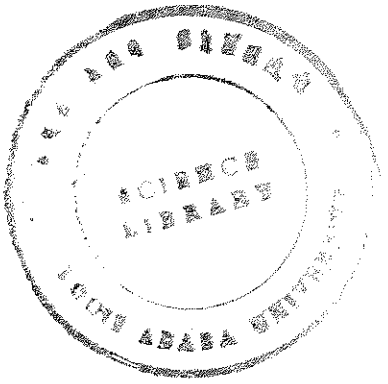
ADDIS ABABA UNIVERISITY SCHOOL OF GRADUATE STUDIES

Temporal Trends in HIV Incidence Among Pregnant Women Attending Antenatal Care Clinics between 1995 and 2003 in Addis Ababa

By:

Tigist Aklilu



A Thesis presented to the School of Graduate Studies of Addis Ababa University in Partial fulfillment of the requirement for the degree of Master of Science in Parsitology

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STARHS- Serologic Testing Algorithm for Recent HIV Seroconversion

Tat- Trans-activator gene

Vif- Viron infectivity factor

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Abstract

HIV infection is routinely diagnosed by various immunoassays that detect the presence of anti-HIV antibodies. But these methods do not distinguish recent from established infections. The previously devised method called Ig G-capture BED-EIA (Subtype B, E, D, Enzyme Immuno Assay) is used to distinguish new from established infection based on standardized optical density values, which is a measure of antibody titer in the serum using cross-sectional specimen. The objective of this study was to identify recent HIV-1 infection using STARHS assay and estimate HIV-1 incidence among pregnant women attending antenatal care clinics in Addis Ababa and to compare the performance of 'in-house' incidence assay kit with commercial assay using 1078 cross-sectional samples collected from pregnant women attending Antenatal Care Clinics (ANC) based on Standardized Optical Density (OD-n) values. Larger percentage of recent infection was observed among women attending the inner city health centers as compared to the outer city health centers and among women aged 15 - 19 years. A decline in HIV incidence was observed in recent years among women of young age groups attending the inner city health centers. Significant correlation was observed between the two assays. There were 84 common samples which had an OD-n value of less than 0.8 and 954 samples with standardized optical density of greater or equal to 0.8 when tested by both commercial and house -made kits. But forty samples (3.7%) had discrepant results. An agreement was also observed between the OD-n values of the two assays, with a correlation coefficient (r) of 0.76.

1. Introduction

The Human Immuno Deficiency Virus (HIV) belongs to group lentivirus, family retroviridae (Gallo and Montaniger, 1988) and it is identified and characterized as the causative agent of Acquired Immuno Deficiency Syndrome (AIDS) (Bare-Sinoussi *et al.*, 1996).

Two distinct types of viruses have been identified in man: HIV -1 and HIV –2. HIV-1 is the global pandemic and is responsible for most of AIDS cases in the world (Neghavi *et al.*, 1999). Though potentially important world wide, HIV-2 infections remain uncommon outside West Africa and they have proved far less virulent than HIV-1 infections (Lallement *et al.*, 1994).

HIV-1 has three distinct groups. M (main), O (outlier) and N (not M -not - O). The M group constitutes the major group of HIV-1 and accounts for most cases of HIV infection worldwide. Members of M group are further divided in to nine "pure" nucleotide sequence defined subtypes (A, B, C, D, G, F, H, J, K: two recombinant subtypes, E and I) having specific geographic regions (Robertson *et al.*, 2000).

HIV infects CD4 T-cells, antigen presenting cells, monocytes, macrophages, langerhan cells, kuffer cells and microglia cells that have CD4 marker like CD4 T-cells. Thus, the virus, has the ability of destroying the immune system, the function of which is to protect the body against infection, and causes a slowly progressive and inevitably total disease (Fauci *et al.*, 1988).

The commonest mode of transmission of the virus throughout the world is by sexual intercourse (Adler, 2001). Heterosexual contact, which is, the predominant mode of HIV transmission, accounts for at least 80 percent of all cases (Barkley, 1993), perinatal transmission accounts for approximately 10 percent of all HIV-1 transmission (Ryder and Temmerman, 1991) and HIV transmission through blood transfusion represents approximately 10 percent of the infection

(Heymann, 1991). Transfusion is the most efficient way to transmit HIV-1: 90% of recipients of seropositive blood become infected.

AIDS is the end stage manifestation of infection with the virus (HIV). The first AIDS cases were diagnosed in 1981 among young homosexual man in the United States (WHO, 1992). But the blood obtained in 1959 from an adult Bantu man in the Democratic Republic of Congo represents the oldest known cases of HIV-1 infection in the world (Zhu *et al.*, 1988).

AIDS continues to be an increasingly complex and dangerous global burden since its recognition (Louis and Bailey, 1993). At the global level, the number of people living with HIV continues to grow from 35 $X10^6$ in 2001 to 38 $X10^6$ in 2003. Nearly 5 $X10^6$ new HIV infections occurred in 2003 and AIDS killed almost 3 $X10^6$ people in the same year. The epidemic claimed the lives of over 20 $X10^6$ people since the first cases of AIDS were identified in 1981 (UNAIDS, 2004). The sub-Saharan Africa is by far the worst affected region. In 2003,an estimated 26.6$X10^6$ people were living with the virus, including the 3.2 $X10^6$ who became infected during the past year and AIDS killed approximately 2.3 $X10^6$ people in 2003 (UNAIDS, 2003). In 2003 alone an estimated 3 $X10^6$ people became newly infected in this region (UNAIDS, 2004).

Young people between the ages of 15-24 are both the most threatened globally accounting for half of all new cases of HIV (UNAIDS, 2004). This indicates that, the disease normally affects younger adults, especially those in the most economically productive phase of their lives. Because of this, the epidemic has become a major obstacle for health, social, and economic development (Karl and Leithaeuser, 2001).

1.1. Overview of HIV infection in Ethiopia

Ethiopia is one of the Sub-Saharan Africa countries, which is severely affected by HIV (Abebe *et al.*, 2001). The first two seropositive samples in Ethiopia were detected in 1984 while testing a collection of sera from 167 hospital patients in Addis Ababa (Tsega, *et al.*, 1988) and the first AIDS cases were diagnosed in 1986 in the capital city of Ethiopia, Addis Ababa (Lester, *et al.*, 1988).

The rate of HIV infection is increasing dramatically since the first report of two cases of HIV in 1984. Like in other African countries, HIV infection initially spread along the main trading roads. In 1988, a survey carried out among sex-workers and truck drivers working on principal traffic routes of Ethiopia has documented HIV prevalence of 17% and 13% respectively (Mengistu *et al.*, 1990). In 1989, a test carried on blood donors of urban areas of the country was only 3.6% (MOH, 1994). A few years later, the infection has already reached all main urban areas of the country. Researches that were conducted on the progression of the HIV epidemic indicated that the prevalence of HIV infection is high in major urban areas of the country. But some recent data indicated that the epidemic has also spread to the rural areas of the country at a fast rate (Mekonnen *et al.*, 2003).

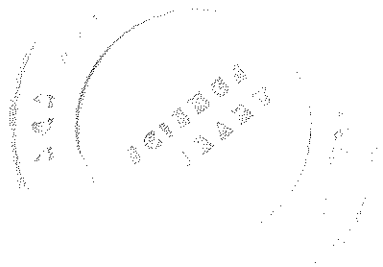
Ethiopia, like most countries in the world, has adopted the sentinel surveillance of HIV to monitor the spread of the virus in the population. Antenatal care clinics (ANC) are a potential sentinel source of information for monitoring the epidemic and assessment of the effectiveness of intervention in a community due to easy accessibility and low non-response (Kwesigabo *et al.*, 1995, Zaba *et al.*, 2000). In Ethiopia, HIV information among ANC attendees has been available since 1989 (UNAIDS, 2000).

Sentinel surveillance performed among pregnant women attending ANC in Addis Ababa resulted in higher values, although a decline was observed in recent years. 17.8% in 1996, 16.5% in 1997, 14.3% in 2000 and 15.6% in 2001 (Tsegaye *et a*l., 2002).

The number of persons living with HIV/AIDS in 2001 was estimated as 2.2 million including 2 million adults and 200,000 children. The highest prevalence of HIV is seen in the group 15-24 years representing 'recent infection' (UNAIDS 2001). AIDS is now the leading cause of death in the age group 15-49, killing adults in the most productive and reproductive phases of their lives (Mekonnen, *et al.*, 2003). According to UNAIDS (2004), the estimated number of adults and children living with HIV/AIDS at the end of 2003 was 1.5 million and the number of adults and children who died of AIDS during 2003 was120, 000.

Very few studies have been done in Ethiopia to estimate incidence, which is a measure of spread of a disease, of HIV infection in Ethiopia. Available information indicates that HIV/AIDS is spreading in Ethiopia at an alarming rate. The cohorts studied by the Ethio Netherlands AIDS Research Project at Wonji Shoa Sugar Factory and Akaki Textile Factories is one among the few cohorts in sub-Saharan Africa. The incidence of these two cohorts between 1997-1999 was 0.18 and 0.63 per 100 persons per year for Wonji and Akaki Cohorts respectively (Mekonnen *et al.*, 2003). According to Ministry Of Health Report (2004), the prevalence of HIV infection among young women (15 –24 years) attending antenatal care clinic in 2003 was 8.6%. As sexual debut often occurs in this age group, this prevalence is sometimes used as proxy for recent infections.

HIV-1 subtype C has become the most prevalent sub type in the HIV-1 pandemic accounting for the majority of all circulating sub types in the world (Esparz and Bhamarapravati, 2000). This subtype also accounts for the intense epidemic observed in Ethiopia (Abebe, *et al.*, 2001, Abebe, *et al.*, 1997). According to Behavioral Survey Report (BSS) (2002), transmission is almost exclusively through heterosexual contact as, the case elsewhere in Africa.



1.2. The Virus

HIV-1 has a complex genomic organization, which differs from the other retroviruses by the presence of multiple accessory genes in addition to the structural *gag, pol* and *env* genes.

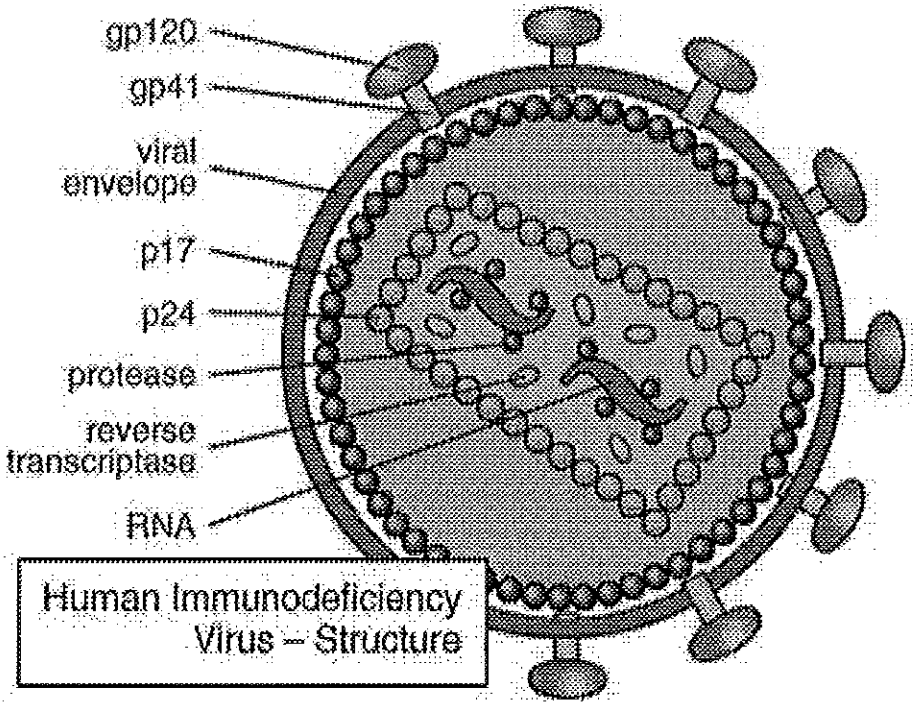


Fig 1. Schematic representation of an HIV virion

The *gag* gene encodes the core nucleocaspid polypeptides and this inner part is comprised of the major casipd protein, p24. The caspid structure also contains viral *pol* gene products as well as the protease, reverses transcriptase and integrase (Gelderblam *et al.*, 1991) and envelops glycoprotein, gp 120 and gp 41 which are virally encoded. These envelope complexes facilitate the entry of the virus to the host cell.

The virus possesses additional six genes, named *vif, vpu, vpr, tat, rev, nef* a feature that makes it unique among retroviruses (Stites and Terr, 1991). *Tat* and *rev* genes encode nuclear proteins,

which are essential for virus replication. *Nef* is a negative regulator and reduces production of the virus). *Rev* regulates viral replication by regulating the expression of viral proteins (Sodorski *et al.*, 1986). *Vif* is virus infectivity factor; facilitates efficient transmission of HIV. *Vpu* is an integral membrane protein and facilitates viral maturation and release (Hahn, 1994, Neghavi, *et al.*, 1999).

HIV begins its infection of a susceptible host cell by binding to the CD4 molecule on the host cell, which serves as a high affinity receptor. Two chemokine receptors, known as CCR5 and CXCR4, were identified as coreceptors to CD4 that permitted virus entry (Weiss, 1996). Following the binding of the outer envelope of the virus (gp120) to CD4 receptors, the virus is internalized (Fauci, *et al.*, 1988, Weiss *et al.*, 1993). Then the genetic material of the virus, which is RNA, is released and undergoes reverse transcription with the help of an enzyme called reverse transcriptase (Stites and Terr, 1991). Once the genetic material of the virus has been changed into DNA, it enters the host cell nucleus, where it can be integrated into the genetic material of the cell. Then the viruses wait for more proteins to be formed by cell to complete the reproductive process. Activation of the host cell results in transcription of viral DNA into messenger RNA (mRNA), which is translated into viral proteins. The new viral RNA forms the genetic material of the next generation of viruses. Following assembly (viral RNA & viral proteins) at the cell surface the virus buds from the cell and is released to infect another cell (Fauci and Lane, 2001).

1.3. Monitoring HIV incidence

As communities evaluate their priorities for prevention efforts they need data relevant to their epidemics (Peterman *et al.*, 1995). The best data for understanding recent changes in HIV epidemic are obtained from determinations of the number of new infections in a defined time period. Incidence, which is a measure of new infections that occur in a population during a specific period, is an important measure of the epidemic (Quan *et al.*, 2002).

Definitive detection of HIV - 1 RNA or p24 antigen in the absence of HIV antibody has been proposed as a means of identifying early infection and a method for estimating incidence (Beyer *et al.*, 1996). However, the duration of this state (p24 positivity - antibody negativity) is quite short (1-2 weeks) with antibodies appearing soon thereafter. The short duration makes it difficult to capture enough people in this phase to make incidence estimate. Testing strategies used to deduce infection during window period has some practical drawbacks because the pre-seroconversion period is relatively short and large sample sizes are required to achieve reasonable confidence intervals for identification of early infection. The use of this methodology is limited to large populations with high incidence (Brookmeyer and Quinn, 1995). The detection of plasma HIV RNA during the period before seroconversion is more sensitive measure for estimating incidence than p24 antigenemia. But the high cost and technology requirements are found as major obstacles to its use for this purpose (Barin *et a*l., 2002).

Serologic monitoring of the HIV-1 epidemic has been generally limited to monitoring seroprevalence, the proportion of persons with HIV-1 antibodies, comprising both those with early infection and those with chronic infection (Barin *et al.*, 2002). The best data for understanding recent changes in transmission are measurements of the number of new infections in a defined time period (incidence) that have been primarily provided by longitudinal cohort studies (Jarlaias *et al.*, 1994). This traditional approach is based on the longitudinal monitoring of seronegative people for seroconversion (Weinstock *et al.*, 1996). That is, incidence estimates are most often calculated by testing a cohort of individuals at two different time periods and observing the number of new infections. These studies are technically difficult and expensive, since they involve repeated sample collection, and testing of these individuals at different time intervals, and may be biased as there might be a loss to follow up of participants and may add uncertainty to incidence measurement (Brookmeyer and Quinn ,1995).

1.4. Markers for early diagnosis of HIV infection

Following the initial burst of viremia, HIV infected individuals generally mount an immune response. This immune response consists of elements of both humoral and cell mediated immunity and is directed against multiple antigenic determinants of HIV as well as against viral proteins produced in infected cells (Stites and Terr, 1991). Antibodies to HIV usually appear 2-6 weeks after infection. Detection of these antibodies forms the basis of most diagnostic, screening tests for HIV infection. The first antibodies directed are those directed against the structural or *gag* proteins of HIV. Antibodies to the gag proteins are followed by the appearance of antibodies to the envelope proteins. In addition, antibodies to regulatory proteins of the virus are produced (Fauci and Lane, 2001).

The different immunoglobulins are used as markers for early diagnosis of HIV infection. There is some evidence for an initial IgM response. Later, IgG and IgA antibodies are produced and there is an exponential increase in their titer, particularly for IgG antibodies over the first 3 to 6 months of infection. Tests that allow detection of IgM and IgG at the same time have been used for diagnosis of early HIV infection (Zijenah and Katzentein, 2002). However, detection of early infection using IgG response in infants born to HIV infected mother is not possible due to persistent maternal HIV specific IgG antibodies (Schupbach, *et al.*, 1994).

Assays which enable the detection of specific HIV-1 IgM, the predominant antibody in the primary immune response, have been proposed for identification of early HIV infection. However the assays have shown little utility, as IgM response to HIV is not consistently produced during early infection (Zijenah and Katzentein, 2002) and due to occasional detection of IgM in patients with long-term infection, which may result from periodic viremia and antigenic stimulation (Parekh and McDougal, 2001).

HIV-1 specific IgA has been investigated as a marker for early diagnosis of HIV infection (Bredberg-Raden, *et al.*, 1995). According to Cooper (1990), the detection of IgA HIV antibodies is an effective method for early diagnosis of HIV infected infants during an early asymptomatic period of their life.

IgE, antibody that does not cross the placenta, is potentially valuable for detection of early HIV infection in children and in adults as high amount of this kind of antibody is produced in the early phase of the infection (Pletcher, *et al.*, 2000). However, this marker is difficult to use in individuals living in Africa, who, as a result of frequent co-infection with parasites, may have high levels of IgE (Gueye-Ndiaye *et al.*, 2000).

1.5. New approaches for detecting recent HIV infections

HIV infection can be divided into categories of recent (within six months after seroconversion) or established infection (more than one year after seroconversion) depending on the quantity of antibody present or their avidities (Suligoi, *et al.*, 2002). According to Parekh (2002), features of antibody response that could be useful in distinguishing infection status include changes in antibody isotype, quantity, avidity and specificity.

Recently the method, STARHS (Serological testing algorithm for recent HIV seroconversion) has been developed which detects recent HIV infection and is used to distinguish incident from established infection using cross-sectional specimens. In this method, a standard commercial diagnostic ELISA is modified to reduce its sensitivity to a point where serum samples with low antibody titers are weakly reactive (Gupta *et al.*, 2003).

The standard ELISA gives information about HIV prevalence (total number of early and long term infections). However, the testing strategy called sensitive / less sensitive testing strategy is

used to distinguish between recent and long term infections using HIV positive specimens (Janssen *et al.*, 1998). This strategy was based on the fact that antibody titers increase gradually during the first few months of infection and then plateau (McRae, *et al.*, 1991). This parameter is used as a tool in order to estimate the relative time that HIV infection occurred. High titer antibodies signal late infection while low titer of antibodies signals early infection (parekh and McDougal, 2001).

Antibodies to various HIV proteins are elicited at different rates after infection. Early in infection, anti-gag (p24) and anti-env- (gp41/gp120) responses are observed, and anti-p24 antibodies decline with the development of clinical AIDS (Parekh *et al.*, 2001, Parekh *et al.*, 2002). Antibodies to envelop glycoproteins remain present until the terminal stages of AIDS, making the env gene products the best antigen to be used in serologic assays. But, in rare cases a loss of HIV antibodies has been observed, although evidence of viral infection can be demonstrated using PCR (Sites and Terr, 1991).

Measuring the level of antibodies to two or more different proteins or peptides is also used as means to differentiate between early and long- term infection. According to Parekh (2001), the envelope proteins, specifically gp41 and its oligomers (gp120/160) were the most useful proteins in distinguishing specimens as recent and long term infections. The gp41 peptides give differential titers and used easily in this enzyme-immunoassay to detect recent infection. Once antibody has appeared to gp41, titters progressively increase during 3-5 months until levels peak at which time they remain fairly constant throughout the remainder of infection.

In the sensitive / less sensitive assay (detuned), an initially reactive sample when tested with the routine assay (ELISA) becomes non-reactive when sample is diluted and tested with less sensitive assay. An individual's serum with established HIV infection would remain reactive following dilution in the less sensitive assay due to high levels of antibody (parekh and McDougal, 2001). The standard ELISA, typically detects HIV antibodies in the blood after 3-4

weeks, the less sensitive detuned version can detect infection only after about 120 days. Thus, a person who tests HIV positive using the standard ELISA, but negative using the detuned ELISA most likely was infected within the past four to six months and this method is of great importance in the identification of early HIV infection.

Figure 2 below shows how HIV antibodies increase over time (theoretical) and how the standard EIA and the LS-EIA (less sensitive Enzyme Immuno Assay) can detect different levels of antibodies.

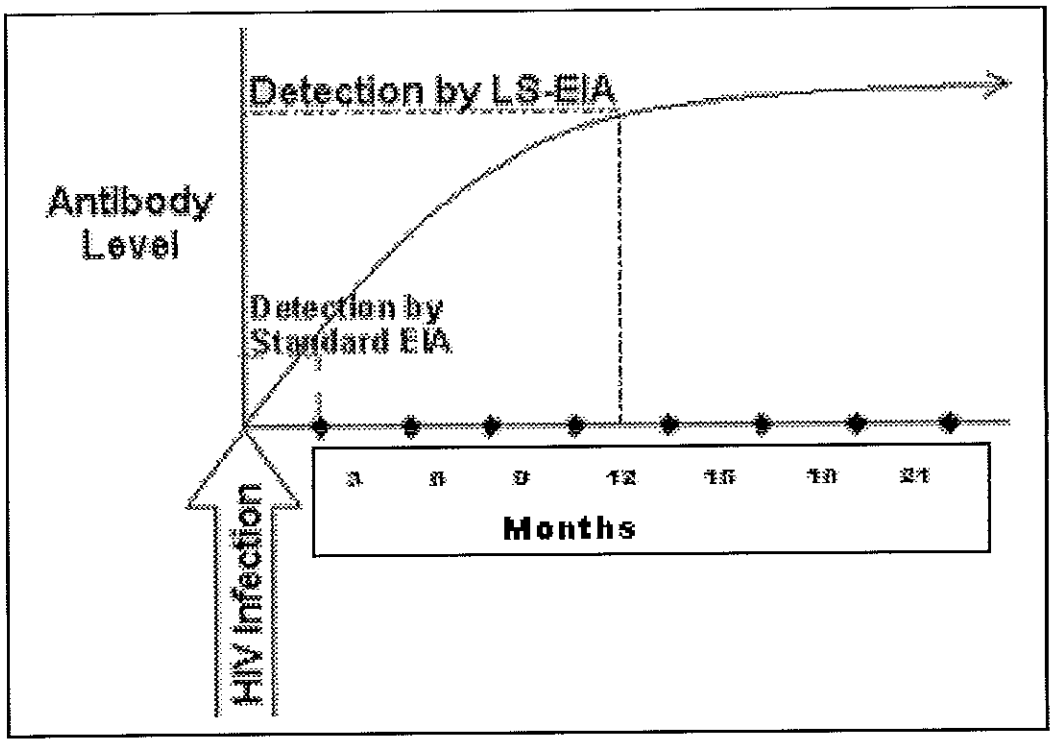


Fig.2.Increment of anti-HIV antibodies over time

More recently, another assay, Ig-G capture BED-CEIA (subtype C, B, E & D Enzyme immunoassay) has been developed. This assay is used to detect an increasing proportion of HIV - IgG in the serum following seroconversion and is designed to perform equivalently in different subtypes (Dobbs *et al.*, 2004). This assay has also been validated for subtype C infection using

Ethiopian seroconverter samples (Meles *et al.*, 2003). The principle of the assay is as follows. The HIV-CEIA is an IgG-capture EIA (see schematic, Figure 3) where representative HIV-IgG and non-HIV-IgG from serum are captured on goat-anti-human IgG coated wells. The relative amounts of HIV-IgG & non-HIV-IgG captured represent IgG antibody populations found in the serum. Indirectly, it measures the proportion of HIV-1 specific IgG in a given specimen with respect to total IgG. Since early seroconverters have a lower proportion of HIV-IgG in the serum, optical density (OD) values are lower on the HIV-CEIA, although the same specimens may have high OD values on regular diagnostic EIAs. (Parekh et al., 2002).

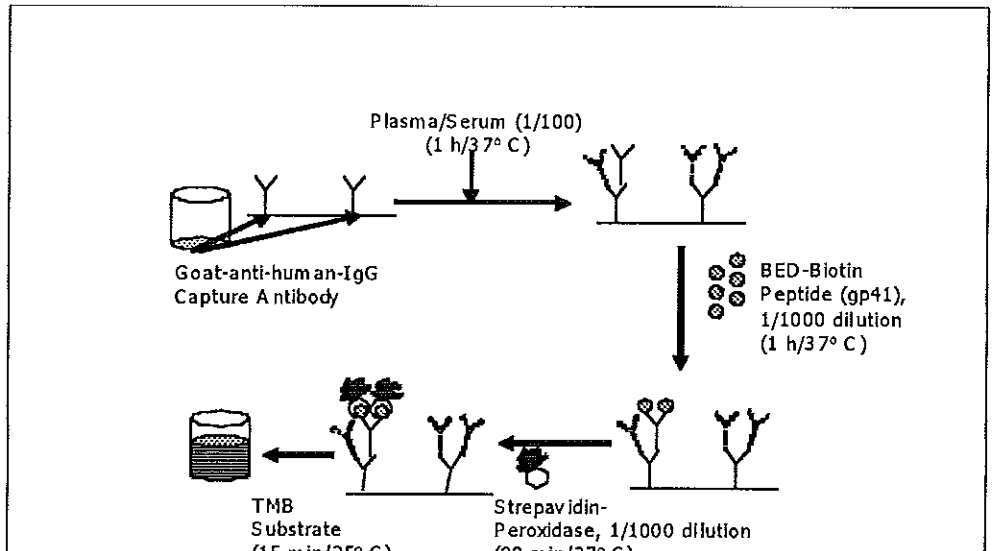


Fig.3 Schematic representations of the principle of Ig G- capture CEIA

Optimal optical-density cut -off (threshold) value is important in classification of HIV infection as recent or long-standing. A specimen was defined as sero -incident if it's OD-n value fell below a certain threshold (cutoff). Specimens registering below that cutoff were defined as having seroconverted within a designated period of time (seroconversion interval) (Parekh *et al.*, 2002).

OD-n of 0.8 corresponds to mean seroconversion duration of 153 days. This cutoff yields few false recent infections in AIDS patients. Thus, OD-n of 0.8 has a better predictive value for classification of HIV infection as early or established infection.

However, the assay has its own limitations. That is since the assay is based on the proportion of HIV- IgG present in total IgG, the differences in IgG concentration in various populations may affect the assay. In addition, patients with AIDS may be misclassified as recent due to combination of high viral antigen with antibody that reduces its availability for titer. Conditions or co- infections that elevate total IgG such as malaria and /or tuberculosis (TB) may also result in false incidence (Parekh and McDougal, 2001).

Antibody avidity is also a means of identifying recent from long-term infection. The method known as Ig-G capture BED-urea EIA has been developed using ELISA. This assay is also used to identify recent infection from established infection. Avidity describes the measurement of binding strength of an antiserum and a complex antigen. It depends on the affinity of the various antibodies present in the serum, and is a description of the overall antigen -antibody interaction (Binley *et al.*, 1997).

Antibody avidity increases progressively with time after exposure to an immunogen (Eisen and Siskind, 1964). Thus, the increase in antibody avidity is hallmark of antibody maturation (Binley *et al.*, 1997). The strength of interaction between the antibody present and antigen in early infection is weak as low avidity HIV-1 antibody comprises the majority of antibodies found in specimen from early infections and the relative avidity of antibody is stronger in established infections and can be estimated serologically based on resistance of the antigen-antibody complex to chaotropic agents (Parekh, *et al.*, 2001, Parekh *et al.*, 2002).

Chaotropic agents are dissociating reagents such as urea, potassium thiocyante and magnesium chloride. The chaotropic agent dissociates low avidity HIV antibody molecules most effectively. Procedurally, samples are incubated with HIV antigen. Then dissociating reagent is added following a wash step. Results are interpreted based on a calculation of avidity index (AI), which is the ratio of the optical density (OD) of dissociating reagent treated specimen to that of the non-treated control. Then, early infection is identified from chronic infection by using this avidity index (AI) (Parekh, *et al.*, 2001, Parekh *et al.*, 2002).

1.6. Significance of detecting recent HIV infection

Detection of recent infection is important for a number of reasons. One of these is minimization of secondary transmission by giving early counseling to the infected person as well as notifying sexual and needle-sharing partners (Janssen *et al.*, 1998, Parekh *et al.*, 2002). A Persons which have recently engaged in high risk behavior with a person with HIV 1 infection may transmit the virus more efficiently than at other times during infection, as there is high rate of viral replication during the early phase of HIV infection. One study reported that individuals had on average viral load above 1 million copies per ml, 13 days after infection (Kaufman, *et al.*, 2000). Hence, detection of early infection is important as it reduces secondary transmission.

Identification of recent infection is vital to HIV prevention effort by identifying who is at greatest risk of acquiring HIV and which communities are in greatest need of health education. In addition, it allows targeting these populations for therapeutic interventions or vaccines (Hu *et al.*, 2003, parekh *et al.*, 2002).

Detecting recent infection is also important to identify HIV subtypes or patterns of drug resistance, which can provide important information about the direction, and dynamics of the epidemic (Parekh *et al.*, 2001).

In general, detecting early infection is important in the prevention effort of HIV infection by allowing timely implementation of interventions such as counseling of patients, management of infection and prevention of transmission.

1.7. Comparison of prevalence and incidence

Prevalence represents the cumulative burden of infections and reflects new infections, migration out of a specific group and deaths while, incidence measures the number of new infections that occur in a population during a specific period (Quan, *et al.*, 2002). . Thus, Prevalence is a measure of extent of a disease while incidence is a measure of spread of a disease in a population.

Information on the prevalence of HIV infection within selected populations is necessary to plan for health and social services. However, prevalence data may not identify those groups in whom new infections are occurring (Schwartz, *et al.*, 2001). Therefore, they are of relatively limited value in evaluating changes in the course of epidemics. But data on trends in HIV incidence provides the most direct assessment of the prevailing HIV epidemic dynamics and the effects of ongoing preventive measures (wawer *et al.*, 1997). Rutherford (2000) also stated that, new cases of HIV infection rather than old cases of HIV infection represent failures of current public health programs and signal a need for refining to prevent the transmission of the virus. Therefore it is of paramount importance to include incidence measurement as part of surveillance programs.

2. Objectives

2.1. General objective

To estimate HIV-1 incidence among pregnant women attending antenatal care clinics in Addis Ababa.

Specific objectives

i) To identify recent HIV-1 infection using STARHS assay, in pregnant women attending antenatal care clinics in Addis Ababa.

ii) To assess temporal trends in the incidence of HIV infection in women attending antenatal care clinics in Addis Ababa.

iii) To compare the efficiency of 'in-house' and commercial kits in HIV incidence testing.

3. Materials and Methods

3.1. Study sites and population

The serum samples in this study were used from the previously collected specimens of sentinel surveillance. The previous study was monitoring trends of the prevalence of HIV infection among women attending antenatal care clinics between 1995 and 2003 (excluding 1998 and 1999) at four health centers in Addis Ababa, namely; Gulele, Kazanches, Tekle-haimanot, and Higher 23.

3.2. Sample size

A total of 7,748 serum samples had been tested for antibodies to HIV between 1995 and 2003. Of these, 15.7 % (1216) were HIV-1 positive. In this study, 1078 HIV positive samples were tested using STARHS assay.

3.3. Laboratory methods

The HIV positive samples were tested twice using 'in-house' and commercial incidence assay kits.

3.3.1. Assay procedure ('in-house' incidence assay kit)

Initial serum screening had been performed using two commercial enzyme linked immunosorbent assay (vironostica HIV uni-form plus O, Organon Teknika, Boxtel, the Netherlands, HIV-1/2 ELISA, Murex, Dartford, UK) (Tsegaye et al., 2002). After testing all specimens had been stored at -80°C. All positive specimens that were collected for the last seven years were removed from deep freezer (-80°C), thawed and tested with STARHS assay (IgG capture BED enzyme immunoassay) as follows. Briefly, pre-coated goat anti-human Ig-G antibody plates and reagents (BioSource International, Camarillo, CA) were taken out from a refrigerator and allowed to come to room temperature. 1 in 100 dilution of controls (Negative Control (NC), High Positive Control (HPC), Calibrator (CAL), and Low Positive Control (LPC)) and specimens were prepared in titer tubes. Then, 100 µl of 1 in 100 dilutions was transferred to IgG plate using a multi-channel pipette and incubated for one hour at 37°C.
Next to that, the plate was washed four times with wash buffer (0.01 M phosphate buffered saline, pH 7.2 [PBS] & 0.05% [v/v] Triton X-100). The BED biotin peptide (gp-41) was diluted (1:1000 dilution) in diluent buffer (100 ml wash buffer & 3 g bovine serum albumin) and 100 µl was added to each well, and the plate was incubated for one hour at 37°C. Wells were washed again four times and 100 µl of 1 in 1800 dilution of streptavidin HRP conjugate was added to each well. This enzyme conjugate was incubated for 90 minutes at 37° C followed by four washes. Finally tetra methyl benzidine (TBM) substrate was added, and the plate was incubated for 15 minutes at 25°C (incubator). Color development was stopped by addition of 100 µl of 2M sulphuric acid. The optical density (OD) values were read at 450 nm with 630 nm as reference wavelength using spectrophotometer.

Normalized optical density values (OD-n) that are calculated as the ratio of Specimen OD/median CAL OD (for the initial plate run) or median specimen OD/median CAL OD (for the confirmatory plate run) were used to minimize run-to-run variability and increase reproducibility (Parekh, *et al.*, 2002, Dobbs *et al.*, 2004).

3.3.2. Assay procedure (commercial kit)

500uL of diluent buffer was added to each tube using a multi-channel pipette and 5 uL of control (Negative control, calibrator, low positive control, and high positive control) and specimen were added to designated tube. Using a multi-channel pipette, the diluted controls and specimens were mixed five times and 100uL of each diluted control and specimens were transferred to test plate (goat anti-human immunoglobulin coated micro well plate). Then, the plate was covered with plate sealer and incubated for 1hour at 37°C. Ten minutes before the end of this incubation step, 1:1000 dilution of HIV-1 BED peptide was prepared in diluent buffer (3 % BSA in wash buffer). Then, the plate was washed four times with 300uL/well of the prepared wash buffer with a ten second soak between each wash. After the final wash, the plate was wrapped in absorbent paper and tapped upside down to remove any remaining wash buffer. Next to this, 100ul of the diluted HIV-1 BED Peptide was added using a multi-channel pipette and covered with a plate sealer and incubated for 1hour at 37°C. Dilute streptavidin HRP conjugate 1:1000 dilution was prepared in diluent buffer. The plate was washed four times and 100uL of diluted conjugate was added to each well using multi-channel pipette. Then, it was covered with a plate sealer and incubated for 90 minutes at 37 °C followed by four washes. 100uL of TMB substrate was added to each well using a multi-channel pipette and incubated in a 25 °C incubator for exactly 15 minutes for color development. Finally, 100uL of stop solution was added to each well to stop the reaction. Optical density was read using a spectrophotometer set at 450 nm wavelengths with reference wavelength at 630 to 650nm.

Validity of the test procedure was checked by using controls (Negative control, calibrator, low and high positive controls), which have their own range of values.

Raw OD or OD-n of any one control, which falls outside of the following limits, were repeated.

Table 1. Range of values showing validity of the test procedures.

	NC	CAL	LPC	HPC
Min	0.040	0.500	0.200	0.900
Max	0.250	1.200	0.800	2.200

NC - Negative Control

HPC - High Positive Control

CAL - Calibrator

LPC - Low Positive Control

Specimens with normalized optical density (specimen OD/calibrator OD) of 1.500 or less were tested again by preparing 3 replicate dilutions of each specimen. Finally, retested samples that had a mean standard optical density (SOD) of more than 0.800 were defined as reactive and those specimens that had a mean standard optical density of 0.800 or less were defined as non-reactive (Janssen, et al., 1998).

3.4. Data analysis

The Statistical analysis was performed using SPSS (Statistical package for the social Science). The result was classified according to age and sites of blood collection (inner city and outer city) for the respective years. A P value of less than 0.05 was considered indicative of statistical significance.

HIV incidence was calculated using the following formula (Parekh, *et al.*, 2002)

$$I = \frac{(365 / W)\ N\ inc \quad x \quad 100}{N\ neg + (365 / W)\ N\ inc/2}$$

W = Window period

N inc = number of recent HIV infection

N neg = number of HIV seronegatives

The 95 % confidence interval (CI) for the incidence estimate was determined as:

$$95\ \%\ CI = I + / - 1.96\ \frac{.....I.....}{(SQRT\ of\ Ninc)}$$

I = Incidence

SORT of N inc = Square root of number of recent HIV infections

4. Results

Comparison of 'in-house' and commercial incidence assay kits

There were 84 common samples that had an OD-n value of less than 0.8 and 954 samples with standardized optical density of greater or equal to 0.8 when tested by both commercial and 'in-house' kits. But forty samples (3.7%) had discrepant results. Out of 1078 samples tested with the in-house incidence assay kit, 14 samples were false reactive samples (OD-n < 0.8) and 26 were false non-reactive (OD-n ≥ 0.8) samples. An agreement was also observed between the raw OD-n values of the two assays, with a correlation coefficient (r) of 0.76.

Table 2. Comparison of normalized optical density values (OD-n) of HIV positive samples (n=1078) measured using 'in-house' and commercial incidence assay kits.

	Commercial			
		OD-n < 0.8	OD-n ≥ 0.8	Total
'In-house'	OD-n < 0.8	84	26	110
	OD - n ≥ 0.8	14	954	968
	Total	98	980	1078

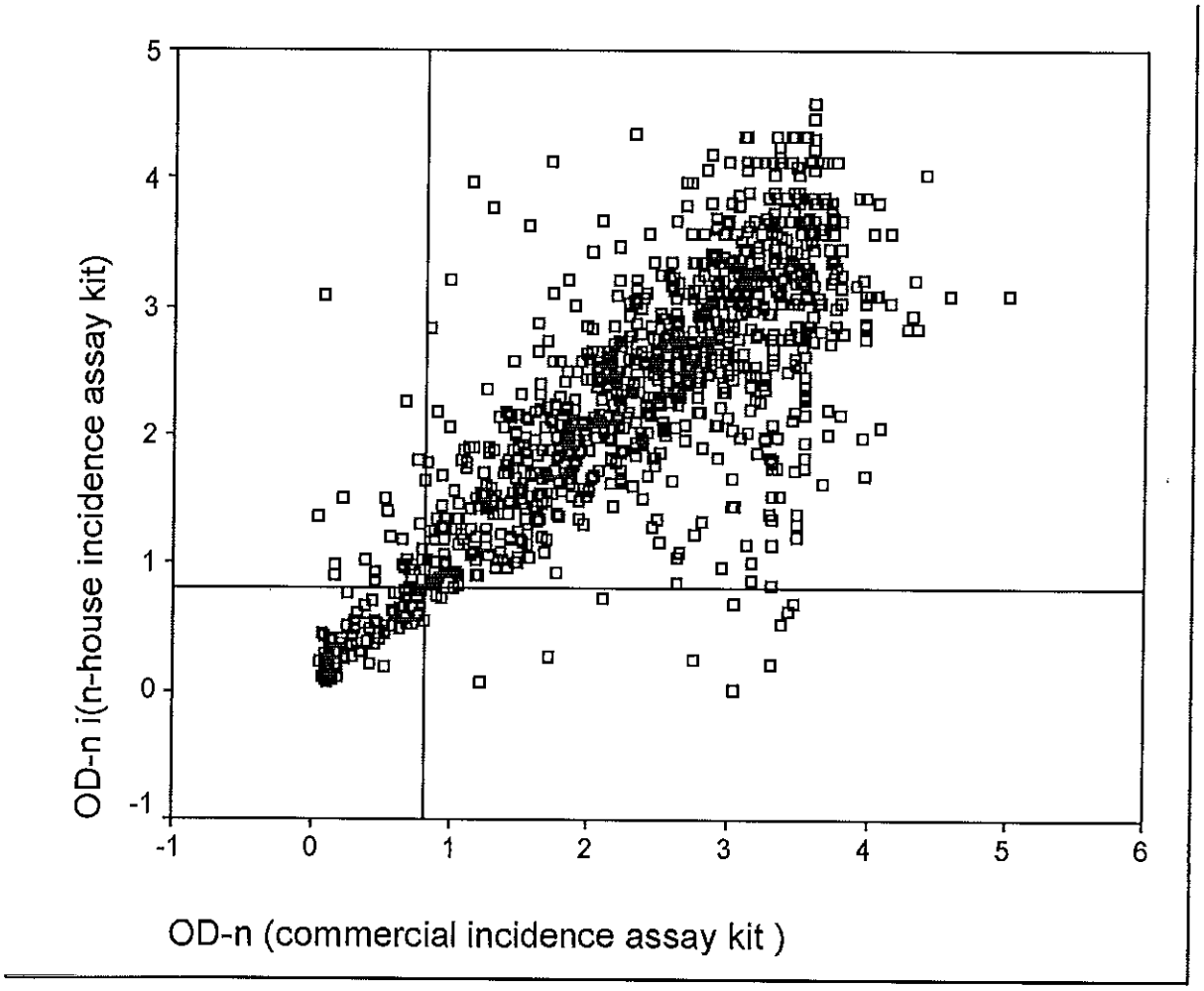


Fig. 4. Comparison of normalized optical density values (OD-n) of HIV positive samples (n=1078) measured using ' in-house' and commercial incidence assay kits. The horizontal and vertical lines represent the cutoff (0.8) for both incidence assay kits.

Although significant correlation existed between the performance of the 'in-house' and commercial incidence assay kits, the commercial assay kit picked relatively more recent infections. As a result, further analyses of the study samples was done by using the commercial kit.

Out of 1078 HIV pregnant women tested, 10.2 % (110) had recent infections while 968 (89.8 %) had long-standing HIV infections. In the inner city health centers, 68 recent infections (10.4%) and 587 (89.6 %) long-standing infections were observed while in the outer city health centers 42 (9.9%) recent seroconverters were found (Table 2).

Table 3.The overall Proportion of recent and long-standing HIV infections in pregnant women attending the inner and outer city health centers in Addis Ababa, 1995 - 2003.

Type of infection	Inner city		Outer city		Overall	
	Frequency	%	Frequency	%	Frequency	%
Recent infections	68	10.4	42	9.9	110	10.2
Long-standing infections	587	89.6	381	90.07	968	89.8
Total	655	100	423	100	1078	100

Overall, the largest proportion of recent seroconverters (17.86%) was observed among women aged between 15-19 years attending both the inner and outer city health centers. Women aged between 20-24 years followed this with 10.04 %. 9.84 % of women aged above 29 years were recent seroconverters. The lowest percentage (8.15%) of seroconverters was observed among women aged between 25-29 years. The difference observed in the percentage of recent infections among women of the different age groups was significant.

An overall declining temporal trend of recent infection was observed in all sites from the year 1995 to 2000 (14.29 % in 1995, 12.90 % in 1996, 11.11 % in 1997, and 7.05 % in 2000). It was then elevated to 9.68% in 2001 and declined again to 6.30 % in 2003.

HIV incidence among pregnant women aged 15 to 19 years declined from 1996 to 2001 and elevated in 2002 among pregnant women attending the inner city health centers (Kazanches and Tekle-Haimanot). No recent infection was observed in 2003, implying that the annual HIV

incidence may have gone down to 0%. A decreasing temporal trend in HIV incidence was also observed among women aged between 20 -24 years. Incidence of HIV infection among women aged between 25-29 years showed a declining trend from 1995 to 2000. However, this was followed by an increasing trend in recent years except the year 2002. The lowest HIV incidence was observed among older women (> 29 years). HIV incidence among women of this age group remained fairly constant from 1995 to 2000 and declined in 2001.A peak HIV incidence was observed among women of this age group in the year 2002 (Table 3).

Women attending the inner city health centers aged between 15 to 19 years had the highest proportion of recent HIV infection (16.3 %) over the seven years period. Women of age group 25-29 followed with a 10 % rate and those aged 20-24 years and women aged over 29 years (9.3%).

Table 4.HIV incidence among pregnant women of different age groups attending the inner city health centers in Addis Ababa between 1995 and 2003.

Year	Age group	%recent infections	Annual incidence %)	95 % CI (%)
1995	15-19	15.4 (2/13)	5.4	-2.1 - 12.8
	20-24	12.3 (7/57)	9.7	2.5 - 16.9
	25-29	18.2 (6 /33)	11.5	2.3 - 20.6
	>29	11.1 (1/9)	2.3	-2.2 - 6.8
1996	15-19	18.5 (5/27)	14.1	1.7 - 26.3
	20-24	8.7 (4/46)	6.6	0.13 - 13.1
	25-29	12.9 (4/31)	9.9	0.19 - 19.6
	>29	10 (1/10)	2.2	-2.1 - 6.5
1997	15-19	20 (2/10)	7	-2.7 - 16.7
	20-24	8.1 (3/37)	4.1	-0.53 - 8.7
	25-29	11.4 (5/44)	7.3	0.91 - 13.8
	>29	11.1 (1/9)	2.4	-2.3 - 7
2000	15-19	20 (1/5)	3.1	-3 - 9.2
	20-24	10.8 (4/37)	5.2	0.1 - 10.3
	25-29	3.4 (1/29)	1.6	-1.6 - 4.8
	>29	9.1 (1/11)	2.3	2.2 - 6.9
2001	15-19	10 (1/10)	2.9	-2.7 - 8.6
	20-24	12.9 (4/31)	4.9	0.1 - 9.8
	25-29	8.8 (3/34)	5	-0.7 - 10.6
	>29	0 (0/9)	0	0
2002	15-19	20 (2/10)	5	-1.9 - 11.8
	20-24	7.3 (3/41)	3.8	-0.5 - 8.1
	25-29	6.7 (2/30)	3.5	-1.3 - 8.2
	>29	25 (2/8)	6.1	-2.4 - 14.5
2003	15-19	0 (0/5)	0	0
	20-24	3.3 (1/30)	2.3	-2.2 - 6.9
	25-29	6.5 (2/31)	6.8	-2.6 - 16.2
	>29	0 (0/8)	0	0

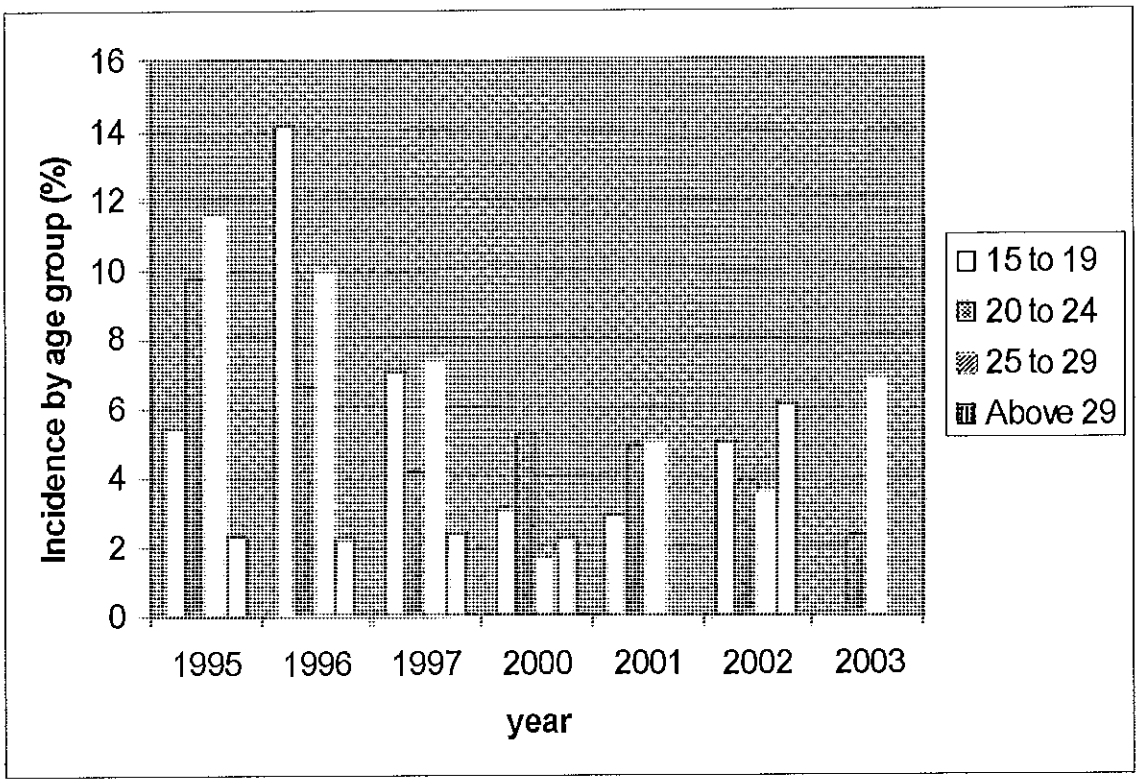


Fig.5. Age specific temporal trends of HIV incidence among pregnant women attending the inner city health centers in Addis Ababa between 1995 and 2003.

In the outer city health centers (Gulele and Higher-23), an increasing trend of HIV incidence was observed among young pregnant women aged 15-19 years from 1996 to 2000.Then a declining trend followed this except the year 2002. An increasing temporal trend in the recent year was observed among women aged 20-24 years. Among women aged between 25-29 years, HIV incidence declined from 1996 to 2000 and increased from 2001 to 2003. Incidence among women aged over 29 years decreased from 1996 to 2000 and elevated in 2001(Table 4).

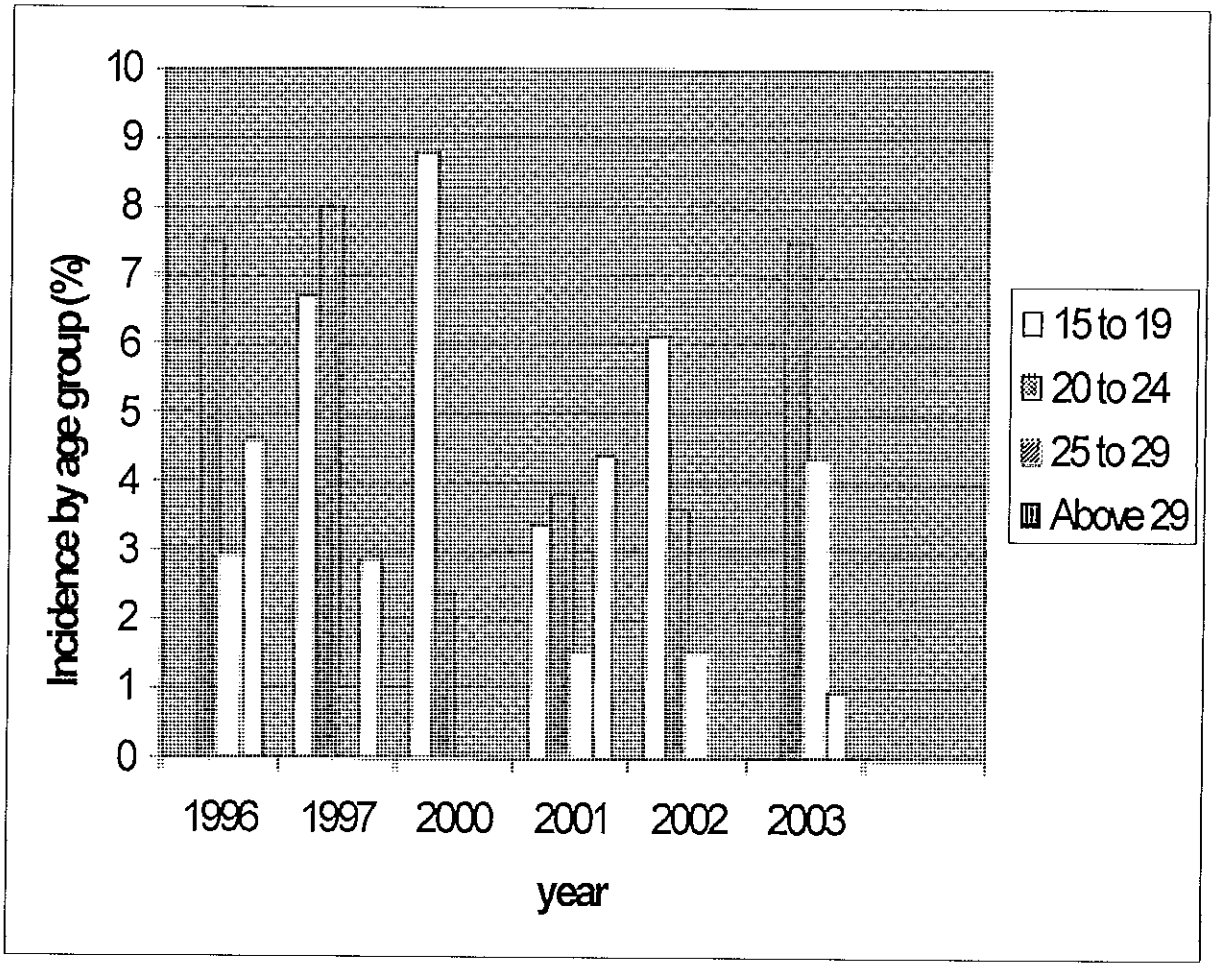


Fig 6 .Age specific temporal trends in HIV incidence among pregnant women attending the outer city health centers in Addis Ababa between 1996 and 2003.

Table 5. HIV incidence among pregnant women of different age groups attending the outer city health center in Addis Ababa between 1996 and 2003.

Year	age group	%recent infections	annual incidence (%)	95 % CI (%)
1996	15-19	0 (0 / 7)	0	0
	20-24	16.7 (6/36)	7.5	1.5 -13.5
	25-29	9.5 (2/21)	2.9	-1.1 - 7
	>29	25 (2/8)	4.6	-1.8 - 10.9
1997	15-19	20 (2/10)	6.7	-2.6 - 15.9
	20-24	15.2 (7/46)	8	2.1 - 13.8
	25-29	0 (0/25)	0	0
	>29	12.5 (1/8)	2.9	-2.8 - 8.5
2000	15-19	33.3 (2/6)	8.8	-3.4 - 21
	20-24	5.4 (2/37)	2.4	-0.92 - 5.7
	25-29	0 (0/22)	0	0
	>29	0 (0/9)	0	0
2001	15-19	16.7 (1/6)	3.4	-3.2 - 9.9
	20-24	10.3 (3/29)	3.8	- 0.5 - 8.1
	25-29	4.2 (1/24)	1.5	-1.5 - 4.5
	>29	16.6 (2/12)	4.4	-1.7 - 10.4
2002	15-19	66.6 (2/3)	6.1	-2.4 - 14.5
	20-24	10 (3/30)	3.6	-0.5 - 7.8
	25-29	5.9 (1/17)	1.5	-1.4 - 4.4
	>29	0 (0/6)	0	0
2003	15-19	0 (0/0)	0	0
	20-24	13 (3/23)	7.5	-0.99 - 16
	25-29	6.7 (1/15)	4.3	-0 4 - 12.6
	>29	6.7 (1/15)	0.95	-0.91 - 2.8

Like the inner city health centers, in the outer city health centers the highest proportion of recent infection was observed among women aged 15 to 19 years. But, this was followed by women aged between 20-24 years (11.9 %) and the lowest proportion was observed among those aged 25-29 years (4 %).

The proportion of recent infection in the inner city declined from 14.3 % in 1995 to 8.5 % in 2000 and elevated to 10.1 % in 2002. Likewise, in the outer city the proportion of recent infection declined from 13.9 % in 1996 to 5.4 % in 2000.Then it elevated to 10.7 % in 2002 and declined to 9.4 % in 2003.

Table 6. Summary of HIV incidence among pregnant women of all age groups attending the inner and outer city health centers in Addis Ababa.

Site	year	%recent infections	annual incidence (%)	95 CI (%)
Inner city	1995	14.3 (16/1112)	7.8	4 - 11.6
	1996	12.3 9 (14/114)	7.7	3.7 - 11.7
	1997	11 (11/100)	5.2	2.1 - 8.2
	2000	8.5 (7/82)	6.4	1.7 - 11.2
	2001	9.5 (8/84)	3.9	1.2 - 6.6
	2002	10.1 (9/89)	4.3	1.5 - 7.1
	2003	4.1 (3/74)	1.3	0.18 - 2.8
outer city	1996	13.9 (10/72)	4.5	1.7 - 7.5
	1997	11.2 (10/89)	4.6	1.7 - 7.5
	2000	5.4 (4/74)	1.8	0.04 - 3.6
	2001	9.9 (7/71)	3.2	0.82 - 5.5
	2002	10.7 (6/56)	2.7	0.53 - 4.8
	2003	9.4 (5/53)	2.3	0.28- 4.3

HIV incidence among pregnant women of all age groups attending the outer city health centers was 4.5 % in 1996, 4.6 % in 1997, 1.8 % in 2001, 2.7 % in 2002 and 2.3 % in 2003 and the annual incidence in the inner city showed declining trend from 1995 to 1997.

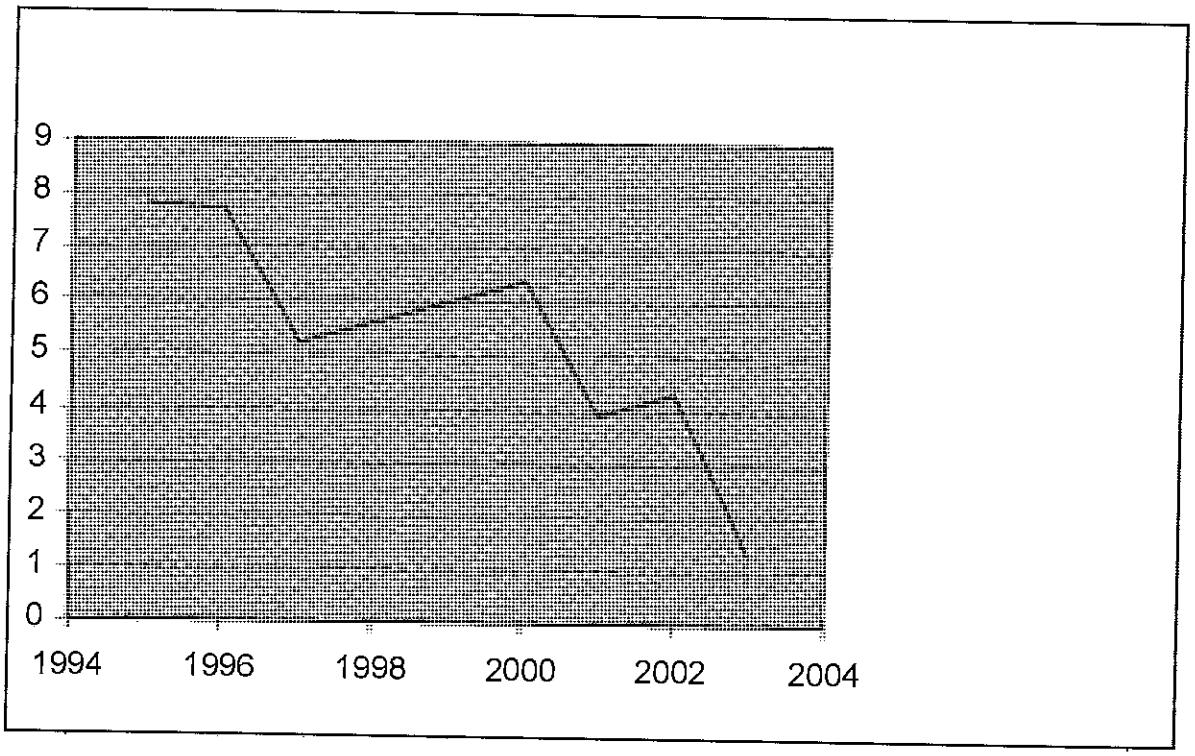


Fig.7.Temporal trends of HIV incidence among pregnant women attending the inner city health centers in Addis Ababa.

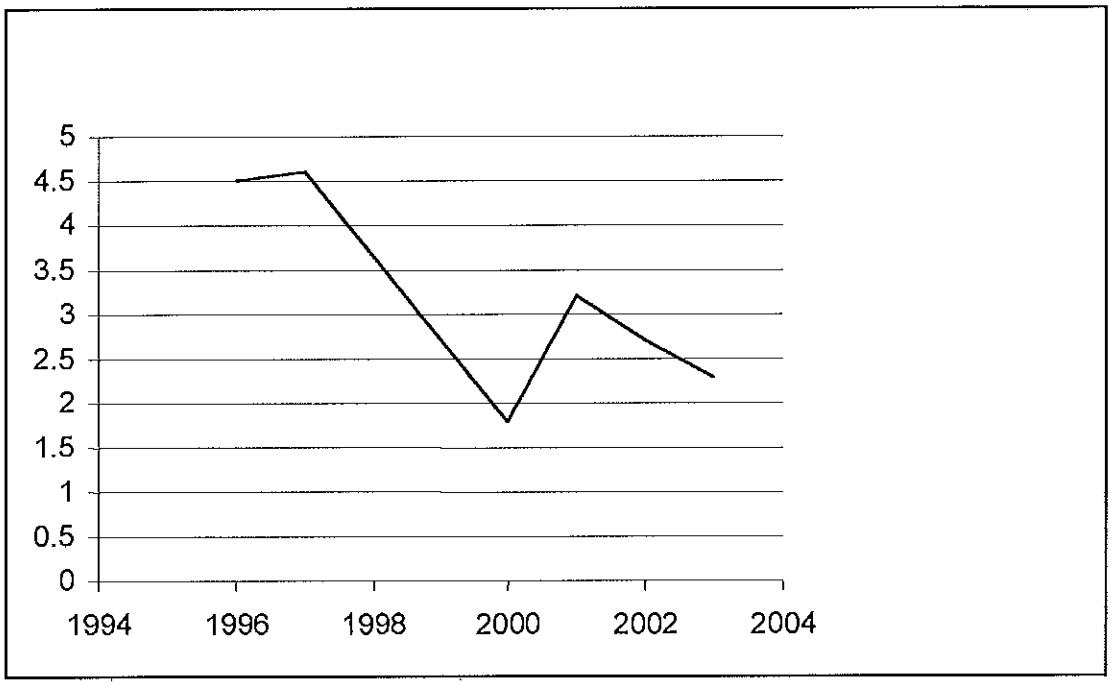


Fig.8. Temporal trends of HIV incidence among pregnant women attending the outer city health centers in Addis Ababa.

The overall incidence in the inner and outer city health centers was 7.7 % in 1995, 5.9 % in 1996, 4.9 % in 1997, 2.5 % in 2000, 3.5 % in 2001, 3.5 % in 2002 and 1.8 % in 2003.

HIV incidence among women attending at kazanches health center declined from 1996 to 2003. There was also steady reduction in HIV incidence among women attending Tekle-Haimanot antenatal care clinic. The same temporal trend was observed among women attending Gulele health center. But an increasing trend of HIV incidence was observed at Higher 23 health center (Table 6).

Table 7.HIV incidence among women attending antenatal care clinics at different sites.

Site	Year	% recent infections	Incidence (%)	95 % CI
Kazanches	1995	13.1 (8/61)	7.9	2.4 - 13.5
	1996	13.7 (7/51)	8.7	2.3 - 15.2
	1997	15.2 (7/46)	6.5	1.7 - 11.2
	2000	4.3 (2/46)	1.9	-0.8 - 4.6
	2001	9.3 (4/43)	4.1	0.1 - 8.1
	2002	12.2 (5/41)	4.8	0.6 - 9
	2003	5.3 (2/38)	3.2	-1.2- 7.5
Teklehaimanot	1995	15.7 (8/51)	7.6	2.3 - 12.9
	1996	11.1 (7/63)	6.9	1.7 - 12
	1997	7.4 (4/54)	3.8	0.08 - 7.6
	2000	13.9 (5/36)	4.6	0.5 - 8.7
	2001	9.8 (4/41)	3.8	0.08 - 7.4
	2002	8.3 (4/48)	3.8	0.08 - 7.5
	2003	2.8 (1/36)	0.62	-0.6 - 1.8
Gulele	1996	15.9 (7/44)	6.4	1.7- 11.2
	1997	12.2 (6/49)	5.7	1.1 - 10.2
	2000	4.3 (2/47)	1.9	-0.8 - 4.6
	2001	7.9 (3/38)	2.8	-0. 4 - 6
	2002	7.1 (2/28)	1.7	-0.7 - 4.2
	2003	6.3 (2/32)	1.5	-0.6 - 3.5
Heigher-23	1996	10.7 (3/28)	2.6	-0.3 - 5.5
	1997	10 (4/40)	3.6	0.07 - 7.1
	2000	7.4 (2/27)	1.7	-0.7 - 4.1
	2001	12.1 (4/33)	3.5	0.07 - 6.9
	2002	14.3 (4/28)	3.7	0.07 - 7.2
	2003	14.3(3/21)	3.7	0.5 - 7.8

5. Discussion

A significant correlation was observed in the performance of 'in-house' and commercial incidence assay kits. However the relative superiority of the commercial assay over the 'in-house' kit is due to the fact that the 'in-house' kit, reagents were prepared by mixing different chemicals in laboratory for the test while the commercial incidence assay kit contains standardized reagents. Thus, the chance of making measurement error is less in the commercial assay as compared to the' in-house' incidence assay kit.

Larger proportion of recent infections was observed among pregnant women attending the inner city health centers, which is in line with the fact that the high risk group, the sex-workers, are in larger numbers in the inner cities as compared to the outer cities. Similarly, community-based study conducted in 1994 in Addis Ababa had also showed the prevalence of HIV infection to be higher in the inner city than in the outer city (Fontanet *et al.*, 1998). Furthermore, According to Behavioral Surveillance Survey report (BSS, 2002), conducted in Addis Ababa, there were some variations in the inner and outer city in the knowledge and misconceptions about HIV/AIDS.

The lowest proportion of recent infection was observed among older pregnant women (> 29 years) attending the inner city health centers in most of the calendar years (1995, 1996, 2001, and 2003) is in agreement with the report of Ministry of Health (2002) that showed advanced HIV infection to be more prevalent in the older age groups of women in general.

Our finding that the proportion of recent infection was much higher in 15-19 age groups as compared to women of higher age groups is in agreement with the global picture reported by UNAIDS (2000). This may be explained by the report of the ministry of health (2002), which showed that women who become pregnant at younger age have more risky sexual behavior than non-pregnant women of the same age in the general population. One such risky behavior is that sexual activity by girls often involves older partners that may have already been HIV infected.

The relatively higher HIV incidence among the younger (15 - 24) pregnant women attending the outer city health centers is similar to the report from South Africa where incidence of HIV-1 was highest in young women in their late teens and early twenties (Rollins *et al.*, 2002).

On the other hand the present data indicated declines in HIV incidence in recent years among young women (15 to 24 years) of the inner city. This finding is in agreement with that of ministry of health report (2004) that showed declining HIV incidence rate in cities over the last few years, which may be indicative of some behavioral changes in the urban population. This is supported by the increased level of awareness about the disease, the tremendous increase in condom distribution and the increasing utilization of VCT services by different social groups. The present findings are also supported by the declining temporal trend in HIV prevalence that was observed among women of young age groups attending the Addis Ababa inner city health centers (Tsegaye, *et al.*, 2002). Our finding supports the idea that, declines in prevalence among young age groups suggest declines in incidence, given that the effects of HIV infection associated reduction in fertility or increase in mortality are less pronounced in recently infected individuals (Zaba, *et al.*, 2000).

Such a declining trend in HIV incidence among persons aged less than 25 years has also been shown in San Francisco, where there are effective HIV control measures (Schwartz, *et al.*, 2001).

HIV incidence also declined from 1996 to 2000 among older women (>29 years) attending the outer city health centers. This observation is consistent with the finding of Taha (1998), where HIV incidence declined steadily in older pregnant women in urban Malawi, where intervention programmes focusing on risk behavior reduction, condom promotion and STD control have been instituted. Such programmes have been underway since 1987 in Ethiopia (Kebede *et al.*,2000) .

The observed increased trend of HIV incidence in recent years among pregnant women aged between 25 and 29 years and decreased trend among women aged below 25 years may indicate that the HIV prevention and control strategy has given more attention to the young as compared to the older age groups

The limitation of this study is that people with AIDS may be classified as being found at the earlier stage of HIV infection due to low antibody production as a result of disease progression and due to poor initial immune response and people with vigorous response could be mis-classified as having long-standing infections when they are actually recent seroconverters (Parekh and McDougal, 2001). However, women at the end- stage of acquired immunodeficiency syndrome are less likely to be pregnant and would be unlikely to affect the predictive value of incidence assays used in this study.

Lack of socio-economic information, history of earlier pregnancy, information on other co-infections etc on the study subjects did not allow analysis of confounding factors in the interpretation of the results.

6. Conclusion

The highest proportion of recent infection was observed among women aged 15- 19 years while the lowest percentage of recent infection was observed among older women. This finding suggests that the disease is affecting people at the most productive phase of their life.

Measuring the number of new infections in a defined time period (incidence) is important to understand recent changes in transmission. HIV incidence is declining in recent years among young women in the inner city and this suggests that the transmission is showing a declining trend in Addis Ababa.

As determining HIV incidence using the traditional method (longitudinal study) is difficult, the use of standardized optical density values on cross-sectional specimens has made the estimation of HIV incidence very practicable.

There is significant agreement in the performance of the two HIV incidence assay kits, the 'in-house' and the commercially developed one. Thus, the incidence of HIV infection can be determined by using cheaper assays optimized under routine laboratory conditions.

7. Recommendations

- Detecting recent HIV infections is very important in the control efforts of AIDS. Thus, HIV surveillance systems should incorporate this method for detecting recent HIV infection.

- For more effective prevention of HIV/AIDS, data related to HIV incidence should be linked with behavioral factors.

- HIV incidence estimations must be done in populations in other cities and the rural setting using this technique.

- For maximum accuracy, CD4 cell counts and clinical information should be available to augment incidence determinations.

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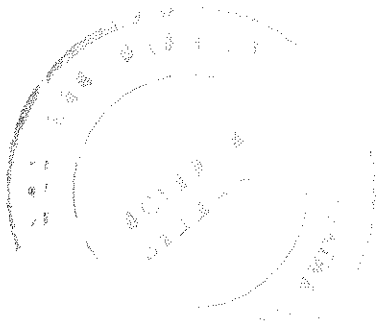

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Declaration

I, the undersigned declare that, this Msc thesis is my original work & has not been presented for a degree in any other university, & that all sources of material used for the thesis have been duly acknowledged.

Investigator: Tigist Aklilu

Signature 

Date of submission 04/07/05

Addis Ababa, Ethiopia

Advisor

Name Signature

Dr. Beyene Petros