ANALYSIS OF SERUM FOR ANTIBODIES TO CYTOMEGALOVIRUS IN INDIVIDUALS WITH SCHIZOPHRENIA AND BIPOLAR DISORDER

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ABBREVIATIONS

AAU	Addis Ababa University
AHRI	Armauer Hansen Research Institute
AIDS	Acquired Immunodeficiency Syndrome
CIDI	Composite international diagnostic interview
CMV	Cytomegalovirus
CNS	Central nervous system
COI	Cut-off Index
COX-2	Cyclooxygenase-2
CSF	Cerebrospinal fluid
DEAFF	Detection of early antigen fluorescent foci
DNA	Deoxyribonucleic acid
DSM	Diagnostic and statistical manual of mental disorders
EBV	Epstein-Barr virus
ELISA	Enzyme-linked Immunosorbent assay
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HEL	Human embryo lung fibroblast
HIV	Human Immunodeficiency Virus
HSV	Herpes simplex virus
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IRB	Institutional review board
OD	Optical density
OPHCC	Office of Population and Housing Census Commision
PCR	Polymerase chain reaction
PP65	Phosphoprotein 65
SMRI	Stanley Medical Research Institute
TMB	Tetra-methyl benzidine
USA	United States of America

ABSTRACT

Background: *Several evidences suggest that Cytomegalovirus (CMV) may play an etiological role in some case of schizophrenia and bipolar disorder. Studies have reported that some individuals experiencing initial episodes of major mental disorders have increased levels of immunoglobulin G (IgG) to CMV.*

Objective*: To determine the magnitude of CMV infection in individuals with schizophrenia, bipolar disorder and healthy controls by using serologic diagnostic methods.*

Materials and Methods*: A case control study conducted from March to May of 2009. A total of 844 serum samples were analysed for the presence and level of IgG to CMV using enzyme linked immunosorbent assay (ELISA). Sera were collected from 216 individuals with schizophrenia, 199 with bipolar disorder and 429 close relatives and other healthy controls, recruited from Butajira district. Seropositivity summarized using percentage and antibody level was summarized using mean. Logistic regression was used for cross tabulated data and linear regression for comparing antibody level among groups. All test of significance was two tailed and, $p < 0.05$ is considered significant.*

Results*: 99.3% of tested sera were positive for IgG to CMV. However, serum levels of IgG to CMV in individuals with schizophrenia [$\beta = 0.21$; 95% CI (0.03, 0.38)] and bipolar disorder [$\beta = 0.20$; 95% CI (0.02, 0.37)] were higher compared to healthy controls. Younger individuals with schizophrenia (<20 years) had higher level of IgG to CMV compared to matched healthy controls [$\beta = 0.64$; 95% CI (0.10, 1.19)].*

Conclusion*: This study provided serologic evidence that infection with CMV may be associated with some cases of schizophrenia and bipolar disorder. Additional studies should be directed at further analysis of antibodies to CMV in the sera and CSF of individuals with recent onset of psychosis.*

Key words: *CMV, Schizophrenia, Bipolar disorder, Butajira, Ethiopia*

CHAPTER I: INTRODUCTION

1.1. Introduction

Psychotic disorders are now recognized to be important public health problems. Although schizophrenia and other major psychotic disorders are relatively uncommon, the financial burden they impose on society can be high. This is because of their early onset in life and significant impairments of long duration (Kebede and Alem, 1999). The burden of psychotic disorders falls particularly heavily upon low and middle income countries (The Academy of Medical Sciences, 2008). Prevalence studies conducted in Ethiopia based on Composite International Diagnostic Interview (CIDI) showed lifetime prevalence of schizophrenia to be 0.4% in Addis Ababa (Kebede and Alem, 1999) and 0.47% in Butajira (Kebede *et al*., 2003). The prevalence of bipolar disorder also reported to be 1.83% in isolated island community in Zeway (Fekadu *et al*., 2004).

Generally it is believed that multiple factors including genetic, infections, season of birth, urban birth, socioeconomic status, prenatal or birth complications may play a role in the etiology of schizophrenia and bipolar disorder (Yolken and Torrey, 1995; Leweke *et al*., 2004; Torrey *et al*., 2006; Niebuhr *et al*., 2008a; Tandon *et al*., 2008). There are also several aspects of psychotic disorders that suggest a possible infectious origin, one is the observation of individuals sometimes present with the clinical symptoms of psychosis in the course of developing or shortly after having had a known infectious disease. Much of the recent research has focused on the potential relationship between the development of schizophrenia and infections with or antibodies to Herpes simplex family viruses and *Toxoplasma gondii* (Leweke *et al*., 2004; Torrey *et al.,* 2006; Amminger *et al.,* 2007; Niebuhr *et al.,* 2008b; Yolken and Torrey, 2008).

As significant causes of encephalitis, viruses such as CMV are prime interest for schizophrenia research (Srikanth *et al.,* 1994; Yolken and Torrey, 1995; Leweke *et al.,* 2004; Torrey et al., 2006; Amminger *et al.,* 2007; Niebuhr *et al.,* 2008a; Niebuhr *et al.,* 2008b; Yolken and Torrey, 2008). Both CMV and schizophrenia have a worldwide distribution and an increased prevalence in lower socioeconomic groups (Yolken and

Torrey, 1995; Torrey *et al.*, 2006). Studies have reported that some individuals experiencing initial episodes of schizophrenia have increased levels of IgG to CMV, but not to other herpes viruses, in their sera and cerebrospinal fluid (Leweke *et al.,* 2004). CMV is also reported to be neurotrophic and has an affinity for limbic system, one of the areas of the brain thought to be affected in schizophrenia (Torrey and Peterson, 1974). In addition there is a study reported that treatment with valacyclovir, the orally administered L-valyl ester of acyclovir, can result in some degree of symptom improvement in individuals with schizophrenia who have serological evidence of CMV infection (Dickerson *et al.,* 2003). In another study, celecoxib, a selective inhibitor of Cyclo-oxygenase-2(COX-2) was administered in conjunction with risperidone to individuals with schizophrenia result in a significant improvement in their symptoms (Muller *et al.,* 2002). Inhibition of COX-2 blocks the replication of CMV (Zhu *et al.,* 2002), and this mechanism may account for the observed symptom improvement in these individuals (Martelius *et al.*, 2002). Although laboratory-based research focusing on CMV as possible etiologic agents for schizophrenia has long history (Yolken and Torrey, 1995; Torrey *et al.,* 2006), ascertaining the nature of a possible etiologic association between infection and schizophrenia is highly challenging. There have been few consistent findings between studies, which could be due to many factors, including the heterogeneity of schizophrenia itself and the use of different immunologic assays across the studies (Niebuhr *et al.*, 2008b). In addition, most existing studies conducted so far have used small sample size (Torrey *et al*., 2006; Niebuhr *et al.,* 2008a). On the other hand, bipolar disorder is the least studied regarding association with CMV infection (Torrey *et al.*, 1982; Hinze-Selch, 2002).

The present study was conducted with the aim of assessing the association of CMV infection with psychotic episodes by evaluating serum samples for the presence and level of IgG to *Cytomegalovirus* collected from individuals with schizophrenia, bipolar disorder, their close relatives and other healthy controls.

1.2. Background of the Project

Schizophrenia and bipolar disorder are chronic neuropsychiatric diseases (Torrey *et al.*, 2006; Niebuhr *et al*., 2008a) that usually begin in young adulthood and have various degrees of severity. Some cases relapse and remit, while others are continuously symptomatic. Bipolar disorder tends to stabilize with age, while many individuals with schizophrenia exhibit clinical improvement in later years; neither disease has a progressive downhill course characteristic of many dementias. The predominant symptoms of schizophrenia and bipolar disorder are auditory hallucinations, delusional and illogical thinking, and affective symptoms that may range from mania to depression (Yolken and Torrey, 1995). It has been established that both genetic and environmental factors play a role in the etiology of schizophrenia; the latter include an excess of winter-spring births, an excess of urban births and an excess of births in lower socioeconomic groups (Yolken and Torrey, 1995; Leweke *et al.*, 2004; Torrey *et al*., 2006; Niebuhr *et al*., 2008a; Tandon *et al*., 2008).

Genetic factors and gene-environment interactions together contribute over 80% of the liability for developing schizophrenia, and a number of chromosomal regions and genes have been linked to the risk for developing the disease. However, despite intensive research and spectacular advances in molecular biology, no single gene variation has been consistently associated with a greater likelihood of developing the illness and the precise nature of the genetic contribution remains obscure at this time. Environmental factors linked to a higher likelihood of developing schizophrenia include cannabis use, prenatal infection, malnutrition, perinatal complications, and a history of winter birth; the exact relevance or nature of these contributions is, however, unclear. How various genetic and environmental factors interact to cause schizophrenia and via which precise neurobiological mechanisms they mediate this effect is not understood. Etiological heterogeneity, complex patterns of gene-gene and gene-environment interaction, and inadequately elucidated schizophrenia pathophysiology are among the explanations invoked to explain our inadequate understanding of the etio-pathogenesis of schizophrenia (Tandon *et al.*, 2008).

The hypothesis that schizophrenia and bipolar disorder are caused by infectious agents was first formulated in the 19th century. As early as 1845, the noted French neurologist Jean

Esquirol wrote: ‘‘many authors assure us that mental alienation is epidemic. It is certain that there are years when, independently of moral causes, insanity seems suddenly to extend to a great number of individuals’’ (In: Yolken and Torrey, 1995). Recent serological studies in people with schizophrenia have focused on members of the human *Herpesviridae* family and the protozoan *Toxoplasma gondii* because of their ability to establish persistent infection within the central nervous system as well as the occurrence of neurological and psychiatric symptoms in some individuals infected with these agents (Leweke *et al.,* 2004; Torrey *et al.*, 2006; Amminger *et al.*, 2007; Niebuhr *et al.*, 2008b).

1.2.1. Epidemiology of Schizophrenia and Bipolar Disorders

Schizophrenia and bipolar disorder are devastating disorders of the central nervous system with worldwide distribution. In United States of America (USA) the annual incidence of schizophrenia averages 15 per 100,000; the point prevalence averages approximately 4.5 per population of 1000, and the risk of developing the illness over one's lifetime averages 0.7%. Schizophrenia runs in families and there are significant variations in the incidence of schizophrenia with urbanization, male gender, and a history of migration being associated with a higher risk for developing the illness (Tandon *et al.*, 2008). Studies conducted in developing countries reported a range of point prevalence for schizophrenia of 0.22-0.28% for rural India, 0.25-0.59% for urban India, 0.14-0.56% for urban China, 0.08-0.46% for rural China and 0.3% for Addis Ababa (Kebede and Alem, 1999). Kebede and Alem (1999) also reported lifetime prevalence for schizophrenia 0f 0.4% based on a survey in Addis Ababa, in this study representative sample of an inner-city community was surveyed by the use of the CIDI. Kebede *et al.* (2003) study of a predominantly rural population in south central Ethiopia has reported lifetime prevalence of schizophrenia to be 0.47%. In similar study conducted in isolated Island community, Zeway, using structured interview by Fekadu *et al*. (2004) reported prevalence of bipolar disorder to be 1.83%.

1.2.2. *Cytomegalovirus*

Cytomegalovirus was first isolated in 1956 from salivary gland, adenoid tissue and liver biopsy using cell culture by three independent investigators. Weller, one of the investigators, named the new virus after its cytopathic effect, which produced large, swollen, refractile cells causing cytomegaly. For the first 180 million years of its existence, CMV managed to avoid eradication by co-evolving with its host. Now in the past 50 years (a blink of the eye in evolutionary terms), it has been faced with changes of unprecedented rapidity as the human population has greatly increased its longevity, and become immunocompromised, either iatrogenically for the purposes of sustaining organ transplantation, or because of the HIV pandemic (Griffiths, 2006).

1.2.3. Virologic Characteristics

Cytomegalovirus is a member of β-*Herpesvirinae* in the subfamily *Herpesviridae* and known to be present in saliva, cervical secretions, breast milk, semen, and human lymphocytes. CMV shares many attributes with other herpes viruses, including genome, virion structure, and the ability to cause latent and persistent infections. CMV is a double-stranded linear deoxyribonucleic acid (DNA) virus with 162 hexagonal protein capsomeres surrounded by a lipid membrane (Figure 1.1). CMV has the largest genome of the herpes viruses, ranging from 230-240 kilo base pairs. The number of virus encoded proteins and the complexity of their functions in the life cycle of this virus are reflected in the size of its genome (Britt and Boppana, 2004). Replication of the virus may be divided into immediate early, delayed early, and late gene expression based on time of synthesis after infection. The DNA is replicated by rolling circles. In vitro, CMV replicates in human fibroblasts. CMV is a lytic virus that causes a cytopathic effect in vitro and in vivo. The pathologic hallmark of CMV infection is an enlarged cell with viral inclusion bodies. Cells that exhibit cytomegaly are also seen in infections caused by other β-herpesvirinae. The microscopic description given to these cells is most commonly an "owl's eye". Although this considered diagnostic, such histological findings may be minimal or absent in infected organs (Wills, 2009).

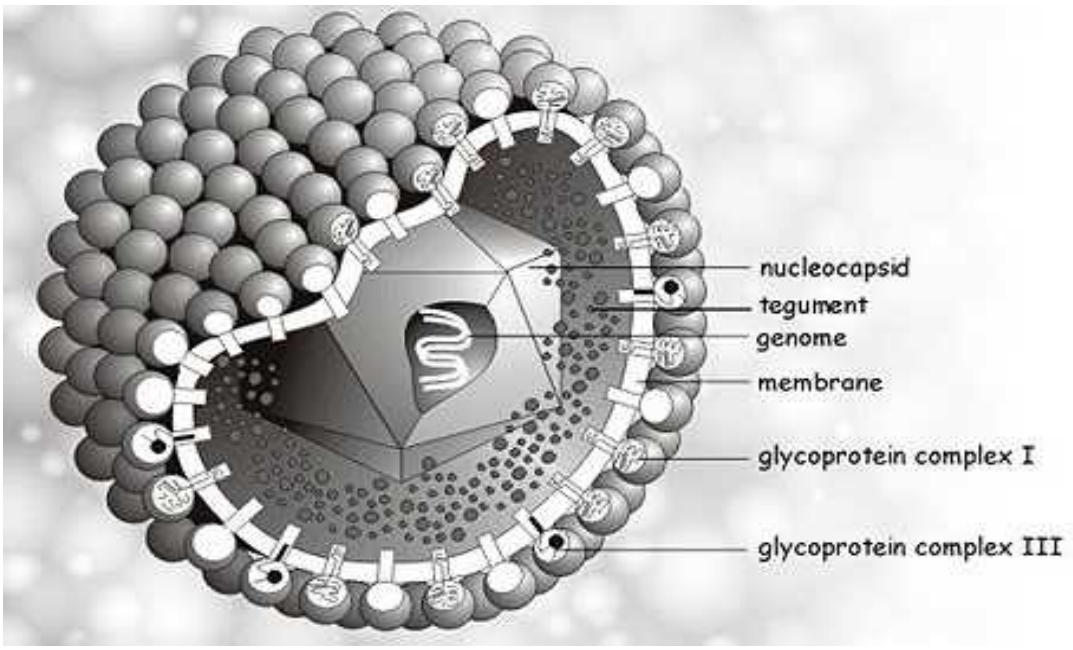


Figure 1.1: Representation of *Cytomegalovirus* (Human Cytomegalovirus study group, 2007)

1.2.4. Epidemiology of CMV Infection

CMV is a ubiquitous agent. Although the age of infection varies worldwide, most people are infected with CMV at some point in life. Transmission of CMV occurs from person to person, in the form of close contact with a patient who is excreting the virus. It can be spread through the placenta, blood transfusions, organ transplantation and breast milk. It also may be spread through sexual contact. Risk factors for infection include patients who attend or work at daycare centers, individuals who had blood transfusions and having multiple sexual partners (Ross *et al.*, 2006). In developing countries, most infections are acquired during childhood; whereas, in developed countries, up to 50% of young adults are seronegative (Wills, 2009). The age adjusted CMV seroprevalence in individuals 6 years and older in the USA was 58.9%, seroprevalence increased steadily from 36.3% among 6-11 year olds to 90.8% in those 80 years and older. When adjusting for age only, seroprevalence differed substantially by race: 51.2% among non-Hispanic white persons, 75.8% among non-Hispanic black persons, and 81.7% among Mexican Americans (Staras *et al.*, 2006). By age 11 in Israel (Sarov *et al.*, 1982) and Saudi Arabia (Ashraf *et al.*, 1985) nearly 100% of

the population were CMV seropositive, compared with 40% in the USA (Staras *et al.*, 2006). Adjei *et al.* (2008) reported seroprevalence of CMV among healthy Human Immune Deficiency Virus (HIV) negative blood donors and HIV/Acquired Immune Deficiency Syndrome (AIDS) patients in Ghana, 77.6% and 59.2% respectively. In related seroprevalence study of antibodies to *Cytomegalovirus* among Prospective Blood Donors in Jos, Nigeria was 92% (Alao *et al*., 2008).

1.2.5. Clinical Significance

When the host is infected, CMV DNA can be detected with polymerase chain reaction (PCR) in all the different cell lineages and organ systems in the body. Upon initial infection, CMV infects the epithelial cells of the salivary gland, resulting in a persistent infection and viral shedding. Infection of the genitourinary system leads to clinically inconsequential viruria. Despite ongoing viral replication in the kidney, renal dysfunction is rare except in renal transplant recipients, in whom CMV is rarely associated with glomerulopathy and possible graft rejection (Wills, 2009).

CMV is usually an asymptomatic infection. In immunocompetent individuals, symptomatic disease usually manifests as a mononucleosis syndrome. However, in patients immunocompromised by HIV, solid-organ transplantation and bone-marrow transplantation clinically significant CMV disease frequently develops. Additionally, congenital transmission from a mother with acute infection during pregnancy is a significant cause of neurological abnormalities and deafness in newborns. Symptomatic disease in immunocompromised individuals can affect almost every organ of the body, resulting in fever of unknown origin, pneumonia, hepatitis, encephalitis, myelitis, colitis, uveitis, retinitis, and neuropathy. As with other herpes viruses, CMV establishes a latent infection in the host (Rafailidis *et al.*, 2008; Wills, 2009).

1.2.6. CMV, Schizophrenia and Bipolar Disorder

Recent research has focused on infectious agents as potential players in the etiologic pathway of psychiatric illnesses such as schizophrenia. Due to their potential neurotropism and latency, viral organisms in particular are considered possible agents in many chronic

central nervous system (CNS) disorders. Encephalitis and other conditions leading to CNS inflammatory changes often present with symptoms that are difficult to distinguish from new onset schizophrenia. As significant causes of encephalitis, viruses such as CMV are prime interest of schizophrenia research (Torrey *et al.*, 2006; Niebuhr *et al.*, 2008b). CMV is neurotrophic and has an affinity for limbic system, one of the areas of the brain thought to be affected in schizophrenia (Torrey and Peterson, 1974). Earlier studies included large numbers of individuals with depression and bipolar disorder, but recent studies have focused more on individuals with schizophrenia (Yolken and Torrey, 1995).

Between 1973 and 1992, 14 serologic studies were reported on CMV antibody levels in patients with chronic schizophrenia; all reported no significant difference in levels between patients and controls. However, these studies were limited by the use of complement fixation or other less sensitive assays, by using problematic control groups, and, in particular, by studying patients who had been unwell for many years (Yolken and Torrey, 1995; Torrey *et al*., 2006). Additional serologic studies have been carried out in patients with a more recent onset of illness. In India, Srikanth *et al*. (1994) reported elevated CMV antibody levels in 6 of 35 individuals with an onset of psychosis within the previous month compared with 35 controls undergoing minor surgical procedures. In Germany, a comparison was made between 86 individuals with schizophrenia, 29 of whom were experiencing their first episode, and 85 unaffected individuals (well matched in terms of socioeconomic and geographical variables) recruited from the general population; significantly more patients than control individuals had antibodies to CMV (Chi-square 10.29; $p = 0.001$) but not to other herpes viruses. In Baltimore, USA, a comparison was made between 415 outpatients with schizophrenia and 164 unaffected matched controls recruited from the community. The patients with schizophrenia were significantly more likely to be seropositive for CMV than the control individuals (42% vs. 23%; odds ratio 2.1, $p = 0.009$) (Torrey *et al.*, 2006).

CMV IgG and Immunoglobulin M (IgM) antibody levels were compared in Germany by Leweke *et al*. (2004) for both the serum and CSF of 36 first episode, never treated individuals with schizophrenia, ten individuals with schizophrenia who were currently medication free but had been treated in the past, 39 individuals with recent onset

schizophrenia who were receiving medication and 73 unaffected control volunteers. For both the serum and CSF, CMV IgG antibody levels were significantly higher (serum $p < 0.001$; CSF $p < 0.004$) in the individuals with schizophrenia that had never been treated compared with the unaffected controls. Particularly noteworthy was the gradual decrease in antibody levels in both the serum and CSF from patients who were never treated to those who had previously been treated, to those currently receiving treatment, suggesting that the medication decreased the antibody response to CMV.

Valacyclovir, the orally administered L-valyl ester of acyclovir, is rapidly converted to acyclovir after ingestion. Acyclovir is converted to its monophosphate derivative by kinases encoded by several different herpesviruses but not by enzymes present in uninfected human cells. The monophosphate is further phosphorylated to the corresponding triphosphate by cellular enzymes, resulting in the inhibition of viral DNA synthesis and viral replication by a number of mechanisms. Clinical trials have documented that valacyclovir is effective in preventing infections due to *Herpes simplex virus type 1*(HSV-1), *Herpes simplex virus type 2* (HSV-2), and CMV in susceptible individuals (Ormrod *et al.*, 2000). Treatment with valacyclovir over 16 weeks can result in some degree of symptom improvement in individuals with schizophrenia who have serological evidence of *Cytomegalovirus* infection; no such improvement was seen for individuals who were seropositive for HSV-1, HSV-2, *Epstein-Barr virus* (EBV) or *Herpes simplex virus type 6* (HSV-6). The biological mechanisms of the clinical response to valacyclovir are not known with certainty but are likely to be related to its effect on the replication of *Cytomegalovirus*; however it was not possible to determine the effect of latent *Cytomegalovirus* infection on patient symptoms (Dickerson *et al*., 2003).

In another study celecoxib, a selective inhibitor of Cyclo-oxygenase-2 was administered in conjunction with risperidone in a double-blind trial of 50 patients with an acute exacerbation of schizophrenia. Those receiving the adjunctive celecoxib had a significant improvement in their symptoms, which was greatest between weeks 2 and 4 ($p = 0.001$) (Muller *et al*., 2002). It is known that CMV infection increases COX-2 expression (Martelius *et al.*, 2002) and inhibition of COX-2 blocks the replication of CMV (Zhu *et al*., 2002), this mechanism may

account for the improvement shown by patients in this study. Bipolar disorder is the least studied regarding association with CMV infection (Hinze-Selch, 2002). Torrey *et al.* (1982) evaluated serum from 178 individuals with schizophrenia, 17 individuals with bipolar disorder, and 41 controls using enzyme immune assay, and identified 11% (20 of 178) individuals with schizophrenia, 18% (3 of 17) bipolar disorder, and no controls had antibody to CMV.

1.2.7. Laboratory Diagnosis of CMV Infection

a. Serology

Serology is a very useful method to ascertain whether patients have a history of CMV infection or not. However, a significant rise in IgG or IgM titers was not observed before the onset of CMV syndrome, which means serology is not useful for the early diagnosis of CMV infection (Tanabe *et al.*, 1997). CMV IgM detection is a very sensitive marker for primary infection, but, unfortunately, it is not specific for primary infection. CMV IgM may be detectable for many months following primary infection and may also be produced following re-infection or reactivation. Likewise, detection of increasing CMV IgG levels over time is an unreliable approach for distinguishing primary from non-primary CMV infection, since most seropositive patients show high IgG levels in the first serum sample collected for testing. Measurement of CMV IgG avidity has proven to be a powerful tool for distinguishing primary from non-primary CMV infection. Defined as the strength with which the IgG attaches to antigen, IgG avidity matures with the length of time following primary infection. Thus, IgG produced within the first 3 to 5 months following primary infection exhibits low avidity, whereas IgG produced several months or years later exhibits high avidity (Prince and Leber, 2002). CMV IgG is produced early in primary infection and persists lifelong. The various methods used for detecting CMV IgG includes complement fixation test, immuno fluorescence test, latex agglutination, ELISA and Radio immune assay (Wong, 2008).

b. Virus isolation and cell culture

Urine, saliva, blood and biopsy samples can be used for virus isolation. Urine should be collected in a sterile container without additives. Saliva samples should first be soaked on to a swab which is then broken off into transport medium. Blood should be collected into a heparinzed bottle containing 500 units of heparin. Tissue biopsies should be placed in sterile plastic containers. The specimens can be treated in the different ways. Human embryo lung fibroblasts (HEL) are most commonly used for cell culture. The specimen is inoculated into HEL cells and kept for 28 days with a blind passage at 14 days. CMV produces a typical focal cytopathic effect (Figure 1.2) such as intranuclear inclusions and cytomegaly (Wong, 2008; Wills, 2009).

c. Detection of early antigen fluorescent foci (DEAFF)

This is a method used for the early diagnosis of CMV infection. In immunocompromised patients, a sensitivity of 78% and a specificity of 100% have been claimed. The specimen is inoculated into cell culture which is examined 24 hours later by immunofluorescence for expressed CMV encoded early proteins (Figure 1.2). The monoclonal antibodies must be able to cover most, if not all strains of CMV (Wong, 2008).

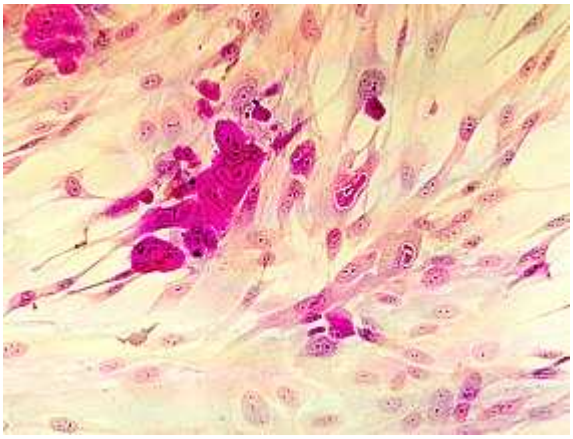
a  b 

Figure 1.2: Cytopathic effects of CMV in cell culture (a), CMV centrifugation culture and fixed and stained 16 hrs after inoculation showing viral proteins in nuclei of infected human fibroblast cells (b) (Wong, 2008).

d. Histopathology

The hallmark of CMV infection is the finding of intranuclear inclusions consistent with herpes virus infection. CMV infection may be confirmed using in situ hybridization or direct or indirect staining of intranuclear inclusions using CMV-specific antibodies linked to an indicator system (eg, horseradish peroxidase, fluorescein) (Tanabe *et al.*, 1997; Wills, 2009).

e. Electron microscopy

Virions in the urine of congenitally infected infants may be visualized by Electron microscopy in up to 80% of case. In immunocompromised individuals though, the viral titers are generally lower than neonates and other herpesviruses are often present in the urine (Tanabe *et al*., 1997).

f. Detection of CMV DNA by PCR

The use of PCR in the diagnosis of CMV infection had been widely studied. PCR offers the advantages of being rapid and sensitive. However, its inherent sensitivity poses a problem since latent CMV genomes, which are present in practically all seropositive individuals, may be detected. Therefore, it is critical to adjust the sensitivity of the PCR so that latent genomes are not detected (Tanabe *et al.*, 1997; Wong, 2008; Wills, 2009).

g. CMV antigenemia test

The test is based upon the detection of phosphoprotein 65 (pp65), a structural protein expressed on the surface of infected polymorphonuclear leukocytes. The number of infected leucocytes present had been reported to correlate with the severity of infection. The main advantage of this test is that it is very rapid so that a result can be available within the same day. As a result, this test is now widely used especially in the monitoring of transplant recipients (Tanabe *et al.*, 1997; Wills, 2009).

1.2.8. Treatment and Prevention of CMV Infection

The drug of choice for treatment of CMV disease is ganciclovir. Ganciclovir is a nucleoside analogue that inhibits DNA synthesis. CMV does not contain a thymidine kinase and the phosphorylation of ganciclovir to ganciclovir monophosphate is performed by UL97 protein

(Faulds and Heel, 1990; Markham and Faulds, 1994). Oral ganciclovir has serum levels 5-10 times less than Intravenous ganciclovir, making oral ganciclovir a less-than-optimal agent for the management of active CMV disease. Because of the low serum levels of the oral form, it has mainly been used for prophylaxis of CMV disease. The major adverse effects of ganciclovir therapy are neutropenia and thrombocytopenia. Neutropenia is managed by dose reduction and/or the addition of growth factors [i.e., granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF)] (Wills, 2009).

Preventive measures for CMV infection are primarily based on good personal hygiene, especially hand washing with soap and water after contact with diapers or oral secretions (particularly with a child who is in day care). Mothers advised to continue breastfeeding, since the demonstrated benefits of breast milk outweigh the minimal risk of acquiring CMV from the breastfeeding mother. Whenever tissue products used and blood transfusions performed, prior screening for CMV should be performed (Center of Disease Control and Prevention, 2008).

No vaccine is available for use in humans. Several candidate CMV vaccines have been developed and tested in clinical trials in humans, but none has proven safe and effective in preventing CMV infection or disease (Arvin *et al*., 2004).

1.2.9. Significance of the Study

Several evidences suggest that CMV may play an etiological role in some cases of schizophrenia and bipolar disorder. Epidemiologically, CMV infection and schizophrenia have a worldwide distribution and an increased prevalence in lower socioeconomic groups (Yolken and Torrey, 1995; Torrey *et al*., 2006). Studies have reported that some patients experiencing initial episodes of schizophrenia have increased levels of IgG to CMV, but not to other herpes viruses, in their sera and CSF (Leweke *et al*., 2004). Treatment with antipsychotic medications may result in a decrease in CMV antibodies, while treatment with anti-herpes virus and anti-inflammatory medications may reduce symptoms in some individuals with schizophrenia (Dickerson *et al*., 2003). There is also some overlap in the genes that are thought to operate in CMV infections and schizophrenia (Carter, 2008). The strongest argument against the role of CMV in schizophrenia is the absence of the traditional CMV neuropathological changes in the brains of individuals with schizophrenia; however, neuropathological studies of CMV have mostly been conducted in immune-compromised individuals (Torrey *et al.*, 2006).

In Ethiopia there were no studies that have examined the potential association of *Cytomegalovirus* infection and major psychotic episode. Thus, the present study was undertaken to elucidate the association of CMV infection and major psychotic episode by analyzing antibody to CMV from stored sera collected from individuals with schizophrenia or bipolar disorder, their close relatives and other healthy controls.

1.3. OBJECTIVES OF THE STUDY

General objective

- To determine the magnitude of CMV infection in individuals with schizophrenia, bipolar disorder, their close relatives, who are parents and siblings, and other healthy controls by using serologic diagnostic methods

Specific objectives

- To detect the presence of IgG antibody to CMV in individuals with schizophrenia, bipolar disorder, their close relatives and other healthy controls using ELISA in order to know the association between CMV infection and psychotic episodes.
- To compare the level of IgG antibody to CMV among individuals with schizophrenia, bipolar disorder, their close relatives and other healthy controls.

CHAPTER II: MATERIAL AND METHODS

2.1. Study design

The study is a case control study. Subjects were selected based on their disease status and controls are close relatives and other healthy individualists living in their neighborhood.

2.2. Study population and area

The study subjects were 216 individuals with schizophrenia, 199 individuals with bipolar disorder, 349 close relatives who are parents and siblings of the patients, and other 80 healthy controls from their neighborhood. The study subjects were recruited from Meskan and Mareko (Butajira) district, 135 km south of Addis Ababa. The district had a population of 227,135 during the 1994 censes. Forty five percent the population, over 100,000, belongs to the age group between 15 and 49 years at the time of sample collection (Office of Population and Housing Census Commission (OPHCC), 1994). Blood was collected for previous genetic study (NERC approved RDHE/59042/2002, February 4, 2002 and RDHE/33-79/2007). The genetic study is on progress at Institute of Psychiatry, London. The stored sera were analyzed for antibodies to infectious agents: CMV, HSV-1, HSV-2 and *Toxoplasma gondii*. This study focused only on analysis of sera for CMV infection from March to May of 2009.

Demographic data and other relevant information about the study participants were retrieved from stored information from genetic study (Appendix I).

Working definitions

a. **Individual with schizophrenia:** Individual who fulfill the criteria for schizophrenia on structured interview described by diagnostic and statistical manual of mental disorder fourth edition (DSM-IV)

b. **Individual with bipolar disorder:** Individual who fulfill the criteria for bipolar disorder on structured interview described by DSM-IV

c. **Relatives:** Healthy parents or siblings (with respect of mental disorder) of individuals with schizophrenia or bipolar disorder

d. **Healthy control**: Healthy neighborhoods (with respect of mental disorder) of individuals with schizophrenia or bipolar disorder

2.3. Sample collection, handling and transport

No new blood samples were collected in this study. Frozen serum, from blood collected from informed and consented study subjects from the previous study had been used. Sera were obtained from the blood by centrifugation. All sera were aliquoted and stored at -20^{o}C at Armauer Hansen's research institute.

2.4. Serum analysis

VIR-ELISA ANTI-CMV-IgG ELISA test kits (Viro-Immuno Labor-Diagnostica GmbH, Germany) was used for qualitative and semi-quantitative determination of IgG class antibody to CMV in sera collected from the study subjects. Serological assay was done at AHRI where the sera were kept frozen.

The purified, homogeneous antigen is fixed to each well of the micro-titer strips.100µl of diluted patient sample (1:101with ready to use sample diluents) is added to the well, any specific antibodies present in the patient's sample are bound during the first incubation (18-25^{o}C for 30 minutes). After removing unbound material by washing four times with wash buffer, the presence of specific antibodies is detected using Anti-human IgG/IgM/IgA peroxidase conjugate during the second incubation (18-25^{o}C for 30 minutes). Excess peroxidase conjugate is then removed by washing four times and tetra-methyl benzidine (TMB) substrate is added and incubated at 18-25^{o}C in the dark for 10 minutes, this result in the development of a blue color. The enzyme reaction is terminated by the addition of a stop solution (100µl of 0.95N H_2SO_4). The intensity of the yellow color developed, when it is read within 10 minutes, is proportional to the concentration of antibodies in the sample (IBL-America, 2007). The absorbance of the well contents is read at 450nm wave length on Multiskane EX reader (Thermo Lab systems Vantaa, Finland).

2.5. Qualitative results calculation

Cut-off value for each micro titer plates was calculated by addition of the absorbance of Negative control and Cut-off control. The ratio of the absorbance of serum samples to the

Cut-off value defines the Cut-off Index (COI). COI > 1.1 reported as positive, COI < 0.9 reported as negative, and COI values between and equal to 0.9 and 1.1 reported as equivocal and need retesting (IBL-America, 2007)

2.6. Quantitative antibody level normalization

Case and control samples were tested on the same plates. But over the course of the study, samples were assayed on 12 different plates. To control for potential systematic error introduced by plate-to plate variation and to ensure that observed differences in optical density (OD) are due to differential expression and not experimental artifacts, data were normalized using the robust median normalization method which combines the within-plate and between-plates variance using the following equation (Wit and McClure, 2004)

$$S_{ijk} = (R_{ijk} - M_k)/\sqrt{(V_k + V_b)}$$

Where R_{ijk} is the raw OD of i^{th} subject's j^{th} blood sample, M_k is the median of all the control samples in plate k, V_k is the variance of R_{ijk} of control samples in plate k, and V_b is the variance of all R_{ijk} between plates. Therefore, S_{ijk} is the scaled score for the i^{th} subject's j^{th} blood sample on plate k.

2.7. Statistical analysis

Each demographic, clinical and laboratory data were entered to Microsoft® office Excel 2007 (© 2006 Microsoft® corporation) and exported to SPSS 15.0 for Windows® (© 2006 SPSS Inc, Chicago, Illinois, USA) for analysis. Logistic regression was used for cross tabulated data and linear regression for comparing antibody level among groups. All test of significance was two tailed and, $p < 0.05$ is considered significant.

2.8. Ethical consideration

Informed consent to use the stored serum had been obtained by field workers from the study subjects in whom blood sample were collected in 2001 (Appendix II and III). For those study subjects who could not consent, because of mental disorder or age consent taken from their parents or guardians (Appendix IV and V). There was not any direct contact between the study subjects and investigator. Results of this analysis have not been linked to the study

subjects' identification in any form (Appendix I). The ethical clearance for using the stored serum was already obtained from the Faculty Research Publication Committee-I (FRPC-I), Faculty of Medicine, Addis Ababa University (December 14, 2007), AHRI/ALERT Ethical Review Committee and (December 21, 2007) and the National Ethical Clearance Committee (NERC) from Ethiopian Science and Technology Agency (Ref no. RDHE/15-84/2008, 29 September 2008) (Appendix VI). The current M.Sc. research project proposal was approved by the Department Research and Ethical Review Committee, Department of microbiology, immunology and parasitology, and subsequently by Institutional Review Board (IRB), Faculty of Medicine, Addis Ababa University (AAU) (March 04, 2009) (Appendix VI) as a formal procedure.

CHAPTER III: RESULTS

3.1. Demographic characteristic of study subjects

Two hundred and sixteen individuals with schizophrenia and their 201 close relatives; 199 individuals with bipolar disorder and their 148 close relatives; and other 80 healthy controls were included in the study. 222/349 (63.6%) of relatives of individuals with schizophrenia and bipolar disorder were parents and the rest were siblings. Patients were chronically mentally ill (average 15 years) and on antipsychotic medication for variable period of time. Age and sex distribution of study subjects is presented in Figure 3.1. 504/844 (59.7%) were males, resulting in overall male to female ratio of 1.5:1. Age of subjects ranged from 15 to 80 years (mean 38.1).

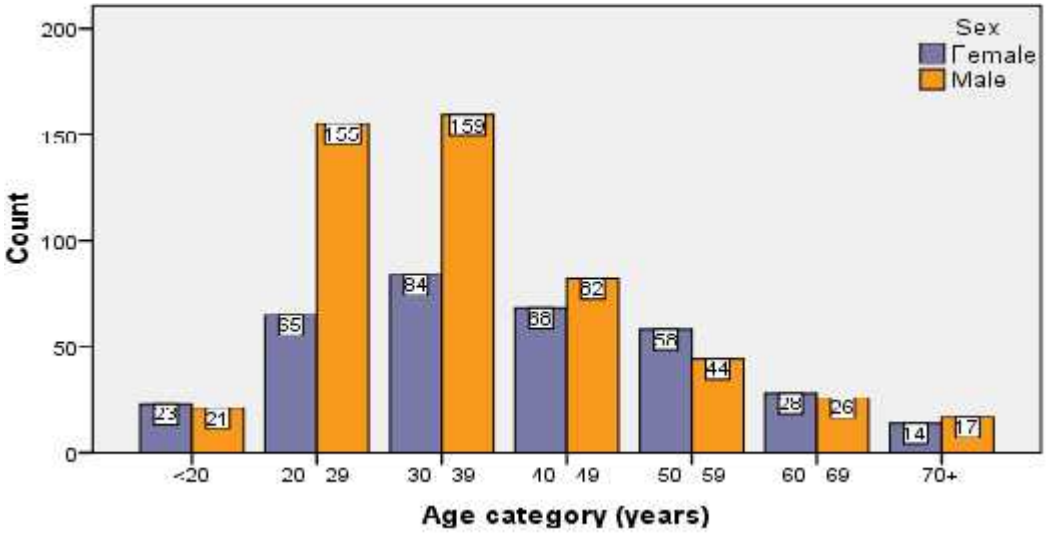


Figure 3.1: Age and sex distribution of study subjects (n=844) (Butajira study on course and outcome of schizophrenia and bipolar disorder, 2009)

Of the 216 individuals with schizophrenia, 173 (80.1%) were males, making male to female ratio of 4:1 and patients age ranged from 17 to 51years (mean 32.7). 104/201 (51.7%) of close relatives of individuals with schizophrenia were males, resulting in male to female ratio of 1.1:1. Age of relatives ranged from 15 to 80 years (mean 48.1). The mean age of individuals with schizophrenia and their close relatives differ significantly after adjusting for

sex [β = -15.1; 95% CI (-17.6, -12.5)]. Age distribution of individuals with schizophrenia and their close relatives is presented in Figure 3.2.

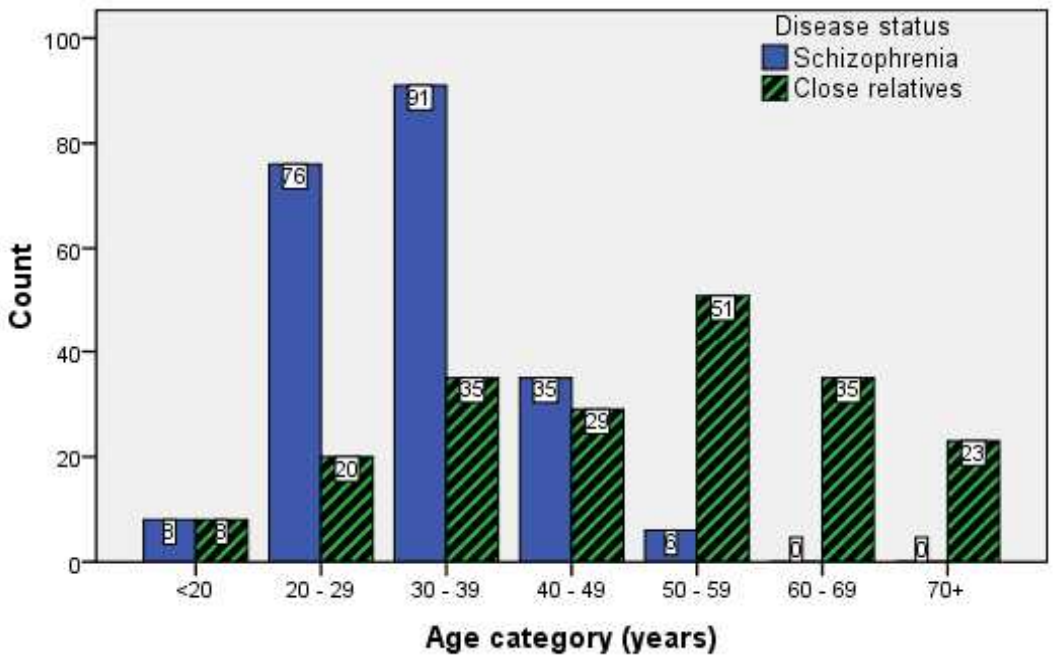


Figure 3.2: Age distribution of individuals with schizophrenia and their close relatives (n=417) (Butajira study on course and outcome of schizophrenia and bipolar disorder, 2009)

Of the 199 individuals with bipolar disorder, 105 (52.8%) were males making male to female ratio of 1.1:1 and patients age ranged from 16 to 50 years (mean 31.6). 79/148 (53.4%) of close relatives of individuals with bipolar disorder were females, resulting in male to female ratio of 0.9:1 and relatives age ranged from 15 to 78 years (mean 45.3). The mean age of individuals with bipolar disorder and their close relatives differ significantly after adjusted for sex [β = -13.6; 95% CI (-16.0, -11.2)]. Age distribution of individuals with bipolar disorder and their close relatives is presented in Figure 3.3.

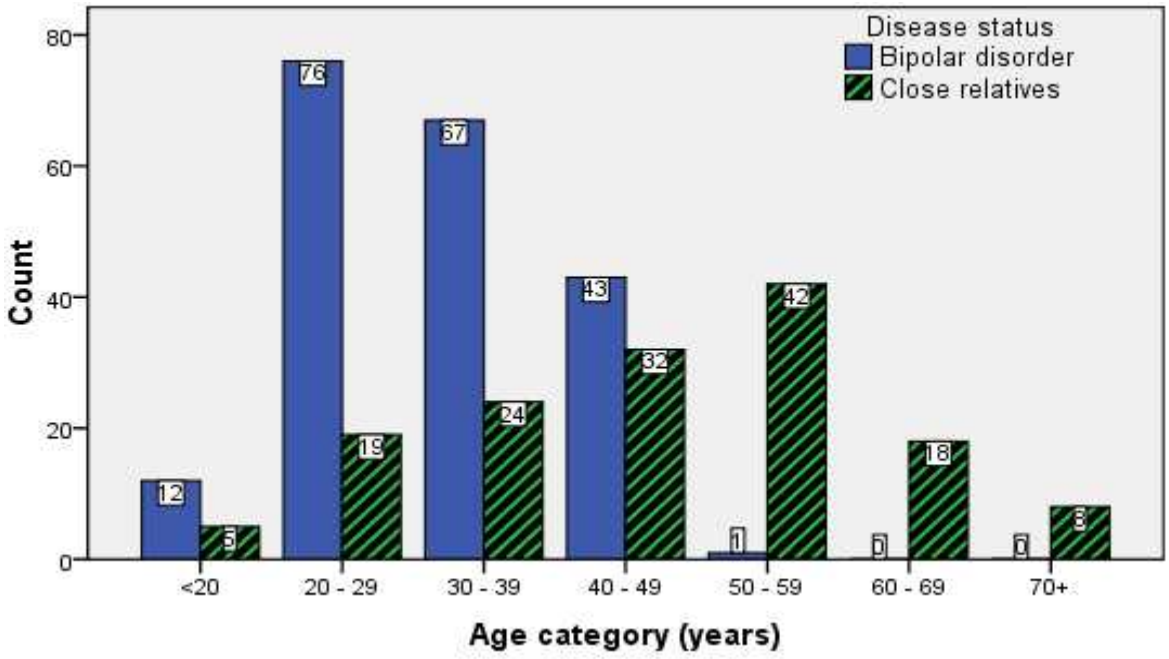


Figure 3.3: Age distribution of individuals with bipolar disorder and their close relatives (n=347) (Butajira study on course and outcome of schizophrenia and bipolar disorder, 2009)

53/80 (66.2%) of non-relative healthy controls were males resulting in male to female ratio of 2:1. The age of healthy controls ranged from 15 to 60 years (mean 30.4). The mean age of individuals with schizophrenia or bipolar disorder, and non-relative healthy controls did not differ significantly [β = 0.3; 95% CI (-0.8, 1.3)]. Age and sex distribution of non-relative healthy controls is presented in Figure 3.4.

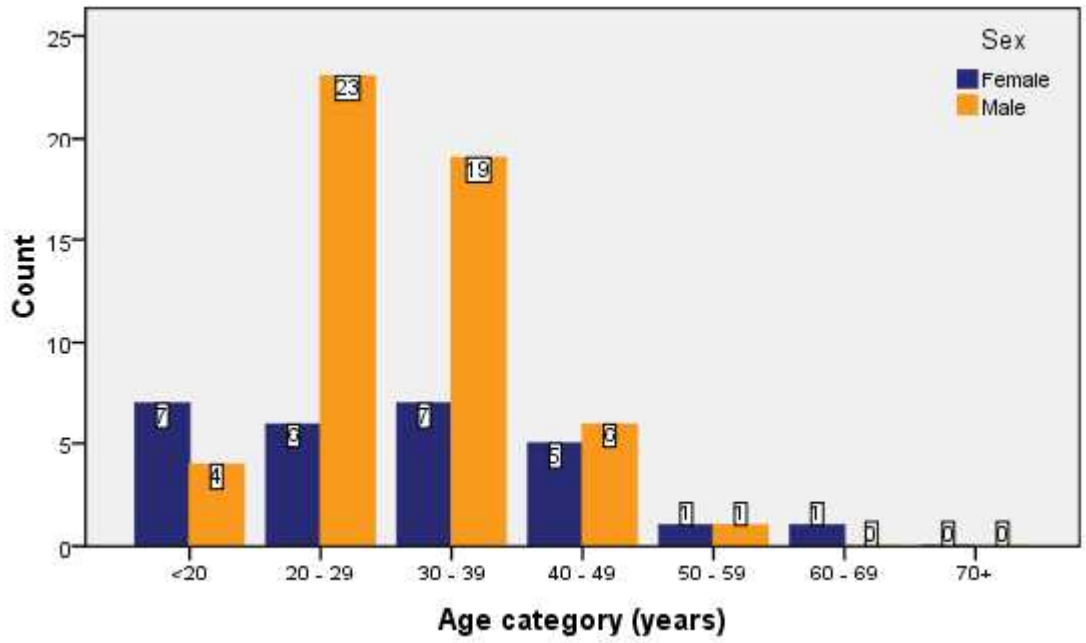


Figure 3.4: Age and sex distribution of non-relative healthy controls (n=80) (Butajira study on course and outcome of schizophrenia and bipolar disorder, 2009)

3.2. Seroprevalence of CMV infection among study subjects

A total of 844 serum samples, one from each study subject, were analyzed using ELISA for IgG to CMV, 838/844 (99.3%) of sera were positive for the antibody. Seropositivity among male and female study subjects were 99% (499/504) and 99.7% (339/340) respectively, and the prevalence did not differ significantly between the two sex groups [odds ratio = 0.29; 95% CI (0.03, 2.53)]. Seroprevalence of IgG to CMV among individuals with schizophrenia or bipolar disorder, close relatives and other healthy controls is presented on Table 3.1. Seroprevalence across the different age groups of study subjects did not vary significantly and near or equal to one hundred percent [odds ratio = 1.52; 95% CI (0.77, 3.03)].

Two hundred fifteen of two hundred sixteen (99.5%) of individuals with schizophrenia, 198/201 (98.5%) of their close relatives and 80/80 (100%) of other healthy controls were seropositive for CMV infection as shown in Table 3.1; comparison across the groups did not feature any statistical significant difference [odds ratio = 1.05; 95% CI (0.27, 4.05)].

Individuals with bipolar disorder and their close relatives presented with seroprevalence of 99.5 and 99.3% respectively (Table 3.1), and comparing these values to seroprevalence in healthy controls (100%) did not exhibit statistical significant difference [odds ratio = 0.65; 95% CI (0.08, 5.09)].

Table 3.1: Seroprevalence of IgG to CMV among individuals with schizophrenia or bipolar disorder, their close relatives and other healthy controls n=844 (Butajira study on course and outcome of schizophrenia and bipolar disorder, 2009)

Seroprevalence of IgG to CMV among study subjects, No. positive (%)						
Age group (years)	Patients with Schizophrenia	Relatives of patients with schizophrenia	Patients with bipolar disorder	Relatives of patients with bipolar disorder	Non-relative healthy controls	**Total**
< 20	8/8 (100.0)	8/8 (100.0)	12/12 (100.0)	5/5 (100.0)	11/11 (100.0)	44/44 (100.0)
20-29	75/76 (98.7)	18/20 (90.0)	75/76 (98.7)	19/19 (100.0)	29/29 (100.0)	216/220 (98.2)
30-39	91/91 (100.0)	34/35 (97.1)	67/67 (100.0)	24/24 (100.0)	26/26 (100.0)	242/243 (99.6)
40-49	35/35 (100.0)	29/29 (100.0)	43/43 (100.0)	32/32 (100.0)	11/11 (100.0)	150/150 (100.0)
50-59	6/6 (100.0)	51/51 (100.0)	1/1 (100.0)	41/42 (97.6)	2/2 (100.0)	101/102 (99.0)
60-69	-	35/35 (100.0)	-	18/18 (100.0)	1/1 (100.0)	54/54 (100.0)
≥ 70	-	23/23 (100.0)	-	8/8 (100.0)	-	31/31 (100.0)
Total	215/216 (99.5)	198/201 (98.5)	198/199 (99.5)	147/148 (99.3)	80/80 (100)	838/844 (99.3)

3.3. Level of IgG to CMV in seropositive study subjects

Out of 844 serum samples, 838 were positive for CMV infection with overall scaled score of mean antibody level of 1.51. Female study subjects presented with higher serum antibody level than males after adjusting for age [β = -0.27; 95% CI (-0.36, -0.17). Study subjects' age was positively correlated with serum level of IgG to CMV ($R = 0.21$; $P < 0.001$) (Figure 3.5).

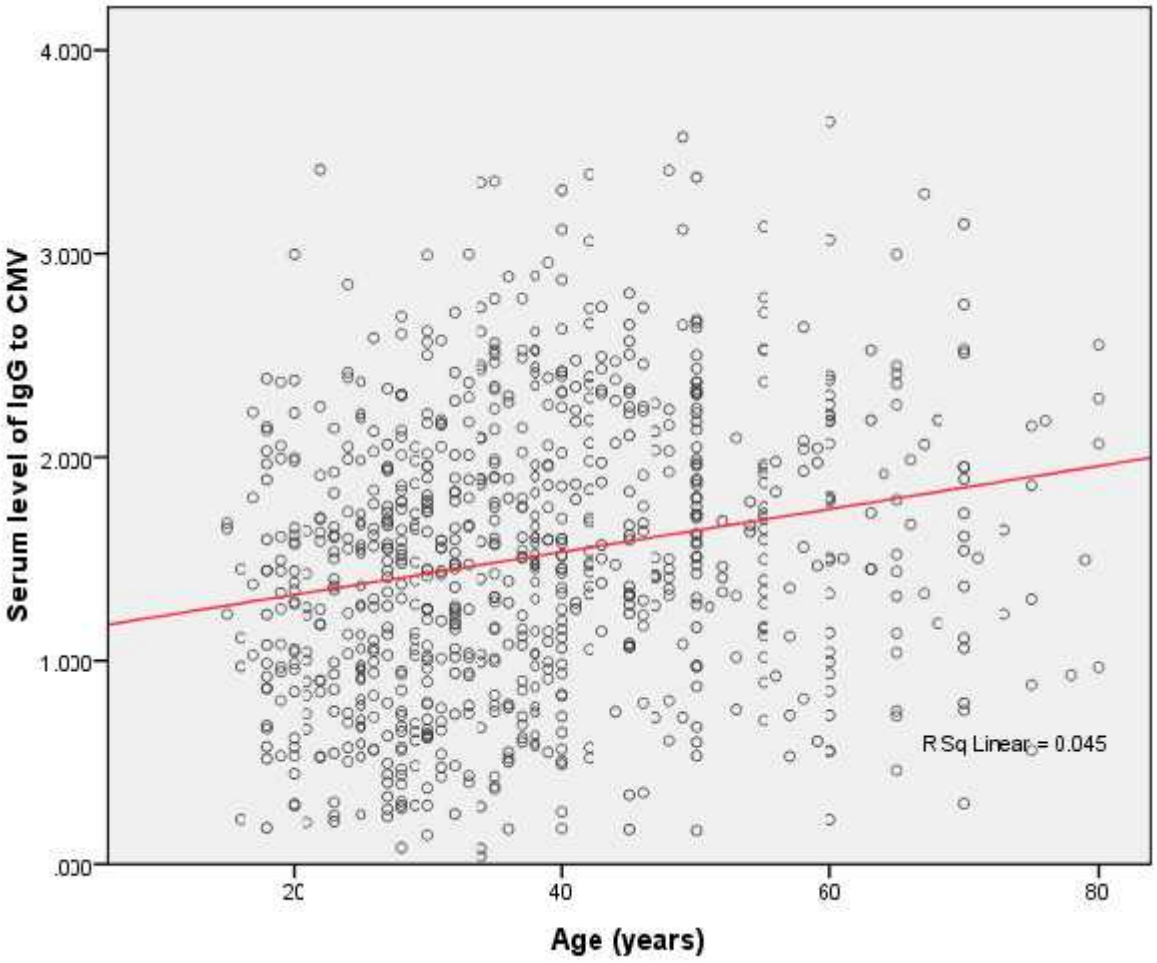


Figure 3.5: Distribution of scaled score of level of IgG to CMV infection with respect of study subjects' age, n=838 (Butajira study on course and outcome of schizophrenia and bipolar disorder, 2009)

3.4. Level of IgG to CMV in seropositive individuals with schizophrenia and controls

After normalizing the recorded optical density, the mean scaled score of serum IgG level in individuals with schizophrenia, their close relatives' and other healthy controls was calculated (Table 3.2). The mean scaled score of antibody level in female and male individuals with schizophrenia were 1.64 and 1.43 respectively; however, the level did not vary significantly [β = -0.21; 95% CI (-0.43, 0.01)]. In addition patients' age and mean scaled score of serum IgG level was positively correlated (R= 0.2; p = 0.005).

On the other hand, female close relatives of individuals with schizophrenia scored higher mean antibody level than their male counterparts after adjusting for age [β = 0.30; 95% CI (0.11, 0.48)]. As relatives' age increased, serum antibody level was also showed to increase [β = 0.01; 95% CI (0.00, 0.01)]. Comparison of the scaled score of serum antibody level in individuals with schizophrenia and their close relatives revealed that, relatives presented with higher score than patients; however, this association was vanished after adjusting for age and sex [β = 0.09; 95% CI (-0.06, 0.25)]. In addition, both male and females individuals with schizophrenia and respective close relatives scored comparable antibody level [Males: β = -0.02; 95% CI (-0.19, 0.14)], [Females: β = -0.12; 95% CI (-0.36, 0.11)].

Comparison of scaled score of serum antibody level in individuals with schizophrenia and non-relative healthy controls showed that, individuals with schizophrenia presented with higher serum antibody level than healthy controls after adjusting for age and sex [β = 0.21; 95% CI (0.03, 0.38)] (Table 3.2).

Table 3.2: Mean scaled score of IgG to CMV in individuals with schizophrenia and controls (Butajira study on course and outcome of schizophrenia and bipolar disorder, 2009)

Seropositive study subjects (No.)	**Mean scaled score of IgG level (SD)**	**β (95% CI)***
Individuals with schizophrenia (215)	1.47 (0.66)	-
Close relatives of individuals with schizophrenia (198)	1.60 (0.68)	0.09 (-0.06, 0.25)**
Non-relative healthy controls (80)	1.26 (0.69)	0.21 (0.03, 0.38)**

* β was calculated in comparison to individuals with schizophrenia

** β adjusted for age and sex

The mean IgG level in individuals with schizophrenia younger than 20 years of age was significantly higher than matched non-relative controls after adjusting for sex [β = 0.64; 95% CI (0.10, 1.19)]. Other age groups also showed that individuals with schizophrenia tended to have higher IgG level; though, the difference was not statistically significant (Table 3.3).

Table 3.3: Mean scaled score of IgG to CMV in individuals with schizophrenia and non-relative healthy controls (Butajira study on course and outcome of schizophrenia and bipolar disorder, 2009)

Age group in years	Disease status (No.)	Mean scaled score of IgG level (SD)	β (95% CI)
< 20	Schizophrenia (8)	1.69 (0.55)	0.64 (0.10, 1.19)*
	Healthy control (11)	1.05 (0.52)	
20-29	Schizophrenia (75)	1.29 (0.59)	0.13 (-0.14, 0.40)
	Healthy control (29)	1.16 (0.69)	
30-39	Schizophrenia (91)	1.51 (0.65)	0.13 (-0.17, 0.43)
	Healthy control (26)	1.38 (0.77)	
40-49	Schizophrenia (35)	1.66 (0.78)	0.40 (-0.13, 0.94)
	Healthy control (11)	1.25 (0.71)	
50-59	Schizophrenia (6)	1.69 (0.69)	0.11 (-1.17, 1.38)
	Healthy control (2)	1.58 (0.25)	

* β adjusted for sex

3.5. Level of IgG to CMV in seropositive individuals with bipolar disorder and controls

The mean scaled score of serum antibody level calculated for individuals with bipolar disorder was 1.51. Females with bipolar disorder presented with higher mean score than male counterparts after adjusting for age [β = -0.30; 95% CI (-0.49, -0.12)]. In addition serum antibody level was positively correlated with patients' age (R = 0.23; p = 0.001). On top of that, female relatives of individuals with bipolar disorder also presented with higher IgG level than male counterparts after adjusting for age [β = -0.30; 95% CI (-0.53, -0.07)].

Mean scaled score of serum antibody level in individuals with bipolar disorder was comparable to their close relatives' score [β = -0.11; 95% CI (-0.26, 0.04)].

In comparison to non-relative healthy controls, individuals with bipolar disorder presented with higher score of antibody level after adjusting for age and sex [β = 0.20; 95% CI (0.02, 0.37)] (Table 3.4).

Table 3.4: Mean scaled score of IgG to CMV in individuals with bipolar disorder and controls (Butajira study on course and outcome of schizophrenia and bipolar disorder, 2009)

Seropositive study subjects' status (No.)	**Mean scaled score of IgG level (SD)**	**β (95% CI)***
Individuals with bipolar disorder (198)	1.51 (0.69)	-
Relatives of individuals with bipolar disorder (147)	1.62 (0.71)	-0.11 (-0.26, 0.04)
Non-relative healthy controls (80)	1.26 (0.69)	0.20 (0.02, 0.37)**

* β was calculated in comparison to individuals with bipolar disorder

** β adjusted for age and sex

CHAPTER IV

DISCUSSION

In the course of developing or after having had some infectious diseases, clinical symptoms of psychosis may be observed (Yolken and Torrey, 2008); such observations raised speculation regarding a possible infectious cause of schizophrenia and bipolar disorder. In recent years, there have been reports suggesting that *Cytomegalovirus*, *HSV-1*, *HSV-2*, and *Toxoplasma gondii* may be etiologically important in schizophrenia (Leweke *et al.*, 2004; Torrey *et al.*, 2006; Amminger *et al.*, 2007; Niebuhr *et al.*, 2008a; Yolken and Torrey, 2008). CMV is neurotrophic and has an affinity for limbic system, one of the areas of the brain thought to be affected in schizophrenia (Torrey and Peterson, 1974). Both CMV infection and schizophrenia have a worldwide distribution and have an increased prevalence in lower socioeconomic groups (Yolken and Torrey, 1995; Torrey *et al.*, 2006).

Studies conducted regarding the association of CMV infection and major psychotic episode so far came with inconsistence findings, and majority of them involved small number of patients and controls. The present study addressed the burden of CMV infection in individuals with schizophrenia, bipolar disorder and healthy controls and tried to clarify some of the ambiguity seen on previous studies. Results from such studies will greatly help in understanding of the etio-pathogenesis of major psychotic episodes and formulating new therapeutic approaches.

In the present study, the mean age of individuals with schizophrenia and bipolar disorder was significantly lower than their respective relatives (Figure 3.2 and 3.3). This was due to majority of relatives were parents. Larger proportion of individuals with schizophrenia in this study were males, this partly explained by males affected earlier in life and more severely than females by schizophrenia (Yolken and Torrey, 1995) and partly by the non-random nature of sample selection.

In the present study, 99.3% of study subjects were seropositive for CMV infection (Table 3.1). The result is comparable with community based studies conducted in Israel (Sarov *et al.*, 1982) and Saudi Arabia (Ashraf *et al.*, 1985) which showed nearly 100% of the

population were infected with CMV. However, our finding was higher than 47.6% seroprevalence rate documented in individuals at ultra-high risk for psychosis *(*Amminger *et al.,* 2007) and community based study in USA which reported 40% infection rate (Staras *et* al., 2006). Our finding was also higher than 77.6% seroprevalence rate reported in HIV-negative blood donors in Ghana (Adjei *et al.,* 2008).

Seroprevalence of 99.5% reported in individuals with schizophrenia in the present study (Table 3.1) was higher than 52.3% and 35.3% (Dickerson *et al.,* 2006) reported for individuals with deficit and non-deficit schizophrenia respectively. Our finding was also higher than 35.9% seroprevalence reported in individuals with schizophrenia in Pittsburgh, USA (Shirts *et al.,* 2008). Seropositivity for CMV infection reported in individuals with bipolar disorder in our study also higher than 18% seroprevalence documented in individuals with bipolar disorder (Torrey *et al*., 1982).

Seroprevalence of CMV infection documented in individuals with schizophrenia, bipolar disorder, their close relatives and other healthy controls did not demonstrated significant difference (Table 3.1). This is in contrary to Torrey *et al.* (2006) observations of significantly more patients than control individuals had antibodies to CMV (Chi-square 10.29; $p = 0.001$) but not to other herpes viruses, when they evaluate 86 patients with schizophrenia, 29 of whom were experiencing their first episode, and 85 unaffected control individuals recruited from the general population in Germany. However, CMV is a ubiquitous agent in most part of the world and most people are infected with CMV at some point in life (Wills, 2009). Thus, difference based on qualitative measures could be difficult to elicit (Niebuhr *et al.,* 2008a) and variation in antibody level between population groups is considered more reliable. Recent studies compared patients and controls sera based on scaled score of antibody level derived from normalization of the recorded optical density (Leweke *et al.,* 2004; Niebuhr *et al.,* 2008a). We also used the same method for quantitative determination of IgG level.

In the present study, female study subjects presented with higher antibody level than male counter parts. This is line with the higher risk of acquiring new and recurrent CMV infection in females due to their major involvement in care of preschool children (Fowler and Pass,

2006). Seroprevalence study conducted in USA also reported higher prevalence in females than males (Staras *et al*., 2006). We also found that serum antibody level steadily increased as the age of study subjects increases (Figure 3.5), this may resulted from recurrent exposure to CMV with age, due to ubiquity of the agent (Wills, 2009).

The relative higher scaled score of anti-CMV IgG level seen on close relatives of individuals with schizophrenia may resulted from the higher mean age of relatives than individuals with schizophrenia (48.1 vs. 32.7 years respectively). However further stratified analysis based on age and sex vanished the observed significant difference (Table 3.2). In contrast, individuals with schizophrenia presented with higher scaled score of antibody level than non-relative healthy controls (Table 3.2). This difference was also maintained in both sexes and younger individuals with schizophrenia. In older age groups also individuals with schizophrenia tended to have higher antibody level (Table 3.3). In the present study, the healthy controls were well matched with the patients in terms of socioeconomic and geographical variables, making it unlikely that the difference was related to differential exposure to CMV. Our findings are in consistence with Torrey *et al*. (2006) observation of 34 patients with a first episode of schizophrenia, schizoaffective disorder or psychosis not otherwise specified and 27 unaffected matched controls. A significantly higher proportion of patients had antibodies against CMV, and the reactivity of the patients' sera, as measured by enzyme immunoassay in optical density, was significantly stronger than that of control individuals. Our finding is also in line with Srikanth *et al*. (1994) report of elevated CMV antibody levels in 6 of 35 individuals with an onset of psychosis within the previous month compared with 35 controls undergoing minor surgical procedures.

We also found that higher level of IgG to CMV in individuals with bipolar disorder in comparison to non-relative healthy controls (Table 3.4). However, there is only limited number of studies done regarding association of CMV infection and bipolar disorder, and majority of them did not consider difference in level of antibody. One such study reported that 18% of patients with bipolar disorder and no controls had antibody to CMV (Torrey *et al.,* 1982). Some researchers believe that bipolar disorder and schizophrenia to be a spectrum of a disorder and appear to share many similarities in the antecedents of the

disorders, such as susceptibility genes on similar chromosomes and an excess of winter-spring births (Torrey and Knable, 1999). Our finding of possible common infectious risk factor for both schizophrenia and bipolar disorder may be the extension of such observations.

In the present study, all patients had been chronically mentally ill and on antipsychotic medication for variable period of time. The effect of chronicity of the illness and antipsychotic medication on the immune system was not controlled in the final analysis. The observed higher level of IgG to CMV in younger patients with schizophrenia may be due to relative short duration of illness and antipsychotic medication use in this age group. Dysfunction of the immune system in individuals with serious mental illnesses had been reported, including immune hyporeactivity as demonstrated by a diminished cutaneous response to exogenous intra-dermal antigens. This suggested that immune system dysfunction may commenced even prior to the use of antipsychotic medications, which have made such research more difficult (Yolken and Torrey, 1995).

Antipsychotic medication may also suppress antibody response. Leweke *et al*. (2004) in Germany demonstrated such an effect by comparing CMV IgG and IgM antibody levels for both the serum and CSF of 36 first-episode, never-treated individuals with schizophrenia, ten individuals with schizophrenia who were currently medication-free but had been treated in the past, 39 individuals with recent onset schizophrenia who were receiving medication and 73 unaffected control volunteers. For both the serum and CSF, CMV IgG antibody levels were significantly higher in the individuals with schizophrenia that had never been treated compared with the unaffected controls. Particularly noteworthy was the gradual decrease in antibody levels in both the serum and CSF from patients who were never treated to those who had previously been treated, to those currently receiving treatment, suggesting that the medication decreased the antibody response to CMV.

LIMITATION OF THE STUDY

All patients in this study were chronically mentally ill and on antipsychotic medication for variable period of time, therefore the effect of chronicity of the illness and antipsychotic medication on antibody response could not be determined. In addition, the number of non-relative healthy controls included in the present study was only eighty.

CONCLUSION AND RECOMMENDATIONS

The current study showed that CMV infection is ubiquitous among individuals with schizophrenia, bipolar disorder and healthy controls, and eliciting association with psychotic episode based on qualitative measures is difficult. However, quantitative methods showed significantly higher level of IgG to CMV in individuals with schizophrenia, especially in the younger age group. Individuals with bipolar disorder also presented with higher antibody level compared to healthy controls.

Thus, the current study provided immunological evidence that infection with *Cytomegalovirus* may contribute to the etio-pathogenesis of some cases of schizophrenia and bipolar disorder. Additional studies should be directed at the further analysis of antibodies to *Cytomegalovirus* in the sera and CSF of individuals with recent onset of psychosis as well as at the direct detection of nucleic acids and proteins derived from CMV in these samples. The identification of CMV is of particular importance since antiviral agents are available which can inhibit the replication of the virus within the CNS. This will result in development of new modalities for the prevention and treatment of schizophrenia and bipolar disorder in a variety of patient populations.

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Appendix I: Data collection format for detection of antibodies to CMV infections from sera collected from individuals with schizophrenia, bipolar disorder and healthy Controls

I.	General Information
Code	001. Age _____ (yrs) 002. Sex _____ 003. Address _____
II.	Clinical Data
004.	History of mental illness 1. Yes 2. No
If yes	
005.	Date of diagnosis of mental illness (DD/MM/YY)_____(E.C.)
006.	Diagnosis of mental illness 1. Schizophrenia 2. Bipolar disorders 3. Others
007.	Duration of antipsychotic treatment_____(months)
III.	Laboratory Data
008.	ELISA-Serology for anti-cytomegalovirus IgG antibody 1. Positive 2. Negative 3. Borderline/Intermediate 4. Titer _____

Appendix II: Information sheet for study subjects (English version)

It is remembered that you volunteered to participate in a genetic study about 6 years ago. Part of the blood you gave during that study was stored at AHRI laboratory in Addis Ababa. In this particular study we would like to use the serum, the liquid/watery, part of the blood you gave during that study for an etiological research. The serum has been stored for so long because your consent to use it for this purpose was so important.

a. **Purpose**: The purpose of this study is look for possible infectious etiological agents.

b. **Duration**: We will use the serum only once for this particular study and will not use for anything else.

c. **Procedures to be carried on**: We will do an investigation for toxoplasmosis, CMV and HSV type I and II at AHRI laboratory

d. **Risks associated with the study**: There is no risk of using the serum to you.

e. **Benefits of the study**: There will be no financial or other direct benefit to you. But your consent for us to use the stored serum sample to test for body defense elements against the diseases mentioned above will help us better understand if the diseases have any relationship to schizophrenia and bipolar disorders.

f. **Compensations**: There will be no compensation for using this serum.

g. **Confidentiality of your information**: The results of the lab findings will be kept confidential and could only be accessed by the researchers. There will be no personal information to be attached to your data.

h. **Termination of the study**: We will respect your decision if you later on change your mind and inform us not to use your sample for the test. Your withdrawal of consent will not affect your right to receive medication you used to get free from the project or continue in the cohort.

I would also like to inform you that this study is approved by the FRPC-I of the Medical Faculty, Addis Ababa University and the Ethiopian Science and Technology Agency, Ethical Review Committees. Their address is

1. Faculty of Medicine Addis Ababa University

   Office of Associate Dean, Postgraduate Programs and Research

   P.O. Box 9086. Addis Ababa, Ethiopia

   Tel. 251-011-551-28-765

2. Ethiopian Science and Technology Agency
   P.O.Box. 2490

   Tel. 251-011-551-13-44

   E-mail: estc@ethionet.et

The address of the principal investigator is: Yared Tedla, MD

Department of Microbiology, Immunology and Parasitology

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P.O.Box. 9086, Addis Ababa, Ethiopia

Tel: +251-091-1407021

E-mail: yared1972@yahoo.com

Appendix II: Information sheet for study subjects (Amharic version)

<u>የመረጃ መስጪያ ቅፅ</u>

ከ 6 ዓመት በፊት ለዘር ሐረግ ጥናት ለመሳተፍ ፈቃደኛ ሆነው የደም ናሙና የሰጡ መሆኑ ይታወቃል፡፡ በወቅቱ ከተወሰደው የደም ናሙና ላይ ፈሳሻማውን ክፍል በአዲስ አበባ አለርት ሆስፒታል ላቦራቶሪ አኑረነዋል፡፡ በዚህ በአሁኑ ጥናት የደም ናሙናዎን ፈሳሽ ወይም ውሃማ ክፍል የአእምሮ ሕመም መነሻ ምክንያት ጥናት ልናደርግበት አስበናል፡፡

ሀ. <u>የጥናቱ አላማ</u>፡-ይህ ጥናት ለአእምሮ ሕመም ምክንያት ሊሆኑ የሚችሉ ኢንፌክሽኖች ካሉ ለማወቅ ይረዳናል፡፡

ለ. <u>የሚፈጀው ጊዜ</u>፡- ይህንን ናሙና ለአንድ ጊዜ ብቻ ለዚህ ጥናት እናውለዋለን፡፡ ከዚህ የተለዬ ለምንም ጉዳይ አንጠቀምበትም፡፡

ሐ. <u>አጠቃቀሙም</u>፡- ቶክሶፕላዝማ ፣ሳይቶመጋሎ እና ሔርፐስ የተባሉ ቫይረስ ለማግኘት የሚረዳ የላቦራቶሪ ምርመራ እናደርግበታለን፡፡

መ. <u>ሊደርስ የሚችል አደጋ</u>፡-ይህንን ናሙና በመጠቀማችን በርሶዎ ላይ የሚደርስ ምንም ዓይነት አደጋ የለም፡፡

ሠ. <u>የሚያገኙት ጥቅም</u>፡- ይህንን ናሙና በመጠቀማችን የምንሰጥዎ ምንም አይነት የገንዘብና ቁሳቁስ ጥቅም አይኖርም፡፡ ይሁን እንጂ በላቦራቶር ተቀምጦ የሚገኘው የደም ናሙናዎን ፈሳሽ ወይም ውሃማ ክፍል ላይ የሰውነት መከላከያ አካል የሆኑ ንጥረ ነገሮች እንዳሉ ለማየት የርሶ ፈቃድ አስፈላጊ ከመሆኑም በላይ ሺዞፍሬኒያና ባይፖላር ዲስኦርደር የተባሉ የአእምሮ ህመም አይነቶች ከኢንፌክሽኖች ጋር ያላቸውን ግንኙነት ለማጥናት ይረዳናል

ረ. <u>ሚስጥራዊነት</u>፡- ከላቦራቶር የሚገኘው ውጤት በሚስጥረ የተጠበቀ ይሆናል፡፡ የግልዎ የሆነ ምንም አይነት መረጃ ከውጤቱ ጋር አይያያዝም፡፡

ሰ. <u>ፈቃደኛነትዎን ስለማቋረጥ</u>፡- ከዚህ ጥናት ራስዎን የማግለልና ናሙናዎን እንዳንጠቀም ፈቃደኝነትዎን የመሠረዝ መብትዎና የተጠበቀ ነው፡፡ በዚህ ጥናት ላይ ፈቃደኛ አለመሆንዎ ከዚህ ቀደም በምንሰጥዎ የህክምና ክትትል አገልግሎት ላይ ምንም ተፅዕኖ አይኖረውም፡፡

ይህ ጥናት በህክምና ፋኪልቲና በኢትዮጵያ ሳይንስና ቴክኖሎጂ ኤጀንሲ የምርምር ኮሚቴዎች ታይቶ የፀደቀ ነው።

አድራሻ ማወቅ ካስፈለገዎ

1. ህክምና ፋከሊቲ፤አዲስ አበባ ዩንቨርስቲ

ድህረ ምረቃ ፕሮግራምና ምርምር የተባባሪ ዲን ቢሮ

የመ.ሳ.ቁ. 9086 አዲስ አበባ

ስልክ 251-011-551-28-765

2. ኢትዮጵያ ሳይንስ ቴክኖሎጂ ኤጀንሲ

የመ.ሳ.ቁ. 2490 አዲስ አበባ

ስልክ 251-011-551-13-44

ኢሜል estc@ethionet.et

3. የዋናው ተመራማሪ አድራሻ

ዶ/ር ያሬድ ተድላ አዲስ አበባ ዩንቨርስቲ ማይክሮባይሎጂ ኢሚኖሎጂና ፓራሲቶሎጂ ት/ክፍል

የመ.ሳ.ቁ. 9086 አዲስ አበባ

ስልክ 251-91-1407021

ኢሜል yared1972@yahoo.com

Appendix III: Consent form for study subjects (English Version)

Mr/Mrs/Miss _____

My name is _____

Having read/heard the information about the purpose of this study I would like to ask for your consent to participate in this study – entitled "Analysis of sera for antibodies to *Toxoplasma gondii*, CMV and HSV type I, II in individuals with schizophrenia and bipolar disorders.

I would like that you confirm your agreement by signing your name if you agree.

Signature of Study subject _____Date_____

Signature of the researcher_____Date_____

Signature of witnesses 1. _____Date_____

2. _____Date_____

Appendix III: Consent form for study subjects (Amharic Version)

<u>የፈቃደኝነት መጠየቂያ ቅጽ</u>

አቶ/ወ/ሮ _____

እኔ ስሜ _____**ይባላል፡፡**

የሰጠንዎ መረጃ አንብበዎል ወይም ሰምተዎል፡፡ በመሆኑም “የደም ናሙናን ቶክሶፕላዝማ ፣ሳይቶመጋሎ እና ሔርፐስ የተባሉ ህዋሳት ምርምር ጥናት„ ለማዋል ፈቃደኝነትዎን እንጠይቃለን፡፡

ፈቃደኛ ከሆኑ ደግሞ ለጥናቱ የተስማሙ መሆንዎን በፊርማዎ እንዲያረጋግጡልን እንፈልጋለን፡፡

የህመምተኛ/ ዘመድ ፊርማ _____**ቀን**_____

የተመራማሪው ፊርማ _____**ቀን**_____

የምስክሮችፊርማ 1._____**ቀን**_____

2._____**ቀን**_____

Appendix IV: Information sheet for study subject's parents/guardians (English Version)

It is remembered that your daughter/son volunteered to participate in a genetic study about 6 years ago. Part of the blood he/she gave during that study was stored at AHRI laboratory in Addis Ababa. In this particular study we would like to use the serum, the liquid/watery, part of the blood he/she gave during that study for an etiological research. The serum has been stored for so long because your consent to use it for this purpose was so important.

a. **Purpose**: The purpose of this study is look for possible infectious etiological agents.

b. **Duration**: We will use the serum only once for this particular study and will not use for anything else.

c. **Procedures to be carried on**: We will do an investigation for toxoplasmosis, CMV and HSV type I and II at AHRI laboratory

d. **Risks associated with the study**: There is no risk of using the serum to your son/daughter.

e. **Benefits of the study**: There will be no financial or other direct benefit to your son/daughter. But your consent for us to use the stored serum sample to test for body defense elements against the diseases mentioned above will help us better understand if the diseases have any relationship to schizophrenia and bipolar disorders.

f. **Compensations**: There will be no compensation for using this serum.

g. **Confidentiality of your information**: The results of the lab findings will be kept confidential and could only be accessed by the researchers. There will be no personal information to be attached to the study subject's data.

h. **Termination of the study**: We will respect your decision if you later on change your mind and inform us not to use the sample for the test. Your withdrawal of consent will not affect the right of the study subject to receive medication he/she used to get free from the project or continue in the cohort.

I would also like to inform you that this study is approved by the FRPC-I of the Medical Faculty, Addis Ababa University and the Ethiopian Science and Technology Agency, Ethical Review Committees. Their address is

1. Faculty of Medicine Addis Ababa University

   Office of Associate Dean, Postgraduate Programs and Research

   P.O. Box 9086. Addis Ababa, Ethiopia

   Tel. 251-011-551-28-765

2. Ethiopian Science and Technology Agency
   P.O.Box. 2490

   Tel. 251-011-551-13-44

   E-mail: estc@ethionet.et

The address of the principal investigator is: Yared Tedla, MD

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Faculty of Medicine, Addis Ababa University

P.O.Box. 9086, Addis Ababa, Ethiopia

Tel: +251-091-1407021

E-mail: yared1972@yahoo.com

Appendix IV: Information sheet for study subject's parents/guardians (Amharic version)

<u>የመረጃ መስጪያ ቅፅ</u>

ከ 6 ዓመት በፊት ለዘር ሐረግ ጥናት ለመሳተፍ ፈቃደኛ ሆነው ልጅዎ የደም ናሙና የሰጠ/ች መሆኑ ይታወቃል። በወቅቱ ከተወሰደው የደም ናሙና ላይ ፈሳሻማውን ክፍል በአዲስ አበባ አለርት ሆስፒታል ላቦራቶሪ አኑረነዋል። በዚህ በአሁኑ ጥናት የደም ናሙናውን ፈሳሽ ወይም ውሃማ ክፍል የአእምሮ ሕመም መነሻ ምክንያት ጥናት ልናደርግበት አስበናል።

ሀ. <u>የጥናቱ አላማ</u>፡-ይህ ጥናት ለአእምሮ ሕመም ምክንያት ሊሆኑ የሚችሉ ኢንፌክሽኖች ካሉ ለማወቅ ይረዳናል።

ለ. <u>የሚፈጀው ጊዜ</u>፡- ይህንን ናሙና ለአንድ ጊዜ ብቻ ለዚህ ጥናት እናውለዋለን። ከዚህ የተለዬ ለምንም ጉዳይ አንጠቀምበትም።

ሐ. <u>አጠቃቀሙም</u>፡- ቶክሶፕላዝማ ፣ሳይቶመጋሎ እና ሔርፐስ የተባሉ ቫይረስ ለማግኘት የሚረዳ የላቦራቶሪ ምርመራ እናደርግበታለን።

መ. <u>ሊደርስ የሚችል አደጋ</u>፡-ይህንን ናሙና በመጠቀማችን በልጅዎ ላይ የሚደርስ ምንም ዓይነት አደጋ የለም።

ሠ. <u>የሚያገኙት ጥቅም</u>፡- ይህንን ናሙና በመጠቀማችን ለልጅዎ የምንሰጠው/የምንሰጣት ምንም አይነት የገንዘብና ቁሳቁስ ጥቅም አይኖርም። ይሁን እንጂ በላቦራቶር ተቀምጦ የሚገኘው የደም ናሙናዎን ፈሳሽ ወይም ውሃማ ክፍል ላይ የሰውነት መከላከያ አካል የሆኑ ንጥረ ነገሮች እንዳሉ ለማየት የርሶ ፈቃድ አስፈላጊ ከመሆኑም በላይ ሺዞፍሬኒያና ባይፖላር ዲስኦርደር የተባሉ የአእምሮ ህመም አይነቶች ከኢንፌክሽኖች ጋር ያላቸውን ግንኙነት ለማጥናት ይረዳናል

ረ. <u>ሚስጥራዊነት</u>፡- ከላቦራቶር የሚገኘው ውጤት በሚስጥረ የተጠበቀ ይሆናል። የጥናቱ ተሳታፊዎች የሆነ ምንም አይነት መረጃ ከውጤቱ ጋር አይያያዝም።

ሰ. <u>ፈቃደኛነትዎን ስለማቋረጥ</u>፡- ከዚህ ጥናት ልጅዎን የማግለልና ናሙናውን እንዳንጠቀም ፈቃደኝነትዎን የመሠረዝ መብትዎና የተጠበቀ ነው። በዚህ ጥናት ላይ ፈቃደኛ አለመሆንዎ

ከዚህ ቀደም ለልጅዎ በምንሰጠው/ጣት የህክምና ክትትል አገልግሎት ላይ ምንም ተፅዕኖ አይኖረውም።

ይህ ጥናት በህክምና ፋኪልቲና በኢትዮጵያ ሣይንስና ቴክኖሎጂ ኤጀንሲ የምርምር ኮሚቴዎች ታይቶ የፀደቀ ነው።

አድራሻ ማወቅ ካስፈለገዎ

1. ህክምና ፋከሊቲ፤አዲስ አበባ ዩንቨርስቲ

   ድህረ ምረቃ ፕሮግራምና ምርምር የተባባሪ ዲን ቢሮ

   የመ.ሳ.ቁ. 9086 አዲስ አበባ

   ስልክ 251-011-551-28-765

2. ኢትዮጵያ ሳይንስ ቴክኖሎጂ ኤጀንሲ

   የመ.ሳ.ቁ. 2490 አዲስ አበባ

   ስልክ 251-011-551-13-44

   ኢሜል estc@ethionet.et

3. የዋናው ተመራማሪ አድራሻ

   ዶ/ር ያሬድ ተድላ አዲስ አበባ ዩንቨርስቲ ማይክሮባይሎጂ ኢሚኖሎጂና ፓራሲቶሎጂ ት/ ክፍል

   የመ.ሳ.ቁ. 9086 አዲስ አበባ

   ስልክ 251-91-1407021

   ኢሜል yared1972@yahoo.com

Appendix V: Consent form for study subject's parents/guardians (English Version)

Mr/Mrs/Miss _____

My name is _____

Having read/heard the information about the purpose of this study I would like to ask for your consent to allow your daughter/son to participate in this study – entitled "Analysis of sera for antibodies to *Toxoplasma gondii*, CMV and HSV type I, II in individuals with schizophrenia and bipolar disorders.

I would like that you confirm your agreement by signing your name if you agree.

Signature of Parent/Guardians _____Date _____

Signature of the researcher _____ Date _____

Signature of witnesses 1._____Date_____

2. _____Date _____

Appendix V: Consent form for study subject's parents/guardians (Amharic Version)

<u>የፈቃደኝነት መጠየቂያ ቅጽ</u>

አቶ/ወ/ሮ _____

እኔ ስሜ _____**ይባላል፡፡**

የሰጠንዎ መረጃ አንብበዎል ወይም ሰምተዎል፡፡ በመሆኑም የልጅዎን የደም ናሙና ቶክሶፕላዝማ ፣ሳይቶመጋሎ እና ሔርፐስ የተባሉ ህዋሳት ምርምር ጥናት„ ለማዋል ፈቃደኝነትዎን እንጠይቃለን፡፡

ፈቃደኛ ከሆኑ ደግሞ ለጥናቱ የተስማሙ መሆንዎን በፊርማዎ እንዲያረጋግጡልን እንፈልጋለን፡፡

የህመምተኛ ዘመድ ፊርማ _____**ቀን** _____

የተመራማሪው ፊርማ _____ **ቀን** _____

የምስክሮች ፊርማ 1. _____**ቀን** _____

2. _____ **ቀን** _____

DECLARATION

I, under signed, declare that this M.Sc. thesis is my original work, has not been presented for a degree in any other University and that all sources of materials used for this thesis have been duly acknowledged.

M.Sc. Candidate Yared Tedla Gebrehiwot

Signature _____

Date and place of submission _____

Addis Ababa, Ethiopia

Supervisor Yimtubezinash W/Amanuel (MD, M.Sc., PhD)

Signature _____

Date and place of submission _____

Addis Ababa, Ethiopia

Supervisor Daniel Asrat (MD, M.Sc., PhD)

Signature _____

Date and place of submission _____

Addis Ababa, Ethiopia