Sero-Prevalence and Associated Risk Factors for the Reactivation of Cytomegalovirus among Hiv-1 Positive Individuals on Highly Active Anti Retroviral Therapy in Addis Ababa, Ethiopia

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This is to certify that the thesis prepared by Yemane Abreha entitled: **"*Sero-Prevalence And Associated Risk Factors For The Reactivation Of Cytomegalovirus Among Hiv-1 Positive Individuals On Highly Active Anti Retroviral Therapy In Addis Ababa, Ethiopia."***And submitted in partial fulfillment the requirements for the degree of masters of Science In applied genetics complies with the regulations of the university and meets the accepted standards with respect to originality and quality. I assure that this thesis was not presented in other Universities by other researcher.

Declared by

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Abbreviations and Acronyms

AIDS -Acquired immunodeficiency syndrome

AU -Arbitrary unit

CMV -Cytomegalovirus

DBS -Dried blood spots

DNA- Deoxyribonucleic acid

ELISA -Enzyme-linked immune sorbent assay

HAART -Highly active antiretroviral therapy

HIV -Human immunodeficiency virus

HLA -Human leukocyte antigen

IDU- Injecting drug user

MSM -Men who have sex with men

NASBA -Nucleic acid sequence based amplification

NAT -Nucleic acid test

NK -cells Natural killer cells

PCP -Pneumocystis carinii pneumonia (Pneumocystis jiroveci pneumonia)

PCR- Polymerase chain reaction

RNA -Ribonucleic acid

CD4+- T-lymphocyte bearing CD4 receptor

CDC -United States Centers for Disease Control and Prevention

SOT-Solid organ Transplant

WHO -World Health Organization

CMIA- chemiluminescent Micro particle immunoassay

IgG- Immunoglobulin G type

IgM- Immunoglobulin M type

OIs-Opportunistic infections

Abstract

The end result of human immunodeficiency virus (HIV) is the depletion of the immune system that leads to OIs like HCMV. Sero-prevalence of human cytomegalovirus (HCMV) infection in HIV-infected patients is high, more than 95% in immune compromised people even in the era of highly active antiretroviral therapy (HAART). The objective of this study was to assess the sero-prevalence and associated risk factors for the reactivation of HCMV infection among HIV-1 patients on HAART and to identify the common associated risk factors on these populations. A cross sectional study design on the samples of HIV-1 patients on HAART was taken place. This study was carried out using PCR abbot system (Abbot System) for the determination of HIV-1/viral load (RNA copies/ml) and chemiluminescent Micro particle immunoassay (CMIA) for the qualitative detection of IgG and IgM antibody to HCMV in human plasma. Out of the total 76 participants recruited for this study 46(60%) were female participants 30(40%) were male participants median age was 38.5, mean age 37.5, and the most frequent age was 40.The HCMV sero-prevalence (anti-HCMV IgG) was 76/76(100%) and 3/76(3.9%) for anti-HCMV IgM. Previous history of lower CD4 or recent CD4 decrease as well as higher HIV-1/viral load was not significantly related ($p > 0.05$) ($p = 0.317$ for IgM) to HCMV positivity. Rather other previous immunologic and clinical profiles were correlated ($p < 0.05$). The highest HCMV prevalence shows the endemicity of the virus and serological tests for HCMV should be taken into account as a diagnostic tool. This study strongly recommends that HCMV comprehensive study should be taken place in Ethiopia to note the actual figure of prevalence of HCMV in HIV-1 patients on ART naïve and taking ART as well as HCMV serological tests should be taken into account.

1. Introduction

1.1. Backgrounds

The human immunodeficiency virus (HIV) epidemic remains one of the greatest global health challenges of the 21st century. In the absence of an effective vaccine or curative therapy, According to the Joint United Nations Program on HIV/AIDS (UNAIDS, 2013), 35.3 million people worldwide were estimated to be living with this deadly virus by end of 2012 of which 23.6 million (67%) were in sub-Saharan Africa. In 2014, 2.6 million children aged <15 years worldwide were living with human immunodeficiency virus (HIV-1), of whose 88% lived in sub-Saharan Africa. The same year, there were 220 000 new infections and 150 000 deaths among children, of which the majority can be attributed to opportunistic infections (OIs) (UNICEF, 2016).

Antiretroviral therapy (ART) a Combination Therapy, Combined Antiretroviral Therapy (cART), Highly Active Antiretroviral Therapy (HAART) to treat HIV infection has been available since 1996 for individuals diagnosed with AIDS and has allowed long-term control of the disease with increased survival, and decreased morbidity and mortality rates (AIDS Info Net ,2014). HAART has increased life-expectancy and quality of life dramatically, with persistent suppression of HIV viraemia. Overall HIV-related mortality decreased from 29.4% per years in 1994 to 8.8% in 1997. However, even in the ART era, HIV-related OIs are still an important cause of hospitalization and death, which may be the result of late diagnosis and low levels of CD4+ T cell counts (Pallela *et al.,* 1998).

Human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) is a major public health problem in sub-Saharan Africa. HCMV has been reported to enhance HIV replication and accelerate the progression of HIV infection to AIDS (Adeola *et al*., 2015).

Recent studies suggest that co infections such as CMV can exacerbate HIV-related chronic immune activation during ART, and might augment the size of the latent HIV reservoir (Gianella *et al.,* 2016). HIV infects cells bearing the CD4 antigen receptor, the most important being T – helper lymphocyte (CD4 T = cells). These cells regulate cellular and humeral-immunity by interacting with other T – lymphocytes, B – lymphocytes, macrophages and natural killer cells. When CD+ 4 positive T – cells are depleted, immune defenses are weakened (Cheesbrough, 2005).

It has been reported that decrease in CD4+ count is partially responsible for major immunodeficiency's that leads to most of the OIs among HIV infected individuals. The most common OIs/co-infections in HIV infected individuals are tuberculosis, chronic diarrhea, candidiasis, HSV-2, HCMV, HCV and HBV (Saha *et al*., 2011).

In a healthy human body, microbes live in a dynamic equilibrium with the host. Each new invading microbe in particular, a virus (or viruses) resets this balance in an attempt to create favorable conditions for its own existence, leading to beneficial or detrimental conditions for other microbes. In response to some invading microbes, however, the host fails to reset this equilibrium, and such microbes become pathogens (Andrea *et al*., 2009).

Human herpes viruses are the major targets of therapy in both settings. The herpes viruses, particularly HCMV, herpes simplex virus (HSV) and varicella-zoster virus (VZV) act as opportunistic pathogens as cell-mediated immunity declines (Wood *et al*., 1996).

HCMV is the largest and most complex of the eight human herpes viruses. The DNA sequence of strain AD169 was the first complete HCMV genome to be published (Chee *et al*., 1990). Revisions have since been made to the initial AD169 genome sequence, and in recent years full genome sequences for several other HCMV strains from both laboratory-adapted viruses and viruses derived from pathological specimens have been published. Currently, the strain Merlin (GenBank Accession number AY446894.2), with a genome size of 235,646bp, is widely regarded as the consensus HCMV reference genome. Wild type HCMV strain Merlin contains at least 170 protein-coding genes (Gatherer *et al*., 2011).

Evidence from high income countries suggests that HCMV infection among HIV infected individuals may cause end organ disease, which is an AIDS defining condition and HCMV may alter the outcome of HIV infection, other than causing end organ diseases (Helene *et al*., 2016).
HCMV remains in a latent state in monocytes, in the immune-compromised host, infection with HCMV strains from the donor organ or reactivation of recipient strains can occur leading to increases in viral load in the blood and commensurate disease development (Soderberg *et al*., 1997).

HCMV infection is the most frequently observed opportunistic ocular viral infection in patients infected with human immunodeficiency virus (HIV). The incidence of HCMV retinitis in patients with acquired immune deficiency syndrome (AIDS) is reported to be between 24% and 30% and is associated with a low CD4+ T-lymphocyte count (Kuperman *et al*., 1998).

A synergistic effect may worsen the immunologic profile and could potentially translate into a more rapid disease progression as trans-activation of HIV-1 gene expression and release of rays of different cytokines by HCMV infected-cells could activate the latent HIV pro-viral DNA (Mujtaba *et al*., 2003).

Quantitative HCMV DNA detected in the plasma of HIV-infected patients with CD4 counts-100 cells/ml is a predictor for HIV disease progression, HCMV disease and death. 43.5% of the patients presented with positive viraemia, but only 7.4% had end organ HCMV disease but recently, new quantitative PCR assays have been developed with increased sensitivity. The threshold of detection has decreased from 400 - 20 copies/ml, in plasma samples (Casado *et al*., 1999).

The risk for mother-to-infant transmission of HCMV may be higher among infants born to women dually infected with HCMV and HIV in a study of 440 infants born to HIV infected U.S. women, the overall rate of in utero infection was 4.5% higher than the <2% rate of in utero infection in the general U.S. population (Thomas *et al*., 2009).

In contrast to other viral causes of hepatitis, patients with HCMV are an icteric, and their aspartate transaminase and alanine transaminase levels rarely go above five times their normal ranges. Other laboratory abnormalities found in association with acute HCMV infection include anemia, thrombocytopenia, and positive cold agglutinins (Gregory H *et al*., 2003).

In Ethiopia, CD4 count less than 200/mm3 and advanced WHO clinical stages of the disease were found to be predictors of OIs (Damte D *et al*., 2013). A study showed low congenital Cytomegalovirus infection among newborns, although there was high sero-prevalence of HCMV infection among mothers at our center; and is likely to be a reflection of the overall high prevalence among adult Ethiopians In Ethiopia, more effort is needed to screen for congenital infectious viral disease as well as usage of advanced techniques should be taken into consideration *(*Mamuy*e et al., 2016).*

1.2. Statement of the problem

HCMV is an AIDS-defining WHO stage 4 opportunistic infection for both adults and children, seen when the CD4 T-cell count falls below 100 cells/μl and as an immune reconstitution syndrome after starting highly active antiretroviral therapy (HAART). The HCMV is considered the most important viral opportunistic pathogen in patients with acquired immune deficiency syndrome (AIDS).

Epidemiological studies showed that infections caused by HCMV occur frequently across countries in the world and CMV sero-prevalence in general population is estimated to range between 60% and 90% in developed countries, even higher rates (>90%) in developing countries. The primary problem here is that there is no diagnostic setup for human cytomegalovirus (HCMV) in blood transfusion throughout the country taking into account that thousands of blood units are given to the different patients there are also hospitals which started an organ transplant in Ethiopia but none of them have this test for their recipients to prevent and treat their patients. Moreover, for around 30 years HIV/AIDS followed by opportunistic infection is major health concern in Ethiopia and there are no comprehensive studies concerning this problem. The other major problems are no efforts nationwide to fill the gap of the problem. Even there is no any clear figure showing this problem except pocket of study or some related articles. Taking in to account these obstacles assessment of the distribution of HIV/AIDS related opportunistic infection like HCMV in Ethiopia will improve prevention program. This study is an attempt to fill the gap with the objective of assessment of the sero-prevalence, and associated risk factors for the reactivation of HCMV among HIV- 1 positive individuals in Addis Ababa Ethiopia.

1.3. Significance of the study

The magnitude of the problem in Ethiopia has not been adequately investigated and still remains a major health problem. The prevalence of infection in a given population can only be reliably estimated by laboratory testing as clinical symptoms are usually absent. HCMV sero-positivity is considered as the best laboratory measure of past infection. Determination of local HCMV sero-prevalence is important especially in regions where the infection is perceived to be common. This will assist identifying risk factors for its acquisition and developing prevention strategies. The first and most important input of this study is to show (figure out) for the first time sero prevalence and of HIV-HCMV co-infection in HIV-1 patients. The second is, to correlate this outcome with late and recent clinical and immunological profiles of HIV-1 patients in Ethiopia. Therefore this study is so much significant to provide information about the distribution and correlation of HIV-1 opportunistic infections and may help to policy makers to set the prevention program related to this problem besides to this the diagnosis and treatment of HIV-1 opportunistic infections like HCMV will be considered as one of the top infections that cause mortality.

2. Objectives of the research project

2.1. General objectives

- To assess the sero-prevalence and associated risk factors for reactivation of HCMV among HIV-1 patients on HAART

2.2 . Specific objectives

- To evaluate the sero- prevalence of HCMV infection in HIV-1 patient on HAART
- To identify associated risk factors of HCMV infection on HIV-1 patients on HAART
- To establish the HCMV level of infection with CD4 count and viral load of HIV -1 on HAART

3. Literature review

3.1 Human immune deficiency virus (HIV)

Human retroviruses were unknown until the 1980's. First discovery of HIV-2 in West Africa and its similarities with simian immunodeficiency viruses (SIVs), first identified in captive macaques in North America, raised the possibility of a link between human and non-human primate lenti-viruses (Daniel et al., 1985). The two known types of HIV—HIV-1 and HIV-2 belong to a family of primate lenti-viruses whose other members infect African green monkeys (SIVagm), sooty mangabey monkeys (SIVsm), mandrills (SIVmnd), sykes monkeys (SIVsyk), and chimpanzees (SIVcpz) (Hayami et al., 1994)

The subtypes and HIV are retroviruses and the etiologic agents of AIDS. HIV belongs to a large family of ribonucleic acid (RNA) lent viruses. These viruses are characterized by association with diseases of immune-suppression or central nervous system involvement and with long incubation periods following infection before manifestations of illness become apparent (Grabowski *et al*., 2017)

HIV prevalence is substantially higher among key populations. Compared to the general population, for example, HIV prevalence is 22 times higher among people who inject drugs as well as in low- and middle-income countries, MSM and Female sex workers (compared to all women of reproductive age) are 19 and 13.5 times more likely to have HIV, respectively, than the background population (Baral *et al*., 2012).

Heterosexual transmission remains the dominant mode of transmission and accounts for about 85% of all HIV-1 infections. Southern Africa remains the epicenter of the pandemic and continues to have high rates of new HIV-1 infections (UNAIDS, 2006).

Although overall HIV-1prevalence remains low in the emerging epidemics in China and India, the absolute numbers, which are fast approaching those seen in southern Africa, are of concern. Outside of sub-Saharan Africa, a third of all HIV-1 infections are acquired through injecting drug use, most (an estimated 8·8 million) of which are in Eastern Europe and central and southeast Asia (Hayes and Weiss, 2006).

Data on HIV infection cases are more useful for determining the populations that need prevention and treatment services, as well as forecasting ART needs. Therefore, surveillance must move from reporting cases of AIDS to reporting cases HIV infection, which captures data on any clinical stage of HIV infection (WHO, 2009).

ART is able to restore the patients' immunity, increasing the number of CD4+ T lymphocytes to their normal values, and it is also able to reduce the HIV viral load In spite of the therapeutic and preventive measures available for the control of infection, epidemiological data indicate that a significant number of individuals are still infected by HIV, particularly youths (Zambarak *et al*., 2001).

Antiviral drugs, other than those with anti-retroviral activity, are used in persons with human immunodeficiency virus (HIV-1) infection for two purposes: treatment or prevention of viral infections that cause disease in persons with immunodeficiency, and to suppress viruses that might act as co-factors and promote replication of HIV-1 itself (Wood et al., 1996).

3.1.1 Human immune deficiency virus (HIV) pathogenesis

HIV RNA viruses containing reverse transcriptase, a polymerase that synthesizes pro-viral DNA from RNA. This pro-viral DNA becomes integrated into the host's cellular genome, leading to a persistent infection. There are 2 main classes of the AIDS virus: HIV-l and HIV-2. The most common virus found, particularly in the United States, is HIV-l. This is also the more virulent form and is associated with a faster immunologic deterioration and clinical progression (Hidalgo *et al*., 2000).

Human Immunodeficiency Virus (HIV) the causative agent of Acquired immunodeficiency syndrome (AIDS) is deadly and which currently has no cure whose case fatality rate is well above 95 % in the absence of treatment Since the outbreak of the HIV pandemic, it is estimated that over 30 million people worldwide have died due to AIDS of which more than 70% were from sub-Saharan Africa (Stain, 2008).

HIV was originally designated human T-lymph tropic virus (HTLV)-III, lymph-adenopathy-associated virus (LAV), or AIDS-associated retrovirus (ARV) (Fauci, 1988). AIDS is induced by the HIV virus. Therefore, it is specifically referred to as HIV/AIDS because other factors such as corticosteroids, cancer chemotherapy, and alkylating agents can also produce AIDS-like symptoms (Stine, 2000).

Many people living with HIV or at risk for HIV do not have access to prevention, care, and treatment, and there is still no cure. HIV primarily affects those in their most productive years; a third of new infections are among young people (ages 15-24). HIV not only affects the health of individuals, it impacts households, communities, and the development and economic growth of nations. Many of the countries hardest hit by HIV also suffer from other infectious diseases,

food insecurity, and other serious problems (The Global HIV/AIDS Epidemic, 2017). Eastern and Southern Africa is home to more than half (53%) of all people living with HIV, as well as more than half of the children living with HIV (62%) (UNAIDS, 2014). Despite the significant impact, new infections in the region have declined by 29% since 2010. Almost all of the region's nations have generalized HIV epidemics—that is, their national HIV prevalence is greater than 1%. In eight countries, 10% or more of adults are estimated to be HIV-positive. South Africa has the highest number of people living with HIV in the world (7.1 million). Swaziland has the highest prevalence in the world (27.2%).

The HIV epidemic in Ethiopia is heterogeneous by sex, geographic areas and population groups. Among women and men combined, HIV prevalence is seven times higher in urban areas than in rural areas (2.9%versus 0.4%) (CSA, 2018). HIV prevalence is 3.6 percent among women in urban areas compared with 0.6 percent among women in rural areas. Seven out of the nine regional states and two city administrations have HIV prevalence above 1 percent. Looking at HIV prevalence by region, it is highest in Gambella (4.8 %), followed by Addis Ababa (3.4%), Dire Dawa (2.5%), and Harari (2.4%) (ICF, 2018).

3.1.2 Human cytomegalovirus (HCMV) prevalence

HCMV is a prevalent pathogen; with 50-100% of the general population showing prior exposure by serology. In Patients with HIV the second most common context in which a family physician will encounter the clinical sequelae (pathology) of HCMV infection is in patients with HIV who have a CD4 T-lymphocyte count of Less than 50 cells per mm3 in the era before HAART (Gregory H *et al*., 2003*).* Immune status, reflected by CD4 (%CD4+ or absolute count) is usually more informative. This is reflected in the most up-to-date WHO recommendations on ART for children and adults to decide the clinical stage (WHO stage) (WHO, 1987)

Sero-prevalence is lowest in Western Europe and in the United States, generally below 80%. Studies from both Europe and the US have identified non-white ethnicity and low socioeconomic status as risk factors for HCMV infection. HCMV sero-prevalence is highest in South America, Africa and Asia, where generally more than 90% of the populations are infected. Studies from non-HIV-1 infected individuals in Africa have found CMV sero-prevalence between 78% and 100 % (Arne B *et al.*, 2007).

Antiretroviral therapy (ART) is able to restore the patients' immunity, increasing the number of CD4+ T lymphocytes to their normal values, and it is also able to reduce the HIV viral load. In spite of the therapeutic and preventive measures available for the control of infection, epidemiological data indicate that a significant number of individuals are still infected by HIV-

1, particularly youths (Silva *et al.*, 2015). Blood transfusion is the most important mode of prenatal/post-natal spread of HCMV to neonates. In different parts of India, serological surveys have shown 80.9% prevalence of HCMV IgG antibodies in women of childbearing age (Chakravarti *et al*., 2009). A serological survey of over 20,000 women in London found 54.4% of these women revealed that HCMV transmissions occur among people of all ages, races, and socioeconomic classes, throughout both the modernized and developing parts of the world (Gaytant MA *et al*., 2002).

HCMV is a ubiquitous with prevalence ranging from 40 -100%. This variation in sero-prevalence of HCMV IgM observed in several studies may probably be due to epidemiological and methodological differences (Notomi *et al*., 2000).

Some patients do not have a sustained response to antiretroviral agents for multiple reasons including poor adherence, drug toxicities, drug interactions, or initial acquisition of a drug resistant strain of HIV-1. Therefore OIs continue to cause substantial morbidity and mortality in patients with HIV-1 infection despite use of ART (Miller *et al*., 1999; Dore *et al*., 2002).

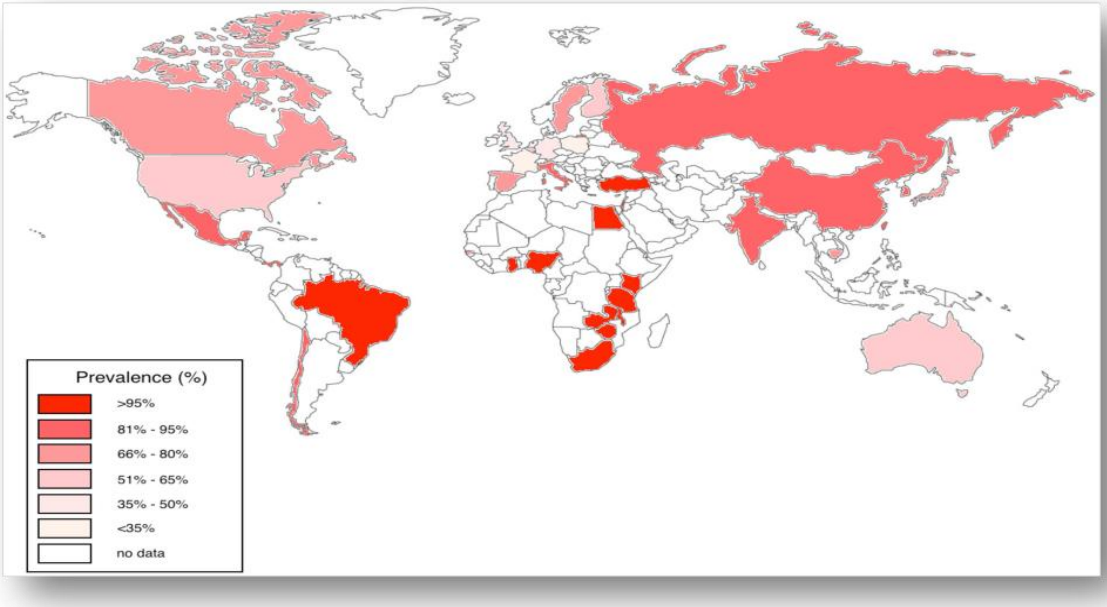

Figure 1: | Worldwide HCMV sero-prevalence rates indults. Aged 16–50 years published between 2005 and 2015. **Source: *(*Emily Adland *et al., 2015)***

Africa has been reported to have the highest IgG prevalence rate of HCMV. For instance Egypt and Western Sudan recorded prevalence rate of 96.0% and 72.2% respectively (MJ Cannon *et al*., 2010). And Nigeria reported 100% HCMV among HIV patients (Kida I M *et al*., 2014). However there are also reports somehow lower HCMV prevalence inside the same country in

Africa, the above Nigerian 100% not consistent in other teaching Hospital in Lagos, Nigeria which revealed 75% HCMV prevalence (Olajumonk *et al*., 2014).

3.1.3 Common Diagnostic Methods for HIV and HCMV

HIV as well as HCMV can be present in a variety of body fluids; however blood and blood components have the greatest concentration of viral components. There are multiple HIV laboratory testing methods available.

Table: 1 Common Diagnostic Method for HIV and HCMV

Common diagnostic methods for HIV	Common diagnostic methods HCMV
(I)Serology: These serologic tests for HIV antibody make use of the human immunologic response to HIV infection in which antibodies, primarily directed against HIV proteins and glycoprotein such as gp120 and gp160, appear after acute HIV infection. The sensitivity and specificity of EIA testing by standard methods using serum exceeds 99%.the EIA is gold standard.	**(I)Serology:** Serological diagnosis of CMV infection can be accomplished by dosing the IgM and IgG antibodies. The firs antibody to appear is IgM, which may be present in the patient's serum for a long period of time after the infection. Moreover, this antibody may reappear after re infection, including infection by different strains of the virus, demonstrating that IgM positivity is not diagnostic of a primary or recent infection with CMV. The IgG antibody appears in the blood after 6 to 8 weeks of infection and can persist indefinitely, although with fluctuation in its levels.
(II) POCT: Point-of-care tests use of rapid serum EIA methods, defined as any test that yields results in less than 30 minutes, provides accuracy nearly as good as routine EIAs. POCTs are typically based upon immune-chromatography, and they usually detect HIV-1 and HIV-2 antibodies using synthetic antigens, and less often are engineered to detect p24 antigen.POCTs have a sensitivity of ≥98%	**(I)Antigenemia (pp65):** The CMV antigenemia test is a rapid method for the detection of CMV phagocytized by neutrophils in the peripheral blood. In particular, monoclonal antibodies to CMV pp65 protein are used as an early and specific marker of active infection. The blood sample should be collected with anticoagulant, and the results are expressed as the number of poly-merphonuclear cells infected in relation to the total number of polymorphonuclear cells counted.
(III) PCR (polymerase chain reaction): is based upon the amplification of HIV-1 RNA in plasma. It is possible for this test to detect the presence of HIV-1 RNA up to 11 to 12 days prior to ELISA and 3 to 6 day before the p24 antigen is detected. Thus, NAT has been utilized as a means for reducing the "window" period to only 10 to 12 days from HIV infection to serologic positivity for screening blood product donations. Such tests can potentially detect levels of HIV-1 RNA as low as 5 to 40 copies/mL. NAT is useful for point-of-care (POC) testing in low resource health care settings.	**(III) QPCR:** Viral load quantification in CMV by quantitative PCR is the main alternative option for the diagnosis of viral replication and for decision making regarding preemptive treatment and monitoring the response to treatment. This test is carried out using the real-time PCR (RT-PCR) technique, which provides better accuracy, a faster response time, higher efficiency and a lower risk of contamination compared with conventional PCR. Quantification of the viral load can be conducted using plasma, whole blood or cerebrospinal fluid. In addition to the test's high sensitivity, limited concordance has been observed between broncho-alveolar lavage positivity by PCR and systemic infection.
(IV) P24 ASSAY: The HIV-1 p24 assay detects the core antigen p24 which is produced by the HIV-1 gag gene. This test is essentially the reverse of the enzyme immunoassay for HIV-1 antibody, because the methodology makes use of an antibody to HIV. p24 coated on a solid phase that "captures" the p24 in a patient specimen. An enzyme conjugated second antibody to p24 is then added and a standard enzyme immunoassay method used for detection. The p24 assay can be utilized on non-lipemic or non-hemolyzed serum, on plasma, or on cerebrospinal fluid. The p24 antigen ian be detected in 12 to 17 days after initial HIV-1 infection.	**(IV)Histology:** The analysis of a biopsy of the affected tissue is useful in the diagnosis of invasive disease, both based on the presence of intracellular viral inclusion and based on the detection of CMV antigens by immune-histo-chemistry or DNA hybridization, together with an inflammatory response. Due to the impossibility of histological analysis, involvement of the central nervous system can be determined based on the presence of CMV in the cerebrospinal fluid, as detected by RT-PCR.

Adopted from: (Azevedo *et al*., 2015) and (WHO/AIDS case definition, 2006)

3.1.4 WHO Clinical Staging, Major Symptoms and CD4 count of HIV

WHO clinical staging is WHO based clinical and immunological classification of HIV infected individuals according to clinical as well as immunological manifestations taken from the Cassese. Based on this they are classified as:

Table: 2 WHO clinical staging major symptoms and CD4 count of HIV

Clinical stage	Clinical Symptoms	CD4 count
I (Asymptomatic)	Asymptomatic Persistent generalized lymphadenopathy	**>500**
II (Mild)	-Unexplained moderate weight loss (<10% of presumed measured body weight) -Recurrent respiratory tract infections (sinusitis, tonsillitis, otitis media and pharyngitis) -Herpes zoster -Angular cheilitis -Recurrent oral ulceration -Papular pruritic eruptions -Seborrhoeic dermatitis -Fungal nail infections	**350-499**
III (Advanced)	-Un explainedi severe weight loss (>10% of presumed or measured body weight) -Unexplained chronic diarrhea for longer than one month -Unexplained persistent fever (above 37.5°C intermittent or constant, for longer than one month) -Persistent oral candidiasis -Oral hairy leukoplasia -Pulmonary tuberculosis -Severe bacterial infections (such as pneumonia, empyema, pyomyositis,bone or joint infection, meningitis or bacteraemia) -Acute necrotizing ulcerative stomatitis, gingivitis or ----Periodontitis -Unexplained anemia (<8 g/dl), neutropaenia (<0.5 × 109 per litre) and/or chronic thrombo-cytopaenia (<50 × 109 per litre	**200-349**
IV (Sever)	-HIV wasting syndrome, Pneumocystis pneumonia, -Recurrent severe bacterial pneumonia, Chronic herpes simplex infection (orolabial, genital or anorectal . of more than one month's duration or visceral at any site) -Oesophageal candidiasis (or candidiasis of trachea, bronchi or lungs) Extra pulmonary tuberculosis. -Cytomegalovirus infection (retinitis or infection of other organs) Central nervous system toxoplasmosis -Extra pulmonary cryptococcosis including meningitis -Disseminated non-tuberculous mycobacterial infection -Progressive multifocal leukoencephalopathy -Disseminated mycosis (extrapulmonary histoplasmosis orcoccidiomycosis) -Recurrent septicaemia (including non-typhoidal *Salmonella*)	**<200**

Adopted from: - (Klatt C, 2018) and (WHO/AIDS case definition, 2006).

3.2.1 Human Cytomegalovirus (HCMV) other strains and genome organization

HCMV is a double-stranded DNA virus that has several close relatives in animals. HCMV was first isolated in tissue e culture in 1956. Because the virus produced large, swollen, refractory cells, it was named cytomegalovirus, and it is also the largest known human virus. HCMV is a member of the herpes virus group (table 1) (Arne B et *al.*, 2013).

Table 2: Human Herpes-virus (HHV) classification **(Source: Daniel Cochrane 2009)**

Name	**Description**	**subfamily**
HHV-1	**Herpes simplex virus 1(HSV-1)**	**Alpha**
HHV-2	**Herpes simplex virus 2(HSV-2)**	**Alpha**
HHV-3	**Varicella zoster virus (VZV)**	**Alpha**
HHV-4	**Epstein Bar virus (EBV)**	**gamma**
HHV-5	**Human cytomegalovirus (HCMV)**	**Beta**
HHV-6	**Human herpes virus 6 (HHV-6)**	**Beta**
HHV-7	**Human herpes virus 7 (HHV-7)**	**Beta**
HHV-8	**Kaposi' sarcoma associated virus (KSHV)**	**gamma**

Herpes-viridae is a family of large and complex viruses ubiquitous in nature and infecting diverse Animal species. The family is classified into a-, b-, and g-herpes-virus subfamilies on the basis of shared biological properties (i.e., host range, replication kinetics, and ability to spread in culture) (chee *et al*., 1990) and genetic relatedness. Herpes-viruses genomes vary considerably in size (125–245 kb), G revealed a set of common ‘‘core’’ genes that are conserved in the viral DNA sequence (Bu+ C content (32–75%), and sequence organization, but these characteristics are independent of subfamily classification. DNA sequence and genetic analysis of viruses from each subfamily have tcher *et al.*, 1998).

HCMV, the prototype member of the herpes-virus subfamily *Beta-herpes-virinae* has a worldwide distribution and infections with this virus are extremely common. The double-stranded DNA (dsDNA) genome of wild-type HCMV strains has a size of around 235 kb, which is longer than all other human herpes-viruses and (Butcher et al., 1998) one of the longest genomes of all human viruses in general. It has the characteristic herpes-virus class E genome architecture, consisting of two unique regions (unique long UL and unique short US), both flanked (figure 2) by a pair of inverted repeats (terminal/internal repeat long TRL/IRL and internal/terminal repeat short IRS/TRS) (Andrew *et al., 2003).*

HCMV have a unique four-layered structure. A core containing the large, double-stranded DNA genome, with an approximate size of 200-250 kbp, is enclosed by an icosapentahedral

capsid which is composed of capsomers. The capsid is surrounded by an amorphous protein coat called the tegument (Khanna *et al.,* 2009). It is encased in a glycoprotein-bearing lipid bilayer envelope like other herpes viruses. Three classes of viral genes are expressed in a permissive cultured cell infected by human cytomegalovirus; immediate early (IE), early, and late. There are two IE proteins (IE1 & IE2) that have overlapping sequence. The immediate early 72-kDa IE1 and 86-kDa IE2 proteins are the first proteins that are expressed at the start of infection (Marjan *et al., 2007).*

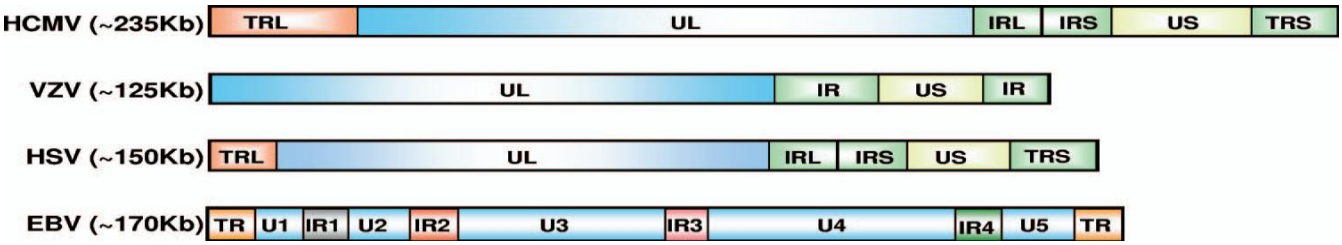

Figure 2: Comparative schematic genome organizations of human herpes viruses. HCMV,VZV, varicella zoster virus; HSV, human simplex virus; EBV,Epstein-Barr virus. The lettering within the individual regions of the genome depicts the following features: terminal repeat long (TRL), unique long (UL), unique short (US), internal repeat long (IRL), internal repeat short (IRS), terminal repeat short (TRS), and internal repeat (IR).
(**Source: Tania Crough and Rajiv Khanna: 2009)**

3.2.2 Human cytomegalovirus (HCMV) pathology and prevention

Defective cell- mediated immunity in HIV infected subjects makes them susceptible to reactivation of latent micro organisms and even lead to reactivation of such an OIs which not only impose a varying range of morbidities, but it also causes a high cost for affected individuals and related society (Alireza *et al.,* 2013).

Among HIV-positive children in Africa, the majorities are co infected with HCMV by their first birthday (figure 1) and almost all are co infected by the time they reach their teens. The growing epidemic of co infected adolescents may have significant clinical impact in these populations. In comparison, a study from North America reports a lower rate of early co infections, finding that 40–50% of HIV/HCMV-co infected children had acquired HCMV before their first birthday (Emily *et al*., 2015).

The presence of latent virus in monocytes forms a circulating reservoir that may sustain full viral replication when differentiation into macrophages occurs, whatever the site(s) of latency, reactivation of latent virus is the critical step in the pathogenesis of CMV infection (Grefte *et al* .,1994)

The persistence of HCMV is related to equilibrium between the host immune response and the evasion mechanisms developed by the virus thus HCMV interferes with the initiation of adaptive immune responses, as well as with immune effectors HCMV has developed several

evasion strategies to interfere with selected functions of dendrite cells (DCs). Monocyte infection by HCMV inhibits their differentiation into macro-phages and into CD1a-positive DCs; a phenomenon that does not require viral replication (Wivin *et al*., 2004).

Prevention strategies include universal antiviral prophylaxis or pre-emptive therapy that is based on sequential viral load monitoring in blood. Both approaches prevent CMV disease in at-risk patients, but they are limited by the toxicities of available anti-CMV drugs but early treatment of viremia (i.e., pre-emptive therapy) may allow some reconstitution of protective immunity (Kimberly and Sankar, 2015)

Optimal prevention, diagnosis and treatment of active CMV infection enhance immunologic outcome. Methods to prevent CMV include universal prophylaxis and preemptive therapy; each has its merits, and will be compared and contrasted (Kotton, 2013)

The incidence of CMV disease in different patient groups is not uniform and in most groups has declined as more aggressive management strategies have been usedfor example, early treatment of active infection or prophylactic treatment with anti-virals. The predominant disease in patients with AIDS has been retinitis (Pillay *et al*., 1995).

3.2.3 Human cytomegalovirus (HCMV) co infection with human immunodeficiency Virus (HIV-1)

HCMV has been known to establish latency following primary subclinical infections in immune-competent individuals. In these individuals reactivation may occur periodically without any observed sequels. However, in deficiency of host-derived antiviral immune responses reactivation often causes pathological changes that may results into invasive disease. Long-lasting immunity in response to HCMV primary infection serves to control subsequent HCMV reactivation in the host which is essential for preventing uncontrolled viral replication and serious HCMV disease (Miriam *et al.*, 2017).

The normal range of CD4+ T helper cells in healthy person is 500-1600 cells/µl of blood, which gradually depletes with progression of HIV infection rendering the patient susceptible to OIs (Akashdeep, 2013).

The CD4+ T helper cell count drops by approximately 50-100cells/µl of blood per year. With gradual decline in CD4+ T cell count, symptomatic phase with opportunistic infections manifesting thrush, weight loss and fatigue starts appearing. Once CD4+ T cell count reaches below 200cells/µl, risk of OIs increases dramatically and patient is said to have progressed to AIDS (Amanpreet, 2013).

HIV-induced disruption of the equilibrium between a human host and its virone leads to two grave consequences: reactivation of ubiquitous symbiotic viruses, which start to replicate to higher levels and infection by new viruses (Brainster *et al*., 2016). In this imbalanced system, both symbiotic viruses and the new invaders cause different pathological conditions, including some ‘‘AIDS-defining’’ ones (Andrea *et al*., 2009)

HIV induces immune-suppression and facilitates all herpes viruses’ development, including HCMV. On the other hand, HCMV promotes HIV Pathogen city, either by introducing into the cell its trans-activation proteins, that activate also HIV pro-viral DNA, or by stimulating production of inflammatory cytokines which activate HIV DNA. Thus, HCMV acts as a cofactor for HIV, with higher viral production and a more rapid progression to acquired Immune-deficiency Syndrome (AIDS) (Arne *et al.*, 2012).

The strong HCMV association to inflammation and T-cell immune response skewing played a significant role in HIV disease progression in the early days of HIV epidemic. Another report indicates that a strong correlation between peak HIV viral load and peak HCMV viral load (Fane *et al*., 2016).

Studies have shown that HCMV viral load is suspected to be the cause of death in most of patients already diagnosed as AIDS and is a significant risk for morbidity and mortality in HIV- HCMV co- infected patients however HAART plays in dramatic decline of HCMV infection and a significant survival of HIV patients (Alireza *et al*., 2013).

CD4+T cells and CD3+ T cells, natural killer cells & antibodies that re-organize surface antigens play a crucial role in the immune response to HCMV, preventing the development of HCMV disease in the immune-competent host (Arne *et al*., 2012) A synergistic effect may worsen the immunologic profile and could potentially translate into a more rapid disease progression as trans-activation of HIV-1 gene expression and release of rays of different cytokines by HCMV infected-cells could activate the latent HIV pro-viral DNA. The risk of HCMV is highest when CD4 counts are below 50 cells/µl and is rare with more than 100 cells/ µl Upto 30% of people develop retinitis and in additional 5-10% develop disease in other organs. Usually decrease in CD4+T cell counts leads to reactivation of these infections (Lalivati *et al.*, 2015).

Factors such as a drastic decrease of the CD4+ T lymphocyte count (<100 cells/mm3), with a consequent loss of immune resistance, as well as the long persistence of HCMV in the organism contribute to the high morbidity and mortality rates exhibited by patients with HIV/AIDS Under such circumstances, the patient becomes more vulnerable to the opportunistic diseases caused

by HCMV especially by reactivation of its latent form. When ART is not performed, infection by HCMV can be associated with severe clinical manifestations (Silva *et al*., 2015).

HCMV replication was sometimes asymptomatic and self-limited that the positive predictive value of finding HCMV DNA in plasma and white blood cells by PCR was 60% and 40%, respectively with High titers of circulating HCMV DNA increased the risk of end-organ disease (Kathryn *et al.*, 2004).

HCMV disease is typically seen when HIV viral load is >100,000 copies/ml of plasma or when p24 antigen is increased along with a low CD4 count. HCMV disease in HIV patients most often manifests as retinitis, esophagitis and enteritis (Ross *et al*., 2011). Other manifestations include peripheral neuropathy, pneumonitis, gastritis or hepatitis and colitis. In HIV infected children, the presence of HCMV infection was associated with more rapid progression into AIDS and death (chakravarty *et al*., 2009).

HCMV disease occurs mainly as a reactivation of latent virus in patients who are sero-positive for HCMV. Since 85%-95% of HIV-infected patients have antibodies to HCMV, other markers are required to detect reactivation (Battes *et al*., 2016). It has been shown that the level of immunodeficiency, as measured by the CD4+ lymphocyte count, is of prognostic significance with respect to the occurrence of HCMV disease in HIV-infected patients. HCMV viremia is both a marker of advanced immune-suppression related to HIV infection and independently predictive of the development of HCMV disease. The risk of developing HCMV disease is significantly higher for patients with CD4+ lymphocyte counts of < 100/mm3 (Gerard., *et al* 1997).

Materials and methods

4.1. Study area and population

The study population was all registered HIV-1 infected patients on HAART in Addis Ababa city referred EPHI for HIV-1 viral load determination. The study was conducted at Ethiopian public health institute (EPHI) and international clinical laboratories (ICL). ICL is an advanced private laboratory found in Addis Ababa capital of Ethiopia.

4.2. Study Source

All HIV-1patients coming to EPHI for routine HIV-1/viral load determination, samples were selected by statistically random sampling since the collected samples within two months are 1500 and the key was approximately 20 using the 76 sample size.

4.3. Study design and period

Cross sectional study was conducted among the samples of HIV-1 Positive individuals who have all the data needed for routine viral load at EPHI from November 2018- February 2019.

4.4. Study Variables

Socio demographic characteristics, HCMV-associated factors in and HIV-1 infected patients were considered as explanatory variables; while opportunistic infection status of HIV-infected patients was considered as outcome variables of this study.

4.5. Sample size and sampling techniques

Sample size was calculated using expected prevalence formula, $\mathbf{N = \frac{Z^2 . P (1 - P)}{D^2}}$

Where

N = sample size

P = prevalence of HCMV antibodies (in sub Sahara) =94.8%

Z = standard normal distribution at 95% confidence limit =1.96

D = allowable error = (5% = 0.05)

- Therefore, Since $N = \frac{Z^2 . P (1 - P)}{D^2}$ was $\frac{1.962 \times 0.948 \times (1 - 0.948)}{(0.05)^2}$

$\frac{1.962 \times 0.948 \times (1 - 0.948)}{(0.05)^2}$ then $\frac{3.8416 \times 0.948 \times 0.052}{0.0025}$

$\frac{3.8416 \times 0.948 \times 0.052}{0.0025}$ that is, $\frac{0.1893755}{0.0025}$ **N** = **76 samples**.

Therefore, total of 76 HIV-1 infected patients using the 94.8 % prevalence of HCMV in HIV infected individuals (Bates *et al., 2016*) in sub Saharan Africa. Individuals were included in the

study considering 95% confidence interval and 5% non response rate. Study participants were selected through systematic random sampling method among HIV-1infected patient's samples on HAART during the study period.

4.6. Inclusion and exclusion criteria

Inclusion criteria

All the HIV-1 positive selected samples receiving HAART with complete data and in both sexes and all age groups were included in this study.

Exclusion criteria

HIV-1 positive samples with incomplete data of one of the variables and HIV-unknown status were excluded from this study.

4.7. Data collection tools

The data collection tools were the national HIV-1 viral load request form for capturing demographic and clinical data since the entire samples referred to EPHI for HIV-1viral load are with complete data there is no need of questionnaire for HIV-1 data collection. With the help of the professionals, first the documents were pre-assessed for their reports then the variables specific to HIV-1 were selected by the molecular laboratory professionals using the data's found from ART data's each data was cross matched if there is assessment bias. We were using random lottery method sampling (for HIV-1) among the samples stored at the EPHI deep freezer after HIV-1 viral load determination.

4.8. Insuring data quality

The data collectors and principal investigator (PI) were expected to have the knowledge and additional short training was given about the reviewing of this documentation. In analytical case; most of the processes was performed by the investigator and senior researchers even though the technicians were participated that is; molecular examination (viral load determination) of the samples and the files in the computer as well as hard copy files were checked for all the results to insure data quality. But in the case of post analytical which were reporting and recording the investigator with senior researchers and the data base manager were responsible though there was aback check-up of the printed results.

4.9. Data processing and analysis

Data for HIV-1 positive was done using double entry by checking if there would be wrong data and clerical error. Analysis and interpretation was done using spss-version-20.

4.10.1 HIV-1 Viral Load Determination: (abbot *i* system)

HIV-1 viral load was determined by quantifying the amount of HIV-1 RNA in plasma sample. The Abbott Real Time HIV-1 assay was used, which uses RT-PCR to generate amplified product from the RNA genome of HIV-1 in clinical specimens. An RNA sequence that is unrelated to the HIV-1 target sequence is introduced into each specimen at the beginning of sample preparation. This unrelated RNA sequence is simultaneously amplified by RT-PCR, and serves as an internal control (IC) to demonstrate that the process has proceeded correctly for each sample. The amount of HIV-1 target sequence that is present at each amplification cycle was measured through the use of fluorescent-labeled oligonucleotide probes on the Abbott m2000r instrument. The amplification cycle at which fluorescent signal is detected by the Abbott m2000rt was proportional to the log of the HIV-1 RNA concentration present in the original sample. The lower detection limit of the assay is (figure 3)150 RNA copies per ml of plasma for 0.2ml sample volume for Abbott plat form and 20 RNA copies per ml of plasma for 1.1 ml sample volumes for Cobas plat form.

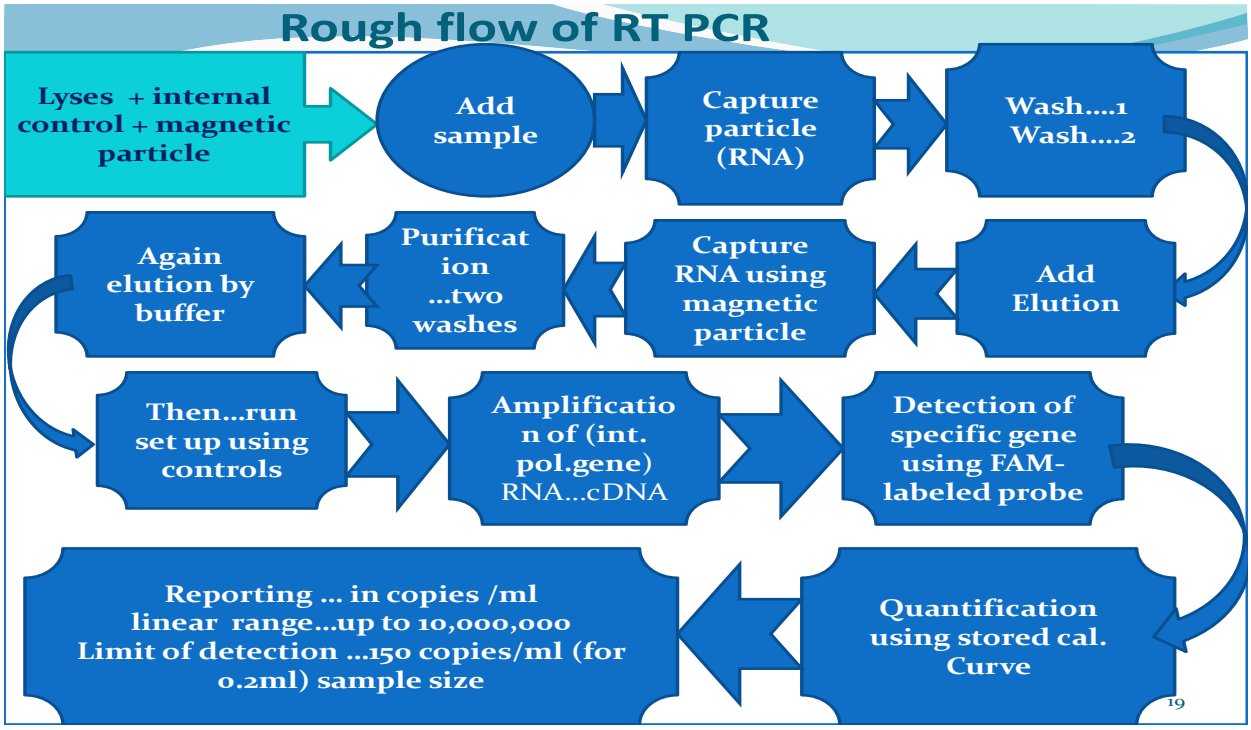

Figure 3: Simplified laboratory work flow of HIV-RT PCR quantitative determination

4.10.2. Chemiluminescent micro-particle immunoassay (ARCHITECT HCMV-IgG/IgM test)

The antibodies specific for HCMV(serologic test) was analyzed using the ARCHITECT HCMV IgG/M assay which is a chemiluminescent micro-particle immunoassay (CMIA) Abbott (Abbot laboratories Chicago, USA) for the qualitative detection (for both IgG and IgM) and semi-quantitative determination (for IgG only) antibodies to HCMV in human serum and plasma. Plasma sample of 3 ml was collected into a sterile un-anti-coagulated bottle, which was

centrifuged, plasma-separated into a sterile bottle and stored at –80 ^{0}C. Plasmas were tested for IgG and IgM HCMV (figure 4) by the chemiluminescent micro-particle immunoassay (CMIA) Abbott (Chicago, USA), according to the manufacturer's instructions.

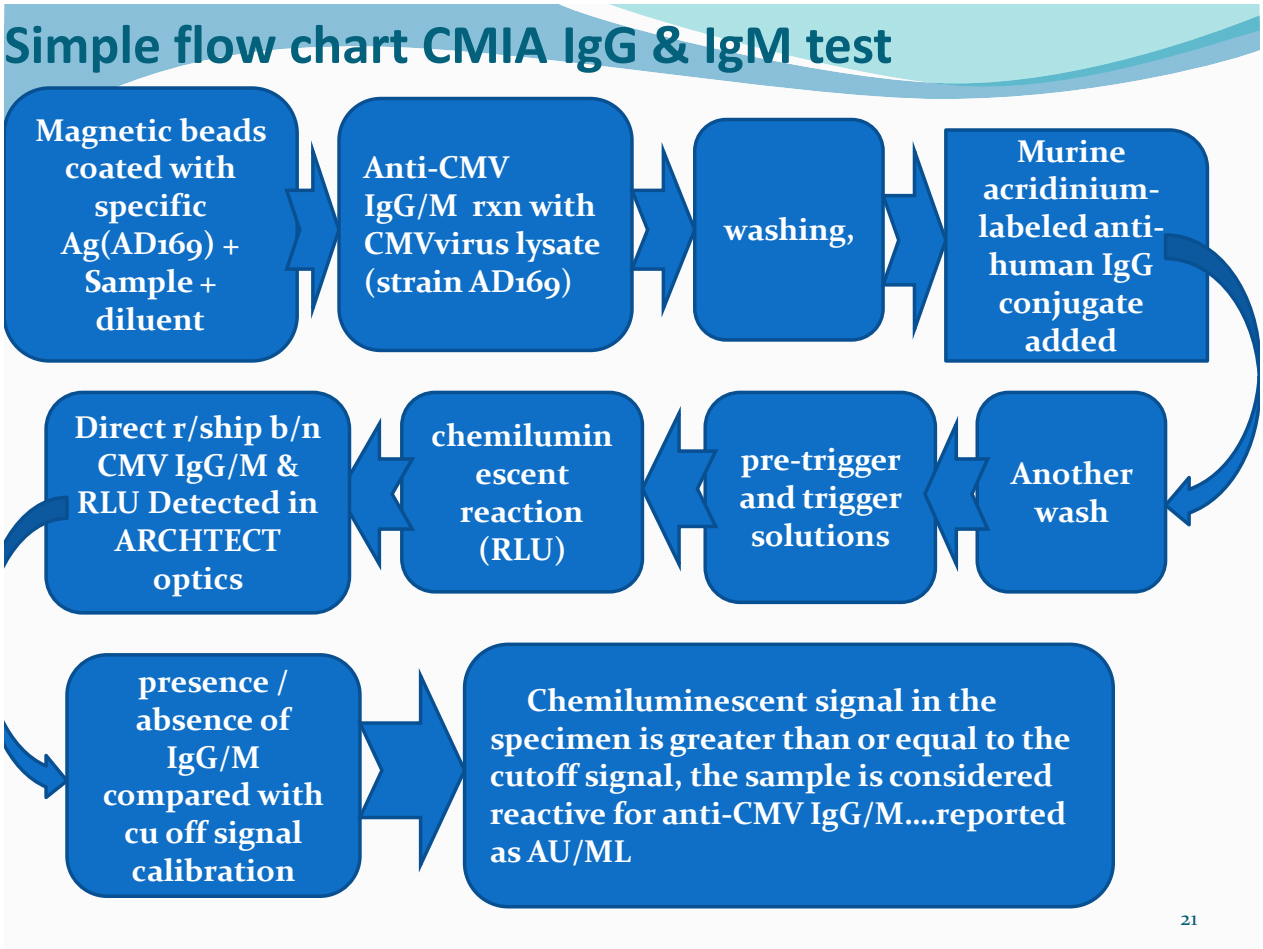

Figure 4: Simplified laboratory work flow of CMIA IgG and IgM qualitative test

Ethical clearance

This study was obtaining an ethical clearance from Scientific and Ethics Research Office (SERO), Ethiopian Public Health Research Institute (EPHI) (annex V) and Addis Ababa University College of natural and computational science (AAU) (annex VI). And this was expected to fulfill the formality and the confidentiality of the data. And the data was analyzed with password locked computer which is permitted only to principal investigator (PI).

5. Result and discussion

Socio-demographic and other factors

Out of the total 1500 samples coming in two months 76 plasma samples of HIV-1 positive patients on HAART referred to the EPHI molecular laboratory were randomly selected from the Samples ready for routine HIV-1/viral load determination. Then their viral load is determined. Of the samples, 46(60%) were female participants and 30(40%) male participants (Female to male ratio is 1:1.53). The minimum age found was 5 and the maximum was 57.

Table 4: General characteristics of participants

CHARACTERISTICS	FREQUENCY	PERCENT (%)	GENDER	
Gender				
Male	30	40	Male	Female
Female	46	60		
Age group				
<18	4	5.3	2	2
18-29	11	14.5	2	9
30-39	24	31.6	6	18
>=40	37	48.7	20	17
WHO clinical stage				
I	72	94.7	29	43
II	4	5.3	1	3
III-IV	0	0	0	0
Most Recent CD4 count				
<100	1	1.3	1	0
100-350	24	31.6	11	13
351-500	19	25	9	10
>500	32	42.1	10	22
Reason for viral load				
First viral load	25	32.9	8	17
Annual viral load	45	59.2	22	23
Suspected ART failure	6	7.9	0	6
ART adherence				
>95% (good)	70	92.1	28	42
85-95%(fair)	6	7.9	2	4
<85%(poor)	0	0	0	0
Baseline CD4 count				
<100	14	18.4	7	7
100-350	42	55.2	16	26
351-500	15	19.7	5	8
>500	12	15.8	2	5

The age categories of the participants were <18 years of age 4(5.3%), 18-29 years 11(14.5%), 30-39 years 24(31.6%) and those with >=40 years of age were 37(48.7%) while the age most frequently found were 40 and is found in the category of >=40 (48.7%) and followed by age 30-39 and that is the mean age category since the mean age is 37.5 also the general socio

demographic is (table 4) in the median age is 38.5 found at age category of 30-39. The mean recent CD4 is 503.2cells/mm^3, and mean baseline CD4 is 278.3cells/mm^{3,} highest recent CD4 is 1280 cells/mm^3, lowest is 98cells/mm^3 while the highest baseline CD4 is 1670cells/mm^{3,} the lowest is 22cells/mm^3 the median recent CD4 is 430cells/mm^3 and median baseline is 206.5cells/mm^3.

In case of ART adherence 70(92.1%) participants were in good (table 3) adherence status to their drugs (>95% adherent) while the rest 6(7.9%) were in fair adherence (85-95% adherent). The adherence status in the age categories shows that <18= 4(5.3%), all at good level, 18-29= 11(14.5%) 10 at good level and 1 at fair level, 30-39 =24(31.6%), 22 at good level 2 at fair level and the age group >=40=37(48.7%) 34 at good level while 3 at fair level which is also the most adherent age group found in the participants. This result is in consistent with (Mangare *et al*., 2018) which reveals HIV immune suppressed patients have shown positive increase in CD4+ count when strictly (adherent) taking ART. Though the indicated highest HCMV IgG and the IgM sero-positivity was found in similar way in the CD4 count range of >=320cells/mm^3 that was 2/3(67%) of the IgM positive and 51/76(67%) of the IgG positive however, in a study in Myanmar (Lilavati *et al*., *2015)* the IgM positive was found in the CD4 cont of <= 200cell/mm^3 which indicates a clear deterioration in immunity unlike this study this may be because of the good adherence patients to their drugs in our study. Their high mean CD4+ count recorded in the study among the HIV infected patients on HAART was good since all the study participants were on HAART and this may be due to the awareness and availability of monitoring facility to the patients.

The ART adherence was also interestingly "good" in both of the gender participants, out of the 46/76(60%) female participants 42/76(55.2%) were at good adherence while 4/76(5.2%) were at fair adherence in case of male 30(40%) participants 28/76(36.8%) were at good adherence while the rest 2/76(2.6%) were at fair adherence. Here there is no any gender or age category that is put in <85% (poor) adherence during their ART course. Though this contributes to the immune restoration of patients study it is difficult to compare with other studies since there were no complete socio-demographic data's in our study.

Table 5: Age and gender distribution of HIV infected individuals to their ART adherence

Participant age	Gender		ART adherence and gender				Total
			F		M		
	F	M	>95%	85-95%	>95%	85-95%	
<18	2(2.6%)	2(2.6%)	2		2		(5.3%) 4
18-29	9(11.8%)	2(2.6%)	8	1	2		(14.5%) 11
30-39	18(23.9%)	6(7.9%)	17	1	5	1	(31.6%) 24
>=40	17(22.4%)	20(26.3)	15	2	19	1	(48.7%)37
Total	46(60%)	30(40%)	**42**	**4**	**28**	**2**	(100%) **76**

HCMV IgG, IgM and Viral load outcomes with the socio-demographic distribution

The results of the immune status of HIV-1 is the CD4 T-lymphocytes together with HIV-1/viral load, so in plasma samples collected from HIV-1infected patients (table 4 with HCMV IgG and IgM) indicates a significant amount of HIV-1/viral RNA copies 15/76 (19.8%) although the 4(5.3%)/15(19.8) were <1000 RNA copies/ml but there was no association with the categories of age (p= 0.667) even though it indicates low significance of immunity deterioration to cause HCMV reactivation. This is in consistent with the study taken place by (Erice *et al.*, 2003) Cytomegalovirus (CMV) and human immunodeficiency virus (HIV) burden. Which revealed HIV viral load (VL) of >10,000 RNA copies/ml is associated with new CMV immune suppression in HAART recipients.

The result also shows CIMA (chemiluminescent micro-particle assay) IgG and IgM test a 76/76(100%) IgG antibody prevalence and 3/76 (3.95%) that of IgM antibody prevalence. Here the study showed the highest prevalence of HCMV IgG (chronic or late infection) and low incidence reactivation (since the 3/76 IgM positive were also positive for IgG) or acute infection (IgM) but still no association with the age groups ($p > 0.05$) for IgG and ($p = 0.681$) for IgM.

This outcome is more similar with the result recorded in university of Benin Nigeria (Ojide *et al*., 2013) which reveals an IgG prevalence of 98.8% and a little difference with IgM prevalence showing 7.0% in the same University hospital in Benin in HIV infected patients on HAART. The chronic infection may be because of HCMV is found everywhere at any age and the difference in the acute infection may be due to social awareness and drug adherence.

This study also showed a clear increase in the number of positive participants as age increases 4(5.3%) at the group of **<18** to 37(48.7) at the age group of **>= 40**, so the result is consistent with the study result in most developed countries, human HCMV sero-prevalence steadily increases after infancy. About10-20% of children are infected before puberty, while in adults the prevalence of antibodies ranges from 40-100% and study result in Iran (Mehrkhani *et al., 2010).* And in similar way the prevalence of HCMV IgG antibodies in three selected General Hospitals in Kaduna State (*Aliyu et al.*, 2016) in Nigeria among HIV-infected individuals the overall sero-prevalence of HCMV IgG among the studied population was found to be 99.4% (177/178) meaning that the average of the three Hospitals but two out of the three Hospitals were found to have 100% IgG prevalence. Besides to this a study in Amazon region Belen, Brazil in (Silva *et al.*, 2015) shows that 239/240(99.6%) and to the study which was taken place in west Africa Guinea-Bissau in 2018 that reveals a 138/138(100%) IgG prevalence (Helene L *et al*., 2018). But in case of the acute infection or reactivation (IgG + IgM) antibodies this study reveals low prevalence of antibodies 3/76(3.9%) which indicates low acute infection or reactivation of the latent infection. This result is somehow separated from the a study separately

taken place in Ebony state Nigeria (Ifeanyi, *et al.*, 2017) that reveals a 4/124 (11.3%) IgM prevalence and the other two African countries Sudan and Nigeria which reveal a 24% and 11.8% prevalence of HCMV IgM respectively and the sero-prevalence of developing countries (8-8.5%) even though it is on the range of the world prevalence which is 0-10% (Omar *et al.*,2016). These differences could be due to infecting strain differences and/or socioeconomic.

Because all the participants in this study were HIV-1 positive strictly on HAART at the same time in an early clinical stage (WHO stage I and II) and their HCMV IgG (late infection) result was 100% IgM (acute infection) was 3.9%, however in studies in Ilory Nigeria, twenty (11.1%) of the 180 HIV-1 sero-positive subjects were positive for anti-HCMV IgM antibody while 169(93.9%) were positive for anti-CMV IgG antibody which indicates similar differences (Adeola., *et al* 2015).

Table 6: Prevalence of CMV IgG and IgM antibodies and HIV/ viral load at age and gender categories

Gender and age category		Number and percent	Total	Viral load statues			p-value	CMV status		p-value	CMV status		p-value
								IgG			IgM		
				UD	<1000	>1000	0.364	pos	neg	0.999	pos	neg	0.681
<18 (N=4)	M	2(2.6%)	4 (5.3%)	4	0	0	.	2	0	.	0	2	.
	F	2(2.6%)					.	2	0	.	0	2	.
18-29 (N=11)	M	2(2.6%)	11 (14.5%)	8	0	3	.	2	0	.	0	2	.
	F	9(11.8%)					.	9	0	.	0	9	.
30-39 (N=24)	M	6(7.9%)	24 (31.6%)	18	2	4	.	6	0	.	2	4	.
	F	18(23.7%)					.	18	0	.	0	18	.
>=40 (N=37)	M	20(26.3%)	37 (48.7%)	31	2	4	.	20	0	.	0	20	.
	F	17(22.4%)					.	17	0	.	1	16	.
Total (N=76)	M	30(40%)	76 (100%)	61	4	7	.	30	0	.	2	28	.
	F	46(60%)					.	46	0	.	1	45	.

N.B: Neg =negative, pos= positive

HCMV (IgG and IgM) antibody outcomes and associated factors

The results of the HIV -1 viral load also indicates that 61/76(80.3%) had no detectable amounts of viral RNA copies/ml, 37/76(48.7%) were females and 24/76(31.6%) were males, 4(5.3%) were <1000 viral RNA copies/ml and 11(14.5%) were >1000 viral RNA copies/ml. Out of the total participants sent to the molecular laboratory 25(32.9%) were sent for their first viral load, 45(59.2%) were for annual viral load and the rest 6(7.9%) were for suspected ART failure during their courses of ART follow up. Participants who had a viral load of <1000 RNA copies /ml were only 4/76(5.3%) 3 female and 1 male and again only one female participant had 514 viral RNA copies /ml but the rest had less than 150 RNA copies /ml. Participants who had >1000 viral RNA copies /ml 11/76(14.5%) those 6/76(7.9%) females and 5/76(6.6%) males indicated in (table 7) the highest undetectable viral RNA found in the age group of >=40 that

was 31/76(40.8) followed by the age group of 30-39 that holds 18(23.7%) but the 11/76(14.5%) participants were also found in the age categories >30 years except the 3 participants found in the age of 18-29. That indicates there was few suspected immune impairment or reactivation of the opportunistic infection (like HCMV). So, this is consistent with most studies in patients on HAART which indicate that correlation between CMV and HIV peak viral loads suggest that CMV plays an important role in HIV in sub-Saharan, Africa (Emily *et al*., 2015). And another study by (Brantsæter *et al.*, 2013) which reveals HCMV replication may drive HIV replication to higher levels (>10,000), which again may cause more rapid HCMV replication, thus creating a repeating circle, the result may be HCMV disease, but also faster progression of HIV disease to new AIDS defining conditions or death.

Table 7: The viral load of participants on HAART

Category	Frequency	Percent	Female	Male	P-value (0.773)
Undetectable	**61**	**80.3%**	**37(48.7%)**	**24(31.6 %)**	**.**
<1000	**4**	**5.3%**	**3(3.9 %)**	**1(1.3 %)**	**.**
>1000	**11**	**14.5%**	**6(7.9 %)**	**5(6.6 %)**	**.**
Total	**76**	**100%**	**46 (60%)**	**30(40 %)**	**.**

The other most important immunologic (associated risk) factor of HCMV reactivation and disease in HIV-1 infected patients is that CD4 T-lymphocyte count/mm^3 there have been two important measures taken specifically to evaluate how the ART reconstitutes (recovers) the immune system of an HIV-patient taking the drugs. By measuring the initial CD4 cont at the starting of the ART and monitoring every 6 months comparison of these immunologic risk factors (table 8) is very important.

Table 8: The baseline and recent CD4 results of participants t

Gender	*Baseline and recent CD4 results*							
	<100		100-350		351-500		>500	
	BSL	RCT	BSL	RCT	BSL	RCT	BSL	RCT
M	7	1	16	11	5	9	2	10
F	7	0	26	13	8	10	5	22
Total	14	1	42	24	13	19	7	32

NB: BSL-Baseline and RCT-recen

These are the baseline(the initial CD4 result) and the recent CD4 counts (most nearest CD4 result) out of the 76 HIV-1 patients on HAART 14 (7 male and 7 female) participants were in a very low (<100cells/mm^3) baseline CD4 count but only 1 female participant had very low recent CD4 count. Comparing those CD4 results is still crucial on low category of CD4 (100-350cells/mm^3) total of 42/76(55.3%) participants (16 males and 26 females) at this (100-

350cells/mm^3) CD4 count but the number of participants whose recent CD4 count at this level was only half 24/76(31.6%) participants (11male and 13 female). Going to the average and higher CD4 results significant increase in the number of participants is observed. In the range 351- 500 cells/mm^3 the difference in the number of participants changed significantly from half to almost more than number which was from baseline to recent CD4 (42 versus 24 to 13 versus 19) at the highest CD4 count (>500 cells/mm^3) it can be seen that clear significant difference in the number of participants total baseline CD4 is 7 and recent is 32 more than four times increase in participants. This clear immunologic (associated risk factor) improvement is an indicator for the low level IgM prevalence (reactivation) though there was high level IgG prevalence. This study reveals that the new infection (IgM) positivity is lower (3.9%) their CD4 also ranges 320-370 cell/mm^3 and this range also is different from most findings and the general fact that HCMV reactivation begins at <100 cells/mm^3. But CD4 T cell number was significantly higher in HIV-infected HCMV negative patients than in HIV-infected HCMV positive patients in a study taken place in Taijan, china (Jiang *et al., 2018).* It has also been in Bandung, Indonesia stated that HCMV diseases are associated with a compromised immune system in HIV infected patients. The disease typically occurs in HIV-infected patients with a CD4 count of <50 to 100 cells/mm^3. However, it has also been reported that HCMV disease occurred in patients with a CD4 count of >100 cells/ mm3 (Sufiawati *et al*., 2013). That is similar with the prevalence of most studies taken place in African countries (Bates *et al*., 2016) and other developing countries outside of Africa and Studies in Omdurman Teaching Hospital, Khartoum's Teaching Hospital and Basher Hospital, Khartoum in 2017 In the State Sudan, that was taken place on a total of 81 HIV sero-positive cases tested and 100% positive for IgG antibodies (Khalid A Enan *et al*., 2017).

The two most important immunological profiles (associated risk factors) that contribute a major role in the reactivation of HCMV are the HIV-1 viral load and the most recent CD4 count in this study their relationship should be inverse (as Viral load in RNA copies/ml increases CD4 count cells/mm should decrease) and vice versa (p-value 0.003) so, this study showed that their inverse relationship (table 9) after their good ART course.

Table 9: The relationship of viral load and recent CD4 count

Recent CD4	*HIV viral load categories*			*P value*	*Total*
	Undetectable	<1000	>=1000	(0.003)	
<100	0	0	1	.	1
100-350	14	2	8	.	24
351-500	16	1	2	.	19
>500	31	1	0	.	32
Total	61	4	11	.	76

The HIV profiles of the (3.9%) 3/76 (IgG + IgM) positive patients are revised in the (table 8) as in the table indicated, There is no participant who was sent for viral load because of suspected ART failure and nor was any whose CD4 count is below 100 cells/mm3 in addition to this the adherence is good and WHO stage grouped in I. This result is somehow separated from the a study separately taken place in Ebony state Nigeria (Ifeanyi, *et al*., 2017) that reveals a 4/124 (11.3%) IgM prevalence and the other two African countries Sudan and Nigeria which reveal a 24% and 11.8% prevalence of HCMV IgM respectively and the sero-prevalence of developing countries (8-8.5%) even though it is on the range of the world prevalence which is 0-10% (Omar *et al*.,2016). But very similar on the other way to the study at Amazon region in Brazil which reveals a 2.1% IgM prevalence and to the study taken in Guinea Bissau (Grønborg *et al*.,2018) which reveals very low IgM antibody prevalence or incidence of 4/138(2.8%). The similarity could be the living styles of the countries and the difference may be due to virus strains that infect the cases.

Sero-positivity of specific HCMV IgM antibodies in relation to different age groups revealed that HCMV Antibodies are high between age groups 31 – 40 years. Another study in Malaysia showed a slight difference in both the IgG and IgM, (IgG= 84%, IgM = 7.2%) although the IgG is still high (Saraswathy *et al*., 2011). This trend could be attributed to the fact that the above mentioned age groups represent active and sexually matured youths with the tendency towards sexual promiscuity and its resultant likelihood of high infection rates.

Table 10: HIV clinical profiles of the CMV-acute (IgM) infections

No.	*Age/ gender*	*Reason for Viral load*	*Viral load*	*Recent CD4*	*ART adherence*	*WHO clinical stage*	*ART duration*	*HCMV (IgM) statues*
1	35/F	1st viral load	8456	320	>95%	I	1	Pos
2	38/M	1st viral load	UD	379	>95%	I	3	pos
3	49/M	Annual vl.	UD	351	>95%	I	6	pos

CD+4 count < 100 cell/µl, HIV/viral load >1000 RNA copies/ml and suspected ART failure can cause high prevalence of specific HCMV IgM antibodies which are main immunologic factors to increase intern the HCMV viral load that showed the risk of HCMV disease, but the recent CD4 count of the three IgM positive patients is (table 10) 320-379 cells/mm^3, HIV-1/viral load for the two undetected while one was 8456 RNA copies/ml, all are categorized in WHO clinical stage I. Which is none AIDs defining stage, and there was no one also with suspect of ART failure or all were in a good ART adherence. However the prevalence of two African countries Sudan and Nigeria which reveal a 24% and 11.8% prevalence of HCMV IgM respectively and the sero-prevalence of developing countries is (8-8.5%). But is on the range of the world prevalence also is up to 10% (Omar *et al*., 2016). So these results seem to be normal infections or new strain acquisition.

6. Conclusion and recommendation

The HCMV sero-prevalence was 76/76(100%) for HCMV anti-IgG in HIV-1 patients recruited for this study and incidence was 3/76(3.9%) IgM antibody. Previous history of lower baseline CD4 or recent CD4 decrease as well as higher HIV-1/viral load was significantly related to HCMV positivity of none of the above results. Rather previous immunologic and clinical profiles (WHO staging, drug adherence and ART regimen) were correlated. And good adherence to the current ART has significant positive input for the lower acute infection of HCMV in Ethiopia. As well as the highest HCMV IgG antibody prevalence shows the endemicity of the virus in this specific population and serological tests for HCMV should be taken into account as a diagnostic tool in the settings of blood transfusion (national blood bank), organ transplant, and when measuring the effectiveness of ART in Ethiopia.

this study strongly recommends that HCMV comprehensive (nationwide) study should be taken place in Ethiopia to note the actual figure of prevalence of HCMV in HIV-1 patients on ART naïve and taking ART, organ transplant patients and pregnant women as well as serological tests should be taken into account in Ethiopia in the settings of blood transfusion (national blood bank), organ transplant pregnant women and their infants since these settings are the main sources of infection and reactivation of HCMV in HIV-1 patients in majority of developing countries as well as when measuring the effectiveness of antiretroviral therapy on immune restoration and might have an impact on the effectiveness of future treatment approaches (in case of HAART).

7. References

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Annex I

EHIOPIAN PUBLIC HEALTH INSTITUTE NATIONAL REFERRENCE LABORATORY (EPHI-NRL) HIV-1 VIRAL LOAD DETERMIATION

HIV-1 /viral load determination test (Abbott *i* system)

Principle of test

The Abbott Real-Ti*m*e HIV-1 assay uses RT-PCR to generate amplified product from the RNA genome of HIV-1 in clinical specimens. An RNA sequence that is unrelated to the HIV-1 target sequence is introduced into each specimen at the beginning of sample preparation. This unrelated RNA sequence is simultaneously amplified by RT-PCR, and serves as an internal control (IC) to demonstrate that the process has proceeded correctly for each sample. The amount of HIV-1 target Sequence that is present at each amplification cycle is measured through the use of fluorescent labeled oligo-nucleotide probes on the Abbott *m*2000*rt* instrument. The probes do not generate signal unless they are specifically bound to the amplified product. The amplification cycle at which fluorescent signal is detected by the Abbott *m*2000*rt* is proportional to the log of the HIV-1 RNA concentration present in the original sample.

Sample Preparation and reagents

The purpose of sample preparation is to extract and concentrate the target RNA molecules to make the target accessible for amplification, and to remove potential inhibitors of amplification from the extract.

The Abbott *m*2000*sp* instrument prepares samples for the Abbott Real-Ti*m*e HIV-1 assay using the Abbott *m* Sample Preparation System (4 × 24 Preps) reagents. The *m*2000*sp* uses magnetic particle technology to capture nucleic acids and washes the particles to remove unbound sample components. The bound nucleic acids are eluted and transferred to a 96 deep-well plate. The nucleic acids are then ready for amplification. The IC is taken through the entire sample preparation procedure along with the calibrators, controls, and specimens.

Reagent Preparation and Reaction Plate Assembly

The Abbott *m*2000*sp* combines the Abbott Real-Ti*m*e HIV-1 amplification reagent components (HIV-1 Oligo-nucleotide Reagent, Thermo-stable rTth Polymerase Enzyme, and Activation Reagent). The Abbott *m*2000*sp* dispenses the resulting master mix to the Abbott 96-Well Optical Reaction Plate along with aliquots of the nucleic acid samples prepared by the Abbott *m*2000*sp*. The plate is ready, after manual application of the optical seal, for transfer to the Abbott *m*2000*rt*.

Amplification

During the amplification reaction on the Abbott *m*2000*rt*, the target RNA is converted to cDNA by the reverse transcriptase activity of the thermo-stable rTth DNA polymerase. First, the HIV-1 and IC reverse primers anneal to their respective targets and are extended during a prolonged incubation period. After a de-naturation step, in which the temperature of the reaction is raised above the melting temperature of the double-stranded cDNA: RNA product, a second primer anneals to the cDNA strand and is extended by the DNA polymerase activity of the rTth enzyme to create a double stranded DNA product. During each round of thermal cycling, amplification products dissociate to single strands at high temperature allowing primer annealing and extension as the temperature is lowered. Exponential amplification of the product is achieved through repeated cycling between high and low temperatures, resulting in a billion-fold or greater amplification of target sequences. Amplification of both targets (HIV-1 and IC) takes place simultaneously in the same reaction. The target sequence for the Abbott RealTi*m*e HIV-1 assay is in the *polintegrase* region of the HIV-1 genome. This region is highly conserved. The IC target sequence is derived from the hydroxyl-pyruvate reductase gene from the pumpkin plant, *Cucurbita pepo*, and is delivered in an Armored RNA particle that has been diluted in negative human plasma.

Detection

During the read cycles of amplification on the Abbott *m*2000*rt*, the temperature is lowered further to allow fluorescent detection of amplification products as the HIV-1 and IC probes anneal to their targets (real-time fluorescence detection). The HIV-1 probe has a fluorescent moiety that is covalently linked to the 5´ end. A short oligo-nucleotide (quencher oligo-nucleotide) is complementary to the 5´ end of the HIV-1 probe and has a quencher molecule at its 3´ end. In the absence of HIV-1 target, the HIV-1 probe fluorescence is quenched through hybridization to the quencher oligo-nucleotide. In the presence of the HIV-1 target sequence, the HIV-1 probe preferentially hybridizes to the target sequence, dissociating from the quencher oligo-nucleotide, allowing fluorescent detection. The IC probe is a single-stranded DNA oligo-nucleotide with a fluorophore at the 5´ end and a quencher at the 3´ end. In the absence of IC target sequences, probe fluorescence is quenched. In the presence of IC

target sequences, probe hybridization to complementary sequences separates the fluorophore and the quencher and allows fluorescent emission and detection. The HIV-1 and IC specific probes are each labeled with a different fluorophore, thus allowing for simultaneous detection of both amplified products at each cycle. The amplification cycle at which fluorescent signal is detected by the Abbott *m*2000*rt* is proportional to the log of the HIV-1 RNA concentration present in the original sample.

Quantization

A calibration curve is required to quantitate the HIV-1 RNA concentration of specimens and controls. Two assay calibrators are run in replicates of 3 to generate a calibration curve. The calibration curve slope and intercept are calculated from the assigned HIV-1 RNA concentration and the median observed threshold cycle for each calibrator and are stored on the instrument. The concentration of HIV-1 RNA in specimens and controls is calculated from the stored calibration curve, and the results

are automatically reported on the *m*2000*rt* workstation. The Abbott Real-Ti*m*e HIV-1 Negative Control, Low Positive Control, and High Positive Control must be included in each run to verify run validity. The *m*2000 System verifies that the controls are within the assigned ranges.

PREVENTION OF NUCLEIC ACID CONTAMINATIO N

The possibility of nucleic acid contamination is minimized because:

• Reverse transcription, PCR amplification, and oligo-nucleotide hybridization occur in a sealed 96-Well Optical Reaction Plate.

• Detection is carried out automatically without the need to open the 96-Well Optical Reaction Plate.

• Aerosol barrier pipette tips are used for all pipeting. The pipette tips are discarded after use.

• Separate dedicated areas are used to perform the Abbott Real-Ti*m*e HIV-1 assay.

REAGENTS

The Abbott Real-Ti*m*e Reagents are intended for single-use only and unused reagents should be discarded.

Abbott Real-Ti*m*e HIV-1 Amplification Reagent Kit (List No. 6L18-90)

1. **INTERNAL CONTROL** Abbott Real-Ti*m*e HIV-1 Internal Control (List No. 2G31Y) (4 vials, 1.2 ml per vial) Noninfectious Armored RNA with internal control sequences in negative human plasma. Negative human plasma tested and found to be nonreactive for HBsAg, HIV RNA, HCV RNA, HBV DNA, anti HIV-1/HIV-2, and anti-HCV. Preservatives: 0.1% Pro-Clin 300 and 0.15% Pro-Clin 950.

2. **AMPLIFICATIO REAGENT PACK** Abbott Real-Ti*m*e HIV-1 Amplification Reagent Pack (List No. 6L18) Four packs of single-use reagents, 24 tests/pack. **Unused reagents should be discarded.**

Each pack contains:

• 1 bottle (0.141 ml) Thermo-stable rTth Polymerase Enzyme (2.9 to 3.5 Units/µl) in buffered solution.

• 1 bottle (1.10 ml) HIV-1 Oligo-nucleotide Reagent. Synthetic oligo-nucleotides (4 primers, 2 probes, and 1 quencher oligonucleotide), and dNTPs in a buffered solution with a reference dye. Preservatives: 0.1% Pro-Clin 300 and 0.15% Pro-Clin 950.

• 1 bottle (0.40 ml) Activation Reagent. 30 mm manganese chloride solution. Preservatives: 0.1% Pro Clin 300 and 0.15% Pro Clin 950.

Abbott Real-Ti*m*e HIV-1 Control Kit (List No. 6L18-80) and Lot-specific Kit Card

1. CONTROL (-) :Abbott Real-Ti*m*e HIV-1 Negative Control (List No.2G31Z) (8 vials, 1.8 mL per vial) Negative human plasma tested and found to be nonreactive for HBsAg, HIV RNA, HCV RNA, HBV DNA, anti-HIV-1/HIV-2, and anti‑ HCV. Preservatives: 0.1% Pro Clin 300 and 0.15% Pro Clin 950.

2. CONTROL (L): Abbott Real-Ti*m*e HIV-1 Low Positive Control (List No.2G31W) (8 vials, 1.8 mL per vial) Noninfectious Armored RNA with HIV-1 sequences in negative human plasma. Negative human plasma tested and found to be nonreactive for HBsAg, HIV RNA, HCV RNA, HBV DNA, anti-HIV-1/HIV-2, and anti HCV. Preservatives: 0.1% Pro Clin 300 and 0.15% Pro Clin 950.

3. **CONTROL (H)**: Abbott Real-Ti*m*e HIV-1 High Positive Control (List No.2G31X) (8 vials, 1.8 mL per vial) Noninfectious Armored RNA with HIV-1 sequences in negative human plasma. Negative human plasma tested and found to be nonreactive for HBsAg, HIV RNA, HCV RNA, HBV DNA, anti‑ HIV-1/HIV-2, and anti HCV. Preservatives: 0.1% Pro Clin 300 and 0.15% Pro Clin 950.

Abbott Real-Ti*m*e HIV-1 Calibrator Kit (List No. 6L18-70) and Lot-specific Kit Card

1. CAL (A): Abbott Real-Ti*m*e HIV-1 Calibrator A (List No. 2G31A) (12 vials, 1.8 mL per vial) Noninfectious Armored RNA with HIV-1 sequences in negative human plasma. Negative human plasma tested and found to be nonreactive for HBsAg, HIV RNA, HCV RNA, HBV DNA, anti-HIV-1/HIV-2, and anti HCV. Preservatives: 0.1% Pro-Clin 300 and 0.15% Pro-Clin 950.

2. **CAL (B):** Abbott Real-Ti*m*e HIV-1 Calibrator B (List No. 2G31B) (12 vials, 1.8 mL per vial) Noninfectious Armored RNA with HIV-1 sequences in negative human plasma. Negative human plasma tested and found to be nonreactive for HBsAg, HIV RNA, HCV RNA, HBV DNA, anti-HIV-1/HIV-2, and Anti HCV. Preservatives: 0.1% Pro-Clin 300 and 0.15% Pro-Clin 950.

Aerosol Containment

To reduce the risk of nucleic acid contamination due to aerosols formed during manual pipetting, aerosol barrier pipette tips must be used for all manual pipetting. The pipette tips must be used only one time. Clean and disinfect spills of specimens and reagents as stated in the Abbott *m*2000*sp* and Abbott *m*2000*rt* Operations Manuals.

Contamination and Inhibition

The following precautions should be observed to minimize the risks of RNase contamination, cross-contamination between samples, and inhibition:

• Wear appropriate personal protective equipment at all times.

• Use powder-free gloves.

• Change gloves after having contact with potential contaminants (such as specimens, eluates, and/or amplified product).

• To reduce the risk of nucleic acid contamination due to aerosols formed during pipetting, pipettes with aerosol barrier tips must be used for all pipetting. The length of the tip should be sufficient to

Prevent contamination of the pipette barrel. While pipetting, care should be taken to avoid touching the pipette barrel to the inside of the sample tube or container. The use of extended aerosol barrier pipette tips is recommended.

• Change aerosol barrier pipette tips between ALL manual liquid transfers.

• The Abbott *m*Sample Preparation System (4 × 24 Preps) reagents are single use only. Use new reagent vessels, reaction vessels, and newly opened reagents for every new Abbott RealTi*m*e HIV-1 assay run. At the end of each run, discard all remaining reagents from the worktable as stated in the Abbott *m*2000*sp* Operations Manual and the Abbott *m* Sample Preparation System (4 × 24 Preps) product information sheet.

STORAGE INSTRUCTIONS:

Abbott Real-Ti*m*e HIV-1 Amplification Reagent Kit (List No. 6L18-90)

The Abbott Real-Ti*m*e HIV-1 Amplification Reagent Pack and Internal Control vials must be stored at – 10°C or colder when not in use. Care must be taken to separate the Abbott Real-Ti*m*e HIV-1 Amplification Reagent Pack that is in use from direct contact with samples, calibrators and controls.

Abbott Real-Ti*m*e HIV-1 Control Kit (List No. 6L18-80)

The Abbott Real-Ti*m*e HIV-1 Negative and Positive Controls must be stored at – 10°C or colder.

Abbott Real-Ti*m*e HIV-1 Calibrator Kit (List No. 6L18-70)

The Abbott Real-Ti*m*e HIV-1 Calibrator A and Calibrator B must be stored at – 10°C or colder.

INSTRUMENT PROCEDURE

The Abbott Real-Ti*m*e HIV-1 application files must be installed on the Abbott *m*2000*sp* and Abbott *m*2000*rt* systems from the Abbott Real-Ti*m*e HIV-1 *m*2000 System Combined Application CD-ROM prior to performing the assay. For detailed information on application file installation, refer to the Abbott *m*2000*sp* and Abbott *m*2000*rt* Operations Manuals, Operating Instructions section.

SPECIM EN COLL ECTIO N, STORAGE, AND TRANSPO RT TO THE TEST SITE

Specimen Collection and Storage

Human plasma (ACD-A and EDTA) specimens may be used with the Abbott Real-Ti*m*e HIV-1 assay. Follow the manufacturer's instructions for processing plasma collection tubes. Freshly drawn specimens (whole blood) may be held at 15 to 30°C for up to 6 hours or at 2 to 8°C for up to 24 hours, prior to centrifugation. After centrifugation, remove plasma from cells. Plasma specimens may be stored at 15 to 30°C for up to 24 hours or at 2 to 8°C for up to 5 days. Plasma specimens may be stored at – 20°C for up to 60 days; if longer storage is required, plasma specimens must be stored at – 70°C or lower.36,37 Multiple freeze-thaw cycles should be avoided and should not exceed 3 freeze/thaw cycles. Thaw plasma specimens at 15 to 30°C or at 2 to 8°C. Once thawed, if plasma specimens are not being processed immediately, they can be stored at 2 to 8°C for up to 6 hours.

Specimen Transport

Ship specimens frozen on dry ice. For domestic shipments, specimens should be packaged and labeled in compliance with applicable local, state, and federal regulations covering the transport of clinical, diagnostic, or biological specimens.

ABBOTT REALTI*M*E HIV-1 ASSAY PROCEDURE

The Abbott Real-Ti*m*e HIV-1 assay provides four sample volume options (0.2 mL, 0.5 mL, 0.6 mL, and 1.0 mL). See assay protocol step 9 and **Interpretation of Results** section.

ASSAY PROTOCOL

Laboratory personnel must be trained to operate the Abbott *m*2000*sp* and *m*2000*rt* instruments. The operator must have a thorough knowledge of the applications run on the instruments and must follow good laboratory practices.

1. Thaw assay controls and IC at 15 to 30°C or at 2 to 8°C. Thaw calibrators at 15 to 30°C or at 2 to 8°C only if performing a calibration run; see **QUALITY CONTROL PROCEDURES** section of this package insert for description of assay calibration. Once thawed, assay controls, IC, and calibrators can be stored at 2 to 8°C for up to 24 hours before use.

2. Vortex each assay calibrator and each control 3 times for 2 to 3 seconds. Ensure that the contents of each vial are at the bottom after vortexing by tapping the vials on the bench to bring liquid to the bottom of the vial. Ensure bubbles or foams are not generated; if present, remove with a sterile pipette tip, using a new tip for each vial.

3. Thaw amplification reagents at 15 to 30°C or at 2 to 8°C and store at 2 to 8°C until required for the amplification master mix procedure. Once thawed, the amplification reagents can be stored at 2 to 8°C
for up to 24 hours if not used immediately.

NOTE: Use 1 bottle of m Lysis Buffer, 1 vial of IC, and 1 Real-Time HIV-1 Amplification Reagent Pack to support up to 24 reactions. Use a second set of reagents to support 25 to 48 reactions, a third set of reagents to support 49 to 72 reactions, and a fourth set of reagents to support 73 to 96 reactions WITH THE EXCEPTIO N OF m MICROP ARTICLES. USE ONLY 2 BO TTLES OF m MICROP ARTICLES WHEN PROCESSING 25 TO 96 SAMPL ES.

4. Invert gently the Abbott *m* Sample Preparation bottles to ensure a homogeneous solution without generating any bubbles. If crystals are observed in any of the reagent bottles upon opening, allow the reagent to equilibrate at room temperature until the crystals disappear. Do not use the reagents until the crystals have dissolved. Ensure bubbles or foam are not generated; if present, remove with a sterile pipette tip, using a new tip for each bottle.

5. Vortex each IC vial 3 times for 2 to 3 seconds before use. Ensure bubbles or foam are not generated; if present, remove with a sterile pipette tip, using a new tip for each vial.

6. Use a calibrated precision **PIP ETTE DEDICATED FOR INTERNAL CONTROL USE ONLY** to add 500 µl of IC to each bottle of m Lysis Buffer. Mix by gently inverting the container 5 to 10 times to minimize foaming.

7. **A maximum of 96 reactions can be performed per run.** A negative control, a low positive control, and a high positive control are included in each run, therefore allowing a maximum of 93 specimens to be processed per run.

8. Thaw specimens at 15 to 30°C or at 2 to 8°C. Once thawed, specimens can be stored at 2 to 8°C for up to 6 hours if not processed immediately.

9. Check sample volume. The Abbott Real-Ti*m*e HIV-1 assay minimum sample volume and associated rack requirements on the Abbott*m*2000*sp* are described below.

CAUTIO N: Steps 10 and 11 must be done in the order described. Vortex the specimens first, and follow with centrifugation. ***If these two steps are not performed in this order, then invalid results may occur.***

10. Vortex each specimen 3 times for 2 to 3 seconds.

11. **Centrifuge specimens at 2000*g* for 5 minutes before loading on the Abbott *m*2000*sp* worktable.**

NOTE: The "*g*" refers to *g* force, not revolutions per minute (rpm).

12. Aliquot each specimen into clean tubes or vials if necessary. Refer to the Abbott *m*2000*sp* Operations Manual for tube sizes. Avoid touching the inside of the cap when opening tubes. Take care not to disturb contents of the tube while removing the tube from the centrifuge and that the bottom of the tube is not touched by the pipette tip. Ensure that the newly aliquotted sample retains the minimum volume indicated in the preceding table.

13. Place the low and high positive controls, the negative control, the calibrators, if applicable, and the patient specimens into the Abbott *m*2000*sp* sample rack.

14. Place the 5 ml Reaction Vessels into the *m*2000*sp* 1 ml subsystem carrier.

15. Load the Abbott *m* Sample Preparation System reagents and the Abbott 96 Deep-Well Plate on the Abbott *m*2000*sp* worktable as described in the following table and in the Abbott *m*2000*sp* Operations Manual, Operating Instructions section.

16. Select the appropriate application file from the Run Sample Extraction screen that corresponds to the sample volume being tested. Initiate the sample extraction protocol as described in the Abbott *m* 2000s*p* Operations Manual, Operating Instruction section.

17. Enter calibrator (needed if a calibration curve has not been stored on the *m*2000*rt*) and control lot specific values in the Sample Extraction**:**

Worktable Setup, Calibrator and Control fields

Lot specific values are specified in each Abbott Real-Ti*m*e HIV-1 Calibrator and Control Kit Card.

NOTE: *Verify the values entered match the values* provided in the lot specific kit cards.
NOTE: The Abbott *m*2000*sp* Master Mix Addition protocol (step 21) must be initiated within 1 hour after completion of Sample Preparation.
NOTE: Change gloves before handling the amplification reagents.

NOTE :The plate-fill setup will automatically be enabled in the following run conditions:

• A batch size of 49 to 95 samples is processed and any valid samples are detected in column locations 7 through 12.

• A batch size of 49 to 95 samples is processed and any samples are detected with a Well Status of "Error."

• A full batch size of 96 samples is processed and any samples are detected with a Well Status of "Error." In these conditions, Reagent Carrier 2 should remain in place, minimally containing the reagent vessel for Elution Buffer (Reagent Carrier 2, location 6). If this reagent vessel has been unloaded, place a new reagent vessel containing the Elution Buffer label within Reagent Carrier 2, location 6. This reagent vessel may remain empty and system fluid will be added to the reagent vessel.

NOTE: System instructions for use of the automated plate-filling feature are found in the *m*2000*sp* Operations Manual (List No. 9K20-04 or higher), Section 5, Operating Instructions,

Sample Extraction – Closed Mode.

18. Load the amplification reagents and the master mix vial on the *m*2000*sp* worktable after sample preparation is completed. Each Amplification Reagent Pack supports up to 24 reactions.

NOTE: A second Amplification Reagent Pack is required if performing 25 to 48 reactions. A third Amplification Reagent Pack is required for 49 to 72 reactions. A fourth Amplification Reagent Pack is required for 73 to 96 reactions.

19. Ensure that the contents are at the bottom of the vials prior to opening the amplification reagents by tapping the vials in an upright position on the bench.

20. Remove and discard the amplification vial caps.

21. Select the appropriate deep well plate from the Run Master Mix Addition screen that matches the corresponding sample preparation extraction. Initiate the Abbott *m*2000*sp* Master Mix Addition protocol. Follow the instructions as described in the Abbott *m*2000*sp* Operations Manual, Operating Instructions section. **The *m*2000*rt* protocol must be started within 50 minutes of the initiation of the Master Mix Addition protocol.**

NOTE: If the run is aborted for any reason subsequent to Step 21, a new 96-well PCR plate must be used if the Abbott *m*2000*sp* Master Mix Addition Protocol (Step 21) will be repeated

22. Switch on and initialize the Abbott *m*2000*rt* instrument in the Amplification Area.

NOTE: The Abbott *m*2000*rt* requires 15 minutes to warm-up.

NOTE: Remove gloves before returning to the sample preparation area.

23. Seal the Abbott 96-Well Optical Reaction Plate after the Abbott *m*2000*sp* instrument has completed addition of samples and master mix according to the Abbott *m*2000*sp* Operations Manual, Operating Instructions section. Export completed PCR plate results to a CD.

24. Place the sealed optical reaction plate into the Splash-Free Support Base for transfer to the Abbott *m* 2000*rt* instrument.

25. Place the Abbott 96-Well Optical Reaction Plate in the Abbott *m*2000*rt* instrument. Import *m*2000*sp* test order via CD per the Import Order instructions in the Abbott *m*2000*rt* Operations Manual, Operating Instructions section.

PO ST PROCESSING PROCEDURES

1. Remove the Abbott 96 Deep-Well Plate from the worktable and dispose of according to the Abbott *m*2000*sp* Operations Manual.

2. Place the Abbott 96-Well Optical Reaction Plate in a sealable plastic bag and dispose according to the Abbott *m*2000*rt* Operations Manual along with the gloves used to handle the plate.

3. Clean the Splash Free Support Base before next use, according to the Abbott *m*2000*rt* Operations Manual.

QUALITY CONTROL PROCEDURES

Abbott *m*2000*rt* Optical Calibration

Refer to the Calibration Procedures section in the Abbott *m*2000*rt* Operations Manual for a detailed description of how to perform an Abbott *m*2000*rt* Optical Calibration. Optical calibration of the Abbott *m*2000*rt* instrument is required for the accurate measurement and discrimination of dye fluorescence during the Abbott Real-Ti*m*e HIV-1 assay. The following Abbott *m*2000*rt* Optical Calibration Plates are used to calibrate the Abbott *m*2000*rt* instrument for the Abbott Real-Ti*m*e

HIV-1 assay:

• FAM -Plate (Carboxy fluorescein)

• ROX -Plate (Carboxy-X-rhodamine)

• VIC-Plate (Proprietary dye)

Assay Calibration

For a detailed description of how to perform an Assay Calibration refer to the Abbott *m*2000*sp* and *m*2000*rt* Operations Manuals, Operating Instructions sections. A calibration curve is required to quantitate the HIV-1 RNA concentration of specimens and controls. Two assay calibrators are run in replicates of 3 to generate a calibration curve (HIV-1 concentration versus the threshold cycle [CT] at which a reactive level of fluorescent signal is detected). The calibration curve slope and intercept are calculated and stored on the instrument. The concentration of HIV-1 RNA in a sample is calculated from the stored calibration curve. Results are automatically reported on the *m*2000*rt* workstation.

Follow the procedure for sample extraction, master mix addition, amplification and detection protocols as stated in the Abbott *m*2000*sp* Operations Manual and the Abbott *m*2000*rt* Operations Manual.

Once an Abbott Real-Ti*m*e HIV-1 calibration is accepted and stored, it may be used for 6 months. During this time, all subsequent samples may be tested without further calibration unless:

• An Abbott Real-Ti*m*e HIV-1 Amplification Reagent Kit with a new lot number is used.

• An Abbott *m* Sample Preparation System (4 × 24 Preps) with a new lot number is used.

• An Abbott Real-Ti*m*e HIV-1 application file for a different sample volume is used.

• A new Abbott Real-Ti*m*e HIV-1 application specification file is installed.

• Pure Dye optical recalibration of the Abbott Real-Ti*m*e HIV-1 assay specific dyes (FAM, VIC, or ROX) is performed per the Calibration Procedures section of the *m*2000*rt* Operations Manual.

Detection of Inhibition

An IC threshold cycle [CT] assay validity parameter is established during a calibration run. A defined, consistent quantity of IC is introduced into each specimen, calibrator, and control at the beginning of sample preparation and detected on the Abbott *m*2000*rt* instrument to demonstrate proper specimen processing and assay validity. The IC is comprised of an RNA sequence unrelated to the HIV-1 target sequence. The median amplification cycle at which the IC target sequence fluorescent signal is detected in calibration samples establishes an IC CT validity range to be met by all subsequent processed specimens and controls. An error is displayed when a specimen or control fails to meet this specification. Refer to the *m*2000*rt* Operations Manual for an explanation of the corrective actions for the error. Specimens whose IC CT value exceeds the established range must be retested starting with sample preparation.

Negative and Positive Controls

A negative control, a low positive control, and a high positive control are included in each test order to evaluate run validity. The lot specific values for the low positive control and high positive control are specified on each Abbott Real-Ti*m*e HIV-1 Control Kit Card and must be entered into the assay test order when a run is performed.

An error is displayed when a control result is out of range. Refer to the Abbott *m*2000*rt* Operations Manual for an explanation of the corrective actions for the error. If negative or positive controls are out of range, all of the specimens and controls from that run must be reprocessed, beginning with sample preparation. The presence of HIV-1 must not be detected in the negative control. HIV-1 detected in the negative control is indicative of contamination by other samples or by amplified product introduced during sample preparation or during preparation of the Abbott 96-Well Optical Reaction Plate. To avoid contamination, clean the Abbott *m*2000*sp* instrument and the Abbott *m*2000*rt* instrument and repeat sample processing for controls and specimens following the **Procedural Precautions**. If negative controls are persistently reactive, contact your Abbott representative.

Monitoring the Laboratory for the Presence of Contamination

It is recommended that this test be done at least once a month to monitor laboratory surfaces and equipment for contamination by amplification product. It is very important to test all areas that may have been exposed to processed specimens, controls, and calibrators, and/ or amplification product. This includes routinely handled objects such as pipettes, the Abbott *m*2000*sp* and Abbott *m*2000*rt* function keys, laboratory bench surfaces, micro-centrifuges, and centrifuge adaptors.

1. Add 0.8 ml RNase-free water to a 1.7 ml RNase-free micro-centrifuge tube.

2. Saturate the cotton tip of an applicator (Puritan or equivalent) in the RNase-free water from the micro-centrifuge tube.

3. Using the saturated cotton tip of the applicator, wipe the area to be monitored using a sweeping motion. Place the applicator into the micro-centrifuge tube.

4. Swirl the cotton tip in RNase-free water 10 times, and then press the applicator along the inside of the tube so that the liquid drains back into the solution at the bottom of the micro-centrifuge tube. Discard the applicator.

5. Pipette 0.5 ml of *m* Wash 1 buffer to a clean tube using the pipette dedicated for Internal Control use.

6. Add 20 µl of the *m* Wash 1 buffer to each micro-centrifuge tube.

7. Cap the micro-centrifuge tube.

8. Test the samples according to the assay procedure section of this package insert.

• Transfer liquid from the micro-centrifuge tube to a 5 ml Reaction Vessel.

• Bring the volume to a minimum of 1.5 ml with RNase-free water.

9. The presence of contamination is indicated by the detection of HIV-1 nucleic acid in the swab samples.

10. If HIV-1 nucleic acid is detected on equipment, follow the cleaning and decontaminating guidelines given in that equipment's operations manual. If HIV-1 nucleic acid is detected on surfaces, clean the contaminated areas with 1.0% (v/v) sodium hypochlorite solution, followed by 70% ethanol or water.

NOTE: Chlorine solutions may pit equipment and metal. Use sufficient amounts or repeated applications of 70% ethanol or water until chlorine residue is no longer visible.

11. Repeat testing of the contaminated area by following Steps 1 through 10.

Results-Calculation

The concentration of viral HIV-1 RNA in a sample or control is calculated from the stored calibration curve. The Abbott *m*2000*rt* instrument automatically reports the results on the Abbott *m*2000*rt* workstation. Assay results can be reported in Copies/ml, Log [Copies/ml], International Units (IU)/ml, or Log [IU/ml]; (1 IU = 0.58 copies, 1 copy = 1.74 IU), with WHO 1st International Standard for HIV-1 RNA (97/656).

INTERPRETATION

Sample volume	Result	interpretation
1ml	Not Detected	Target not detected
	< 1.60 Log [Copies/ml][a]	
	1.60 to 7.00 Log [Copies/ml]	Detected
	> 7.00 Log [Copies/ml]	> ULQ[d]
0.6ml	Not Detected	Target not detected
	< 1.60 Log [Copies/ml][a]	
	1.60 to 7.00 Log [Copies/ml]	Detected
	> 7.00 Log [Copies/ml]	> ULQ
0.5ml	Not Detected	Target not detected
	< 1.88 Log [Copies/ml] [b]	
	1.88 to 7.00 Log [Copies/ml]	Detected
	> 7.00 Log [Copies/m]	> ULQ
0.2ml	Not Detected	Target not detected
	< 2.18 Log [Copies/ml] [c]	Detected
	2.18 to 7.00 Log [Copies/ml]	
	> 7.00 Log [Copies/ml]	> ULQ

NB:

a 40 Copies/ml
b 75 Copies/ml
c 150 Copies/ml
d ULQ = upper limit of quantitation

• A result of "Not Detected" signifies that no target was detected.

• A result of "< 1.60, < 1.60, < 1.88, < 2.18 Log [copies/ml]" indicates that target was detected but is less than the lower limit of quantization (LLQ) for the respective input volumes of 1.0, 0.6, 0.5, and 0.2 ml.

• For 1.0 ml and 0.6 ml input volumes, a result of "1.60 to 7.00 Log [copies/ml]" indicates that the target was detected and the concentration falls between 1.6 log copies per ml (LLQ) and 7.0 log copies per ml (ULQ). For a 0.5 ml input volume, a result of "1.88 to 7.00 Log [copies/ml]" indicates that the target was detected and the concentration falls between 1.88 log copies per ml (LLQ) and 7.0 log copies per ml (ULQ). For a 0.2 ml input volume, a result of "2.18 to 7.00 Log [copies/ml]" indicates that the target was detected and the concentration falls between 2.18 log copies per ml (LLQ) and 7.0 log copies per ml (ULQ). Note that no interpretation is reported on the *m*2000*rt* printout when results fall between LLQ and ULQ.

• A result of "> 7.00 Log [copies/ml]" indicates that the target was detected and is greater than ULQ.

Limit of Detection (LOD)

The limit of detection is defined as the HIV-1 RNA concentration detected with a probability of 95% or greater.

Limit of Detection, 1.0 ml Sample Volume is

The LOD claim for the Abbott Real-Ti*m*e HIV-1 assay is 40 copies/ml with the 1.0 ml sample volume procedure. But Probit analysis of the data determined that the concentration of HIV-1 RNA detected with 95% probability was 25 copies/ml (95% CI 20–33).

Limit of Detection, 0.6 ml Sample Volume is

The LOD claim for the Abbott Real-Ti*m*e HIV-1 assay is 40 copies/ml with the 0.6 ml sample volume procedure. But Probit analysis of the data determined that the concentration of HIV-1 RNA detected with 95% probability was 39 copies/ml (95% CI 33–49).

Limit of Detection, 0.5 ml Sample Volume

The LOD claim for the Abbott Real-Ti*m*e HIV-1 assay is 75 copies/ml, with the 0.6 ml sample volume procedure. But Probit analysis of the data determined that the concentration of HIV-1 RNA detected with 95% probability was 65 copies/ml (95% CI 33–49).

Limit of Detection, 0.2 ml Sample Volume

The LOD claim for the Abbott Real-Ti*m*e HIV-1 assay is 150 copies/ml with the 0.6 ml sample volume procedure. But Probit analysis of the data determined that the concentration of HIV-1 RNA detected with 95% probability was 119 copies/ml (95% CI 33–49).

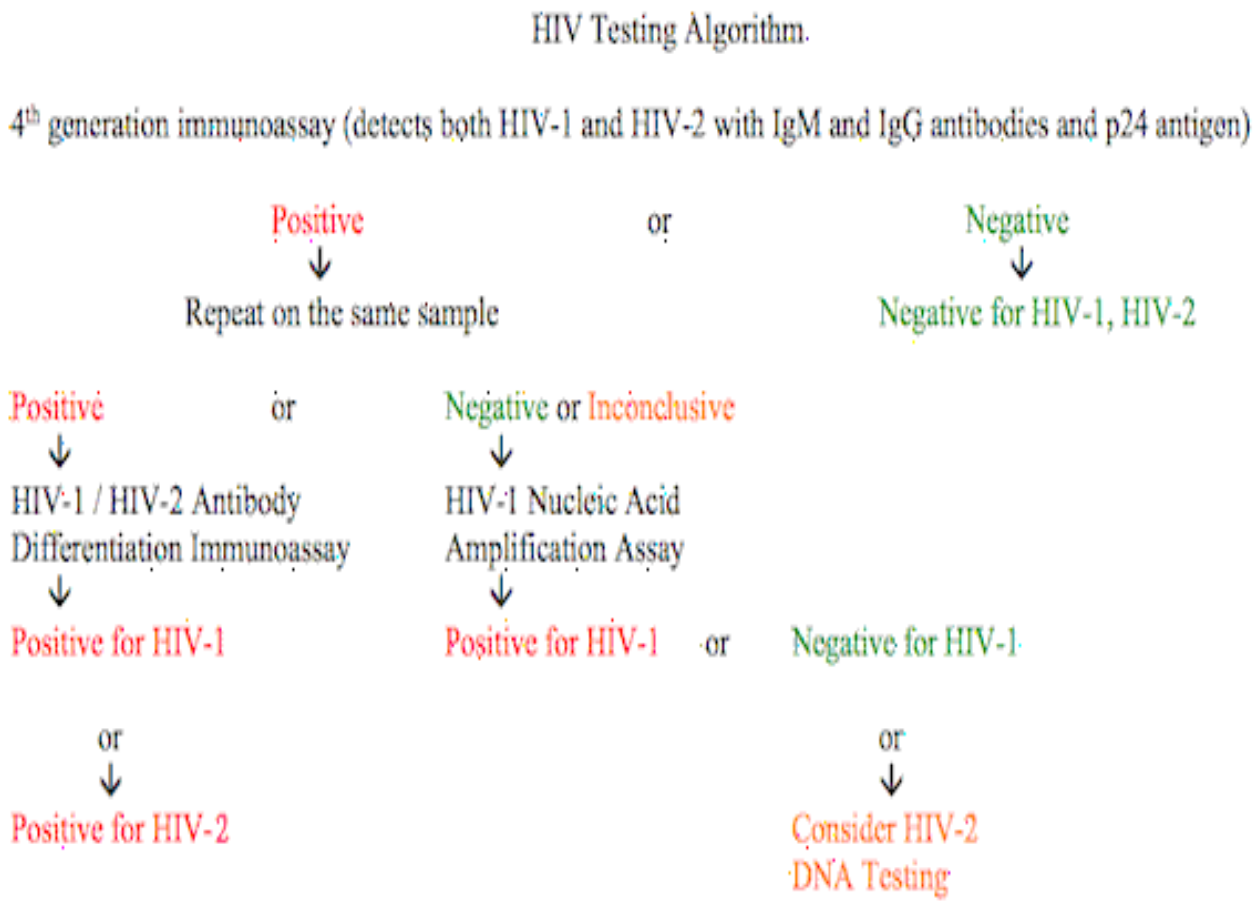
Linear Range

The upper limit of quantization (ULQ) for the Abbott Real-Ti*m*e HIV-1assay is 10 million copies/ml, and the lower limit of quantification is equivalent to the LOD (40 copies/ml for the 1.0 ml and 0.6 ml

sample volume procedure, 75 copies/ml for the 0.5 ml sample volume procedure, and 150 copies/ml for the 0.2 ml sample volume procedure.

Annex II

HIV TESTING ALGORISM



Source: (Klatt C, 2018) and (WHO/AIDS case definition, 2006)

Annex III

FEDERAL MINISTRY OF HEALTH, ETHIOPIA LABORATORY REQUISITION AND REPORT FORM FOR HIV VIRAL LOAD TESTING

Federal Ministry of Health, Ethiopia

Laboratory Requisition and Report form for HIV Viral Load Testing

1. Health Facility Information Internal medicin Specialty center

Facility Name: Bekezata Facility Code: 14/9/155 Facility Tel.No. _____

Region: AA Woreda: Kirkos

Requested by: Name Sr Senait Signature: Sf Date (ET) 29/6/11 (dd/mm/yyyy)

2. Client Information

Unique ART ID: 14/9/155/00019 MRN _____

Sex: ☐M ☐F

Age (years) 68 <1 year (in months) _____

3. Current ART regimen

☐Adult First Line Regimen: _____

Date (ET) Initiated ___/___/___ (dd/mm/yyyy)

☐If pediatric, First Line Regimen: _____

Date (ET) Initiated ___/___/___ (dd/mm/yyyy)

☐Current Second Line Regimen: _____

Date (ET) Initiated ___/___/___ (dd/mm/yyyy)

4. Current ART Adherence

☐ Good >95 %

☐ Fair (85-94%)

☐ Poor <85%

5. Is the client pregnant ☐ Yes or ☐ No **Breastfeeding** ☐ Yes or ☐ No

6. CD4 count history (CD4% for <5 years)

Most recent result _____cells/ul Date (ET) ___/___/___ (dd/mm/yyyy)

Baseline result (pre ART) _____ cells/ul Date (ET) ___/___/___ (dd/mm/yyyy)

7. Current Clinical observations/sympotoms:

WHO (Treatment) Staging: ☐I ☐II ☐III ☐IV

8. Reason for Test

Routine Viral load: ☐First Viral load test (6 months or more on ART) ☐Annual Viral Load (VL) Test

Suspected ART Failure: ☐ Initial Viral load >1000 copies/ml (repeat) ☐ Immounlogical ☐ Clinical

9. To be filled by referring laboratory

Date (ET) Specimen Collected: ___/___/___ (dd/mm/yyyy) Time (ET): _____

Specimen type

☐Whole Blood ☐DBS ☐Plasma

Date specimen sent to Reference laboratory ___/___/___ (dd/mm/yyyy) Time (ET): _____

10. For Testing laboratory use only

LAB ID: 5711617

Date Received (ET): (dd/mm/yyyy)

Specimen quality

☐Acceprable ☐ Unacceptable Reason_____

_____

Test results:

Test Date (ET): 3/4/19 (dd/mm/yyyy)

Test result: Undetectable copies/ml

Tested by MH Signature _____

Dispactch date (ET): ___/___/___ (dd/mm/yyyy)

Reviewed by _____ signature _____

Annex IV

INTERNATIONAL CLINICAL LABORATORIES, ETHIOPIACHEMILUMINESCENT MICRO PARTICLE IMMUNOASSAY TEST *CMV-IgG/IgM ANTIBODY TESTING*

Chemiluminescent Micro particle immunoassay test

Principle

The ARCHITECT CMV IgG and IgM assay is a two-step immunoassay for the qualitative detection and semi-quantitative determination of IgG and/or IgM antibodies to Cytomegalovirus in human serum and plasma with flexible assay protocols, referred to as Chemiflex. In the first step, sample, assay diluents, and CMV virus lysate (strain AD169) coated paramagnetic micro particles are combined. Anti-CMV IgG and/or IgM present in the sample binds to the CMV virus lysate (strain AD169) coated micro particles. After washing, murine acridinium-labeled anti-human IgG conjugate is added to create a reaction mixture. Following another wash cycle, pre-trigger and trigger solutions are added to the reaction mixture. The resulting chemiluminescent reaction is measured as relative light units (RLUs). A direct relationship exists between the amount of anti-CMV IgG in the sample and the RLUs detected by the ARCHITECT *i* System optics. The presence or absence of anti-CMV IgG in the sample is determined by comparing the chemiluminescent signal in the reaction to the cutoff signal determined from a previous calibration. If the chemiluminescent signal in the specimen is greater than or equal to the cutoff signal, the sample is considered reactive for anti-CMV IgG.

Reagents

ARCHITECT CMV IgG Reagent Kit (6C15/16)

Microparticles: 1 or 4 Bottle(s) (6.6 ml per 100-test bottle) CMV virus lysate (strain AD169) coated micro particles in TRIS buffered saline. Minimum concentration: 0.08% solids. Preservatives: Pro-Clin 300 and antimicrobial agents.

Conjugate: 1 or 4 Bottle(s) (5.9 ml per 100-test bottle) Murine acridinium-labeled anti-human IgG in MES buffer. Minimum concentration: 44 ng/ml Preservatives: sodium azide and antimicrobial agents.

Assay diluents: 1 or 4 Bottle(s) (10.0 ml per 100-test bottle) Assay Diluent: CMV IgG assay diluent containing calf serum and MES buffer. Preservatives: ProClin 300 and ProClin 950.

Other Reagents

ARCHITECT *i* Pre-Trigger Solution

PRE-TRIGGER SOLUTION: Pre-Trigger Solution containing 1.32% (w/v) hydrogen peroxide.

ARCHITECT *i* Trigger Solution

TRIGGER- SOLUTION: Trigger Solution containing 0.35 N sodium hydroxide.

ARCHITECT *i* Wash Buffer

WASH BUFFER: Wash Buffer containing phosphate buffered saline solution. Preservatives: antimicrobial agents.

Specimen collection and preparation for analysis

Specimen Types

The specimen collection tubes listed below were verified to be used with the ARCHITECT CMV IgG assay. Other specimen collection tubes have not been tested with this assay.

- Human serum (including serum collected in serum separator tubes)
- Human plasma collected in:
- Plasma separator tubes (lithium heparin)
- Potassium EDTA
- Sodium citrate
- Lithium heparin
- Sodium heparin
- ACD
- CPDA-1
- CPD
- Potassium oxalate/sodium fluoride
- Liquid anticoagulants may have a dilution effect resulting in lower concentrations for individual patient specimens.
- The ARCHITECT *i* System does not provide the capability to verify specimen type. It is the responsibility of the operator to verify that the correct specimen types are used in the ARCHITECT CMV IgG assay.

Storage

- Specimens may be stored on or off the clot, red blood cells or separator gel for up to 14 days refrigerated at 2-8°C.
- If testing will be delayed more than 14 days, remove serum or plasma from the clot, red blood cells, or separator gel. Specimens may be stored for up to 14 days refrigerated at 2-8°C prior to being tested. If testing will be delayed more than 14 days, store frozen (-10°C or colder).
- No qualitative performance differences were observed between experimental controls and nonreactive or reactive specimens subjected to 6 freeze/thaw cycles; however, multiple freeze/thaw cycles should be avoided.

Preparation for Analysis

- Follow the tube manufacturer's processing instructions for serum and plasma collection tubes. Gravity separation is not sufficient for specimen preparation.

- Mix thawed specimens thoroughly by low speed vortexing or by inverting 10 times. Visually inspect the specimens. If layering or stratification is observed, continue mixing until specimens are visibly homogeneous.
- To ensure consistency in results, specimens must be transferred to a centrifuge tube and centrifuged at ≥ 10,000 RCF (Relative Centrifugal Force) for 10 minutes before testing if they contain fibrin, red blood cells, or other particulate matter, they require repeat testing, or they were frozen and thawed.
- Transfer clarified specimen to a sample cup or secondary tube for testing.
- Centrifuged specimens with a lipid layer on the top must be transferred to a sample cup or secondary tube. Care must be taken to transfer only the clarified specimen without the lipemic material.

Assay Procedure

- Before loading the ARCHITECT CMV IgG Reagent Kit on the system for the first time, the micro particle bottle requires mixing to re-suspend the micro particles that have settled during shipment. After the first time the micro particles have been loaded, no further mixing is required.

✓ **Invert the micro particle bottle 30 times.**

- Visually inspect the bottle to ensure micro particles are re-suspended. If micro particles remain adhered to the bottle, continue inverting the bottle until the micro particles have been completely re-suspended.

✓ **If the micro particles do not re-suspend, DO NOT USE. Contact your local Abbott representative.**

- Once the micro particles have been re-suspended, place a septum on the bottle. For instructions on placing septum's on bottles, refer to the **Handling Precautions** section of this package insert.
- Load the ARCHITECT CMV IgG Reagent Kit on the ARCHITECT *i* System.

➢ Verify that all necessary assay reagents are present. Ensure that septum's are present on all reagent bottles. Order calibration, if necessary.

- The minimum sample cup volume is calculated by the system and is printed on the Order list report. No more than 10 replicates may be sampled from the same sample cup. To minimize the effects of evaporation, verify adequate sample cup volume is present before running the test.
- Priority: 75 µl for the first ARCHITECT CMV IgG test plus 25 µl for each additional ARCHITECT CMV IgG test fromthe same sample cup.
- ≤ 3 hours on board: 150 µl for the first ARCHITECT CMV IgG test plus 25 µl for each additional ARCHITECT CMV IgG test from the same sample cup.

- 3 hours on board: additional sample volume is required. For information on sample evaporation and volumes, refer to the ARCHITECT System Operations Manual, Section 5.
- If using primary or aliquot tubes, use the sample gauge to ensure sufficient patient specimen is present.

➢ **Prepare calibrators and controls.**

o Mix ARCHITECT CMV IgG Calibrators and Controls by gentle inversion before use.

o To obtain the recommended volume requirements for the ARCHITECT CMV IgG Calibrators and Controls, hold the bottles **vertically** and dispense 4 drops of each calibrator or 4 drops of each control into each respective sample cup.

➢ **Load samples**.

For information on loading samples, refer to the
ARCHITECT System Operations Manual, Section 5.

➢ **Press RUN**.

➢ For additional information on principles of operation, refer to the ARCHITECT System Operations Manual, Section 3.

➢ For optimal performance, it is important to perform routine maintenance as described in the ARCHITECT System Operations Manual, Section 9. When a laboratory requires more frequent maintenance, follow those procedures.

Results

The ARCHITECT CMV IgG assay uses a 4 Parameter Logistic Curve fit (4PLC, Y-weighted) data reduction method to generate a calibration curve.

Calculation

The ARCHITECT *i* System calculates the Calibrator A through F mean chemiluminescent signal from two Calibrator- A through F replicates, generates a calibration curve and stores the result. The default result unit for the ARCHITECT CMV IgG assay is AU/ml. But in the case of CMV-IgM test the ARCHITECT *i* System calculates the Calibrator 1 mean chemiluminescent signal from three Calibrators 1 replicates and stores the result. Results are reported by dividing sample result by the stored Calibrator 1 result. The default result unit for the ARCHITECT CMV IgM assay is Index. Sample results may also be reported as sample to cutoff (S/CO). The values for Index and S/CO are equivalent.

Interpretation

- Specimens with concentration values < 6.0 AU/ml are considered nonreactive for IgG antibodies to CMV. Individuals with such results are presumed to be not infected with CMV and susceptible to primary infection.

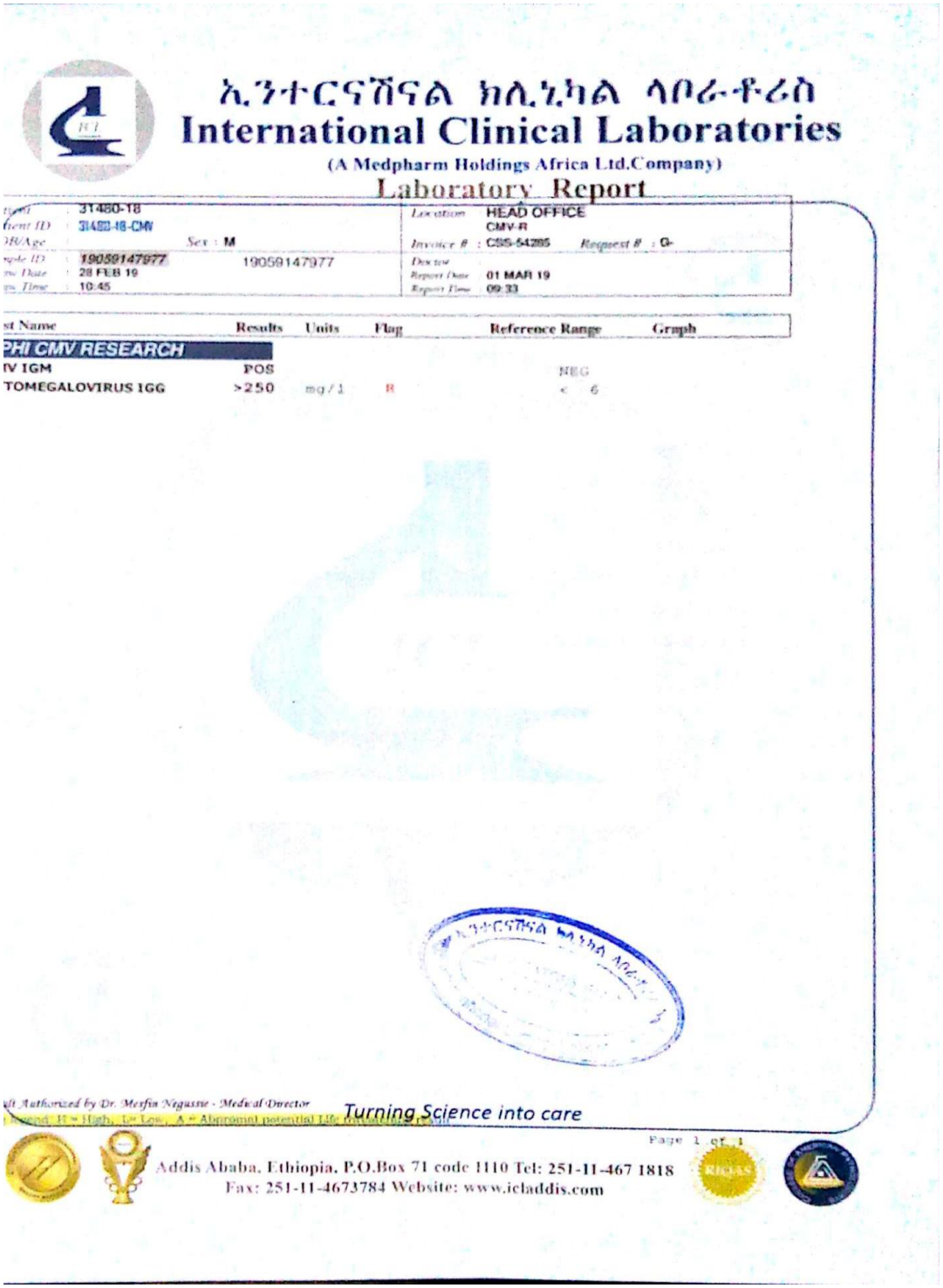
- Specimens with concentration values ≥ 6.0 AU/ml are considered reactive for IgG antibodies to CMV and indicate past or acute infection. Such individuals are potentially at risk of transmitting CMV infection, but are not necessarily currently contagious.
- **NOTE:** It is recommended to confirm results of specimens with concentration values between 6.0 AU/ml and 15.0 AU/ml using a CMV IgM test, or a second sample should be taken, if possible, within a reasonable period of time (*e.g.* two weeks) and used to repeat ARCHITECT CMV IgG testing.

✓ Specimens with concentration values < 0.85 Index are considered nonreactive for IgM antibodies to CMV and indicate the absence of acute infection.

✓ Specimens with concentration values ≥ 1.00 Index are considered reactive for IgM antibodies to CMV and indicate acute infection. Such individuals are potentially at risk of transmitting CMV infection.

Annex V

INTERNATIONAL CLINICAL LABORATORIES, ETHIOPIA LABORATORY REQUISITION AND REPORT FORM FOR CMV-IGG/IGM ANTIBODY TESTING



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International Clinical Laboratories
(A Medpharm Holdings Africa Ltd.Company)
Laboratory Report

	31480-18	Location	HEAD OFFICE
	31480-18-CMV		CMV-R
	Sex : M	Invoice #	C5S-54285 Request # : G-
	19059147977 19059147977	Doctor	
	28 FEB 19	Report Date	01 MAR 19
	10:45	Report Time	09:33

st Name	Results	Units	Flag	Reference Range	Graph
PHI CMV RESEARCH					
IV IGM	POS			NEG	
TOMEGALOVIRUS IGG	>250	mg/l	R	< 6	

Authorized by Dr. Mesfin Negussie - Medical Director

Turning Science into care

Page 1 of 1

Addis Ababa, Ethiopia, P.O.Box 71 code 1110 Tel: 251-11-467 1818
Fax: 251-11-4673784 Website: www.icladdis.com

Annex VI

Ethical clearance from college of natural and computational science Addis Ababa University

COLLEGE OF NATURAL & COMPUTATIONAL SCIENCES
Addis Ababa University

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OFFICE OF THE DEAN
የዲን ጽ/ቤት

Ref. No.
ቁጥር CNSDO/229/10/2018
Date
ቀን January 18, 2018

To Whom It May Concern

The College of Natural & Computational Science Institutional Review Board (CNS-IRB) Committee in its meeting held on 11/01/2018 Minute No. IRB/031/2018 has examined the project proposal entitled "**Molecular characterization and prevalence of cytomegalovirus among HIV-positive individuals in Addis Ababa, Ethiopia**" by Yemane Abreha.

The proposal is approved for implementation.

With regards,

Shibru Temesgen /Dr./
Dean, College of Natural & Computational Science
Addis Ababa University
Addis Ababa

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ኢ.ሜይል/E-mail: dean_cns@aau.edu.et

Please Quote our reference number in you correspondence

"Examine all things; hold fast that which is good" "ሁሉን መርምሩ መልካሙን ያዙ"

Annex VII

Ethical clearance from Ethiopian public health institute scientific and ethical review committee

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Ethiopian Public Health Institute
አዲስ አበባ-ኢትዮጵያ Addis Ababa, Ethiopia

ስልክ-Tel: +251 11 2133499, +251 11 2751522, ፋክስ Fax: +251 11 2758634,
የ.ሣ.ቁ - P. O. BOX: 1242/5654 e-mail: ephi@ethionet.et
www.ephi.gov.et

ቁጥር EPHI 6.13/291
Ref. No
ቀን 05 APR 2018
Date

Institutional Review Board (EPHI-IRB)

Certificate of Protocol Approval

EPHI-IRB Meeting No. *019*

Protocol number: *EPHI-IRB-067-2017*

Protocol Title: Prevalence and Risk Factors of Cytomegalovirus among HIV Positive Individuals in Addis Ababa, Ethiopia.	
Principal Investigator	*Yemane Abreha*
Institute	*EPHI*
Study site/s	*Addis Ababa, Ethiopia*
Elements Reviewed	☐ Attached ☑ *Not attached*
Decision of the meeting	☑ *Approved* ☐ Approved with Recommendation

I. Elements approved-
1. Protocol Version No. *Ver 001*
2. Protocol Version Date: *27 Mar 2018*

II. Obligations of the PI-
1. Should comply with the standard international & national scientific and ethical guidelines
2. All amendments and changes made in protocol and consent form needs IRB approval
3. The PI should report SAE within 10 days of the event
4. End of the study, including technical reports, thesis works and manuscripts should be Reported to the IRB

III. Details of recommendation (if approved with recommendation) _____

Institution Review Board (IRB) Approval date: *27 Mar 2018*
Approval period: from *27 Mar 2018* to *26 Mar 2019*
Follow up report expected in: 3 Months ____ 6 months ✓ 9 months____ one year____

Chairperson, IRB
Signature _____
Date 29 Mar 2018

Director General
Signature _____
Date 04/04/18

Ebba Abate (Dr)
Director General