ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF BIOLOGY

DETECTION OF RECENT HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) INFECTIONS AND ESTIMATING ITS INCIDENCE RATE IN ETHIOPIA.

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOLOGY

(APPLIED GENETICS)

BY

KALEAB KETEMA

JULY 2007

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Acknowledgments

First and foremost I would like to thank God for He has made everything possible.

For his critical reading of the manuscript, kind, fatherly pieces of advice and invaluable suggestions, I would like to express my deepest gratitude to my research advisor Prof. Beyene Petros.

I am also grateful to my external advisor Dr. Dawit Wolday for his help to give me this area of research and his valuable guidance.

I am grateful to Ato Desta Kassa and Ato Belete Tegbaru for spending their valuable time in reading, correcting, and giving valuable comments till the final writing up of the paper. My special thanks also go to Ato Hailu Meles, for his valuable assistance and moral support throughout the course of this work.

I would like to give many thanks to Ato Ermias Hailu, Ato Tesfaye Tilahun, W/t Mulu, Ato Asfaw Beyene and other staff members of the ENARP laboratory for their invaluable technical assistance and advice.

Finally, I would like to sincerely express my thankfulness to my families and friends who were always there when I wanted them. Without their help I would not be able to accomplish my work.

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List of abbreviations

AIDS - Acquired Immune Deficiency Syndrome

ANC – Antenatal Care Clinic

BED-CEIA – Subtype B, E, D Capture Enzyme Immunosorbent Aassay

BSA – Bovine Serum Albumin

CAL – Calibrator

CDC-Center for Disease Control

CSA- Central Statistical Authority

DNA – Deoxyribonucleic acid

ELISA – Enzyme Linked Immunosorbent Assay

EIA- Enzyme Immunosorbent Assay

HIV – Human Immunodeficiency Virus

HIV-1- Human immunodeficiency Virus Type 1

HIV-2 - Human immunodeficiency Virus Type 1

HPC – High Positive Control

IgA - Immunoglobulin A

IgE - Immunoglobulin E

IgG – Immunoglobulin G

IgM - Immunoglobulin M

LPC – Low Positive Control

LS-EIA – Less sensitive Enzyme Immunosorbent Assay

MOH – Ministry Of Health

NC – Negative Control

OD – Optical Density

OD-n – Standardized (Normalized) Optical Density

PHI – Primary HIV Infection

PLWHA – People Living With HIV/AIDS

RNA – Ribonucleic acid

RPR – Rapid Plasma Reagin

SNNPR-Southern Nations Nationalities and Peoples Region

STARHS – Serologic Testing Algorithm for HIV Seroconversion

UAT- Unlinked Anonymous Testing

UNAIDS – United Nations Global Fund for HIV/AIDS

VCT – Voluntary Counseling and Testing

WHO – World Health Organization

Abstract

Several tests have been developed to diagnose HIV/AIDS and detect the presence of anti-HIV-1 antibodies. But, these tests were not able to distinguish recent from established infections which are particularly useful in estimating the incidence of HIV-1. Recently, a simple enzyme immunoassay was developed that detects increasing levels of anti-HIV IgG after seroconversion and can be used for detecting recent HIV-1 infections to estimate incidence. This involves use of the branched peptide that includes gp41 immunodominant sequences from HIV-1 subtypes B, E and D allowing detection of HIV specific antibodies among various subtypes including the HIV-1 subtype C, which is accountable for the intense epidemic observed in Ethiopia. Therefore, the objective of this study was to use this assay to detect recent infections and estimate HIV-1 incidence rates among five regions (Addis Ababa, Tigray, SNNPR, Oromia, and Amhara), specific areas (urban and rural) of these regions and make comparison of HIV-1 incidence rates among different age groups in the selected regions by using cross sectional samples collected from pregnant women who attended Antenatal Care Clinics in the 2005 HIV surveillance in Ethiopia. However, some specimens from persons with longer-term infection could be classified as recent by the assay and this can inflate the incidence estimate. Thus, the incidence estimate was adjusted for misclassifications by using a recently developed formula. The adjustment for misclassification is a dynamic formula correcting for the imputed sensitivity and specificity of the assay based on the analysis of specimens with known dates of seroconversion and maintained the incidence estimate closer to the observed incidence. As a result, the study showed a national adjusted incidence rate of 2.05% (95% CI, 1.83-2.26) in pregnant women aged 15-49 (urban 3.13% (95% CI, 2.71-3.55) and rural 1.24% (95% CI,1.00-1.46)). The highest proportion of recent infections ($P<0.05$) and the largest incidence of HIV-1 (8.4%) (95% CI, 6.13-10.14) was observed in Addis Ababa, the capital city. The lowest incidence of HIV-1 was observed in SNNPR. Overall, higher proportion of recent infections was detected in rural health centers (26.8%) as compared to the urban (22.2%), ($P > 0.05$). Incidence of HIV-1 was found to be higher in the urban areas except for Amhara where the reverse was true. Age wise the highest proportion of recent infections was detected in the age group between 15-19 years (32.2%) ($P<0.05$) with the highest incidence being in the age group 25-29 years (2.33%) (95% CI, 1.90-2.76). In conclusion, the BED HIV-1 incidence assay provides a valuable tool in obtaining information about recent infection and incidence of HIV -1 and thus can be useful for purposes such as the identification of high risk population for enrolling in studies or intervention of early infection. The incidence estimate in the present study can only be considered as relevant for the five regions, not for the whole of Ethiopia because sampling strategy is limited to five regions (Addis Ababa, Tigray, SNNPR, Oromia, and Amhara) which account for the majority of HIV prevalence in Ethiopia and specimens obtained through similar sampling strategies should be used to compare the results obtained by using this assay with those reported by the Ministry of Health.

Keywords: AIDS , Antenatal care clinics , Assay , BED-HIV-1 incidence assay , Ethiopia , gp41, HIV-1 , IgG , Incidence , Misclassifications , Recent infections , Seroconversion

1. Introduction

1.1. Epidemiology of HIV/AIDS

Since its first recognition in 1981, acquired immune deficiency syndrome (AIDS) has become the most devastating disease in the history of mankind. More than twenty five million AIDS related deaths have been reported, making it one of the most destructive epidemics in recorded history (UNAIDS/WHO, 2005). The number of people living with the human immunodeficiency virus (HIV) continues to grow, as does the number of deaths due to AIDS. In 2006, the total number of people living with HIV globally was estimated to be 39.5 million [range: 34.1-47.1]. This included the estimated 4.3 million [range 3.6–6.6] adults and children who were newly infected with HIV in 2006 (UNAIDS/WHO, 2006).

According to the UNAIDS/WHO (2006) epidemic report, 17.7 million [range: 15.1-20.9] women were living with HIV in 2006, which is over one million more than in 2004. The most striking increases in the number of people living with HIV have occurred in East Asia and in Eastern Europe and Central Asia, where the number of people living with HIV in 2006 was over one fifth (21%) higher than in 2004. Sub-Saharan Africa remains hardest hit and is home to 24.7 million [range 21.8-27.7 million] people (two third of the global prevalence) living with HIV, 1.1 million more than in 2004. An estimated 2.8 million [2.4 million–3.2million] adults and children became infected with HIV in 2006, more than in all other regions of the world combined. The 2.1 million [range: 1.8–2.4] AIDS deaths in sub-Saharan Africa represent 72% of the 2.9 million [range: 2.5 - 3.5] global AIDS deaths.

Like most of sub-Saharan Africa, Ethiopia has been experiencing a severe HIV/AIDS epidemic. The first two sero-positive samples were detected in 1984 in Addis Ababa (Tsega *et al.*, 1988) and the first AIDS cases were reported in 1986 in Addis Ababa (Lester *et al*., 1988).

Since its first detection in 1984, the HIV epidemic appears to have gradually increased in Ethiopia (MOH, 2004). Studies that were conducted on the development of the epidemic indicated that the people living in the major urban areas of the country are more affected than in the rural (Abebe *et al.*, 2003; Mekonen *et al*., 2003).

Ethiopia, although published data that depict HIV epidemics on a national scale are limited, is among the leading countries in Africa. The main source for HIV surveillance data in Ethiopia is the antenatal care clinic (ANC) - based HIV sentinel surveillance system, which was started in 1989 (Hladik *et al*., 2006). It is based on unlinked anonymous testing of left-over blood in selected (sentinel) ANC sites, conforming to World Health Organization (WHO)/Joint United Nations Program on AIDS (UNAIDS) guidelines (UNAIDS/WHO, 2003). Other sources of surveillance data include case reporting (AIDS, sexually transmitted diseases), and infrequent surveys of the general population or high-risk groups (Hladik *et al*., 2006).

According to MOH (2006), the national HIV prevalence in Ethiopia in 2005 was estimated to be 3.5% (3% among males and 4% among females). The estimated prevalence was 10.5% (9.1% among males and 11.9% among females) in urban areas and 1.9% (1.7% among males and 2.2% among females) in rural areas. The overall HIV incidence estimate for Ethiopia in 2005 was 0.26% and is projected to remain stable until 2010.

It was estimated in 2005 that 1,320,000 people were living with HIV/AIDS (634,000 were living in rural areas and 686,000 in urban areas) and there were more women living with HIV/AIDS than men in the age group 15-29 years. However, more men were living with HIV /AIDS than women in the age group 30+ years. Besides, an estimated 137, 500 new AIDS cases, 128,900 new HIV infections (353 a day) including 30,300 HIV positive births, and 134,500 (368 a day) AIDS deaths (including 20,900 in children (less than15 years of age)) occurred in 2005. AIDS accounted for an overall estimated 34% of all young adult (15-49 old) deaths and 66.3% of all young adult (15-49 old) deaths in urban Ethiopia (MOH, 2006).

1.2. HIV and Its Life Cycle

1.2.1. The virus

Since the beginning of the AIDS epidemic researchers have made great effort to understand the nature of the disease and its causal agent, the human immunodeficiency virus (HIV). The viruses responsible for AIDS, like the epidemic, have proven to be more complicated and more

unpredictable than first recognized (Hahn *et al.*, 2000) and it is generally agreed that they represent novel, zoonotic introductions in to the human populations in the past 100 years (Hahn *et al.*, 2000).

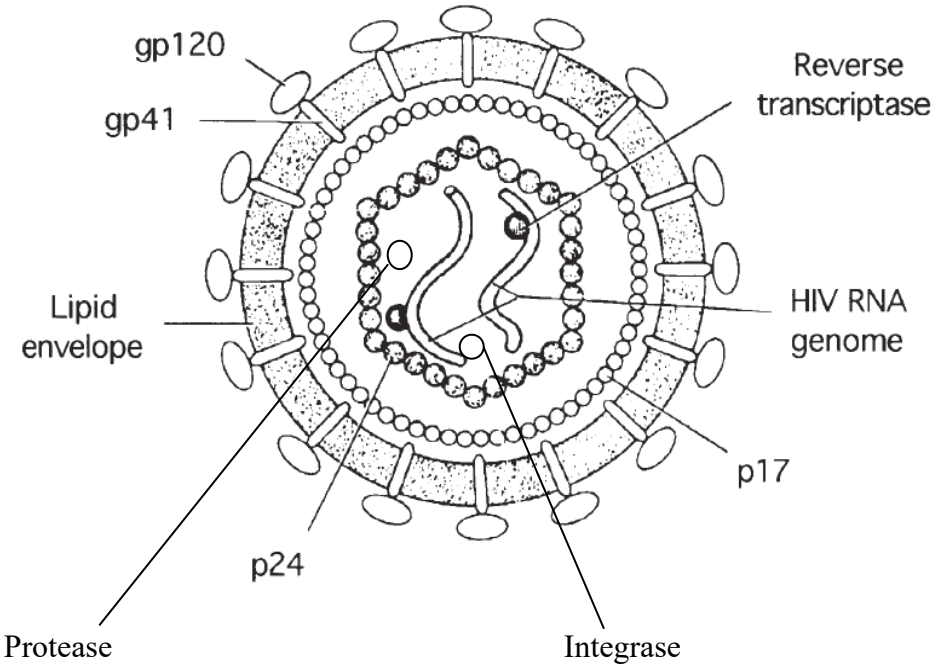


Figure 1: - The structure of the human immunodeficiency virus particle (Adapted from: Catton, 1996)

Human immunodeficiency virus is a retrovirus specifically infecting humans. The HIV viral particle consists of two copies of a 9.6-kilobase RNA genome with associated replicative enzymes: reverse transcriptase, protease, and integrase, within a tubular core composed mainly of the p24 capsid protein (Greene, 1991). Enclosing the core is a lipid envelope through which protrudes the viral glycoprotein receptor gp41 and gp120 complex (Figure 1) (Catton, 1996).

1.2.2. Genetic diversity of HIV

The genome of the human immunodeficiency virus is extremely variable compared with human DNA and with most other pathogens. This results from the high mutation rate because of reverse transcriptase, high viral turnover, viral genomic recombination, and immune and therapeutic selection pressures (Peeters and Sharp, 2000). There are two main types of HIV: type 1 (HIV-1) and type 2 (HIV-2). Based on similarity between the viral genomes, HIV-1 is divided into three

groups: main (M), outlier (O), and the lately discovered group, new (N) **(**Kuiken *et al*., 2000). Presently 9 subtypes (Labeled A-D, F-H, J and K) of the most common HIV-1 variant (group M) as well as several circulating recombinant forms have been recognized (Triques *et al*., 2000; Piot and Bartos, 2002) while groups N and O, which in contrast to group M are largely confined to Gabon, Cameron and neighboring countries, are not subdivided further. HIV-2 is only subdivided into subtypes designated by letters A–F) **(**Kuiken *et al*., 2000).

Figure 2: - Geographical distribution of HIV-1 clades (Source: Weinberg 2004)

According to Piot and Bartos (2002) (Figure 2) A and A/G recombinant variants predominate in west and central Africa while B has been the predominant species in Europe and the Americas. However, with increasing immigration and globalization, at least 25% of new infections in Europe are presently non-B African and Asian variants. Clade C is largely predominant in southern and eastern Africa, India and Nepal and has created the recent epicenters of HIV pandemic by its uncontrolled spread throughout Botswana, Zimbabwe (Janssens *et al*., 1997), Ethiopia (Abebe *et al*., 2001), Malawi, Zambia, Namibia, Lesotho, South Africa, India, Nepal and China. Clade D is generally limited to east and central Africa, with sporadic cases observed in southern and western Africa (Janssens *et al*., 1997). Clade E frequently appears as an A/E mosaic in Thailand, the Philippines, China and Central Africa (Janssens *et al*., 1997; Piot and

Bartos 2002). Clade F has been reported from central Africa, South America and Eastern Europe whereas G and A/G recombinant viruses have been observed in western and eastern Africa as well as in central Europe (Piot and Bartos, 2002). Clade H has only been detected in central Africa (Quinone-Mateu and Arts, 1999) (Figure 2) whereas clade J has been reported exclusively from Central America (Bikandou *et al*, 1999) and clade K has been identified in the Democratic Republic of Congo and Cameroon (Triques *et al*., 2000).

1.2.3. Transmission and life cycle

Although it is highly pathogenic in humans, HIV-1 cannot replicate in most other host species (Gardner and Luciw, 1989). This tropism is determined primarily by whether host cells express the required cofactors. For example, by lacking a functional receptor and appropriate transcriptional machinery, mouse cells do not support infection by HIV-1 (Zheng and Peterlin, 2005). Thus, the organism resists the pathogen via a cell-based incompatibility. However, a pathogen can also be restricted by the presence of dominant inhibitory factors, which attack the incoming virus directly and block its integration into the host genome. This situation also pertains to HIV-1 in mouse cells and represents true "intracellular immunity" (Zheng and Peterlin, 2005). Importantly, this host response is more rapid than both traditional innate or adaptive immunity and can prevent the establishment of the infection (Zheng and Peterlin, 2005)**.**

HIV can only be acquired by direct contact with the blood or other body fluids of infected human beings. There is no evidence to support transmission by biting insects such as mosquitoes, nor of animal or environmental reservoirs of infection. Nor is there any evidence that HIV may be transmitted by ordinary social or household contacts with an infected person. The main routes of transmission are sexual contact, the blood-borne route and vertically from mother to child. Contact of mucous membranes or broken skin with HIV-containing fluids has also occasionally resulted in infection. A number of cofactors may increase the risk of acquiring HIV infection, including many sexually transmitted diseases, especially those producing ulcerative lesions (Catton, 1996)

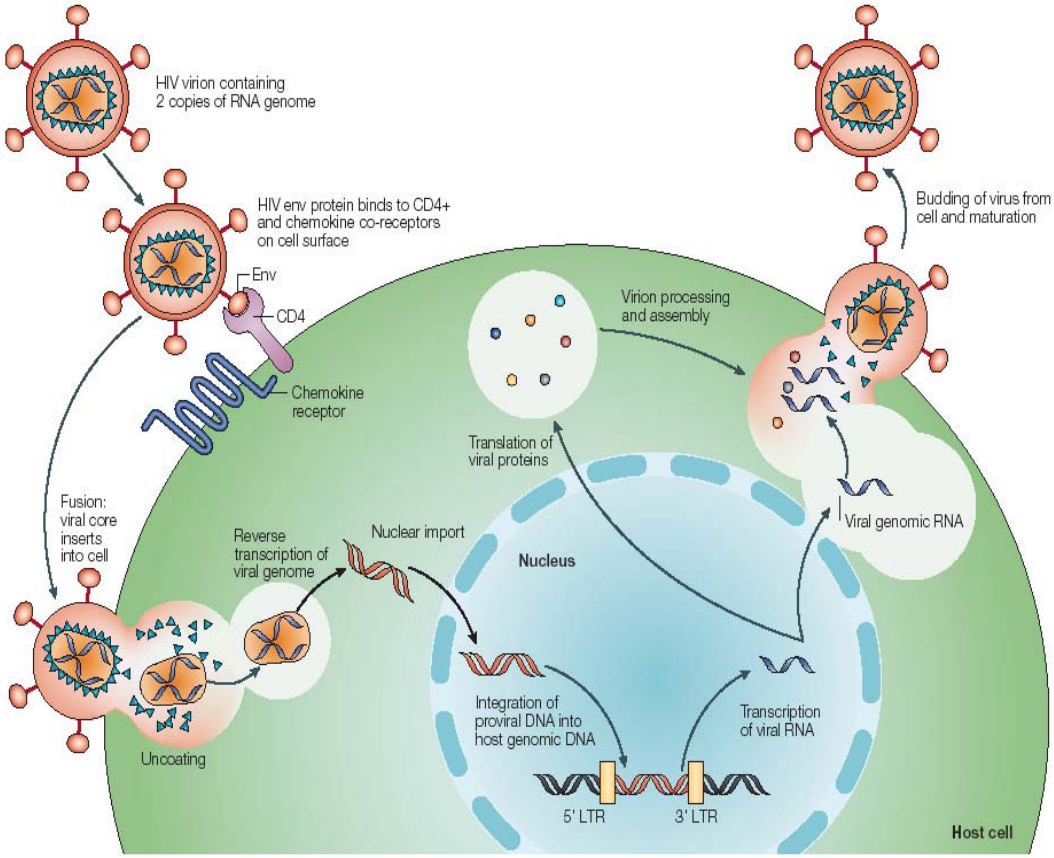


Figure 3: - Key aspects of the HIV life cycle (Source, Rambout *et al*., 2004).

HIV infects CD4 T-cells, monocytes, macrophages, langerhan cells, kupffer cells and microglia cells all of each having CD4 markers. Thus, the virus has the ability of destroying the immune system and causes a slowly progressive and inevitably total disease (Fauci, 1988). Although HIV is able to infect a variety of cell types, AIDS results from the depletion of CD4+ T-helper lymphocyte cells, a key component of the human immune system. HIV begins its infection of a susceptible cell by binding to the CD4 molecule on the host cell, which serves as a high affinity receptor. Two chemokine receptors, CCR5 and CXCR4, were identified as co-receptors to CD4 that permitted viral entry (Weiss, 1996). The virus is then internalized following the binding of the outer envelope of the virus (gp120) to CD4 receptors (Weiss, 1993; Fauci, 1988) and the genetic material of the virus, which is RNA, is released and undergoes reverse transcription with the help of an enzyme called reverse transcriptase (Figure 3) (Volberding *et al*., 2003).

Once the genetic material of the virus has been changed into DNA (Figure 3), 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 the 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 and 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. Markers for early diagnosis of HIV infection

In the weeks following initial infection, there is a transient burst of high titre viremia, which seems to be important in seeding the virus widely to lymphoid tissue in the body and perhaps the brain (Fauci, 1993). This burst of HIV in the peripheral blood quickly clears, probably due to the appearance of a neutralizing antibody response and cell mediated immunity, and trapping of the virus within the lymph nodes (Catton 1996). Antibodies to HIV usually appear 2-6 weeks after infection. Detection of these antibodies forms the basis of most diagnostic and screening tests for HIV infection. The first antibodies directed are those directed against the structural or gag proteins of HIV. Antibodies to gag proteins are followed by the appearance of antibodies to the envelope proteins. In addition, antibodies to regulatory proteins of the virus are also produced (Fauci and Lane, 2001).

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 the diagnosis of early HIV infection (Zijenah and Katzenstein, 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 Katzenstein, 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 Weiblen *et al*. (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 early HIV infection in children and in adults as high amount of this antibody is produced in the early phase of the infection (Fletcher *et al*., 2000). However, this marker is difficult to use in individuals in Africa, who, as a result of frequent co-infection with parasites, may have high levels of IgE (Gueije-Ndiaye, 2002).

1.4. Monitoring HIV incidence

Assessment of methods for disease prevention efforts in a community needs data, which is pertinent to their epidemics (Peterman *et al*., 1995). Determination of the number of new infections in a defined period of time gives the best data for understanding recent changes in HIV epidemic (Quan *et al*., 2002). The detection of new HIV-1 infection (incidence) can provide point estimates of the current state of HIV-1 transmission; help to distribute resources in a timely manner and to observe the impact of intervention (Mekonnen *et al.*, 2003).

Ethiopia has implemented the sentinel surveillance of HIV to monitor the spread of the virus in the population (MOH, 2004). Antenatal Care Clinics (ANCs) are potential sentinel sources of information for monitoring the epidemic and assessment of the efficiency of intervention in a community due to easy accessibility and low non- response (Kwesigabo *et al*., 1995; Zaba *et al*., 2000). It has also been stated that HIV-1 incidence can now be measured using cross-sectional specimens collected for HIV sentinel surveillance (Parekh and McDougal, 2005). However, very few studies have been done in Ethiopia to estimate incidence (Mekonnen *et al.*, 2003).

1.5. Detection of recent HIV infections

There are mainly two broad categories of detecting recent HIV Infections, which are named as "Traditional" and "Laboratory" methods. The traditional method includes longitudinal cohort studies which use those who are at risk for HIV infection and seek repeated HIV testing and people tested at Voluntary Counseling and Testing (VCT) centers more than once. However, it is more difficult to measure incidence in these methods due to reasons such as technical difficulty, expensiveness, requirement of long follow up and may be results are biased (Parekh and McDougal, 2001).

However, in the last few years, several different laboratory methods have been identified to particularly detect recent infections either in the pre-seroconversion phase or in the post-seroconversion phase. Cross sectional specimens, such as those collected for surveillance for assessing HIV prevalence, can be used for this purpose. Laboratory tests during the pre-seroconversion phase include detection of HIV-1 RNA or p24 in the antibody-negative segment of the population, while those in post-seroconversion phase depend on various properties of maturing HIV antibodies to detect recent infection (Figure 4) (Parekh and McDougal, 2005).

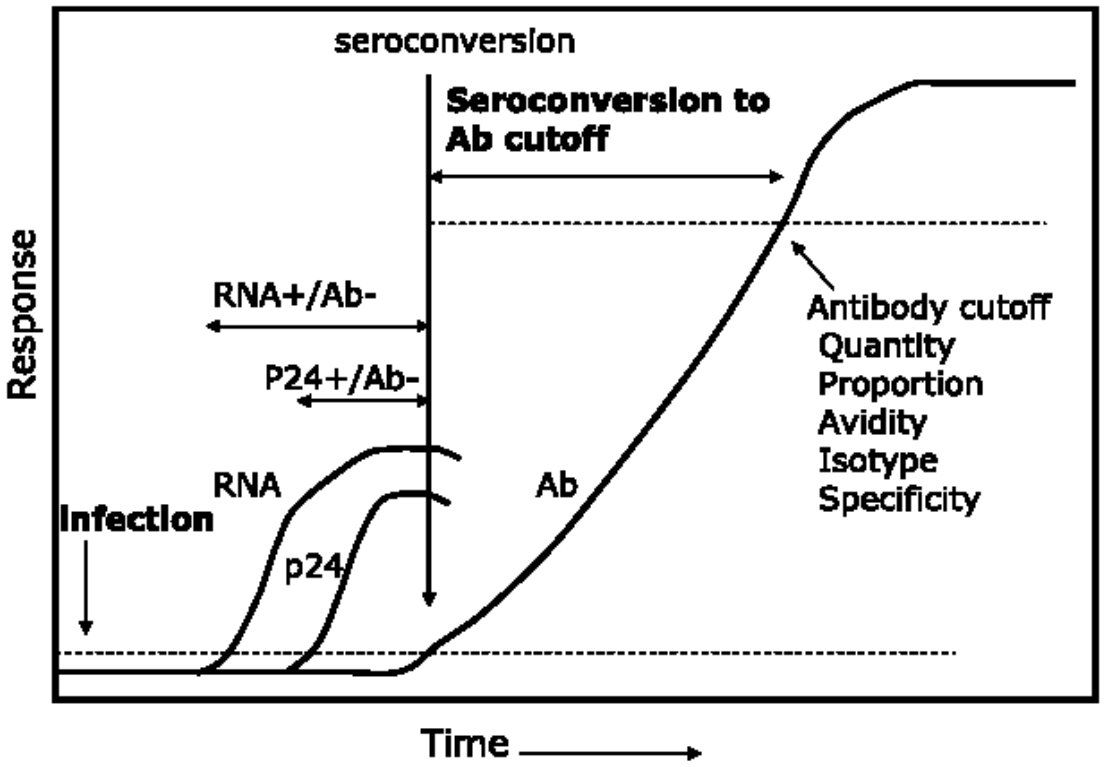


Figure 4: A schematic diagram showing various parameters that define early HIV-1 infection (Source: Parekh and Mc Dougal, 2005)

Detection of HIV-1 RNA or p24 antigen, before development of specific antibodies indicates very recent infection (primary HIV infection or PHI). Some studies (Brookmeyer *et al*., 1995; Brookmeyer and Quinn, 1995; Pilcher *et al.,* 2004) have used these methods to detect recent infections. This approach requires that seronegative people be tested by these methods because the duration of this stage is likely to be very short (about 10-20 days), therefore a large sample size is required to detect enough individuals in this short "window period" and achieve reasonable confidence intervals for incidence estimates. The strategy would also require screening seronegative members of the population (Parekh and McDougal, 2005). Therefore, these strategies are not ideally suited for wide scale implementation for determination of HIV-1 incidence. A test applied to seropositive population post-seroconversion would consequently be more economical and generally more practical (Parekh and McDougal, 2005).

Several tests have been developed or are being investigated for testing seropositive individuals post-seroconversion to detect recent infections. Because this method is performed only on

seropositive individuals, relatively less amount of sample is required. This post seroconversion "window period" refers to the meantime period seropositive individuals may stay below a predefined threshold on the test following seroconversion (Parekh and McDougal, 2005). Subsequently, based on the characteristics (window period) of the test used, annualized incidence can be calculated.

Different assays have been developed for the assessment of recent infections based on the post-seroconvertion principle. The development and application of a less sensitive (LS) 3All-LS assay fore example provided the first practical approach and permitted detection of recent HIV-1 infection to estimate incidence (Janssen *et al*., 1998).

Janssen *et al.* (1998) described a modification of a commercial HIV-1 antibody assay (3A11) from Abbot to detect recent seroconversion. The assay was modified to render it less sensitive (hence termed 3A11-LS) in detecting HIV-1 antibodies by use of increased serum dilution (1/20,000 instead of 1/400) and shortened incubation times (30 min from 120 or 60 min). However, the main limitations of 3A11-LS assay were specimen dilution of 1/20000, which was cumbersome and contributes to high variability and requirement of dedicated equipment (Parekh and McDougal, 2001)

Another commercial assay, Vironostica HIV-1 EIA (Enzyme Immunosorbent Assay) from Organon Technica was similarly modified (Vironostica-LS EIA) and has been used as a replacement to detect recent infections (Rawal *et al*., 2003). The LS-EIAs have been used in several studies to determine HIV-1 incidence (McFarland *et al*., 1999; Weinstock *et al*., 2002; Gouws *et al*., 2002).

There was significant interest in the implementation of 3A11-LS and Vironostica-LS in other parts of the world. However, two separate studies demonstrated that both the 3A11-LS and Vironostica-LS EIAs had significantly different "window periods" in subtypes B or E infected populations from Thailand (Parekh *et al*., 2001; Young *et al*., 2003). The longer window periods (270 and 350 days, respectively) in E infected persons were attributed to the use of subtype B derived antigens in the assays. This indicated that window periods in other divergent HIV-1

subtypes from Africa and Asia are also likely to be different. Due to the presence of two or more HIV-1 subtypes in some parts of the world and their varying epidemiology, it is not practical to use these assays to estimate incidence universally. Therefore, in spite of the importance of estimating HIV-1 incidence, the LS-EIAs have not been implemented widely in other parts of the world due to multiple circulating subtypes and complication of interpreting data (Parekh and McDougal, 2005).

With the observation of the limitations of the previous assays, several alternative assays were devised which are based on the development of humoral responses (Parekh *et al*., 2001) that examined epitope or antigen-specific responses, antibody avidity/affinity, antibody quantity and conformation dependence of antibodies (Parekh and McDougal, 2005). Antibody titers were further examined to asses which specific protein (s) may be the most useful in distinguishing specimens from recent and long-term infections (Parekh *et al., 2001).* Envelope proteins, specifically gp41 and its oligomers (gp120/160) were found to be the most useful. The immunodominant peptide of gp41 resulted in differential titers and can easily be used in an EIA.

From the multitudes of assays examined, the subtype B,E,D (BED)-capture enzyme immunoassay (BED-CEIA) which indirectly measured increasing quantity (proportion) of HIV-IgG out of the total IgG in the serum was developed (Parekh *et al*., 2002). This assay was designed using a branched gp41 peptide (BED) with sequences derived from different subtypes B, E and D to achieve similar performances with the different subtypes (Dobbs *et al*., 2004). Sequences from subtypes B, E and D were found to be sufficiently divergent and representative of the major subtypes of HIV-1 prevalent in the world (Hu *et al*., 2003).

Subsequent validation of the assay with specimens from Africa (Subtype A, C and D) demonstrated that this assay was useful for detecting recent HIV-1 infection and for estimating incidence from a range of different HIV-1 subtypes (Parekh *et al*., 2002). Therefore as HIV-1 subtype C accounts for the intense epidemic observed in Ethiopia (Abebe *et al*., 2001), BED-CEIA would be expected to be better than the other post-seroconversion tests used for incidence assays in Ethiopia.

1.6. Importance of detecting recent HIV infection

Detection of recent infection is important for 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). Individuals who have recently been engaged in high risk sexual contact with a person with HIV-1 infection may transmit the virus more efficiently as there is a high rate of viral replication during the early phase of HIV infection (Kaufman *et al*., 2000).

Identification of recent infection is also vital to HIV prevention efforts by identifying who is at greatest risk of acquiring HIV and which part of the community is in greatest need for health education. In addition, it allows targeting this population for therapeutic interventions or vaccines (Parekh *et al*., 2002; Hu *et al*., 2003). Detection of 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).

Disease incidence, the proportion of people with in a susceptible population who acquire the disease over a given period of time, is difficult to measure and gradually requires prospective follow up in defined cohorts. This problem could be circumvented if there is a means of distinguishing persons with recent versus long-term infections (Parekh *et al*., 2002). Therefore; this study aimed to assess the rate of new HIV-1 infections in Ethiopia.

2. Objectives

General objective:

Detect recent HIV-1 infection and estimate its incidence rate in Ethiopia.

Specific objectives:

1. Determine incidence rates of HIV-1 among regions and among specific areas (Urban vs. Rural) within regions.
2. Make comparison of HIV-1 incidence among age groups in selected regions of Ethiopia.

3. Materials and methods

3.1. Study site and population

3.1.1. Study site

Five regions (Tigray, Amhara, Oromia, SNNPR and Addis Ababa), which have been involved in the 2005 HIV surveillance, were included in this study. In these regions, 63 Antenatal Care Clinics (ANC) were selected for specimen collection.

3.1.2. Study population

The study population included pregnant women of ages 15-49 years who attended the selected ANC sites for the surveillance program. Left-over samples from Rapid Plasma Reagin (RPR) test were collected to be tested for HIV infections using the unlinked anonymous testing following World Health Organization (WHO)/Joint United Nations Program on AIDS (UNAIDS) guidelines (UNAIDS/WHO, 2003).

All positives and 10% of the negatives at testing sites in the regions samples were transported to Ethiopian Health and Nutrition Research Institute (EHNRI) where their HIV status was confirmed using ELISA systems. The study was done on all samples from the five regions which tested positive for HIV infections, while the negative samples were simply used for calculation purposes. The specimens were tested initially with Vironostika-HIV Uniform II Ag/Ab (Biomerieux, The Netherlands) and then the positives were confirmed with Enzygnost HIV-1/2(Dade Behring, Germany). Discrepant results were further confirmed with Murex HIV-1.2.O (Abbott, United Kingdom) (MOH, 2006). Indeterminate specimens were excluded from the study. A total of 1216 seropositive and 23089 seronegative plasma/serum specimens collected from pregnant women were used in the study.

3.2. Laboratory methods

In this study the detection of new infections in the HIV seropositive specimens was done by using BED-CEIA (Calypte Biomedical Corporation, Rockville, Maryland, USA). Briefly, in IgG-capture enzyme immunoassay, the wells of a micro titration plate were coated with goat anti-human IgG. When serum or plasma is added to the wells, anti-HIV-IgG and non-anti-HIV-IgG are captured on the goat-anti-human IgG coated wells (Figure 5). The relative amounts of anti-HIV-IgG and non-anti-HIV-IgG captured represent the total IgG antibody populations found in the serum or plasma. Indirectly, the test measures the proportion of HIV-1 specific IgG in a given specimen with respect to total IgG. Early seroconvertors have a lower proportion of HIV-specific IgG in the serum/plasma than those with long-term infection. Studies have indicated that HIV-specific-IgG may continue to increase for more than two years after seroconversion when tested by this assay (Parekh *et al*., 2002).

The assay was performed at 1/100 dilution of the specimen to detect early or long-term infection. The assay is not affected significantly by variation in dilution, as long as the proportion of HIV- and non-HIV IgG in the diluted specimens remains constant. The format of the assay results in ease of dilution and higher precision (Parekh and McDougal, 2001).

Use of a multi-subtype derived gp41 (B, E, D) antigen in the assay permits equivalent detection of antibodies of different HIV-1 subtypes with similar seroconversion durations (Parekh *et al*., 2002). However, the assay can also be used in populations with divergent HIV-1 subtypes such as A, E, C using uniform criteria (such as cutoff, seroconversion duration or “window period”) (Hu *et al*., 2003).

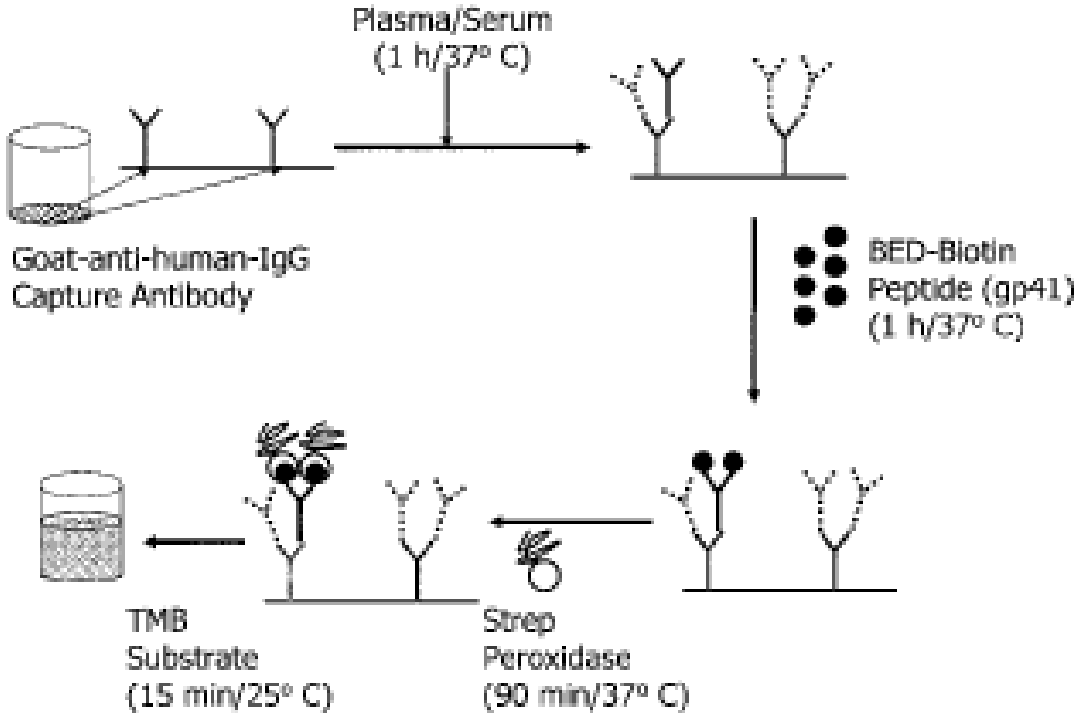


Figure 5: Schematic drawing showing the various steps of BED-CEIA for detection of recent infections.

(NB: Among the antibodies captured, HIV IgG is shown by solid lines, while non-HIV IgG is shown by broken lines. TMB, tetramethylbenzidine; strep, streptavidine) (Source: Dobbs *et al*., 2004)

Wash buffer was prepared by mixing 10x wash buffer concentrate (PH~6.8), which contains 10x concentrate of phosphate buffered saline and detergent, at 1:10 dilution in distilled water (PH~7.4). Then, sample diluent was prepared by adding 3 grams of Bovine Serum Albumin (BSA) for every 100mL of wash buffer. 500µL of the prepared diluent buffer was transferred to each titer tube using a multi-channel pipette. Following this, 5µL of each control (positive and negative control), calibrator (CAL), and samples were added to the appropriate titer tubes, which gives a final dilution of 1: 100. Using a multi-channel pipette, the diluted controls and specimens were mixed five times and 100µL of each mixed sample was transferred from the titer tubes to test plate (goat anti-human immunoglobulin G coated micro-well plate). Then the plate was covered with a plate sealer and incubated for 1hour at 37°C (Figure 5). Ten minutes before the

end of this incubation step, 1:1000 dilution of HIV-1 BED peptide was prepared in diluent buffer (3 grams of BSA in 100mL of wash buffer).

After incubation the plate was washed 4 times using the wash buffer by using micro-well plate washer programmed for this purpose (wash four times with 300µL/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 100µL of the diluted HIV-1 BED peptide was added using a multi-channel pipette. The plate was then covered with plastic tape and incubated for 1 hour at 37°C. Then, the plate was washed 4 times following 1-hour BED incubation. 100 µL of the 1:1000 diluted HRP-conjugate was added to each well using a multi-channel pipette and the plate was covered with plate sealer for incubation for 1.5 hours at 37°C and washed 4 times. Following this, 100 µL of TMB substrate was added to each well using a multi-channel pipette (for color development) and incubated for exactly 15 minutes at 25°C. Finally, 100 µL of stop solution (1 N H_2SO_4) was added to each well using a multi-channel pipette to stop the reaction. Optical density was read using a spectrophotometer set at 450nm (reference 630-650nm). The data was then transferred to HIV-1 BED incidence excel spreadsheet for analysis.

Controls (Negative control, calibrator, low and high positive controls) were used to check the validity of the test procedure; and had their own range of values.

The median OD (Optical density) values (the “middle” values of the 3 ODs), of each of the controls and calibrator were determined and used in the calculation of the normalized OD (ODn).

The median value for each control and calibrator must be within the indicated ranges shown below (Table 1) for a run to be considered acceptable. Thus, tests run with the median OD values of the calibrator or any control falling outside of these limits were considered invalid and rejected and repeated (Calypte Biomedical Corporation, 2004).

Table 1: Acceptable median OD ranges of controls and calibrator used in the HIV screening test of samples (Source: Calypte Biomedical Corporation, 2004).

Range	Negative Control	Calibrator	Low Positive control	High Positive Control
Minimum	0.000	0.380	0.200	0.600
Maximum	0.250	1.350	0.800	2.100

OD normalization decreases run-to-run variability and increases reproducibility (Parekh *et al*., 2002; Dobbs *et al*., 2004). As a result, the normalized OD was determined for the median of the observed OD values and for each control. It was calculated by dividing the median OD of each control by the median OD of the calibrator.

The ODn of control= (median OD of control)/ (median OD of calibrator)
The ODn of sample #1= (OD of sample #1)/ (median OD of calibrator)

For confirmatory testing, the ODn was the median of the triplicate OD values for the specimen divided by the median OD of the calibrator.

Table 2: Minimum/maximum acceptable ODn ranges of controls and calibrator used in the confirmatory HIV test of samples (Source: Calypte Biomedical Corporation, 2004).

Range	Negative control	Calibrator	Low positive control	High positive control
Minimum	0.000	1.000	0.400	1.200
Maximum	0.300	1.000	0.750	1.900

Tests run with the ODn of the median of any control or the calibrator falling outside of these limits (Table 2) were rejected as invalid and repeated.

3.2.3. HIV testing of the surveillance specimens

Serologic diagnosis of HIV infection is based on a multi-test algorithm for detecting antibodies to HIV. Screening tests provide presumptive identification of specimens that contain antibody to HIV. Enzyme immunosorbent assays (EIAs) or simple/rapid immuno-diagnostics are selected for their high sensitivity of detecting antibodies to HIV; and supplemental or confirmatory tests can be used to confirm infection in samples that are initially reactive on conventional EIAs. Alternatively, repetitive testing incorporating EIAs or rapid tests selected for their specificity may be used to confirm whether specimens found to be reactive for HIV antibodies with a particular screening test are specific to HIV. For practical purposes, resource-poor settings depend heavily on EIA and rapid tests for screening and confirmation (WHO/CDC, 2001).

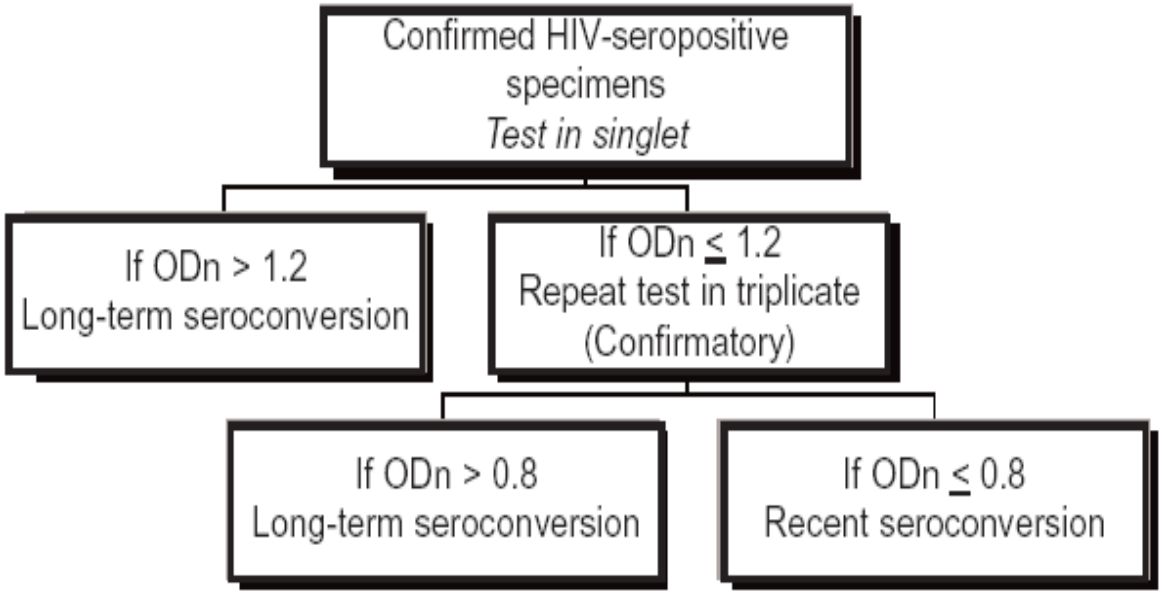
1. The test algorithm used in the 2005 surveillance (MOH, 2006):

   - **Screening**……..Vironostika-HIV Uniform II plus O (Biomerieux, the Netherlands)
   - **Confirmatory**……Enzygnost HIV-1/2 plus (Dade Behring, Germany)
   - **Tiebreaker**……Murex HIV-1/2 (Abbott, United Kingdom)

2. Test result interpretation was decided as follows (MOH,2006):

   - Viro. Positive/Enzy. Positive……Positive
   - Viro. Positive/Enzy. Negative/Murex Positive……....Positive
   - Viro. Positive/Enzy. Negative/Murex Negative……..Indeterminate (excluded from the study)

3.2.4. The algorithm used for testing and interpretation of results (Source: Calypte Biomedical Corporation, 2004):



The formula for incidence estimation

HIV incidence was calculated using the following formula (Parekh *et al*., 2002), which is slightly modified by the US CDC (Calypte Biomedical Corporation, 2004).

$$I = \frac{[365/W]R}{Nneg + [365/W][R/2]} \text{ X } 100$$

Where,

W= Window Period, the seroconversion interval that is the mean-time interval between seroconversion and cutoff ODn (normalized OD) in the BED assay (181 days) (Doobs *et al.*, 2004).

R = number of recent infection.

Nneg= Total testing HIV negative.

I= Incidence.

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

$$95\%\ \text{CI} = \text{I} +/- 1.96\ \frac{I}{\sqrt{R}}$$

Some specimens from persons with longer-term infection could be classified as recent by the assay; this can inflate the incidence estimate. Thus, the calculated incidence value was adjusted for misclassifications by using a recently developed formula (McDougal *et al.,* 2006), Thus,

1. The correction factor F is determined as:

$$F = \frac{[R/P]+\gamma-1}{[R/P][\alpha-\beta+2\gamma-1]}$$

2. Multiply the number of recent infections (R), in the incidence formula by F:

$$I = \frac{[F][365/W]R}{Nneg + [F][365/W][R/2]} \text{ X } 100$$

Where,

F=adjustment factor for sensitivity/specificity adjustment

P= Total testing HIV positive.

α= Sensitivity of BED test for detecting recent (<window period (W)) infection.

β= specificity of the BED test over the period >W to <2W.

γ= Specificity of the BED test over the period >2W.

Values for the imputed variables are based on the analysis of 2532 specimens from 1192 people with known (approximate) date of seroconversion. The values are (McDougal *et al*., 2006):

- α=0.7682
- β=0.7231
- γ=0.9443

3.3. Data analysis

Statistical analysis was performed by using STATA version 7 (STATA corporation, Texas, U.S.A.) and a P value < 0.05 was considered indicative of statistical significance. Incidence data was analyzed by using a spreadsheet available at http://www.calypte.com and the proportion of recent infections among age groups, regions and locations within regions was compared by using Chi- square test.

4. Results

A total of 1216 specimens collected from 5 regions (Addis Ababa, Tigray, SNNPR, Oromia, and Amhara) were tested by using BED-CEIA. In these regions, 63 Antenatal Care Clinics (ANC) were selected for specimen collection. Specific sites (Urban and Rural) of each region and age categories are shown in Tables 3 to 6.

Out of the samples tested by the assay, 289 (23.8%) were found to be recently infected and the rest 927 (76.2%) were longstanding infections. 95.8-100% of the specimens which gave positive results when tested with Vironostica-HIV Uniform II plus O and negative results with Enzygnost HIV-1/2 plus, but positive results with murex HIV-1/2, were characterized as new infections. However, only 10.4-15.9% of the specimens were positive with Vironostica-HIV Uniform II plus O and Enzygnost HIV-1/2 plus and therefore were characterized as new infections (Table 7). Thus, the highest rate of new infections observed in Addis Ababa, correlates with the characteristics (low antibody level) of the specimens collected.

The results of this study have shown the overall proportion of new infection and the adjusted annual incidence rate to be 23.8% and 2.05% (95% CI, 1.83-2.26), respectively. Comparing the overall rural and urban cases, although not significant (P=0.073), women tested in rural sites had higher proportion (26.8%) of recent infection than those tested in urban areas (22.2%). However, higher adjusted incidence rate was observed in pregnant women attending the urban centers (3.13%) than the rural (1.24%) (Table 8).

Table 3: ANC Sentinel Sites in five regions (Tigray, Amhara, Oromia, SNNPR and Addis Ababa) involved in the HIV surveillance in Ethiopia.

Region	Site name		Total
	Urban	**Rural**	
Addis Ababa	Akaki H.c.		**5**
	Gulele H.c.		
	Higher 23 H.c.		
	Kazanchis H.c.		
	Teklehaimanot H.c.		
Amhara	Addis Zemen H.c.	Bibugne H.c.	**17**
	Bahrdar H.c.	Bora H.c.	
	Bahrdar Hosp.	Chara Clinic	
	Estie H.c.	Dangla H.c.	
	Gonder H.c.	Enewari H.c.	
		Haik H.c.	
		Kone H.c.	
		Mertolemariam H.c.	
		Sekela Clinic	
		Tenta H.c.	
		Metema Hosp.	
		Delgi H.c.	
Oromia	Adama Hosp.	Ayra H.c.	**20**
	Alemaya Hosp.	Begi H.c.	
	Chiro Hosp.	Borena Gossa clinic	
	Jimma H.c.	Dadim clinic	
	Mettu H.c.	Dello H.c.	
	Nekemte Hosp.	Derra H.c.	
	Shashemene Hosp.	Gambo Hosp.	
	Moyale H.c.	Toke clinic	
		Chewaka H.c.	
		Amaya clinic	
		Mesela H.c.	
		Kokosa H.c.	
SNNPR	Awassa H.c.	Agam H.c.	**12**
	Dilla Hosp.	Attat Hosp.	
	Hossana Hosp.	Chencha Hosp.	
	Sodo H.c.	Chiri H.c.	
		Sheko H.c.	
		Teza H.c.	
		Gazer H.c.	
		Bechi H.c.	
Tigray	Abi Adi H.c.	Atsibi H.c.	**9**
	Adigrat H.c.	Edaga Arbi H.c.	
	Maychew Hosp.	Workamba H.c.	
	Mekele H.c.	Zana H.c.	
		Semema H.c.	
Total			**63**

Table 4: Overall urban and rural HIV Prevalence among pregnant women of age 15-49 years in five regions (Addis Ababa, Tigray, SNNPR, Amhara, and Oromia) of Ethiopia in 2005.

Region	Total screened	Total HIV positive (%)	Percent Positives	
			Urban	Rural
Addis Ababa	1921	**245 (12.8%)**	245 (100%)	-
Tigray	3130	**137 (4.37%)**	113 (82.4%)	24 (17.5%)
SNNPR	4245	**115 (2.7%)**	68 (59.1%)	47 (40.9%)
Oromia	7321	**244 (3.33%)**	161 (65.9%)	83 (34%)
Amhara	7688	**475 (6.17%)**	211(44.4%)	264 (55.6%)

Table 5: HIV prevalence among pregnant women in different health centers in Addis Ababa in 2005.

Site	Total screened	Percent HIV positive
Kazanchis	341	**19.6%**
Akaki	426	**9.4%**
Teklehaimanot	330	**10.6%**
Gulele	380	**14.7%**
Higher 23	444	**10.6%**

Table 6: Age-related HIV prevalence among pregnant women in five regions (Tigray, Amhara, Oromia, SNNPR and Addis Ababa) of Ethiopia in 2005.

Age group	Total screened	Percent HIV positive
15-19	3704	**3.86%**
20-24	7929	**5.8%**
25-29	7129	**5.97%**
>/=30	5373	**3.26%**

Table 7: Percent recent HIV infections in pregnant women based on sera collected from five regions (Addis Ababa, Tigray, SNNPR, Oromia, and Amhara) by using the initial testing algorithm in 2005.

Region	**Testing**	**Positives N (%)**	**Recent infections N (%)**
Addis Ababa	Viro.Positive/Enzy Negative/Murex Positive	63(25.7%)	63 (100%)
	Viro.Positive/Enzy. Positive	182 (74.28%)	19(10.4%)
Tigray	Viro.Positive/Enzy Negative/Murex Positive	24(17.51%)	23(95.8%)
	Viro.Positive/Enzy. Positive	113 (82.48%)	18 (15.9%)
SNNPR	Viro.Positive/Enzy Negative/Murex Positive	13(11.3%)	13 (100%)
	Viro.Positive/Enzy. Positive	102(88.7%)	13(12.7 %)
Oromia	Viro.Positive/Enzy Negative/Murex Positive	22 (9%)	22(100%)
	Viro.Positive/Enzy. Positive	222(91%)	27(12.16%)
Amhara	Viro.Positive/Enzy Negative/Murex Positive	30 (6.3%)	29 (96.6%)
	Viro.Positive/Enzy. Positive	445 (93.6%)	62(13.9%)

Table 8: Adjusted overall annual HIV incidence among pregnant women attending urban and rural health centers from five regions (Addis Ababa, Tigray, SNNP, Oromia, and Amhara) in 2005.

Site	Prevalence (%)	Recent infection (%)	Adjusted incidence (%)	95% CI (%)
Urban	8.1	22.2%(177/798)	3.13	2.71-3.55
Rural	2.7	26.8%(112/418)	1.24	1.03-1.46
Total	5.0	23.8%(289/1216)	2.05	1.83 – 2.26

When we compare the recent infection and incidence rate among the regions, the highest proportion of recent infections and HIV-1 incidence (33.5% and 8.4%, respectively) was observed in pregnant women attending health centers in Addis Ababa followed by Tigray (29.9 % and 2.38%)and SNNPR (22.6% and 1.02%). However, in Oromia, the proportion of recent infections was found to be 20.1% but the incidence rate was 1.07%. The lowest proportion (19.2%) was observed in Amhara region where the annual incidence was 1.91% (Table 9). The difference observed in the proportion of recent infections among these regions was highly significant (P=0.000).

The incidence of HIV in Addis Ababa was higher than that of the other regions. This was followed by Tigray, Amhara and Oromia. The least was observed in SNNPR.

Table 9: Adjusted annual HIV incidence among pregnant women attending the health centers in the five regions (Addis Ababa, Tigray, SNNPR, Oromia, and Amhara) in 2005.

Region	**Prevalence**	**Recent infection (%)**	**Adjusted incidence (%)**	**95% CI (%)**
Addis Ababa	12.8	33.5%(82/245)	8.4	6.73-10.14
Tigray	4.4	29.9%(41/137)	2.38	1.71-3.05
SNNPR	2.7	22.6%(26/115)	1.02	0.67-1.37
Oromia	3.3	20.1%(49/244)	1.07	0.79-1.36
Amhara	6.2	19.2%(91/475)	1.91	1.57-2.26

Comparing the proportion of recent infections and incidence rates in the five sites in Addis Ababa (Teklehaimanot, Kazanchis, Higher 23, Gulele and Akaki health centers (H.Cs)), the highest percentage of recent infection (47.8%) was observed in Kazanchis health center. Akaki had the proportion of recent infections of (32.5%) followed by Teklehaimanot (28.6%) and Higher 23 (27.7%). Gulele had the lowest proportion (25%) of recent infections of all health centers in the capital. The difference observed in the percentage of recent infections in the five sites in Addis Ababa was not significant (P=0.06) (Table 10).

The highest incidence rate in Addis Ababa was observed in Kazanchis H.c. (20.05%) than the others. It was followed by Gulele H.c. (7.00%), Akaki H.c. (5.85%), Teklehaimanot H.c. (5.73%), and the lowest was observed in Higher 23 H.c. (5.49%) (Table 10).

Table 10: Adjusted annual HIV incidence among pregnant women attending different health centers in Addis Ababa in 2005.

Site	**Prevalence**	**Recent infection (%)**	**Adjusted incidence (%)**	**95% CI (%)**
Kazanchis	19.6	47.8%(32/67)	20.05	13.21-26.89
Akaki	9.4	32.5%(13/40)	5.85	3.07-8.63
Teklehaimanot	10.6	28.6%(10/35)	5.73	2.34-9.11
Gulele	14.7	25% (14/56)	7.00	3.77-10.23
Higher 23	10.6	27.7%(13/47)	5.49	2.71-8.27

When rural and urban sites within regions (Tigray, SNNPR, Oromia and Amhara) were compared, higher proportion of recent infection was observed in the rural areas. The difference in the proportion of recent infections was highly significant in SNNPR (P=0.004), Oromia (P=0.013) and Amhara (P=0.014). However, there was no significant difference in the proportion of recent infections in the rural and urban areas of Tigray (P=0.167). Incidence of HIV -1 was found to be higher in the urban areas (except in Amhara region where HIV incidence was found to be higher in the rural areas) (Table 11).

Table 11: Adjusted annual HIV incidence among pregnant women attending the urban and rural health centers in the four regions (Tigray, SNNPR, Oromia, and Amhara) in 2005.

Region	Site location	Prevalence	Recent infection (%)	Adjusted incidence (%)	95% CI (%)
Tigray	Urban	9.6	27.4(31/113)	4.91	3.35-6.47
	Rural	1.2	41.7(10/24)	0.96	0.37-1.56
SNNPR	Urban	6.4	13.2(9/68)	1.12	0.53-1.7
	Rural	1.5	36.1(17/47)	0.99	0.53-1.44
Oromia	Urban	6.6	15.5(25/161)	1.5	0.94-2.05
	Rural	1.7	28.9(24/83)	0.87	0.55-1.19
Amhara	Urban	8.9	14.2(30/211)	1.8	1.26-2.34
	Rural	5.0	23.1(61/264)	1.96	1.53-2.4

Considering the relationship between recent infection and age groups, the largest proportion of recent infections was observed among pregnant women aged 15-19 years (32.2%) followed by those aged greater than or equal to 30 years (29.1%) and 25-29 years (22.8%). The lowest percentage (19.8%) of infections was observed among women aged 20-24 years. The difference observed in the percentage of recent infections among women of the different age groups was highly significant (P=0.006) (Table 12).

The highest incidence rate was observed in the ages 25-29 (2.33%) years, followed by ages 15-19 years (2.28%) and ages between 20-24 (1.87%) years. The lowest incidence was observed in the ages greater than or equal to 30 years (1.7%).

Table 12: Adjusted annual HIV incidence among pregnant women of different age groups attending health centers in five regions (Addis Ababa, Tigray, SNNPR, Oromia, and Amhara) in 2005.

Age	**% Prevalence**	**Recent infection (%)**	**Adjusted incidence (%)**	**95% C.I. (%)**
15-19	3.9	32.2% (46/143)	2.28	1.67- 2.89
20-24	5.8	19.8% (91/459)	1.87	1.54 - 2.21
25-29	6.0	22.8% (97/426)	2.33	1.90 – 2.76
>/=30	3.3	29.1% (51/175)	1.7	1.27- 2.13

In Addis Ababa, the proportion of recent infections among pregnant women aged 15-19 years was 46.4%. This was followed by those aged 25-29 years (33.7%) and 20-24 years (31.9%). The lowest percentage (28.7%) of recent infections was observed among women aged greater than or equal to 30 years. The difference observed in the percentage of recent infections among women of the different age groups was not significant (P=0.478) (Table 13).

The highest incidence rate in Addis Ababa (10.36%) was observed in the age group 25-29 years, followed by ages 15-19 years (9.68%) and 20-24 (8.31%) years. The lowest incidence was observed in the ages greater than or equal to 30 years (5.15%).

Table 13: Adjusted annual HIV incidence among pregnant women of different age groups attending health centers in Addis Ababa in 2005.

Age	% Prevalence	Recent infection (%)	Adjusted incidence (%)	95% C.I. (%)
15-19	10.3	46.4%(13/28)	9.68	4.42-14.94
20-24	13.2	31.9%(30/94)	8.31	5.34-11.28
25-29	15.2	33.7%(30/89)	10.36	6.65-14.07
>/=30	9.6	28.7%(8/28)	5.15	1.58-8.72

The largest proportion of recent infections in Tigray was observed among pregnant women aged greater than or equal to 30 years (50%) followed by those aged 15-19 years (26.3%) and 20-24 years (25.6%). The lowest percentage (22.9%) of infections was observed among women aged 25-29 years. The difference observed in the percentage of recent infections among women of the different age groups was not significant (P=0.098) (Table 14).

The highest incidence rate in Tigray (2.74%) was observed in the age group greater than or equal to 30 years, followed by those aged 25-29 years (2.73%) and 20-24 (2.09%) years. The lowest incidence was observed in the ages 15-19 years (1.34%).

Table 14: Adjusted annual HIV incidence among pregnant women of different age groups attending health centers in Tigray in 2005.

Age	% Prevalence	Recent infection (%)	Adjusted incidence (%)	95% C.I. (%)
15-19	2.9	26.3%(5/19)	1.34	0.17-2.52
20-24	4.6	25.6% (11/43)	2.09	0.85-3.32
25-29	6.9	22.9% (11/48)	2.73	1.12-4.35
>/=30	2.8	50% (12/24)	2.74	1.19-4.30

In SNNPR the largest proportion of recent infections (30.8%), was observed among pregnant women aged 15-19 years followed by those aged greater than or equal to 30 years (28.6%) and 20-24 years (20%). The lowest percentage (19.6%) of infections was observed among women aged 25-29 years. The difference observed in the percentage of recent infections among women of the different age groups was not significant (P=0.729) (Table 15).

The highest incidence rate in SNNPR (1.9%) was observed in the ages 15-19 years, followed by ages greater than or equal to 30 years (1.07%) and 25-29 (0.91%) years. The lowest incidence was observed in the ages 20-24 years (0.87%).

Table 15: Adjusted annual HIV incidence among pregnant women of different age groups attending health centers in SNNPR in 2005.

Age	**% Prevalence**	**Recent infection (%)**	**Adjusted incidence (%)**	**95% C.I. (%)**
15-19	3.4	30.8%(4/13)	1.9	0.04-3.77
20-24	2.7	20% (7/35)	0.87	0.23-1.51
25-29	2.9	19.6% (9/46)	0.91	0.31-1.50
>/=30	2.1	28.6%(6/21)	1.07	0.21-1.93

The largest proportion of recent infections in Oromia was observed among pregnant women aged 15-19 years (27.6%) followed by those aged 25-29 years (23.9%) and greater than or equal to 30 years (16.2%). The lowest percentage (15.7%) of infections was observed among women aged 25-29 years. The difference observed in the percentage of recent infections among women of the different age groups was not significant (P=0.365) (Table 16).

The highest incidence rate in Oromia (1.86%) was observed in the ages 25-29 years, followed by ages 15-19 years (1.18%) and 20-24 (0.71%) years. The lowest incidence was observed in the ages greater than or equal to 30 years (0.61%).

Table 16: Adjusted annual HIV incidence among pregnant women of different age groups attending health centers in Oromia in 2005.

Age	% Prevalence	Recent infection (%)	Adjusted incidence (%)	95% C.I. (%)
15-19	2.4	27.6% (8/29)	1.18	0.36-2.00
20-24	3.2	15.7% (13/83)	0.71	0.33-1.10
25-29	4.5	23.9%(22/92)	1.86	1.08-2.64
>/=30	2.6	16.2 (6/37)	0.61	0.12-1.09

In Amhara region the largest proportion of recent infections was observed among pregnant women aged 15-19 years (29.6%) followed by those aged greater than or equal to 30 years (29.2%) and 25-29 years (16.6%). The lowest percentage (15.7%) of infections was observed among women aged 20-24 years. The difference observed in the percentage of recent infections among women of the different age groups was significant (P=0.010) (Table 17).

The highest incidence rate in Amhara region (2.39%) was observed in the age group 15-19 years followed by those greater than or equal to 30 years (1.89). the age group 20-24 years had an incidence rate of (1.81%). The lowest incidence was observed in the age group 25-29 years (1.7%).

Table 17: Adjusted annual HIV incidence among pregnant women of different age groups attending health centers in Amhara in 2005.

Age	% Prevalence	Recent infection (%)	Adjusted incidence (%)	95% C.I. (%)
15-19	4.4	29.6%(16/54)	2.39	1.22-3.56
20-24	8.5	14.7%(30/204)	1.81	1.17-2.46
25-29	6.7	16.6%(25/151)	1.7	1.03-2.36
>/=30	3.6	29.2%(19/65)	1.89	1.04-2.73

5. Discussion

In this study, the BED-CEIA was used to detect recent infections and calculate HIV-1 incidence rates from it in pregnant women of age 15-49 years who attended the selected ANC sites of five regions in the 2005 surveillance program in Ethiopia.

Although a population survey would give a clearer picture of HIV epidemic in the general population, it is more time consuming and costlier than sentinel surveillance studies (Saphonn *et al*., 2002). Therefore, in resource-constrained settings, particularly in sub-Saharan Africa, HIV surveillance has focused on estimating HIV prevalence through serological testing of samples collected from sentinel populations (McDougal *et al*., 2005).Also, in countries with generalized epidemics, in which HIV prevalence in the adult population exceeds 1%, HIV surveillance is most often conducted in antenatal clinics, where blood is collected as part of antenatal care and screened for syphilis, anemia, or blood group typing (Hladik *et al*., 2005).

Unlinked anonymous testing (UAT) for the purpose of surveillance, is performed on the blood left over after these routine-screening tests (UNAIDS/WHO, 2003). Ethiopia faces a generalized and expanding HIV/AIDS epidemic with a rising trend in HIV prevalence and stable incidence (Hladik *et al*., 2006) and has implemented sentinel surveillance to monitor the spread of the virus in the population (MOH, 2004).

Pregnant women from antenatal clinics are potential sentinel sources of information for monitoring HIV epidemic and evaluation of the efficiency of intervention in a community due to ease of access and low non-response (Kwesigabo *et al*., 1995; Zaba *et al*., 2000).

The 15 to 49age range was used in this study as it covers people in their most sexually active years (MOH, 2002). While the risk of HIV infection obviously continues beyond the age of 50, the vast majority of those who engage in substantial risk behaviors are likely to be infected by this age (UNAIDS/WHO, 2004). Studies in several African countries also suggest that prevalence in pregnant women attending antenatal clinics is a good proxy measure of prevalence in the 15 to 49 year old population (Wawer *et al*., 1997; Fontanet *et al*., 1998; Flykesnes *et al*., 1998, Kilian *et al*., 1999; Glynn *et al*., 2000).

The overall adjusted incidence rate of 2.05% in pregnant women aged 15-49 years, and the urban (3.13%) and rural (1.24%) incidence rates based on sentinel samples from ANCs (Antenatal Clinics) in five regions (Addis Ababa, Tigray, SNNPR, Oromia and Amhara) are much higher than the report of Ministry of Health (2006)(0.26%; 0.99% in urban areas and 0.12% in rural areas).The discrepancy in the two findings could be due to the difference in the methods used and the sample size. Ministry of health reported the incidence estimates of HIV in the country for 2005 by using samples from pregnant women of ages 15-49 years who attended ANCs throughout the nation and measured incidence through modeling of prevalence data of many years (1989-2005) (MOH, 2004; 2006) whereas the present study used sentinel samples from pregnant women who have attended antenatal clinics in only five of the regions which have been involved in the 2005 surveillance program. Moreover, regions with large population sizes which account for 86.6% of all people living with HIV/AIDS and to 86.7% of the total estimated HIV positive pregnancies (Amhara, Oromia, Addis Ababa, and SNNPR) (MOH, 2006) were included in this study. In countries with limited resource settings, estimating HIV incidence through modeling of serial HIV prevalence has frequently been used in populations and sentinel subgroups to monitor HIV epidemic (Hoff *et al*., 1988; Bucyendore *et al*., 1993). However, surveillance systems focusing on new (incident) infections can provide a more accurate, timely and proximate measure of recent HIV-1 transmission than systems following trends in the prevalence of disease (McDougal *et al*., 2005).

The ability to detect and distinguish recent and long-term HIV-1 infections using laboratory tests has made the measurement of HIV-1 incidence realistic and practical. HIV-1 incidence can now be measured by using cross-sectional specimens collected for HIV sentinel surveillance (Parekh and McDougal, 2005). The IgG-capture BED-CEIA, which detects the increasing anti HIV IgG as a proportion of IgG following seroconversion is specific for population estimates of HIV-1 incidence. Since the assay has the same performance for different subtypes (Parekh and McDougal, 2005), its application for detecting the HIV-1 C subtype in Ethiopia is appropriate. Moreover, it requires a relatively very small volume of serum sample (Parekh *et al*., 2002; Hu *et al*., 2003) and is a very stable assay with minimal variation between multiple operators and plate lots (Dobbs *et al*., 2004).

However, there are reports that show the estimated BED-CEIA incidence to be higher than the incidence measured based on a prospective cohort in the same time period (Hu *et al*., 2003) and in pregnant women in Atlanta, U.S.A. (Nesheim *et al*., 2005). In addition, the UNAIDS reference group on Estimates, Modeling and projections issued a statement in response to preliminary data from population surveys and selected validation studies which demonstrated that the BED assay overestimated HIV-1 incidence by misclassifying a number of individuals with long term infection as recent infection in cross sectional settings (UNAIDS,2005).

Recently, McDougal *et al*, (2006) introduced adjustments for misclassifications in the incidence calculation formula and reported an excellent agreement in the comparison of incidence results that were obtained during follow-up for seroconversion and the BED estimate on the same specimen sets. This confirms that the assay, performs as it was designed to perform. The adjustment for misclassification is a dynamic formula correcting for the imputed sensitivity and specificity of the assay at any given ratio of recent to longer term infection and maintains the incidence estimate closer to the observed incidence (McDougal *et al.,* 2006).

By using adjustment for misclassification, the proportion of recent infections was higher in pregnant women tested in rural areas and the incidence rate was higher among those attending the urban health centers than rural (except for Amhara region where the reverse was true).

The higher incidence in the rural areas of Amhara region than the urban is in agreement with the report of Abebe *et al.* (2003) and that of Ministry of Health (2004), which reported the urban and rural residents of Amhara region to be at higher risk of HIV infection.

The reports of the Ministry of Health (2004) and Hladik *et al*, (2006) have shown that there is high incidence in the urban areas. Similarly, differences in HIV-1 prevalence rates between urban and rural areas are marked in almost all countries in eastern Africa, with urban rates being much higher than those in rural areas (WHO, 2005). This could be explained by the presence of larger numbers of high-risk groups, such as commercial sex-workers, alcohol and drug users, and migrants, in urban areas.

In the present study, the highest proportion of recent infections and incidence rate were observed in pregnant women attending health centers in Addis Ababa. This corroborates the reports of Ministry of Health (2004) and Hladik *et al.* (2006) that showed higher levels of HIV-1 incidence rates by 2003 in Addis Ababa (2.05%) and other urban areas (1.75%). Again, the EDHS (Ethiopian Demographic and Health survey) of the year 2005 reported by the Central Statistical Agency (CSA) (2006) and Ministry of Health (2006) indicated that HIV prevalence level was highest in Addis Ababa. This may be due to the high prevalence of high-risk sexual behaviors in towns (MOH, 2004; CSA, 2006).

The lowest Prevalence and incidence in SNNPR corroborates the reports of Abebe *et al*, (2003) and Ministry of Health reported that this region has the lowest prevalence next to Somali region and the lowest incidence next to Somali and oromia regions.

The highest incidence rate in Addis Ababa was observed in Kazanchis Health center (H.C.) followed by Gulele, Teklehaimanot, and Akaki H.C.s. The lowest was observed in Higher 23 H.C. This was consistent with the report of Ministry of Health (2006) except that the incidence in Higher 23 H.C. was higher than that of Akaki H.C. in the present study.

The proportion of recent HIV infection was higher in pregnant women in the age group 15-19 years compared to the higher age groups. For women, there is a clear pattern of higher HIV prevalence with sexual debut at this age range (CSA, 2006). This is also similar to the global picture reported by UNAIDS, (2000) and may be explained by the fact 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 (MOH, 2002).

Higher proportion of recent infections, next to pregnant women of age group 15-19 years was found in the age group greater than or equal to 30 years. This is similar to the report made by Savasta (2004) whereby HIV infection was stated to increase in older age groups.

The highest prevalence of HIV in the 25-29 years age group is similar to the report of Abebe *et al*, (2003) and its highest incidence in the same age group could be associated with the observed highest sexual activity (CSA, 2006) and to the fact that the main mode of transmission of HIV is heterosexual (Quinn, 1996; Fontanet *et al*., 1998). This finding is also consistent with the reports of other studies (UNAIDS, 1998; Demissie *et al*., 2000; Belachew and Gebresilassie, 2001 and Bane *et al*., 2003).

The report of Ministry of Health (2004) that the prevalence of HIV is more pronounced in the younger age groups (15-30 years) supports the lowest incidence that is observed in the age group greater than 30 years in this study.

In Addis Ababa the peak prevalence of HIV was observed among pregnant women aged 25-29 years. This supports the report of Wolday *et al*, (2007) that showed the peak prevalence of HIV in the age group 20-24 years to have shifted to the age group 25-29 years during 2001 and 2003.

6. Limitations of the study

1. The IgG capture BED-CEIA is dependent upon the increasing proportion of HIV IgG in relation to total IgG present in the serum. Thus, conditions or co-infections that elevate total IgG may result in false high incidence (Parekh & McDougal, 2001).

2. The BED-CEIA is prepared for research purposes and for HIV-1 population incidence estimation only. It is not used for diagnostic purposes (Calypte Biomedical Corporation, 2004).

7. Conclusions

1. The highest proportion and incidence rate of HIV infections was observed in Addis Ababa.
2. The overall higher proportion of recent infections was observed in the rural areas than the urban areas. This may suggest that most of the work to reduce the impact of the disease has put its focus in the urban areas despite the fact that majority of the people of Ethiopia is living in the rural areas.
3. In Amhara region higher proportion of recent infections and incidence of HIV was observed in the rural centers than in the urban.
4. The highest incidence observed in the age group 25-29 years could possibly be due to the highest sexual activity reported for this age group.
5. The larger proportion of recent infections in women of age greater than or equal to 30 years in this study may depict that the infection is increasing in the older age groups.
6. The BED HIV-1 incidence assay provides a valuable tool in obtaining information about recent infection and incidence of HIV-1.
7. The BED-CEIA incidence estimate in the present study can only be considered as relevant (appropriate) for the five regions and not for the whole of Ethiopia because sampling strategy is limited to regions which account for the majority of people living with HIV/AIDS and the total estimated HIV positive pregnancies

8. Recommendations

1. The BED HIV assay can be useful for purposes, such as identification of high-risk population for enrolling in studies or intervention of early infection.
2. Preventive strategies should give equal emphasis to all age groups, areas (urban and rural) and regions.
3. The same number of samples should be used to compare the results obtained by using this assay with those reported by the ministry of health.

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Declaration

I, the undersigned, declare that this thesis is my original work and that all sources of material used for the thesis have been correctly acknowledged.

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