown in **Table 9.**

Table 9: Geographic Distribution of HIV negative individuals recruited as controls (n=40)

States	**Number (%)**
Tamil Nadu	28 (70%)
Andhra Pradesh	5 (13%)
Jharkhand	2 (5%)
West Bengal	1 (3%)
Kerala	1 (3%)
Karnataka	1 (3%)
Assam	1 (3%)
Bihar	1 (3%)
Total	**40 (100%)**

LAg Avidity Assay

All blood samples of HIV-1 infected individuals and the HIV negative controls in the study population were tested using the LAg Avidity EIA following the manufacturer's instructions.

The HIV negative controls and samples positive for p24 antigen alone and negative for HIV-1 IgG were tested along with positive samples as internal negative control and all these samples were negative in the assay. The mean normalized optical density (ODn) value of these controls were 0.068 ± 0.03.

The mean ODn value of samples positive for p24 alone were 0.131 ± 0.05.

Both control and p24 positive ODn results fell in the negative control range (0-0.240) according to the LAg avidity EIA.

The ODn values between the controls and HIV-1 infected individuals is depicted in **Fig 13.**

The mean ODn value of samples of HIV-1 infected individuals was 3.549 ± 1.1.

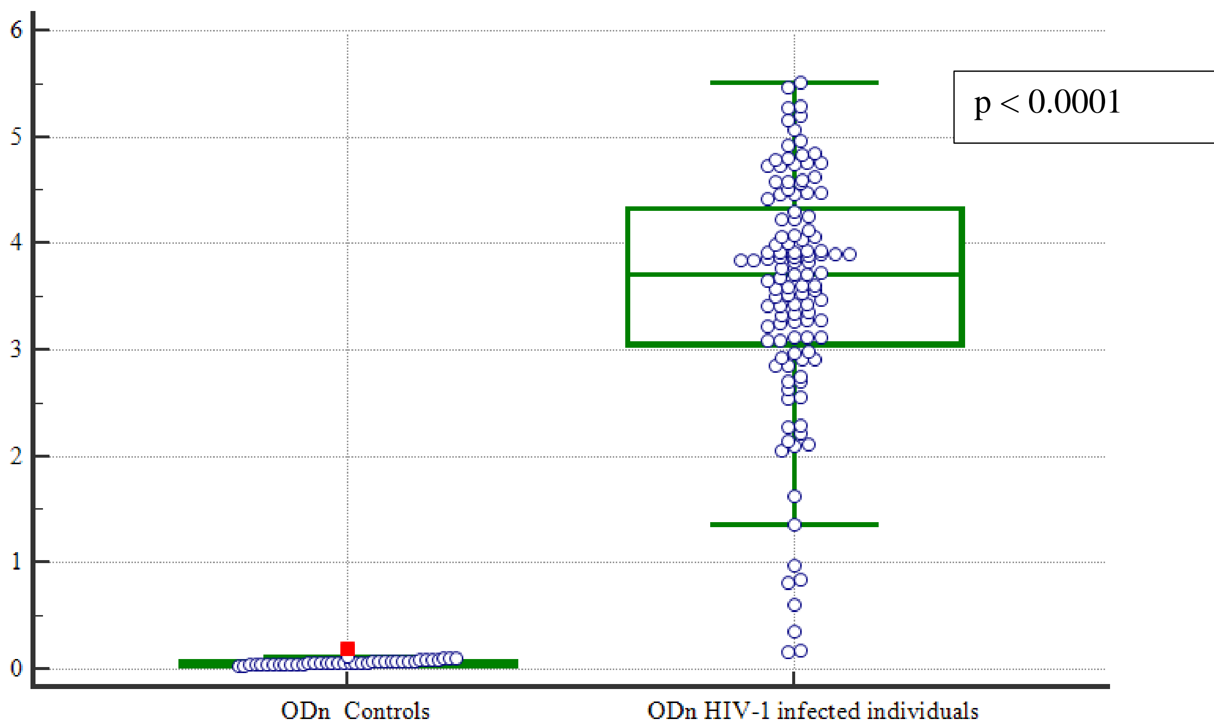


Figure 13: Box and Whisker plot showing the distribution of ODn values of the HIV negative controls (n=40) and HIV-1 infected individuals (n=117) in the study population

Samples of the HIV-1 infected individuals were tested and the results are shown in **Fig 14.**

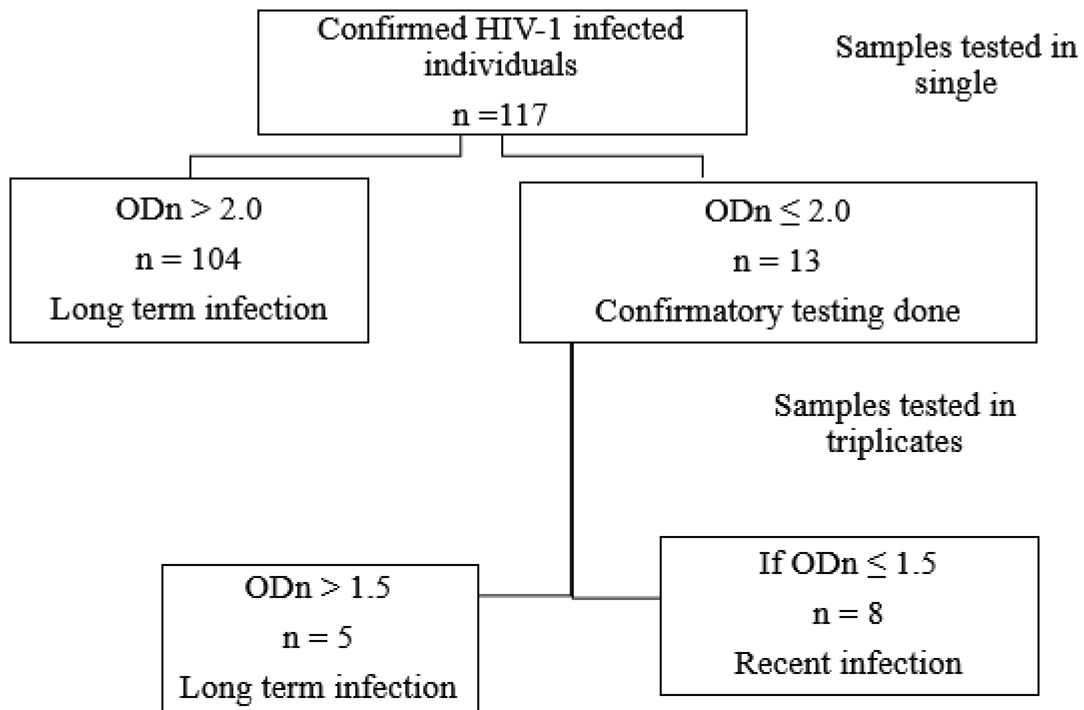


Figure 14: The different ODn value status of the samples from HIV-1 infected individuals in the study population using the LAg Avidity EIA

Of 117 samples tested, 8 (6.84%) were found to recently infected (less than 6 months) as described by the manufacturer.

The comparison between the ODn values of recent infection and long term infection is shown in **Fig 15.**

The mean ODn value was significantly lower for recent infections compared to long term infections (ODn 0.662 ± 0.42 vs 3.761 ± 0.84, p <0.0001).

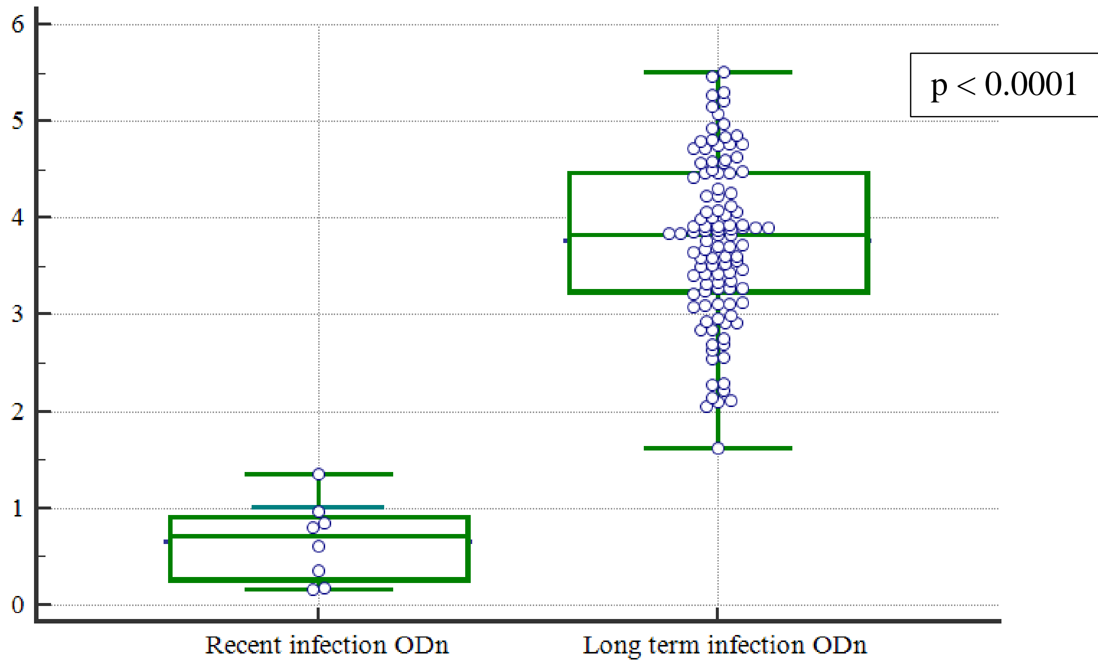


Figure 15: Box and whisker plot between ODn values of recent and long term infection ODn cutoff 1.5 using the LAg avidity EIA

Variability

The inter and intra-assay coefficient of variation for the LAg avidity EIA for individuals with recent HIV-1 infection was carried out and there was no significant difference in % CV observed. This data is shown in **Table 10.**

Table 10: Inter-assay and intra-assay variability of the LAg avidity EIA using samples of individuals with recent infection

Sample ID	Inter – assay coefficient of variation (CV%)	Intra – assay coefficient of variation (CV %)
LAV 8	8%	5%
LAV 31	8%	6%
LAV 43	13%	1%
LAV 48	4%	13%
LAV 55	25%	3%
LAV 104	13%	4%
LAV 110	4%	3%
LAV 117	10%	7%
Mean % CV of the assay	**11%**	**5%**

Among the 8 individuals who were found to be recently infected by the LAg assay 5 got infection form the infected spouse and three got it through sexual exposure with commercial sex workers. The details on these 8 individuals are shown in **Table 11.**

Table 11: Details about individuals who are recently HIV-1 infected (n = 8)

	Age/ Sex*	Marital Status	Occupation	Exposure	Trans-mission from spouse	High risk behavior	State	ODn
1	38/F	Married	Laborer	Sexual	Yes	No	Tamil Nadu	0.807
2	68/M	Married	Business	Sexual	No	Yes	Jharkhand	1.354
3	36/F	Married	Housewife	Sexual	Yes	No	Tamil Nadu	0.166
4	39/M	Married	Business	Sexual	No	Yes	Andhra Pradesh	0.353
5	34/F	Married	Housewife	Sexual	Yes	No	Andhra Pradesh	0.845
6	32/M	Married	Driver	Sexual	No	Yes	Jharkhand	0.183
7	35/F	Married	Laborer	Sexual	Yes	No	Tamil Nadu	0.974
8	39/F	Married	Housewife	Sexual	Yes	No	Jharkhand	0.612

*M= Male, F= Female

In-house avidity assay

The results of in house are interpreted based on the avidity index (AI). The distribution of the AI of the 117 HIV-1 infected individuals is depicted in the **Fig 16.**

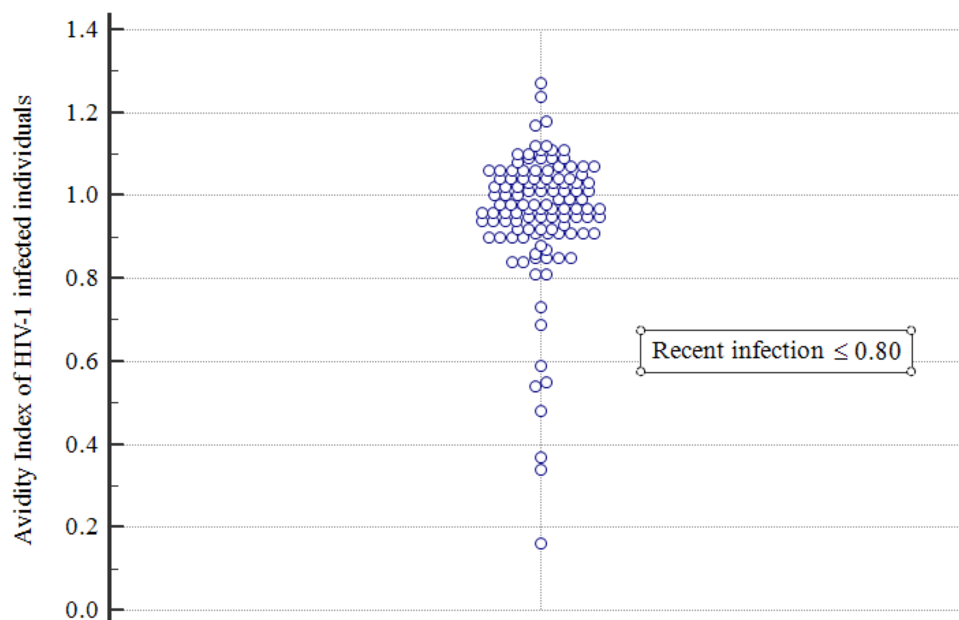


Figure 16: Distribution of Avidity Index for HIV-1 infected individuals (n =117) using the in-house avidity assay

The comparison of the avidity index (AI) between the recent and long term infection in the study population was carried out. The cutoff for HIV-1 infection to be recent and long term was AI ≤0.80. The mean AI is significantly lower for recent than long term infection (0.494 ±0.18 vs 0.992 ±0.09, $p < 0.001$). This data is shown in **Fig 17**.

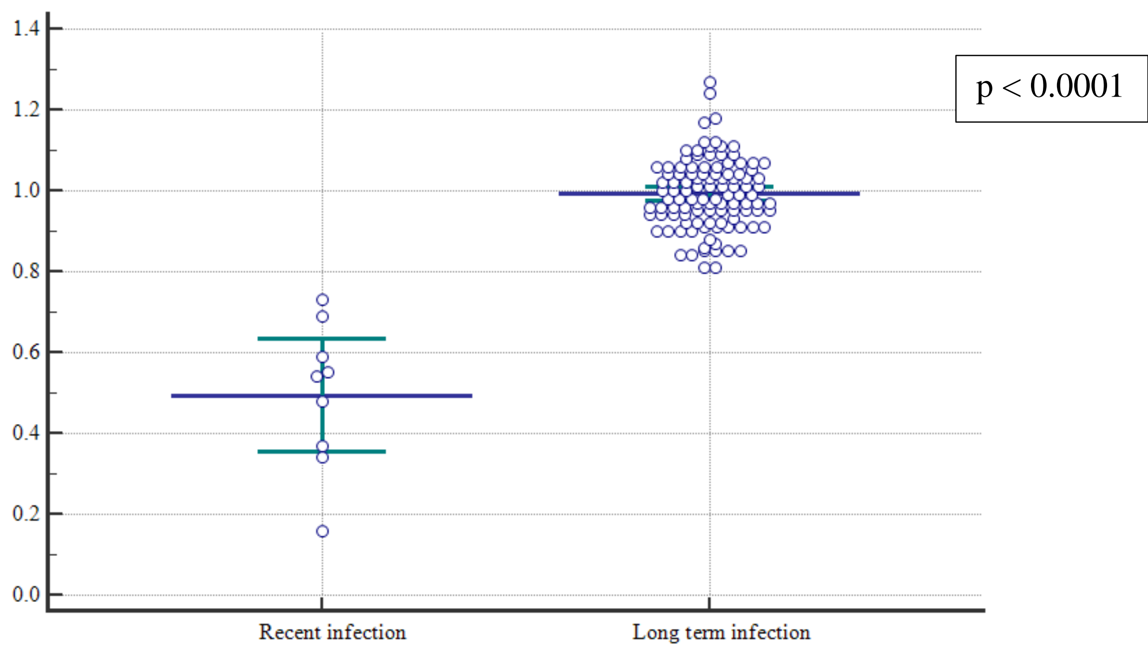


Figure 17:Comparison between recent (n = 9) and long term infection (n = 108) based on Avidity Index (AI) cutoff 0.80 using the in-house avidity assay

The comparison between LAg avidity EIA and in-house avidity assay of 117 samples from HIV-1 infected individuals is shown in **Table 12.**

Table 12: The comparison between LAg Avidity EIA and in-house avidity assay of 117 samples from HIV-1 infected individuals

	LAg Avidity EIA		
	Positive	Negative	Total
In house assay positive	8	1	9
In house assay negative	0	108	108
Total	8	109	117

With LAg avidity EIA as gold standard the accuracy indices of in-house avidity assay was calculated and showed good sensitivity and specificity. This data is shown **Table 13.**

Table 13: The accuracy indices of the in-house avidity assay in comparison with the commercial LAg avidity EIA

Sensitivity	100 % (95% CI 63.06-100%)
Specificity	99.08 % (95% CI 94.99-99.98%)
Positive Predictive value (PPV)	88.89 % (95% CI 51.75-99.72%)
Negative Predictive value (NPV)	100 % (95% CI 96.64-100%)

The degree of agreement between the with LAg avidity EIA and the in-house avidity assay was calculated using the Kappa test and showed a significant agreement between the assays and is shown in **Table 14.**

Table 14: The degree of agreement between the with LAg avidity EIA and the in-house avidity assay calculated using the Kappa test

Degree of agreement (κ)	0.94
Standard deviation (Z)	10.15
Probability (p)	<0.0001

Variability

The inter and intra-assay coefficient of variation of the in-house avidity assay for 5/8 samples of the individuals with recent infection is shown in **Table 15.**

Table 15: Inter-assay and intra-assay variability of the in-house avidity assay for samples of individuals with recent infection

Sample ID	Inter-assay coefficient of variation (CV%)	Intra-assay coefficient of variation (CV%)
LAV 8	8.0%	0.70%
LAV 31	12.8%	13.53%
LAV 43	2.4%	2.13%
LAV 48	0.4%	1.08%
LAV 55	1.8%	2.12%
LAV 104	21.5%	14.23%
Mean % CV	**7.82%**	**5.6%**

Western Blot

In every run of Western Blot, a non-reactive, weak reactive and strong reactive sample was tested. In negative control, only serum band was formed. Weak reactive control had less bands compared to strong reactive controls, the bands were more in number in strong reactive controls. The intensity of the band was also more. When reading was taken the numbered end of the strip was placed at the bottom as shown in **Fig 18 a.** And the various bands were identified using the aid provided by the manufacturer which is shown in **Fig 18 b.** The bands for which intensity was 2+, 3+ and 4+ was considered as positive for antibodies to that particular antigen

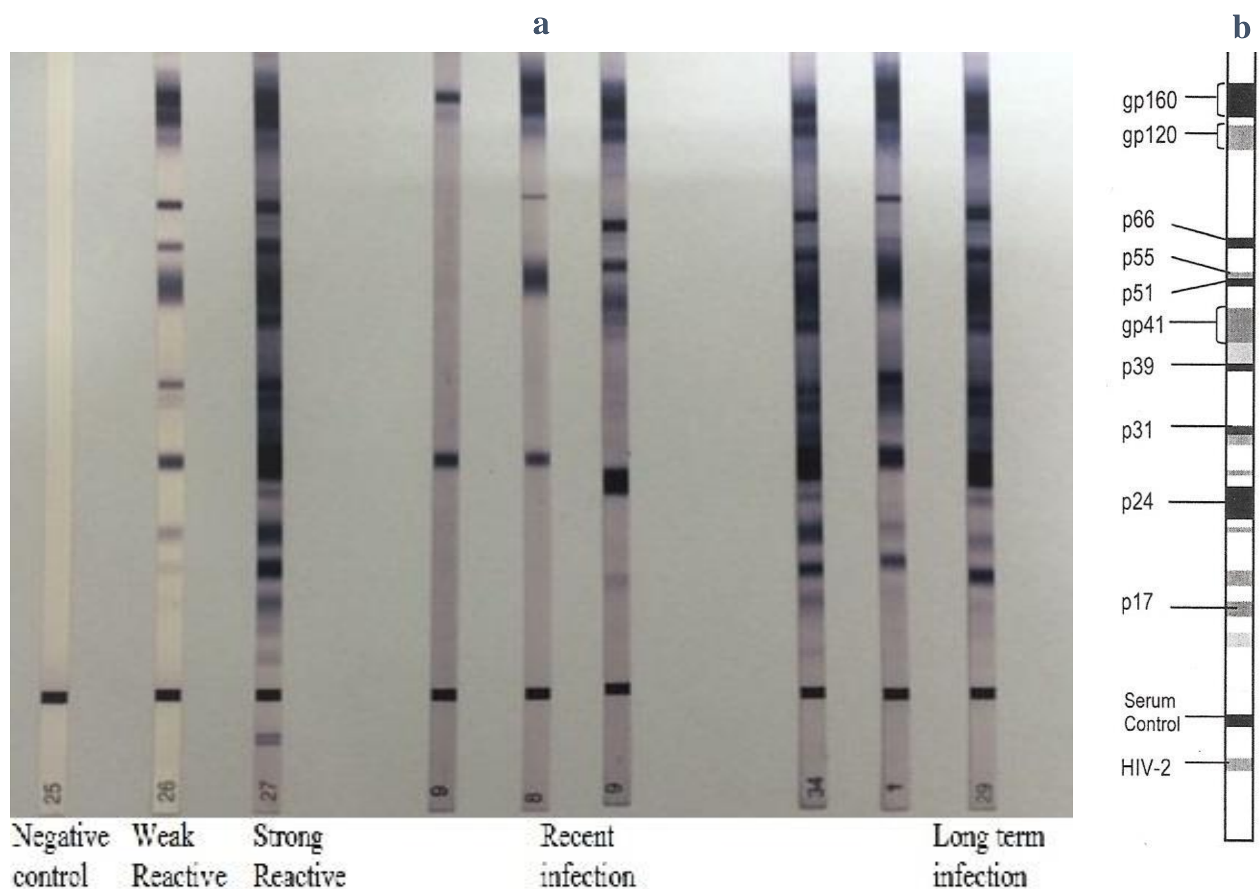


Figure 18: Western Blot of the negative control, weak reactive, strong reactive, samples of individuals with recent and long term infection. In negative control, only serum band was formed. Weak reactive control had less bands compared to strongly reactive controls, the bands are more in number. The intensity of the band was also more. In recent infections the bands were fewer and intensity of bands is less when compared to long term infection. The figure b shows a card which was used to identify the various bands which had developed.

Western blot analysis of the samples of HIV-1 infected individuals with recent infection showed weak band or no band against pol proteins – p31, p51 and p66, when compared to those with long term infection. Summary of the western blot analysis in HIV-1 infected individuals (n=117) is shown in **Table 16.**

Table 16: Western Blot reactivity to the different antigens in HIV-1 infected individuals with recent (n = 8) and long term infection (n = 109)

Antibodies to	Recent infection (n = 8)		Long term infection (n = 109)		p value
	Negative/Weak*	Positive#	Negative/Weak*	Positive#	
p17	7 (87.5%)	1 (12.5%)	78 (71.6%)	31 (28.4%)	0.57
p24	0	8 (100%)	8 (7.3%)	101 (92.7%)	--
p31	6 (75%)	2 (25.0%)	24 (22%)	85 (78%)	0.004
p39	7 (87.5%)	1 (12.5%)	72 (66.1%)	37 (33.9%)	0.39
gp41	4 (50.0%)	4 (50.0%)	3 (2.8%)	106 (97.2%)	--
p51	6 (75.0%)	2 (25.0%)	39 (35.8%)	70 (64.2%)	0.07
p55	8 (100%)	0	83 (76.1%)	26 (23.9%)	0.26
p66	3 (37.5%)	5 (62.5%)	12 (11%)	97 (89%)	0.10
gp120	1 (12.5%)	7 (87.5%)	0	109 (100%)	--
gp160	0	8 (100%)	0	109 (100%)	--

*Weak = Intensity of band is 1+, #Positive = Intensity of the band 2+ and more

Group 3 – HIV-1 infected individuals before ART and after responding to ART, (n = 25)

Demography

In group 3, HIV-1 infected individuals before treatment with ART and following initiation of ART (n = 25) majority were men (76%), the gender distribution of these individuals is shown in **Fig 19.**

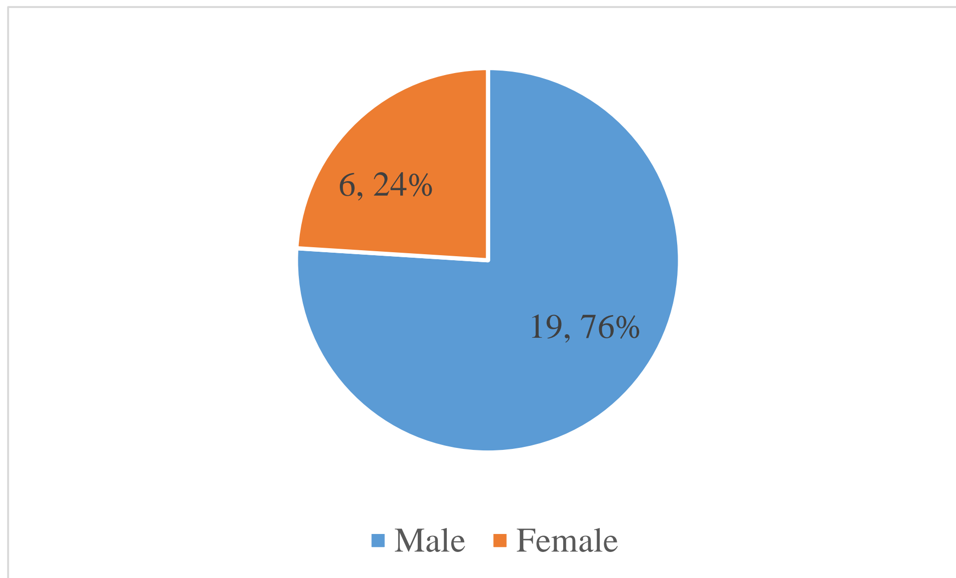


Figure 19: The gender distribution of the HIV-1 infected individuals (n = 25) of group 3 - Before treatment with ART drugs and following the initiation of treatment and responding to the same.

We used archived paired serum samples of HIV-1 infected individuals before treatment with ART and 6-12 months following initiation of ART and responding to the same. The viral load of these individuals after responding to 6-12 months of ART were below detection limit or the logarithmic difference of the viral load before and after ART were more than 1. The viral loads of HIV-1 infected individuals before treatment with ART and following the initiation of treatment after and responding to the same is shown in **Table 17.**

Table 17: The viral loads (VL) of the HIV-1 infected individuals before ART and following initiation of ART (n = 25)

Age (Years)	Sex*	VL before ART	VL after ART	log diff of VL	Treatment period
36	M	156,491	2,857	1.74	1 year
25	F	68,210	1,575	1.64	1 year
46	F	52,767	1,012	1.72	1 year
62	M	77,043	754	2.01	6 months
27	M	87,202	279	2.49	1 year
39	M	131,461	251	2.72	1 year
44	M	728,815	218	3.52	1 year
35	M	169,337	179	2.98	1 year
34	M	12,947	<72	2.26	1 year
30	M	4,462	<72	1.80	1 year
41	M	188	<72	0.42	1 year
36	M	552	<72	0.89	1 year
52	M	47,413	<72	2.82	1 year
43	M	21,717	<72	2.49	1 year
30	M	35,212	<72	2.70	1 year
49	M	121	<72	0.23	1 year
38	M	25,107	<72	2.55	1 year
44	M	121	<72	0.23	1 year
41	M	21,884	<72	2.49	1 year
45	F	646,481	<72	3.96	1 year
34	M	21,206	<72	2.48	1 year
66	M	68,009	<72	2.98	1 year
30	F	26,996	<72	2.58	1 year
40	F	3,724	<72	1.72	6 months
26	F	2,917	<72	1.61	1 year

*M = Male, F = Female

The mean ODn values of samples from HIV-1 infected individuals (n=25) responding to treatment was significantly lower than the samples prior to treatment (ODn = 3.977±1.1 Vs ODn = 4.306± 0.97, p=0.0038) as shown in **Fig 20.**

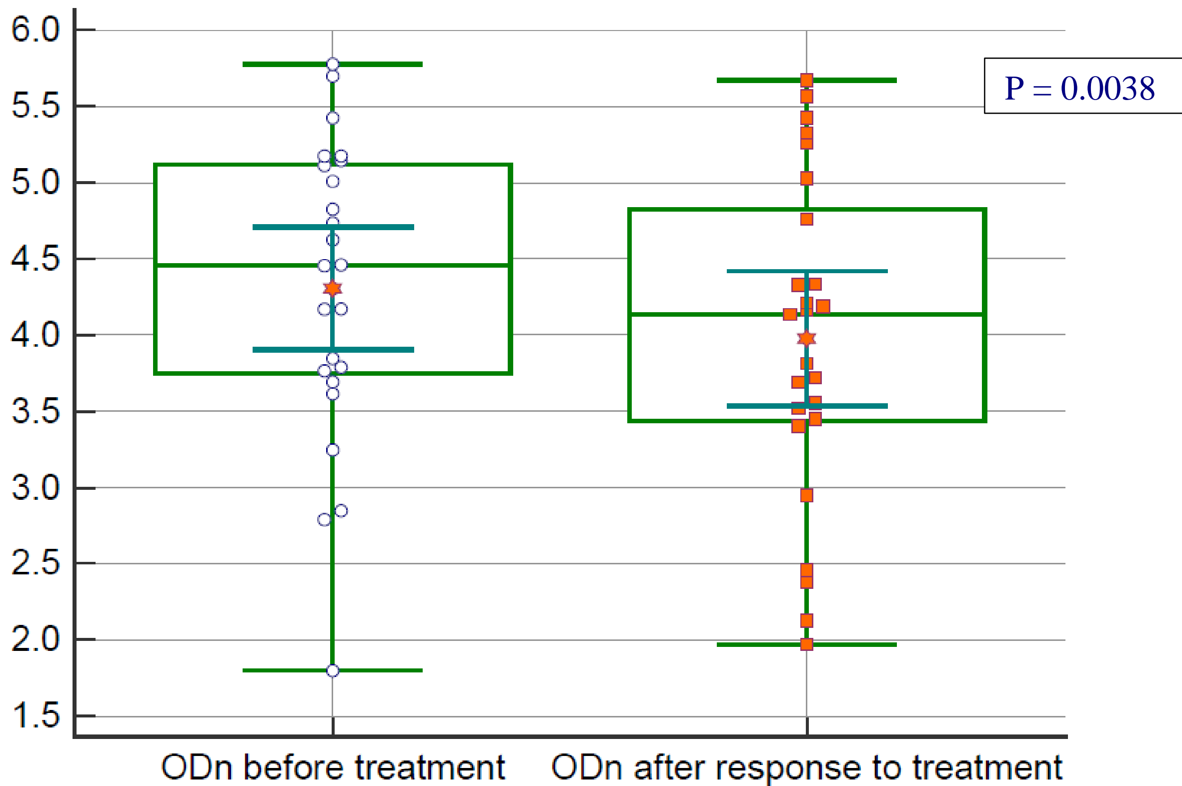


Figure 20: Box and whisker plot of ODn value of HIV-1 infected individual before ART and after responding to ART (n=25)

Group 4 – HIV-1 infected individuals responding to ART and following treatment failure (n = 7)

HIV-1 infected individuals (n = 7) responding to ART had viral load below detection limit. The viral loads of HIV-1 infected individuals when they were responding to ART and after they developed virological failure is shown in **Table 18**.

Table 18: HIV-1 infected individuals responding to ART and after developing virological failure (n = 7)

Age	Sex	VL* after responding ART	Treatment in years	VL* after virological failure
49	Male	<40	8	129,233
42	Female	<40	5	32,293
46	Male	<40	4	827,829
48	Male	<40	9	191,885
45	Female	<40	9	1,108,938
30	Male	<40	5	21,174,000
43	Male	<72	4	528,647

*VL = viral load

There was an increase in the mean ODn values when individuals (n=7) responding to ART had virological failure compared to when they were responding to ART (ODn = 4.128±0.6 vs ODn = 3.144±1.2, p = 0.057) is shown in **Fig 21.**

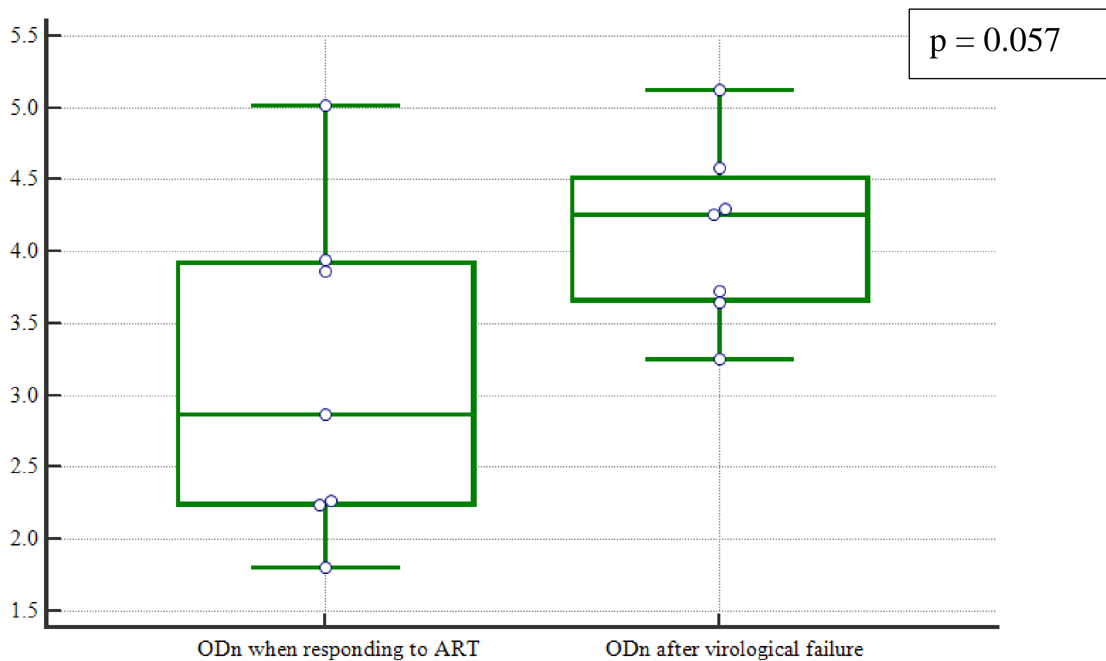


Figure 21 : Box and whisker plot of ODn values in HIV-1 infected individual when they were responding to ART and after developing virological failure (n=7)

6. Discussion

Human Immunodeficiency Virus infection is a major health problem worldwide. It has claimed more than 35 million lives so far. In 2015, there were about 36.7 million people living with HIV globally(2). There is no known cure available for HIV infection but there are many effective antiretroviral drugs to control the infection and help in prevention. HIV-1 is known to cause a more severe disease than HIV-2(11). The knowledge about the routes of transmission helps us to introduce effective strategies for prevention. The awareness of the spread of HIV infection among different groups is important, so that immediate interventions can be introduced in the most effective manner to reduce the spread of infection. HIV incidence is the rate at which a population acquires HIV infection and it measures the magnitude of current HIV transmission. In order to know HIV-1 incidence, we need accurate assays for differentiating between recent and long term infections (12). In 2015, the number of new HIV infections was about 2.1 million worldwide(2). India had an estimate of 86,000 new HIV infections(6). Knowing the HIV-1 incidence helps us in program planning, population surveillance, evaluation of the effectiveness of current intervention programs and selecting population for clinical trial for new preventive intervention or early treatment(12).

In our study carried out during August 2015 to July 2016, of 896 volunteers who came to ICTC of a tertiary care centre, 132 (14.73%) was positive for HIV infection. All these individuals were tested according to NACO strategy 3. Among the volunteers who attended the ICTC, HIV-1 infection were seen more in men (57%) compared to women (43%) which was comparable with the national data where 40.5% of the total HIV infection were in women (6). In our study, the infection was more common in men and

women in ages 31-50 years (70%). In India, children (below 15 years) accounted for 12% (10,400 of 86,000) of total new infections while 88% (75,900 of 86,000) new infections were among individuals above 15 years of age(7). The mode of transmission in 96% of the individuals in the study population was through sexual contact. Blood transfusion associated HIV-1 infection was only 2% and in remaining 2% the route of transmission was unknown. In India, the incidence of blood donor sero-reactivity has reduced from 1.2% to 0.2% in blood banks supported by NACO(6). In blood bank a single test of high sensitivity is used for HIV reactivity. Highly sensitive tests increase the chance of false positives but it reduces the chance of missing an HIV infection. So if an individual tested positive in a blood bank screening test, then the individual is referred to ICTC for confirmation(53). In rural areas of certain states like Uttarakhand, Uttar Pradesh, Jharkhand, Bihar and Chhattisgarh, the access to safe blood is limited(6). One of the HIV-1 infected individual who acquired the infection by blood transfusion was from Jharkhand. Sexual transmission was associated with high risk behavior in 57% of the HIV-1 infected individuals (n=117) and 42% acquired the infection from their HIV-1 infected spouses and in 1% high behavior or spouse's history unknown. Among the HIV-1 negative controls, there were more women (60%) than men (40%), this can be probable due to the fact that some of these women have come to get tested as their spouses are positive for HIV infection.

Identification of the recent HIV-1 infection is important because it helps us to assess the incidence of HIV infection in the population(12). It helps us to identify the groups who are at higher risk of acquiring new HIV infection and to direct health campaigns to target them. It helps us in contact tracing and can also help us to the assess the

effectiveness of our intervention programmes(12,15). Clinically it is difficult to identify recent infection as most of them are asymptomatic but highly infectious. Also, one of the components of global strategy for prevention and assessment of HIV drug resistance is to check for transmitted drug resistance in recently infected populations(90).

Janssen et al (57) first reported a laboratory test for differentiating recent HIV-1 infections from established infections. They developed and validated a serological testing algorithm in which HIV-1 infected individuals had reactive result on a sensitive HIV-1 EIA (3A11 assay, Abbott, Illinois, USA) but nonreactive results on a less sensitive (LS) EIA which was a modification of the same EIA (3A11-LS assay). These individuals were identified as having early HIV infection. For the less sensitive EIA, they modified the sample dilution, sample incubation time, and conjugate incubation time. Their assay diagnosed 95% of the recent infection but misdiagnosed 0.4% with long term infection and 2% with late-stage AIDS (severe immunosuppression) as having a recent HIV-1 infection. There were many other studies on less sensitive immunoassays but all assays based on this principle had used antigen from HIV-1 subtype B and not reliable for subtype C prevalent in India(65,66,70).

To overcome this subtype bias, Parekh et al (58) devised BED capture EIA (BED CEIA, (Calypte Biomedical Corporation, Oregon, USA) which detected the increasing levels of HIV-1 specific IgG after seroconversion and which could be used for detecting recent HIV-1 infection. They used synthetic protein which had immunodominant regions of gp41 from HIV-1 subtypes B, E, and D as antigen. The proportion of the HIV-1 immunoglobulin G to total immunoglobulin G gradually increased for 2 years following seroconversion. The assay had high false recency rate (FRR). Moyo et al (95) evaluated

false recent rates of HIV-1 subtype C incidence in Botswana using the BED CEIA (Calypte Biomedical Corporation, Oregon, USA), a Bio-Rad Avidity Index (BAI) assay (modification of the Bio-Rad HIV1/2+O EIA, Bio-Rad Laboratories, Washington, USA). The false recent rate mean values for BED CEIA was 6.05% (95% CI 4.15–8.48), Bio-Rad Avidity Index was 5.57% (3.70–8.0). The inter-assay agreement between BED capture EIA and Bio-Rad avidity index was 92.8% (95% CI 90.1–94.5) for recent/long-term infection.

In the study reported in this thesis, of the recruited population, 8 (6.84%) were found to be recently infected that is ODn value <1.5. According to a study by Duong et al (87) the mean duration of recency (MDR) in LAg avidity EIA (Sedia, Oregon, USA) for ODn = 1.0 was 88-94 days and for ODn = 2.0 was 177-183 days; and the overall proportion of false recent rate increased from 0.6% to 2.5% when cutoff of ODn was increased from 1.0 to 2.0. So in order to balance between longer mean duration of recency and smaller false recency rate (<2%), they suggested a cutoff ODn = 1.5, which represented a mean duration of 130 days(87). In our study, except for one individual all the other recently infected individuals (n = 7) had ODn <1.0 and would have acquired the infection in the last 3 months. While in BED capture EIA, the cutoff for ODn was 0.80, so if ODn ≤ 0.80 it was considered as recent seroconversion and if ODn >0.80 it was considered as long term infection. In BED CEIA, ODn = 0.8 corresponded to a mean duration of recency of 197 days (55). Among the recently infected individuals (n = 8) all of them acquired the infection by heterosexual transmission. 7 (87.5%) of them belonged to the age group 31-40 years and 1 belonged to age group 61-70 years. In them, 5(62.5%) were females and acquired the infection from their HIV-1 infected

spouses and 3 (37.5%) males acquired the infection through high risk behavior. Among the recently infected men, two of them were from Jharkhand, one of them a driver and another business man who travels a lot and have got the infection from commercial sex workers. This suggests the importance of curbing the infection in high risk groups because they serve as a huge pool of HIV infection in our community. From the high risk group, the HIV infection is transmitted to the bridging population which includes long distance drivers and migrant population. From bridge population it goes to their spouses or sexual partners. It is very important to identify the HIV infection in individuals but it is even more important to identify the individuals with recent infection as they will have a very high viral and are more infectious(96,97). Studies show that individuals who are recently infected with HIV tend to be more engaged in high risk behaviour than individuals who has had long term HIV infection(98–100).

There was not much national data available for frequency of recent infections. Hall et al(74) in United States tested 6864 of HIV-1 positive specimens using the BED assay and classified 31% (2133) as recent infection. Compared to our study where we had only 6.83% rent infections, they had 31% recent infection but their sample size was large and covered almost 22 states in United States. One of the reasons for the low incidence in our study was because many of the volunteers in our ICTC were spouses of already diagnosed HIV infected individuals. A study by Simmons et al (101) in Ukraine during 1 year tested 446 newly diagnosed HIV-1 infected individuals using LAg avidity assay, 39 (8.7%) were classified as recent infection with ODn < 1.5. But of these 39, 10 were reclassified as long term infection as their viral load was less than 1000. So, there were 29 (6.5%) individuals with recent infection. Preliminary studies

by Parekh et al(86) and Duong et al(64) using the LAg assay suggested a good reproducibility with coefficient of variation <10%. In our study the mean inter-assay and intra-assay coefficient of variation of LAg avidity assay for samples of individuals with recent HIV infection was 11% and 5% respectively.

One of advantage of the LAg avidity assay over other assays for recent infection was that it used recombinant protein as antigen. This protein contained the major subtypes and recombinants of gp41 immunodominant region of HIV-1 group M. It overcame the limitation of less sensitive assay which used only HIV-1 subtype B and of BED CEIA which used only antigen against HIV-1 subtypes B, E and D. In India, HIV-1 subtype C is more prevalent(32). The wells of LAg are coated with the recombinant antigen in limiting concentrations because at high concentration of antigen, IgG antibodies with low and high avidity bind to the antigen and binding occurs with the help of both binding sites of the antibodies. When the concentration of the antigen is lowered, only binding of antibodies with high avidity is permitted and as concentration of the antigen is limited binding at both sites simultaneously is not possible so the antibodies with low avidity would be able to bind strongly(16). Even though there are many commercial assays for identifying recent infections, we found the need to develop an in-house as the cost of these assays are very high and not affordable by every laboratory. One of the issues we had with the LAg avidity assay was its high cost. So we decided to use an indigenous commercial assay and develop an in-house by modifying it.

Wei et al (16) developed a new recombinant protein which could detect antibodies against all recombinants and major subtypes of HIV-1 group M; the recombinant protein was derived from immunodominant region (IDR) of glycoprotein41 of the

respective subtypes . The recombinant protein (rIDR-M) was expressed in Escherichia coli. It was equally reactive with HIV-1 antibodies of the different subtypes and divergent subtypes B and AE. In their study, they used recombinant protein in two assay formats to measure avidity of the antibody. One was a two-well avidity index that is a single sample required two wells for the test and second assay format was a one-well limiting antigen avidity assay. In both assays they used buffer with pH 3.0 to dissociate antibodies with low avidity. They found that recent HIV-1 infection could be detected with or without dissociation agent when the concentration of the antigen was limited. Also, the one-well limiting antigen avidity EIA permitted more samples to be tested over the two-well avidity index -EIA. And both these assays were better than the available commercial assays as they used a recombinant protein which prevented the subtype based performance(16). The detection ability of the assay was more efficient when dissociation buffer with pH 3.0 was included. In our study, initially the 7M urea solution that we used for the in-house assay had a pH = 6.4 and we found that it didn't dissociate the low avidity antibodies giving us an almost equal OD value with and without urea treatment. When we changed the pH to 3.0, the urea solution worked and it dissociated the low avidity antibodies giving us a decrease in OD value with urea treatment.

Suligoi et al (60) had evaluated the precision and accuracy of the avidity index (AI) calculated on an automated 3rd generation micro particle EIA (AxSYM HIV 1/2gO assay from Abbott). They used guanidine as the dissociation agent for separating the low avidity antibodies, they duplicated each sample and one in phosphate buffered solution and one in 1M guanidine (1:10). They had evaluated the precision by

performing the test on multiple replicates of 8 HIV-1 serum samples and evaluated the accuracy in identifying recent infections (< 6 months of seroconversion) using 216 serum specimens from 47 individuals whose seroconversion dates were known. They evaluated the sensitivity and specificity of the procedure for different avidity index cutoff values. This modification of 3rd generation assay can be an accurate tool for classifying recent and long term infection.

In another study Suligoi et al (59) compared the avidity index between an automated 3rd and 4th generation immunoassays (AxSYM HIV 1/2 gO; Abbott Diagnostics vs Architect HIV Ag/Ab Combo; Abbott Diagnostics). The fourth generation assay detects both HIV-1 p24 antigen and antibodies. They used 142 samples collected from 75 HIV-1 infected individuals whose seroconversion dates were known. They found that both assays showed 91.5% accuracy in identifying recent HIV-1 infection using AI < 0.80 and the 4th generation was found to slightly more sensitive (89.4% vs 84.8%, $p > 0.05$) and but less specific (93.4% vs 97.4%, $p > 0.05$). The correlation (R) between assays was 0.87. The accuracy of AI with Architect HIV Ag/Ab Combo was 94.7%, when they excluded 20 specimens which fell in the gray zone area near the cutoff (0.75 < AI < 0.84) and the agreement between the two assays was found to be 99.2%.

All 117 samples which were tested using LAg avidity assay was used and tested using the in-house avidity assay. This in-house avidity assay was a modification of a second generation enzyme immunoassay (Microlisa, J. Mitra diagnostics, New Delhi) and the wells were coated with gp41, gp120 for HIV-1 and gp36 for HIV-2. The cut off was set as < 0.80 based on study by Suligoi et al (59,60,102). The assay picked up 9 samples with AI <0.80. All the recently infected classified according to LAg avidity assay had

an avidity index of <0.80. The extra sample picked up by the in-house avidity assay had AI=0.73 in the in-house avidity assay and ODn = 2.11 in the LAg avidity assay. The mean AI was significantly lower for recent than long term infection (0.49 ±0.18 vs 0.99 ±0.09, $p < 0.001$). The sensitivity of the in house assay was 100% (95% CI 63-100%) and the specificity of the assay was 99.08% (95% CI 94.99- 99.98%) when compared to the LAg avidity assay. The positive and negative predictive value were 88.89% (95% CI 51.75-99.72%) and 100% (96.64-100%) respectively. The degree of agreement between the two assays was 0.94 with probability of <0.0001. Our in-house avidity assay showed good reproducibility with an inter-assay coefficient of variation of 7.82% and intra-assay coefficient of variation 5.6% for samples of individuals with recent HIV-1 infection.

Gao et al (88) developed a limiting antigen avidity dot immune gold filtration assay. It is rapid membrane based immunodiagnostic assay and compared to the enzyme immunoassay, they were more suitable for onsite testing because it is rapid, economical and convenient. The sensitivity and accuracy of the assay was increased by adding a silver staining. Its agreement with LAg avidity assay was $\kappa = 0.75$ which was less compared to the agreement of the in-house assay used in our study with LAg avidity $\kappa = 0.94$.

Thomas et al (45) studied the maturation of avidity of IgG antibodies to the different antigen gp41, p24 and p17. They found that antibodies to gp41 are more reliable than antibodies to p17 and p24. Antibodies to gp41 reflects time since first detection of antibodies in all cases. Whereas antibodies to p17 and p24 was not so reliable during the same time period. Also, when individual was on ART the avidity of gp41 antibody

remained high but avidity of anti-p24 fell before onset of AIDS. The in-house assay we used in our study had gp41 and C terminus of gp120 coated in the wells. Suligoi et al (59,60) used 1M guanidine as the chaotropic agent to separate the weak bonds between the antibody and antigen and followed the manufacturer's after that. Shepherd et al(15) used 7M urea as chaotropic agent in their study. In our study we used 7M urea at pH=3.0 for dissociation. The sensitivity (100%) and specificity (99.08%) was comparable with the avidity assay developed by Shepherd et al(15), they had sensitivity and specificity of 100% and 98.65% respectively. Their assay was a modification of Genscreen HIV1/2 (Bio-Rad, Marnes-la-Coquette, France).

The in-house assay we used had many advantages over other assays used for classifying recent and long term infections. It is microtitre plate assay which uses equipment available in most basic virology laboratories. It does not require expensive and large automated platform. It is easy to transfer the assay between laboratories and so can be used in local laboratories rather than reference laboratory for testing for recent infection. The in-house assay requires very small amount of sample when compared to automated platforms. It uses a simple and easy modification in the commonly available commercial assay. The quality control issues are overcome through the good quality control of the commercial assay. The good sensitivity for recent infection can help in prioritizing for contact tracing and help to minimize the transmission of the infection in early stages as the individuals are more infectious during this period. The in-house assay in our study is an indigenous assay with sensitivity 100% and specificity 99.5% and detects the presence HIV-1 subtype C which is prevalent in India(103). Standardizing the in house assay also reduces the cost of avidity testing in HIV-1 infection.

Western blot analysis of our study samples of recently infected individuals demonstrated low levels of antibodies to pol and gag proteins compared to those with long term infection. Re et al (17) study showed that even recently HIV-1 infected individuals who were treated had complete immunoblot response to envelope proteins within 6 months. This finding was similar to what we found in our study that even those individuals who were recently infected with HIV-1 were having developed bands to envelope protein. This suggests that antibodies to envelope proteins appear early in the disease and is maintained. In our study, individuals with recent infection had weak or no bands against pol proteins. There was low reactivity to p31, p51 and p66 in individuals with recent infection compared those with long term infection. There was a significant difference in reactivity to p31($p = 0.004$) between individuals with recent and long term HIV infection and the probability value of p51 ($p = 0.07$) and p66 ($p = 0.10$) just fell short of significance. Also the results suggest that antibodies to different antigens appear along the course of the disease and not simultaneously.(11).

One of the aims of our study was look at the effect of antiretroviral treatment on the response to antibody maturation. In paired sample testing of HIV-1 infected individuals, the first sample was collected before initiation of antiretroviral therapy and the second sample was collected after 6 to 12 months and the individuals were responding to on ART. The mean normalized optical density (ODn) value from the paired samples of HIV-1 infected individuals before initiating ART and after responding to ART obtained using the LAg avidity assay showed a significant decrease ($p=0.0038$) in ODn (4.306 ± 0.97 to 3.977 ± 1.1). This may be attributed to the impaired production of the humoral immune response. ART seems to prevent the emergence of IgG antibodies as it

suppresses the viral replication so there is no persistent production of antigens to stimulate the immune system for production of antibodies(17,46).

Suligoi et al (60) determined the effects of ART, advanced stage of the disease and low HIV viral load on the avidity index (AI) by analyzing 15 serum samples from 15 individuals whose seroconversion dates were unknown. These samples were analyzed using a modified version of the automated 3rd generation micro particle EIA (AxSYM HIV 1/2gO assay from Abbott) where they initially took duplicates of a single sample and one he diluted in 1M guanidine and the other in phosphate buffered saline; after this they followed the manufacture's instruction. Their results for the precision of the procedure had total variance of the AI < 10%. They found that the mean avidity index was significantly lower for samples obtained within 6 months of seroconversion than those obtained later (0.68 ±0.16 versus 0.99±0.10; P < 0.0001). The avidity index < 0.6 had a sensitivity of 33.3% and a specificity of 98.4% whereas avidity index < 0.9 had a sensitivity and specificity of 87.9 and 86.3% respectively. They found that ART, low CD4-cell count and low viral load had no apparent effect on the AI.

Study done by Re et al (17) in Italy evaluated the antibody avidity and Western Blot pattern in recently infected HIV-1 individuals with early ART and not on ART. Thirty-six seroconverters were followed over 2 years. They were divided into two groups, one included 19 HIV-1 infected individuals not on treatment and second group included 17 individuals who were treated within 3 months of seroconversion. Their results showed a significant (p=0.03) lower reactivity to HIV-1 pol proteins and gag proteins and a lower mean value of the avidity index (0.66 vs 0.8, treated vs untreated) in individuals on ART. They also found that the treated individuals developed complete

western blot response within 6 months only to env proteins (gp41 and gp120). Antibody avidity maturation requires an active viral replication. Also the early initiation of ART prevents the gradual evolution of the avidity index of HIV-1 specific antibodies.

A study by Selleri et al(46) in Italy evaluated the effect of ART in evolution of antibody avidity in 13 individuals with symptomatic primary HIV infection. Of the 13, 8 individuals were on ART at diagnosis. The avidity index and western blot patterns was analyzed on plasma/ serum sample for one year. Among the 8 individuals, 4 individuals of them stopped ART subsequently. Their results showed that viremia in untreated individuals reached a set point in 4-6 months and in the treated cases there was a suppression of viremia and the viral load was undetectable during treatment. At diagnosis avidity index was low in both untreated (median AI = 0.42, range = 0.33-0.43) and treated individuals (median AI= 0.44, range = 0.40-0.72). The individuals were followed up at 3,6 and 12 months and showed progressive increase in avidity index in untreated individuals but in all the individuals on ART it remained below 0.80(46). In a study by Wendel et al(18), they observed decrease in proportion of HIV-1 specific IgG when the HIV-1 infected individual had a viral suppression associated with antiretroviral therapy which was shown by the median decrease of 0.42 in normalized optical density in BED capture EIA (Calypte Biomedical Corporation, Oregon, USA) per year of antiretroviral therapy. They also found that avidity index was not affected by longer duration of antiretroviral therapy.

In the study reported in this thesis the paired sample testing of HIV-1 infected individuals responding to treatment and had viral loads below detectable level and after they developed virological failure, there was an increase in the mean normalized optical

density (ODn) values (p=0.058) in LAg avidity assay when they had virological failure compared to when they were responding to ART (ODn 4.13±0.6 vs ODn 3.14±1.2). The significance fell short probably because of the small sample size. In a study by Wendel et al(18), they observed different patterns of BED CEIA normalized optical density values in HIV-1infected individuals with viral breakthrough (viral load > 1000 copies/ml) associated with lack of adherence or drug resistance. The BED CEIA normalized optical density (ODn) values increased by a median ODn of 0.52 in HIV-1 infected individuals (n= 9/20) when the viral load went above 1000 copies/ml and these BED CEIA ODn values returned to values before viral breakthrough when viral suppression was re-established. The BED ODn values increased by a median ODn of 0.81 in HIV-1 infected individuals (8/20) at the visit after viral breakthrough (mean 322 days after the viral breakthrough). In the remaining three HIV-1 infected individuals there was no difference in BED capture EIA ODn values with viral breakthrough. They also found that longer duration of viral breakthrough does not affect the avidity index. Selleri et al (46) demonstrated an increase in avidity index in 4 individuals who interrupted the antiretroviral therapy, the avidity index went more than 0.80 in less than 6 months. These HIV-1 infected individuals were on treatment and subsequently stopped. Our results have shown the similar results and establishes the fact that there can be increase in avidity when there was a virological failure.

In conclusion, our study showed that 6.83% of the 117 HIV-1 infected attendees in ICTC are infected recently (less than 6months) by the LAg avidity assay. And the modified commercial assay in-house can be used as an alternative assay for detecting recent infection

7. Summary and Conclusion

Summary

The study was done to find the frequency of recent HIV-1 infections among the volunteers who attended ICTC of a tertiary care centre using LAg avidity EIA and to compare the results with an in-house assay and with HIV western blot antibody pattern. The other objective of the study was look at the effect of antiretroviral therapy on avidity maturation of HIV-1 specific immunoglobulin G among HIV-1 infected individuals.

1. Among the 896 volunteers who were tested in ICTC, 132 (14.73%) were found to be positive for HIV infection.
2. Of 132 individuals who were positive for HIV, 131 (99.24%) were positive for HIV-1 and 1 (0.76%) individual was positive for HIV-2.
3. Eight (6.83%) individuals were found to be recently (< 6 months) infected using the LAg avidity assay among the 117 HIV-1 infected individuals recruited for the study. Among these 8 individuals with recent HIV-1 infection, 7 (87.5%) probably had a recent infection of less than 3 months based on the assay criteria.
4. Of the 117 HIV-1 infected individuals recruited for the study, 40% were from Tamil Nadu, 18% were from Andhra Pradesh and 15% were from West Bengal. Among them 57% were men and 43% were women.
5. Among the HIV-1 infected individuals (n = 117), 70% belonged to the age group 31-50 years.
6. Sexual contact was the predominant route of transmission in 96% of the HIV-1 infected individuals (n = 117). Mode of acquisition of HIV-1 infection in 92.54%

of men was high risk behavior and 86% of women acquired the infection from their HIV-1 infected spouses.

7. The mean normalized optical density (ODn) value of samples obtained with LAg assay from individuals with recent infections were significantly less than those with long term infection (ODn 0.662 ± 0.42 vs 3.761 ± 0.84, $p < 0.0001$).
8. The inter and intra-assay coefficient of variation for the LAg avidity assay were 11% and 5% respectively.
9. The accuracy indices of the in-house assay when compared with the LAg avidity assay were good with a sensitivity of 100% (95% CI 63.06-100%), specificity of 99.08% (95% CI 94.99-99.98%) with positive predictive value of 88.89% (95% CI 51.75-99.72%) and negative predictive value of 100% (95% CI 96.64-100%).
10. The in-house assay had a very good agreement $\kappa = 0.94$ with LAg avidity EIA ($p<0.0001$).
11. The inter and intra-coefficient of variation of the in-house avidity assay was 7.82% and 5.6% respectively for individuals with recent infection.
12. The antibodies against *pol* proteins were less in those with recent infection compared to those with long term HIV-1 infection as detected by the Western Blot assay.
13. The mean normalized optical density (ODn) values in the LAg avidity assay of samples from HIV-1 infected individuals (n=25) responding to treatment was significantly lower than the samples prior to treatment from the same individuals (ODn = 3.977 ± 1.1 vs ODn = 4.306 ± 0.97, $p=0.0038$).

14. There was an increase in the mean normalized optical density (ODn) values obtained from the LAg avidity assay from samples of HIV-1 infected individuals (n=7) when responding to ART had virological failure compared to when the same individuals were responding. (ODn = 4.128±0.6 vs ODn = 3.144±1.2, p = 0.057). The significance fell short probably because of the small sample size.

Limitations

Though LAg avidity assay can predict the mean duration of the recent infection, it cannot identify the exact month of infection. Samples from HIV-1 infected individuals with AIDS were not tested for checking the specificity of the assay.

Conclusion

In conclusion, the frequency of recent HIV-1 infection was 6.83% in newly diagnosed HIV-1 cohort. Antiretroviral therapy had a significant effect on avidity of HIV-1 antibodies, possibly predicting virological failure. The modification of the indigenous assay allows the availability of avidity testing for recent HIV-1 infection with low cost and is a good alternative to commercially available assay as it has good accuracy indices.

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OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA.

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

August 24, 2015

Dr. Diviya Alex
PG Registrar
Department of Clinical Microbiology
Christian Medical College,
Vellore 632 004

Sub: **Fluid Research Grant NEW PROPOSAL:**
Human Immunodeficiency Virus Antibody avidity testing to identify recent infection among the newly diagnosed HIV-1 infected individuals and the effect of anti retroviral therapy on the avidity antibodies - A pilot study.
Dr. Diviya Alex, Emp. No: 21130, PG Registrar, Dept. of Clinical Microbiology, Dr. Rajesh Kannangai, Emp. No: 20093, Prof & Head, Dr. John G Fletcher, Emp. No: 31449, Dr. Priscilla Rupali, Prof & Head, Dept. of Infectious Diseases, Dr. J.V. Punitha, Medicine, Mr. S. Jaiprasath, Emp. No: 31559, Mr. John Paul Demosthenes, Emp. No: 33324, Clinical Virology, Mr. TennisonInbaraj W. Emp. No: 52560, Lab. Technician, ICTCistics.

Ref: IRB Min No: 9527 [DIAGNO] dated 07.07.2015

Dear Dr. DiviyaAlex,

I enclose the following documents:-

1. Institutional Review Board approval 2. Agreement

Could you please sign the agreement and send it to Dr. Nihal Thomas, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Nihal Thomas
Secretary (Ethics Committee), Institutional Review Board

Dr. NIHAL THOMAS
MD.,MNAMS.,DNB(Endo),FRACP(Endo),FRCP(Edin),FRCP(Glasg)
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

Cc: Dr. Rajesh Kannangai, Department of Clinical Virology CMC,

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
Tel : 0416 - 2284294, 2284202 Fax : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

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Ref: IRB Min No: 9527 [DIAGNO] dated 07.07.2015

Dear Dr. Diviya Alex ,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "Human Immunodeficiency Virus Antibody avidity testing to identify recent infection among the newly diagnosed HIV-1 infected individuals and the effect of anti retroviral therapy on the avidity antibodies - A pilot study" on July 07th 2015.

The Committee reviewed the following documents:

1. IRB Application format
2. Consent Form and Patient Information Sheet (English, Tamil, Hindi, Telugu, Bengali)
3. Patient Proforma
4. Cvs of Drs. Diviya Alex, Mr. John Paul Demosthenes, Rajesh Kannangai, Priscilla Rupali, Mr. Tennison Inbaraj W, Jaiprasath, John G Fletcher
5. No of documents 1 – 4

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
Tel : 0416 - 2284294, 2284202 Fax : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
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Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on July 07th 2015 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

Name	Qualification	Designation	Affiliation
Dr. Alfred Job Daniel	D Ortho, MS Ortho, DNB Ortho	Principal, Chairperson-Research Committee, IRB, CMC, Vellore	Internal, Clinician
Dr. Nihal Thomas	MD, MNAMS, DNB(Endo), FRACP (Endo) FRCP(Edin) FRCP (Glasg)	Professor & Head, Endocrinology. Additional Vice Principal (Research), Deputy Chairperson(Research Chairperson), Member Secretary (Ethics Committee), IRB. CMC, Vellore	Internal, Clinician
Dr. Simon Pavamani	MBBS, MD	Professor, Radiotherapy, CMC, Vellore	Internal, Clinician
Dr. Vivek Mathew	MD (Gen. Med.) DM (Neuro) Dip. NB (Neuro)	Professor, Neurology, CMC, Vellore	Internal, Clinician
Dr. Mathew Joseph	MBBS, MCH	Professor, Neurosurgery, CMC, Vellore	Internal, Clinician
Dr. Ranjith K Moorthy	MBBS, MCh	Professor, Neurological Sciences, CMC, Vellore	Internal, Clinician
Dr. Bobby John	MBBS, MD, DM, PhD, MAMS	Professor, Cardiology, CMC, Vellore	Internal, Clinician
Dr. Benjamin Perakath	MBBS, MS, FRCS	Professor, Colorectal Surgery, CMC, Vellore	Internal, Clinician
Dr. Chandrasingh	MS, MCH, DMB	Professor, Urology, CMC, Vellore	Internal, Clinician
Dr. Anup Ramachandran	PhD	The Wellcome Trust Research Laboratory Gastrointestinal Sciences, CMC, Vellore	Internal, Basic Medical Scientist
Dr. Anand Zachariah	MBBS, PhD	Professor, Medicine, CMC, Vellore	Internal, Clinician

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MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Dr. Balamugesh	MBBS, MD(Int Med), DM, FCCP (USA)	Professor, Pulmonary Medicine, CMC, Vellore	Internal, Clinician
Dr. Visalakshi. J	MPH, PhD	Lecturer, Biostatistics, CMC, Vellore	Internal, Statistician
Dr. Rajesh Kannangai	MD, PhD.	Professor, Clinical Virology, CMC, Vellore	Internal, Clinician
Dr. Niranjan Thomas	DCH, MD, DNB (Paediatrics)	Professor, Neonatology, CMC, Vellore	Internal, Clinician
Dr. Jacob John	MBBS, MD	Associate Professor, Community health, CMC, Vellore	Internal, Clinician
Dr. Inian Samarasam	MS, FRCS, FRACS	Professor, Surgery, CMC, Vellore	Internal, Clinician

We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any **adverse events** occurring in the course of the project, any **amendments in the protocol and the patient information / informed consent**. On completion of the study you are expected to submit a copy of the **final report**. Respective forms can be downloaded from the following link: http://172.16.11.136/Research/IRB_Polices.html in the CMC Intranet and in the CMC website link address: http://www.cmch-vellore.edu/static/research/Index.html.

Kindly provide the total number of patients enrolled in your study and the total number of withdrawals for the study entitled: "Human Immunodeficiency Virus Antibody avidity testing to identify recent infection among the newly diagnosed HIV-1 infected individuals and the effect of anti retroviral therapy on the avidity antibodies - A pilot study." on a monthly basis. Please send copies of this to the Research Office (research@cmcvellore.ac.in)

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
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MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Fluid Grant Allocation:
A sum of 50,000/- INR (Rupees Fifty Thousand) will be granted for 1 year and out of which a maximum of Rs 5,000/- can be spent for stationery, printing, Xeroxing and computer charges (if computers used are within the institution)..

Yours sincerely

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr. NIHAL THOMAS
MD.,MNAMS, DNB(Endo) FRACP(Endo) FRCP(Edin),FRCP(Glasg)
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

Cc: Dr. Rajesh Kannangai, Department of clinical Virology , CMC,

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
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BAGAYAM, VELLORE 632002, TAMIL NADU, INDIA

Ref: FG/9527/07/2015

September 4, 2015

Mr. Robby Pria Sundarsingh
Treasurer
Christian Medical College,
Vellore.

Dear Mr. Robby Pria Sundersingh,

Sub: **Fluid Research Grant NEW PROPOSAL:**
Human Immunodeficiency Virus Antibody avidity testing to identify recent infection among the newly diagnosed HIV-1 infected individuals and the effect of anti retroviral therapy on the avidity antibodies - A pilot study.
Dr. Divya Alex, Emp. No: 21130, PG Registrar, Dept. of Clinical Microbiology. Dr. Rajesh Kannangai, Emp. No: 20093, Prof & Head, Dr. John G Fletcher, Emp. No: 31449, Dr. Priscilla Rupali, Prof & Head, Dept. of Infectious Diseases, Dr. J.V. Punitha, Medicine, Mr. S. Jaiprasath. Emp. No: 31559, Mr. John Paul Demosthenes, Emp. No: 33324, Clinical Virology, Mr. Tennison Inbaraj W. Emp. No: 52560, Lab. Technician, ICTCistics

Ref: IRB Min No: 9527 dated 07.07.2015

The Institutional Review Board at its meeting held on July 7th 2015 vide IRB Min. No. **9527** accepted the project for a sum of *Rs. 50,000/- (Rupees Fifty Thousand Only) will be granted for 1 year and out of which a maximum of Rs 5,000/- can be spent for stationery, printing, Xeroxing and computer charges (if computers used are within the institution).*

If overspent the excess should be debited from the respective departmental or Special funds.

Kindly arrange to transfer the sanctioned amount to a separate account to be operated by Dr. Diviya Alex (diviya.m88@gmail.com)and Dr.Rajesh Kannangai(kannangair@cmcvellore.ac.in)

Yours sincerely,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

CC: Dr.Diviya Alex, Clinical Microbiology, CMC
Dr.Rajesh Kannangai, Clinical Virology CMC
File

PATIENT INFORMATION SHEET

1. **Study title: Human Immunodeficiency Virus Antibody Avidity Testing to identify recent infection among the newly diagnosed HIV-1 infected individuals and the effect of Antiretroviral Therapy on the Avidity Antibodies – A pilot study**

Please read this carefully. It tells you important information about the study. A member of the research team will explain you about your participation in this study. If you have any questions about the research or about this form, please ask us. If you decide to take part in this study, you must sign or provide your thumb impression in this form to show your willingness to take part in this study.

Why is this study being done?

Human immunodeficiency virus (HIV) weakens your immune system making you prone to many infections including those which normally doesn't cause infection. HIV is transmitted sexually, through blood and blood products and also from mother to child before, during and after birth. It takes almost 8-10 years for the person to be clinically symptomatic. India has the 3rd largest number of people living with HIV infection globally. The purpose of our study is find whether the person was infected recently. This will help us to find the group of people who are recently infected thereby focus on prevention of HIV infections among them.

What will happen in this study?

Individuals above 18 years, either sex, who are HIV-1 infected individual will be recruited in this study.

If you agree to participate and you meet all the criteria required for the study as patient, a part of the blood sample already collected from you will be used for the study.

Basic information, medical history and details of treatment will be collected from you.

Will I be paid to take part in this study?

You will not be paid for taking part in this research.

What are the risks and possible discomforts from being in this study?

There is no risk or discomforts. The consent is for using a part of the blood sample already collected from you for the study.

What are the possible benefits from being in this study?

By being a part of this study, you will not benefit directly with regard to the immediate management of the disease, but as a result of your participation, we will be able to identify the recently infected individuals among the HIV-1 infected individuals and minimize the transmission of the disease.

If I take part in this research study, how will you protect my privacy?

Information collected from you for this research study will be stored in the investigator's research files. Your name and other information that might identify you will be recorded with a unique code number, protecting your identity and information from others. The research consent form that you sign may be inspected by the regulatory agencies or the Institutional Review Board in the course of carrying out their duties. If the signed research consent form is inspected or copied, the hospital will use reasonable efforts to protect your privacy. The results obtained from this study will be published in the scientific journals, but no information about your identity will be disclosed.

If I have questions or concerns about this research study, whom can I call?

You can contact the following person for your questions about the study

Dr. Diviya Alex

PG Registrar

Department of Microbiology,

Christian Medical College, Vellore - 632004

Phone: 0416 2282588, 9940729250

Informed Consent Form

1. **Study Title: Human immunodeficiency virus antibody avidity testing to identify recent infection among the newly diagnosed HIV-1 infected individuals and the effect of antiretroviral therapy on the avidity antibodies – A pilot study**

Study Number: ___________

Subject's Initials: ________________ Subject's Name: _____________________________________

Date of Birth / Age: ________________________

(Subject)

(i) I confirm that I have read and understood the information sheet dated ___________ for the above study and have had the opportunity to ask questions. [ ]

(ii) I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. [ ]

(iii) I understand that the Sponsor of the clinical trial, others working on the Sponsor's behalf, the Ethics Committee and the regulatory authorities will not need my permission to look at my health records both in respect of the current study and any further research that may be conducted in relation to it, even if I withdraw from the trial. I agree to this access. However, I understand that my identity will not be revealed in any information released to third parties or published. [ ]

(iv) I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s). [ ]

(v) I agree to take part in the above study. [ ]

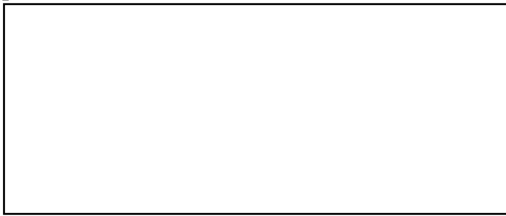
(vi) It may be possible to do special research tests related to HIV in the future if you agree to allow the specimen blood sample you have donated to be saved after the study is finished. These future study bears no relationship to the present study. Your signature below will allow for the specimen to be stored in a central specimen bank with the possibility that it may be used in future tests. The future tests may not directly benefit you, but may provide important medical knowledge.
I agree for you to store the specimen blood sample I have donated for doing special research tests in future. [ ]

Signature (or Thumb impression) of the Subject/Legally Acceptable

Date: _____/_____/______

Signatory's Name: ________________________________ Signature:

Or



Representative: _________________

Date: _____/_____/______

Signatory's Name: ________________________________

Signature of the Investigator: ________________________

Date: _____/_____/______

Study Investigator's Name: _________________________

Signature (or) thumb impression of the Witness: ___________________________

Date: _____/_____/_______

Name and Address of the Witness: ______________________________

PATIENT PROFORMA

1. **Study Title: Human immunodeficiency virus antibody avidity testing to identify recent infection among the newly diagnosed HIV-1 infected individuals and the effect of antiretroviral therapy on the avidity antibodies – A pilot study**

Study number: **Date:**

Name:

Hospital number:

Date of birth / Age: Sex:

Address:

State: **Mother tongue:**

Marital Status:

Occupation:

History of exposure/ organ transplantation/blood transfusion:

Any high risk behavior:

Date of HIV diagnosis:

History/Diagnosed other infections:

Sample collected by

Name:

Signature:

HIV-1 infected individuals

Study No.	Age	Sex	Marital Status	Occupation	Exposure	Transmission from Spouse	High risk behaviour	State	ODn	AI
LAV-1	40	Male	Married	Mechanic	Blood transfusion	No	No	Jharkhand	2.845	0.92
LAV-2	40	Female	Married	Housewife	Sexual	Yes	No	Andhra Pradesh	4.425	0.95
LAV-3	45	Male	Married	Business	Sexual	No	Yes	Andhra Pradesh	3.12	0.98
LAV-4	45	Male	Married	Driver	Sexual	No	Yes	Andhra Pradesh	3.422	0.90
LAV-5	40	Female	Married	Housewife	Sexual	Yes	No	Andhra Pradesh	3.318	0.99
LAV-6	30	Male	Married	Artist	Sexual	Yes	No	Tamil Nadu	3.595	0.96
LAV-7	30	Female	Married	Housewife	Sexual	Yes	No	Bihar	4.464	1.10
LAV-8	38	Female	Married	Labourer	Sexual	Yes	No	Tamil Nadu	0.807	0.34
LAV-9	30	Female	Married	Housewife	Sexual	Yes	No	West Bengal	3.675	1.24
LAV-10	39	Male	Married	Supervisor	Sexual	No	Yes	Tamil Nadu	3.916	0.86
LAV-11	43	Female	Married	Housewife	Sexual	Yes	No	West Bengal	3.561	0.93
LAV-12	46	Male	Married	Business	Sexual	Yes	No	West Bengal	2.927	0.97
LAV-13	40	Female	Married	Housewife	Sexual	Yes	No	West Bengal	2.272	0.85
LAV-14	25	Male	Married	Driver	Sexual	No	Yes	Bihar	4.083	1.02
LAV-15	22	Female	Married	Housewife	Sexual	Yes	No	Bihar	3.433	1.01
LAV-16	44	Female	Married	Nurse	Sexual	Yes	No	West Bengal	4.228	1.10
LAV-17	26	Male	Married	Labourer	Sexual	No	Yes	Tamil Nadu	3.924	1.12
LAV-18	29	Female	Married	Housewife	Sexual	Yes	No	Manipur	4.503	1.07
LAV-19	40	Male	Married	Business	Sexual	No	Yes	Manipur	5.07	0.84
LAV-20	42	Male	Married	Labourer	Sexual	No	Yes	Uttar Pradesh	4.972	1.03
LAV-21	40	Female	Married	Housewife	Sexual	Yes	No	Uttar Pradesh	4.473	1.06
LAV-22	35	Male	Married	Mechanic	Sexual	No	Yes	West Bengal	4.126	1.07
LAV-23	23	Female	Married	Housewife	Sexual	Yes	No	West Bengal	4.766	1.27
LAV-24	35	Female	Married	Housewife	Sexual	Yes	No	Andhra Pradesh	3.917	1.11
LAV-25	25	Male	Married	Driver	Sexual	No	Yes	Jharkhand	5.293	1.03
LAV-26	20	Female	Married	Housewife	Sexual	Yes	No	Jharkhand	4.81	1.06
LAV-27	42	Male	Married	Labourer	Sexual	No	Yes	Tamil Nadu	4.463	0.97
LAV-28	45	Male	Married	Tea shop worker	Sexual	Yes	No	Andhra Pradesh	2.097	0.88
LAV-29	47	Male	Married	Farmer	Sexual	No	Yes	Tamil Nadu	2.914	1.06
LAV-30	22	Female	Widow	Housewife	Sexual	Yes	No	Tamil Nadu	3.702	0.96
LAV-31	68	Male	Married	Business	Sexual	No	Yes	Jharkhand	1.354	0.59
LAV-32	52	Male	Married	Government employee	Sexual	Yes	No	Chhattisgarh	5.509	1.01
LAV-33	53	Female	Widow	Labourer	Sexual	No	Yes	Tamil Nadu	2.991	0.94
LAV-34	42	Male	Single	Labourer	Sexual	No	Yes	Tamil Nadu	4.577	1.09
LAV-35	41	Female	Married	Housewife	Sexual	Yes	No	Tamil Nadu	3.115	0.85
LAV-36	37	Male	Married	Carpenter	Sexual	No	Yes	West Bengal	3.409	1.11
LAV-37	48	Male	Married	Electrician	Sexual	No	Yes	Chhattisgarh	4.063	1.00
LAV-38	35	Female	Married	Housewife	Sexual	Yes	No	Chhattisgarh	4.722	1.12

HIV-1 infected Individuals

Study No.	Age	Sex	Marital Status	Occupation	Exposure	Transmission from Spouse	High risk behaviour	State	ODn	AI
LAV-39	33	Male	Single	Business	Sexual	No	Yes	West Bengal	5.463	1.03
LAV-40	51	Male	Married	Ex BSI	Sexual	No	Yes	Assam	3.22	1.02
LAV-41	47	Male	Married	Hotel labourer	Sexual	No	Yes	Tamil Nadu	2.914	1.08
LAV-42	49	Male	Married	Farmer	Sexual	No	Yes	Tamil Nadu	3.277	0.99
LAV-43	36	Female	Married	Housewife	Sexual	Yes	No	Tamil Nadu	0.166	0.55
LAV-44	51	Male	Married	Labourer	Sexual	No	Yes	Tamil Nadu	3.089	0.99
LAV-45	31	Female	Married	Housewife	Sexual	Yes	No	Tamil Nadu	2.629	0.95
LAV-46	38	Male	Married	Auto consulting	Sexual	No	Yes	West Bengal	4.851	1.09
LAV-47	42	Female	Married	Housewife	Sexual	Yes	No	Jharkhand	4.763	1.07
LAV-48	39	Male	Married	Business	Sexual	No	Yes	Andhra Pradesh	0.353	0.37
LAV-49	39	Male	Married	Salesman	Sexual	No	Yes	Assam	4.795	0.90
LAV-50	26	Male	Married	Tailor	Sexual	No	Yes	West Bengal	4.927	1.04
LAV-51	30	Female	Widow	Housewife	Sexual	Yes	No	Tamil Nadu	4.726	0.95
LAV-52	55	Male	Married	Not known	Sexual	No	Yes	Andhra Pradesh	5.153	0.92
LAV-53	42	Male	Married	Labourer	Sexual	No	Yes	Tamil Nadu	4.571	1.01
LAV-54	58	Male	Married	Business	Sexual	Yes	No	Andhra Pradesh	2.699	1.04
LAV-55	34	Female	Married	Housewife	Sexual	Yes	No	Andhra Pradesh	0.845	0.48
LAV-56	35	Male	Married	Farmer	Sexual	No	Yes	Tamil Nadu	3.766	1.05
LAV-57	45	Male	Married	Government employee	Sexual	No	Yes	Tamil Nadu	3.355	0.95
LAV-58	40	Female	Married	Nil	Sexual	Not sure	No	Tamil Nadu	3.845	0.91
LAV-59	36	Male	Married	Business	Sexual	No	Yes	West Bengal	2.845	0.92
LAV-60	43	Female	Married	Housewife	Sexual	Yes	No	Tamil Nadu	3.582	1.04
LAV-61	47	Male	Married	Tailor	Sexual	No	Yes	Tamil Nadu	3.9	1.03
LAV-62	40	Male	Married	Labourer	Sexual	No	Yes	Karnataka	3.601	1.03
LAV-64	37	Male	Married	Farmer	Sexual	No	Yes	Tamil Nadu	3.497	0.97
LAV-65	48	Male	Married	Hotel Business	Sexual	No	Yes	Andhra Pradesh	3.884	1.04
LAV-66	30	Male	Married	Labourer	Sexual	No	Yes	West Bengal	3.998	0.91
LAV-67	44	Female	Married	Labourer	Sexual	Yes	No	Andhra Pradesh	3.871	0.85
LAV-68	39	Male	Married	Business	Sexual	No	Yes	Uttar Pradesh	5.275	0.94
LAV-69	44	Male	Married	Farmer	Sexual	No	Yes	Andhra Pradesh	3.114	0.94
LAV-70	33	Female	Married	Housewife	Sexual	Yes	No	Andhra Pradesh	3.917	0.96
LAV-72	30	Female	Married	Housewife	Sexual	Yes	No	Andhra Pradesh	3.993	0.98
LAV-73	43	Male	Married	Farmer	Sexual	No	Yes	Tamil Nadu	2.059	0.85
LAV-74	22	Male	Single	Army	Sexual	No	Yes	Tamil Nadu	2.217	0.84
LAV-75	41	Male	Married	Hotel labourer	Sexual	No	Yes	Tamil Nadu	3.863	1.09
LAV-76	30	Female	Married	Housewife	Sexual	Yes	No	Andhra Pradesh	2.541	0.95
LAV-77	38	Male	Married	Auto driver	Sexual	No	Yes	Jharkhand	2.286	0.81
LAV-78	45	Female	Married	Farmer	Sexual	Yes	No	Tamil Nadu	3.42	0.97
LAV-79	48	Female	Married	Housewife	Sexual	Yes	No	Tamil Nadu	3.096	0.95

HIV-1 infected Individuals

Study No.	Age	Sex	Marital Status	Occupation	Exposure	Transmission from Spouse	High risk behaviour	State	ODn	AI
LAV-80	46	Male	Married	Mechanic	Sexual	No	Yes	Andhra Pradesh	3.71	1.18
LAV-81	30	Female	Married	Labourer	Sexual	Yes	No	Tamil Nadu	3.832	1.01
LAV-82	45	Male	Single	Farmer	Sexual	No	Yes	Tamil Nadu	2.75	0.91
LAV-83	46	Male	Married	Labourer	Sexual	No	Yes	Tamil Nadu	3.534	0.95
LAV-84	42	Female	Married	Housewife	Sexual	Yes	No	Andhra Pradesh	2.966	0.81
LAV-85	42	Female	Married	Housewife	Sexual	No	Yes	Jharkhand	2.561	0.96
LAV-86	50	Male	Married	Labourer	Sexual	No	Yes	Tamil Nadu	3.339	0.90
LAV-87	55	Female	Married	Housewife	Sexual	Yes	No	Jharkhand	3.908	1.04
LAV-88	37	Male	Married	Electrician	Sexual	No	Yes	West Bengal	4.03	0.97
LAV-89	35	Male	Married	Labourer	Sexual	No	Yes	West Bengal	3.842	1.06
LAV-90	51	Male	Married	Police	Sexual	No	Yes	Tamil Nadu	3.822	1.06
LAV-91	43	Male	Married	Labourer	Sexual	No	Yes	Tamil Nadu	2.111	0.73
LAV-92	36	Female	Married	Housewife	Sexual	No	Yes	Tamil Nadu	3.272	0.90
LAV-93	38	Female	Married	Housewife	Sexual	Yes	No	Tamil Nadu	3.925	1.04
LAV-94	32	Female	Married	Housewife	Sexual	Yes	No	Jharkhand	4.068	1.01
LAV-95	30	Female	Married	Housewife	Sexual	Yes	No	West Bengal	3.278	1.17
LAV-97	50	Female	Married	Housewife	Sexual	Yes	No	Jharkhand	2.148	0.98
LAV-98	50	Female	Married	Housewife	Blood transfusion	No	No	Tamil Nadu	1.629	1.06
LAV-99	27	Female	Married	Housewife	Sexual	Yes	No	Tamil Nadu	3.475	1.02
LAV-100	32	Male	Married	Business	Sexual	No	Yes	Tamil Nadu	3.518	0.98
LAV-101	58	Female	Married	Housewife	Not known	Not known	No	Tamil Nadu	3.874	1.11
LAV-102	36	Female	Married	Labourer	Sexual	Yes	No	Tamil Nadu	2.702	0.87
LAV-103	53	Male	Married	Labourer	Sexual	No	Yes	Andhra Pradesh	3.607	1.01
LAV-104	32	Male	Married	Driver	Sexual	No	Yes	Jharkhand	0.183	0.16
LAV-105	39	Male	Married	Service	Sexual	No	Yes	West Bengal	4.743	1.07
LAV-106	41	Female	Married	Labourer	Sexual	Yes	No	Andhra Pradesh	3.897	0.92
LAV-107	48	Male	Married	Labourer	Sexual	No	Yes	Jharkhand	4.595	1.04
LAV-108	26	Male	Married	Teacher	Sexual	No	Yes	Tamil Nadu	3.717	0.94
LAV-109	49	Male	Married	Business	Sexual	No	Yes	West Bengal	4.221	0.91
LAV-110	35	Female	Married	Labourer	Sexual	Yes	No	Tamil Nadu	0.974	0.69
LAV-111	40	Male	Married	Business	Sexual	No	Yes	Jharkhand	4.579	1.09
LAV-112	27	Female	Married	Housewife	Sexual	Yes	No	Bangladesh	4.263	0.91
LAV-113	33	Male	Married	Labourer	Sexual	No	Yes	Tamil Nadu	3.25	0.97
LAV-114	29	Male	Single	Private Job	Sexual	No	Yes	Tamil Nadu	3.654	1.03
LAV-115	48	Male	Married	Driver	Sexual	No	Yes	Tamil Nadu	4.474	1.00
LAV-116	36	Male	Married	Business	Sexual	No	Yes	Bihar	4.839	0.97
LAV-117	39	Female	Married	Housewife	Sexual	Yes	No	Jharkhand	0.612	0.54
LAV-118	42	Female	Married	Housewife	Sexual	Yes	No	Andhra Pradesh	4.626	1.00
LAV-119	65	Female	Married	Housewife	Not known	No	No	Tamil Nadu	5.204	0.98
LAV-120	42	Male	Married	Labourer	Sexual	No	Yes	Jharkhand	4.302	0.91

HIV negative controls

S. No	Age	Sex	Marital Status	Occupation	Exposure	Transmission from Spouse	High risk behaviour	State	ODn
1	25	Male	Married	Painter	No	No	No	Tamil Nadu	0.036
2	38	Male	Married	Agriculture	Sexual	No	Yes	Bihar	0.104
3	34	Female	Widow	Housewife	Sexual	Yes	No	Karnataka	0.050
4	29	Male	Married	Driver	No	No	No	Andhra Pradesh	0.043
5	25	Male	Married	Painter	No	No	No	Tamil Nadu	0.053
6	39	Male	Married	Not known	Sexual	Yes	No	Andhra Pradesh	0.068
7	36	Female	Married	Housewife	Sexual	Yes	No	Tamil Nadu	0.075
8	42	Female	Married	Housewife	Sexual	Yes	No	Tamil Nadu	0.083
9	54	Female	Married	Housewife	Sexual	Yes	No	Kerala	0.055
10	24	Male	Single	Painter	No	No	No	Tamil Nadu	0.062
11	22	Male	Single	Student	No	No	No	Andhra Pradesh	0.069
12	48	Female	Single	Housewife	Sexual	Yes	No	Tamil Nadu	0.044
13	25	Female	Single	Tailor	No	No	No	Tamil Nadu	0.053
14	25	Female	Married	Housewife	Sexual	Yes	No	West Bengal	0.070
15	30	Female	Married	Housewife	Sexual	Yes	No	Assam	0.057
16	40	Male	Married	Mechanic	No	No	No	Tamil Nadu	0.099
17	32	Female	Married	Housewife	Sexual	Yes	No	Andhra Pradesh	0.036
18	33	Female	Married	Housewife	Sexual	Yes	No	Tamil Nadu	0.066
19	60	Female	Married	Housewife	No	No	No	Tamil Nadu	0.078
20	18	Female	Single	Student	No	No	No	Tamil Nadu	0.041
21	60	Male	Married	Labourer	No	No	No	Tamil Nadu	0.066
22	30	Female	Married	Labourer	No	No	No	Tamil Nadu	0.091
23	55	Male	Married	Business	Sexual	No	Yes	Tamil Nadu	0.080
24	24	Female	Married	Housewife	No	No	No	Tamil Nadu	0.053
25	29	Female	Single	DME Engineer	No	No	No	Tamil Nadu	0.072
26	60	Male	Married	Labourer	No	No	No	Andhra Pradesh	0.199
27	33	Male	Married	Farmer	No	No	No	Tamil Nadu	0.095
28	33	Male	Married	Electrician	No	No	No	Tamil Nadu	0.037
29	26	Female	Divorcee	Housewife	No	No	No	Tamil Nadu	0.039
30	35	Female	Married	Housewife	Sexual	Yes	Nil	Tamil Nadu	0.077
31	58	Male	Married	Salesman	Sexual	No	Yes	Jharkhand	0.064
32	29	Male	Married	Driver	No	No	No	Tamil Nadu	0.053
33	36	Female	Married	Textile worker	No	No	No	Tamil Nadu	0.051

HIV negative controls

S. No	Age	Sex	Marital Status	Occupation	Exposure	Transmission from Spouse	High risk behaviour	State	ODn
34	27	Female	Married	Housewife	No	No	No	Tamil Nadu	0.093
35	40	Female	Married	Teacher	Sexual	Yes	No	Tamil Nadu	0.060
36	40	Female	Married	Housewife	Sexual	Yes	No	Jharkhand	0.114
37	45	Female	Married	Housewife	No	No	No	Tamil Nadu	0.050
38	36	Male	Single	Computer Technician	No	No	No	Tamil Nadu	0.097
39	39	Female	Married	Housewife	Sexual	Yes	No	Tamil Nadu	0.050
40	44	Female	Married	Housewife	Sexual	Yes	No	Tamil Nadu	0.038