Serological Detection and Molecular Characterization of Cytomegalovirus and its Glycoprotein B (UL55) among Sudanese Renal Transplant Recipients

gB الكشف المصلي والتوصيف الجزيئي للفيروس مضخم الخلايا و الجين السكري لفاريسي الكلي السودانيين (UL55)

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DECLARATION

I declare that this thesis is hereby submitted to Sudan University of Science and Technology for the degree of doctor of philosophy and has not been previously submitted by me for a degree at this or any other university.

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DEDICATION

To My family with love

Hind Haidar
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ABSTRACT

This is analytical cross sectional study conducted at Kidney transplanted association hospital and Ahmed Gassim teaching hospital in Khartoum state in the period from 2013 to 2015, was aimed to detect CMV using ELISA and quantitative real-time PCR (qRT PCR), estimate viral loads and to determine the distribution of gB genotypes among Sudanese renal transplant recipients by DNA sequencing.

One hundred and four renal transplant recipients were included in this study. Blood and serum samples were collected from them, their age ranged from 11 to 72 years with mean age of 37 years. Males were 72(69.2%), while females were 32(30.8%). In this study, 50(48%) of them received their organs in local hospitals, while 54(52%) received their organs in abroad. Most of the renal transplant recipients’ received organs from relative donors 79(76%) and only 25 (24%) received organs from non-relative donors. The mean post-transplantation duration was 54 months, ranged from < than month - 204 months. The majority of post-transplantation duration varies from < than month - 12 months which represents 53(51%), 13 to 24 months 18(17.3%), 25 to 36 months 9(8.8%), 37 to 48 months 8(7.7%), and more than 48 months was 16(15.4%). The majority of recipients under study 79(75.9%) received triple immunosuppressive drugs, 24(23.1%) received two drugs while only one (1%) received one drug.

CMV IgM was detected using ELISA technique in 27(26%) of recipients, while 103(99%) recipients had CMV IgG in their serum. CMV DNA (viremia) was detected in 40/104 (38.5%) of renal transplant recipients using quantitative real time PCR with viral loads ranging from 62 copies /ml (1.8 log10) to 1.43x10^8 copies/ml ( 9 log10) and average of  358 x10^4 copies/ml (6.5 log10). Symptomatic recipients with CMV disease were 17/104 (16.3%) while asymptomatic with CMV infection were 23/104 (22.1%). CMV viremia showed no significant difference (P.value > 0.05) with sex and types of immunosuppressive therapy received by transplant recipients, while there is a significant difference (P.value = 0.05) between high viral loads and types of immunosuppressive therapy
received by transplant recipients. At the same time the correlation between high viral loads (>1000 copies /ml) and development of CMV disease observed significant difference (P.value = 0.00), in which, 14/17 (82%) of patients had clinical symptoms of CMV disease with high viral loads and 22/23 (95.7%) of patients had no clinical symptoms of CMV disease with low viral loads (<1000 copies/ml). The most common presenting symptoms of CMV disease were fever, fever and leucopenia, and gastrointestinal disease. The distribution of gB genotypes in Sudanese renal transplant recipients observed that gb3 was the most frequent genotype (80%) while gB4 was (20%) and no mixed genotypes were detected.

In conclusion, qRT-PCR from plasma samples is very sensitive for detection of CMV replication and was more sensitive than ELISA technique in detecting CMV in renal transplant patients. Viral loads were lower with asymptomatic patients. CMV gB3 was considered the most predominant glycoprotein B genotype in Sudanese renal transplant recipients with CMV disease.
المستخلص

هذه دراسة تحليلية تمت في مستشفى جمعية زاري عالي الكلي السودانى ومستشفى أحمد قاسم التعليمى في ولاية الخرطوم في الفترة من 2013 إلى 2015. هدفت هذه الدراسة لاستخبارات فيروس مضخم الخلايا باستخدام تقنية الأمير وتفاعل البلمرة التنسللى الكمي في الوقت المناسب وتعداد نسخ الفيروس وتحديد التوزيع الجينى لجينات البيرروتين السكرى (gB) لفأرة السنوى في مستشفى البكوى السودانيين باستخدام التنسللى الجينى.

شملت هذه الدراسة مائتان وأربعة من غارسي الكلي. وتم أخذ عينات الدم وصلصال الدم، كانت أعمارهم تراوح بين 11 و 72 سنة وحوتجعل العمر 37 سنة. وكانت نسبة الذكور منهم (69.2%) ونسبة الإناث (30.8%). في هذه الدراسة (48%) من الغارسين تم نقل الأعضاء لهم في مستشفى محلة بينما (52%) نقلت لهم الأعضاء في مستشفى خارج البلاد. معظم الغارسين تلقوا أعضاء من أقربائهم (76%) بينما (24%) منهم فقط تلقوا أعضاء من أشخاص غرباء. وكان متوسط وقت ما بعد الغرس يتعلق في الفئة الاقل من شهر 2-4 شهرا. حيث ان معظمهم يقع في الفئة أقل من شهر 12-12 شهر التي تمثل (51%) وفترة 13-18 شهر 18 (17.3%) و36-48 شهر تمثل (7.7%) وفترة 48 شهر تمثل (15.4%). معظم الغارسين (75.9%) تلقوا علاجات ثالوثية مثبتة للفيروس، ومنهم (23%) منهم تلقوا علاجات ثانوية مثبتة للفيروس.

و (11%) فقط منهم تلقوا علاج واحد مثبت للمنعة.

أظهرت الدراسة عدم وجود فرق ذو دلالة إحصائية (0.05) بين وجود الحمض النووي فيروس (P.value > 0.05) بين وجود الحمض النووي فيروس (P.value < 0.05) في نسخ الفيروس الفيروسية وانواع العلاجات المثبتة للمنعة ووجود فرق ذو دلالة إحصائية بين نسخ الفيروس الفيروسية وانواع العلاجات المثبتة للمنعة. في نفس الوقت ووحظ ان هناك فرق ذو دلالة إحصائية (0.00) في العلاقة بين نسخ الفيروس الفيروسية (أكبر من 1000 نسخة/ مليتر) مع تطور مرض مضخم الخلايا والذي فيه 14/17 (82.4%) مريض لديه إعراض مرض فيروس مضخم الخلايا ونسبة عالية للفيروس. وان (75.7%) من المرضى الذين ليس لديهم إعراض المرض لديهم نسخ متعددة من الفيروس (أقل من 1000 نسخة/ مليتر). الإعراض الأكثر شيوعاً ببيعالمراض. فيروس مضخم الخلايا هي السكري. الحمى ونزعان كريات الدم البيضاء والأعراض المعوية. أظهر التوزيع الجينى لجينات البروتين السكري (gB) في السودان أن الجين (gB) ويتمثل (80%) هو الأكثر شيوعا في السودان بينما (gB) يمثل (20%) وليس هناك أنواع مختلفة.
ونتيجة لذلك نجد أن تفاعل البلمرة التسلسلي الكمي في الوقت المناسب من عينات البلازما مفرط الحساسية للكشف عن تكاثر الفيروس مضخم الخلايا. كما أنه أكثر حساسية من تقنية اليسا في اكتشاف فيروس مضخم الخلايا. الاختلاف الكبير ظهر في نسبة وجود نسخ فيروس مضخم الخلايا مع وجود و عدم وجود الإعراض عند المرضى؛ نسب الفيروس المتدنية وجدت في المرضى الذين ليس لديهم إعراض. أعتبر جين البروتين السكري (gB) الأكثر شيوعا بين غارسي الكلي في السودان المصابين بمرض فيروس مضخم الخلايا.
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LIST OF ABBREVIATIONS

PCR: Polymearase Chain Reaction.
RT- PCR : Real time PCR.
CMV: Cytomegalovirus.
gp : glycoprotein
gB: Glycoprotein B.
gH: Glycoprotein H.
gN: Glycoprotein N.
gO : Glycoprotein O.
SOT : solid organ transplant.
ESRD : End-stage renal disease.
HLA: Human leucocyte antigen.
Cs A: Cyclosporine A.
CD: Cluster of differentiation.
MMF: Mycophenolate mofetil.
TLR: Toll like receptor.
FKBP: FK-binding protein.
TAC : Tacrolimus.
NK: Natural Killer cell.
PTLD: Post-transplant lymphoproliferative disorder.
C5b: Complement component 5b
ATP: Adenosine Tri –Phosphate
DAMPs : Damage-activated molecular patterns.
TCR : T-cell receptor.
RTRs: Transplantation recipients.
HHV: Human herpesvirus.
BKV: BK polyomavirus.
EBV: Epstein-Barr virus.
JCV: JC polyomavirus.
HSV: Herpes Simplex Viruses.
HCMV: Human Cytomegalovirus.
UL: Unique long region
US: Unique short region.
TRL: Terminal repeat long.
IRL: Internal repeat long.
IRS: Internal repeat short.
MCP: Major capsid protein.
SCP: Smallest capsid protein.
TRS: Terminal repeat short.
gC: Glycoprotein complex.
ERGIC: Endoplasmic reticulum-Golgi intermediate compartment.
AD: Antigen domain.
gM: glycoprotein M.
gL: glycoprotein L.
IE: Immediate-early.
MIE: Major Immediate-early.
HIV: Human Immunodeficiency Virus.
AIDS: Acquired Immunodeficiency Syndrome.
MHC: Major histocompatibility complex.
TH: T helper cell.
D/R: Donor/Recipient.

TNF-α: Tumor necrosis factor alpha.

ALA: Anti-lymphocyte antibodies.

BAL: Broncho-Alveolar Lavage.

EDTA: Ethylene diamine tetra acetic acid.

VZV: Varicella Zoster.

TCID₅₀: The tissue culture infective dose 50

CPE: Cytopathic effect.

IFA: Immune-fluorescence assay.

ELISA: Enzyme-linked immunosorbent assay

PBLs: Peripheral blood leucocytes.

qRT-PCR: Quantitative Real Time PCR.

CFT: Complement fixation test.

RIA: Radioimmunoassay.

RFLP: Restriction fragment length polymorphism.

TWBCs: Total white blood cells.
CHAPTER ONE
INTRODUCTION

1.1. Introduction

Chronic kidney disease is usually progressive and may lead to renal failure. Most of the signs and symptoms of renal failure can be relieved by dialysis but renal transplantation is the only real cure for patients with end-stage renal failure. It may restore complete health and function (Rhoades and Bell, 2009).

Human cytomegalovirus (HCMV) is one of the eight herpesviruses that are pathogenic for humans (Olyaee et al., 2005). It is a beta-herpesvirus with a large dsDNA genome (~ 235 000 bp) (Renzette et al., 2014). It has most complex viral genomes, composed of double stranded linear DNA. The virus has a capsid surrounded by a tegument and a glycoprotein envelope. The virus is highly species specific and shows tropism for specific cell types (Dar, 2007).

HCMV is an opportunistic pathogen is ubiquitously distributed in human population (Zhang et al., 2010) and the primary infection is usually asymptomatic (Polz-Dacewicz et al., 2013) and usually causes a latent infection (Zhang et al., 2010).

The seroprevalence of HCMV ranges from 40 to 100% worldwide, with lower rates in Europe, parts of North America, and Australia, and higher rates in Africa and Asia (Kim and Kim, 2011). The viral intrauterine transmission to fetus can lead to stillbirth, abortion, and mental retardation (Zhang et al., 2010).

CMV is a common pathogen which complicates treatment of immunosuppressed patients (transplantation, HIV patients) (Kotton et al., 2013) and chemotherapy recipients (Zhang et al., 2010).

CMV is a major infectious complication of renal transplantation and CMV disease in renal transplant recipients. It has a significant impact on morbidity and mortality and graft survival (Olyaee et al., 2005). In the absence of any form of prophylaxis treatment, HCMV infection develops in 50% to 90% of organ transplant recipients, resulting in symptomatic disease in 7% to 33% of them (Madi et al., 2011b). Three major patterns that observed in solid organ
transplantation recipients are: primary infection develops when a CMV seronegative individual receives cells latently infected with the virus from a seropositive donor, secondary infection or reactivation infection develops when endogenous latent virus is reactivated in CMV-seropositive individual post transplantation. Superinfection or reinfection occurs when a seropositive recipient receives latently infected cells from a seropositive donor and the virus that reactivates post transplantation is of donor origin (Cukuranovic et al., 2012).

Many infected patients develop symptomatic CMV disease, manifested by pneumonia, hepatitis, gastrointestinal ulcers, a non-specific febrile illness associated with leucopenia and thrombocytopenia, or less commonly retinitis. Patients with CMV pneumonia or disseminated infection often die (Olyaee et al., 2005). In addition to direct consequences, CMV acts as an immunomodulator increasing the risk for other opportunistic infections (Kotton et al., 2013) and potentially making CMV infection an important risk factor for the development of acute and chronic allograft rejection (Coaquette et al., 2004), resulting in significant morbidity, graft loss, and adverse outcomes (Kotton et al., 2013).

Quantitative measurement of CMV-DNA levels including PCR testing of plasma or whole blood is the best method for diagnosis of CMV infection in solid organ transplant (Kim and Kim, 2011). The gold standard for diagnosis of CMV is the quantitative nucleic acid testing (QNAT). QNAT-CMV has been performed preferentially by real time PCR, using plasma or total blood (Requião-Moura et al., 2015) which is widely considered as an efficient and highly sensitive technique for the evaluation of HCMV DNA kinetics (Habbal et al., 2009).

Whole blood assays often have higher viral loads than plasma assays (Kim and Kim, 2011), but plasma viral load monitoring is of modest clinical utility for prediction of CMV disease (Garrigue et al., 2008). The presence of CMV DNA in plasma suggests active viral replication due to spread of the virus from the leukocyte into the plasma (Rangbar-Kermani et al., 2011) The highest viral
loads are associated with tissue-invasive disease, while the lowest are observed with asymptomatic CMV infection (Kim and Kim, 2011).

The HCMV genome encodes numerous glycoproteins, of which gB, gH and gN are the most abundant. Genotyping of HCMV is mostly based on sequence variation in surface glycoprotein genes, which often show genetic polymorphism. gB gene, the gH gene and the gN gene have all been utilized for genotyping HCMV, with the gene for the gB glycoprotein having been used most frequently. There are four major gB genotypes (gB1 to gB4). In addition to its important role in viral entry into host cells, cell-to-cell transmission and membrane fusion of infected cells, the gB glycoprotein is also a major target for neutralizing antibodies (Dar, 2007).

Two strategies are commonly used for CMV prevention: universal prophylaxis and preemptive therapy. Universal prophylaxis involves giving antiviral therapy to all “at risk” patients beginning at or immediately after transplant for a defined time period. In preemptive therapy, patients are monitored at regular intervals for early evidence of CMV replication prior to the onset of clinical symptoms by use of a laboratory assay (Cukuranovic et al., 2012).

Today, ganciclovir (GCV) and valganciclovir (VGCV) are the treatment of choice of HCMV in case of infection in solid organ transplant (SOT) recipients, which lead to a decline in the CMV disease and associated morbidity in SOT recipients (Madi et al., 2011a).
1.2. Rationale

Renal failure is a common disease in Sudan; the estimated incidence is 70 -140/ million inhabitation/year in the world. Improved graft survival has made renal transplantation the ultimate choice for the patients with end stage renal disease (ESRD). The prevalence of treated ESRD reported as 106 patients/million population with renal transplantation of 28.4% of them (Banaga et al., 2015).

CMV infection is one of most frequent infectious complications after renal transplantation and a significant risk factor for the development of graft failure and death after renal transplantation (Requião-Moura et al., 2015). In the absence of prophylactic treatment, about 50% renal transplant with active CMV infection will progress a potentially fatal end-organ disease. Early diagnosis and detection of CMV infection and disease is a curial factor that lead to more effective methods of prevention and treatment of the disease (Madi et al., 2011a).

Despite advances in this field in the world, still there is a difficulty in the diagnosis of this disease among transplants in Sudan. Numerous studies have focused on the distribution of CMV gB polymorphism and it is relation to clinical findings of the disease in transplant recipients internationally. However, few studied were performed on CMV in renal transplant recipients in Sudan. No published data were found about CMV genotyping neither in renal transplant recipients nor in other population.

This study helps in detecting CMV infection and disease in Sudanese renal transplant recipients and helps renal centers to avoid a lethal outcome of CMV and to start preemptive therapy at the earliest stage is of extreme significance to avoid loss of human resources by graft loss and death.
1.3. Objectives

1.3.1. General objective

To perform serological detection and molecular characterization of cytomegalovirus and its glycoprotein gB (UL55) among Sudanese renal transplant recipients.

1.3.2. Specific objectives

1. To detect CMV viremia in plasma of renal transplant recipients and; to estimate the viral loads using quantitative real–time PCR assay.
2. To detect CMV IgM and IgG antibodies in serum of renal transplant recipients using ELISA technique.
3. To correlate between CMV viremia, plasma viral loads and sex, post transplantation time, immunosuppressive therapy, and symptoms of CMV disease.
4. To determine the frequency rate of different gB (UL 55) genotypes using CMV DNA sequencing.
CHAPTER TWO
LITERATURE REVIEW

2. Literature review

2.1. Renal transplantation

The urinary system consists of two kidneys, two ureters, the urinary bladder, and the urethra. The formation of urine is the function of the kidneys, and the rest of the system is responsible for eliminating the urine. Body cells produce waste products such as urea, creatinine, and ammonia, which must be removed from the blood before they accumulate to toxic levels and to maintain the normal composition, volume, and pH of both blood and tissue fluid. Kidneys form urine to excrete these waste products (Scanlon and Sanders, 2007).

Chronic kidney disease is usually progressive and may lead to renal failure. Most of the signs and symptoms of renal failure can be relieved by dialysis but renal transplantation is the only real cure for patients with end-stage renal disease (ESRD). It may restore complete health and function (Rhoades and Bell, 2009).

Kidney transplantation is the treatment of choice for the majority of patients with end-stage kidney disease (Sharif, 2016). It offers improved survival and quality-of-life benefits compared with dialysis (Ishibashi and Suzutani, 2012).

Since the first successful kidney transplant in 1954, the scientific advances over the subsequent decades have led to significant improvements in patient/graft survival and quality of life for kidney allograft recipients (Sharif, 2016). Donor organs are obtained from cadavers and living relative donors (e.g., parent, sibling). The success of transplantation depends primarily on the degree of histocompatibility, adequate organ preservation, and immunologic management (Ramanathan et al., 2001).

2.2. Host–graft adaptation

The term “host–graft adaptation” describes the decrease in both donor-specific responsiveness and the risk of rejection in the months after a successful transplantation that is maintained by immunosuppression. Changes in the organ, a loss of donor dendritic cells and a resolution of injury contribute to the adaptation. Regulatory T cells may also be able to control alloimmune responses, by analogy with their ability to suppress autoimmunity (Halloran, 2004).
2.3. Immunosuppressive treatment after renal transplantation

Immunosuppressive agents are used to control the immune response after transplantation of an HLA-mismatched graft. If no immunosuppression is used, the graft will be rejected (Afzali et al., 2010). Chronic allograft injury has replaced acute rejection as the major cause of graft loss in renal transplantation. The more potent immunosuppressive therapy that has successfully reduced the incidence of acute rejection has also resulted in a higher incidence of viral infection (Smith et al., 2010). Triple immunosuppressive regimen, consisting of corticosteroids, azathioprine and cyclosporine A, became the standard immunosuppressive protocol for many transplant centers throughout the world (Sing-Leung, 2001).

2.3.1. Azathioprine (Imuran)

Azathioprine is a purine analogue. It inhibits purine metabolism, leading to DNA inhibition and finally cell proliferation is thereby inhibited. It causes impairing of a variety of lymphocyte functions (Piedras et al., 2013) by blocking CD28 costimulatory signaling (Piedras et al., 2013; Hartono et al., 2013).

2.3.2. Corticosteroids

Steroids are a cornerstone of immunosuppressive therapy in kidney transplantation. More than 95% of transplant recipients are treated with steroids as a usual component of clinical immunosuppressive regimens. Prednisone, prednisolone, and other glucocorticoids are used alone and in combination with other immunosuppressive agents for treatment of transplant rejection and autoimmune disorders. Most immunosuppressive regimens are currently based on the combination of calcineurin inhibitors (cyclosporine A, tacrolimus) with antiproliferative agents (azathioprine, mycophenolate mofetil) and steroids (prednisone) (Piedras et al., 2013).

2.3.3. Cyclosporine A

Cyclosporine (CsA) is the cornerstone of immunosuppression for many years. CsA is a calcineurin inhibitor. Calcineurin is a protein phosphate that is critical for T-cell activation. The effect is exerted through binding to cyclophilins (Watson and Dark, 2012); it inhibits the transcription of pro-inflammatory cytokine genes (e.g. Interleukin-2, interferon-γ) in T cells and thus prevents the proliferation of T cells. High cyclosporine
levels in blood have been associated with an increased risk of developing HCMV infection (Al-Alousy et al., 2011).

2.3.4. Tacrolimus
Tacrolimus (TAC) is a macrolide antibiotic. Like cyclosporine A, tacrolimus binds to an immunophilin, FK-binding protein (FKBP) in the cytoplasm. The resultant tacrolimus-FKBP complex then interacts with calcineurin and inhibits its action in the same manner as cyclosporine A.

2.3.5. Polyclonal anti-lymphocyte globulins and OKT3
Anti-lymphocyte globulins (ATGAM) and thymoglobulin are polyclonal anti-lymphocyte globulins. The effect of is mediated mainly through interacting with a variety of surface markers (e.g. CD45, CD3, CD4) on the lymphocytes. OKT3 is a murine monoclonal antibody against the CD3 complex of molecules on the surface of T lymphocytes. Polyclonal anti-lymphocyte globulins and OKT3 are mainly used for the treatment of severe acute rejection. As both polyclonal anti-lymphocyte antibodies and OKT3 significantly impairs cell-mediated immunity, patients receiving these antibodies are predisposed to opportunistic infections especially CMV infections and malignancies (Sing-Leung, 2001).

2.3.6. Mycophenolate mofetil (Cellcept)
Mycophenolate mofetil has been developed as a replacement for azathioprine for maintenance immunosuppression. It acts by inhibiting inosine monophosphate dehydrogenase, a key enzyme in the de novo purine synthesis pathway, thereby limiting the proliferation of B and T lymphocytes. It reduces the incidence of acute rejection in the first year after transplantation and the need for intensive immuno-suppression to treat rejection. Mycophenolate mofetil may also be useful in treating acute cellular rejection and reversing refractory acute rejection in renal transplant patients and may help to prevent chronic allograft rejection. The major side effect of MMF is increased risk of tissue invasive cytomegalovirus infection (Sing-Leung, 2001).

2.3.7. Rapamycin
The mammalian target of rapamycin (mTOR) inhibitors, everolimus and sirolimus has been used for the prophylaxis of rejection in kidney transplant patients. Rapamycin binds to the same immunophilin as tacrolimus, namely FKBP, to become active. It acts by
inhibiting a key enzyme known as target of rapamycin (TOR), this results in the inhibition of proliferation of activated lymphocytes (Kahan, 2000), NK, and B cells (Johansson, 2014). Rapamycin is reducing acute rejection in renal transplant patients more than azathioprine (Kahan, 2000). Rapamycin may prevent the development of graft atherosclerosis, a hallmark of chronic rejection (Halloran, 2004).

2.3.8. Other immunosuppressive drugs

Other immunosuppressive therapy includes; daclizumab, basiliximab and alemtuzumab (Issa and Braun, 2013). Also aelatacept and alefacept (amevive) (Sing-Leung, 2001), eculizumab (soliris) (Kumar and Gaston, 2011). FK778, Malononitrilamide, FTY 720, CP-690, 550 and Tyrphostin AG 490 (Halloran, 2004).

2.4. Acute and chronic rejection in renal transplant recipients

Acute rejection seems to be a risk factor for chronic rejection in renal transplants (Caltenco-Serrano et al., 2001), which is the major cause of graft loss in renal transplantation (Smith et al., 2010). Chronic rejection is seemed to be a result of the production of cytokines and growth factors by different cell types, leading to a proliferative remodeling of graft vessels along with structural changes in the parenchyma, and gradual deterioration of graft function (Yilmaz et al., 1996).

Late Acute Rejection is often severe and difficult to reverse, with a high risk of subsequent graft loss (Nankivell and Alexander, 2010).

Chronic renal allograft rejection was defined as the gradual deterioration in graft function in the absence of any other disease. It occurs during a span of months to years due to unresponsive to current treatment. It thought to be the end result of uncontrolled repetitive acute rejection episodes or a slowly progressive inflammatory process, its onset may be as early as the first few weeks after transplantation or any time thereafter (Shaikewitz and Chan, 1994). Characterized by interstitial fibrosis, glomerulosclerosis, tubular atrophy and arterial narrowing, is the major reason for the loss of renal allografts after the first post-transplant year (Yilmaz et al., 1996). It’s the most important cause of long-term graft failure and it is a common reason for retransplantation (Ishibashi and Suzutani, 2012).

From a tissue compatibility point of view between the donor and the recipient, that is, through the class I and II MHC antigens, there is a very important determining factor, as
for example the presence of receptor antibodies, specific against the MHC class I donor antigens. In these cases, the grafts are rapidly and irreversibly rejected (Caltenco-Serrano et al., 2001).

2.4.1. Rejection mechanism

The donor kidney is comprised resident immune cells such as dendritic cells that are easily activated to engulf fragments of damaged tissue and pathogens. Resident dendritic cells become activated is through low blood flow to the kidney and to process of removing the kidney, from either a live or a deceased donor (McKay and Steinberg, 2010).

Activation of donor innate immune cells associated with transplantation begins with ischemia/anoxia induced death of donor kidney cells. Dead and dying cells contain immunologically active molecules called damage-activated molecular patterns (DAMPs), like heat-shock proteins, ATP, uric acid, RNA, DNA, as well as proteins. Donor kidney cells contain receptors for DAMPs including toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs), they are thought to trigger the immune events that cause acute rejection. This newly identified innate immune system plays an essential role in the earliest events associated with rejection (McKay and Steinberg, 2010).

The recipient’s innate immune cells (such as neutrophils, natural killer cells, and macrophages) vigorously infiltrate the donor tissue and add to the ischemia-induced tissue injury. The encounter between donor dendritic cells and recipient T cells is the key initiating event of cellular rejection. The two cell types engage each other using cell surface receptors – the human leukocyte antigen (HLA) molecule on the dendritic cell and T-cell receptor (TCR) of the T cell. The T-cell receptor (TCR) and several associated molecules called the CD3 chains. HLA molecules are highly polymorphic and important in transplantation, it allowing presentation of a diverse number of “foreign” peptides. The foreign peptides are derived from the allograft. Foreign proteins can be presented by HLA molecules to either CD4 T cells or CD8 T cells. If the foreign protein is adjoined to HLA class II molecules it will be presented to CD4 T cells. On the other hand, if adjoined to HLA class I molecules, it will be presented to CD8 T cells. Both CD4 and CD8 T cells participate in the rejection process (McKay and Steinberg, 2010).
T helper cells secrete different cytokines, in the case of graft rejection, they function to recruit the army of recipient immune cells aimed at destruction of the foreign allograft. When activated, cytotoxic T cells directly destroy target cells by releasing cytotoxins such as perforin and granzyme. Perforins form pores in the target cell membranes and granzymes enter the target cell and destroy it. Cytotoxic T cells play a role in graft rejection as well as destruction of virally infected tissue (McKay and Steinberg, 2010).

2.5. Viral infections after renal transplantation

Viral infections are a major problem in allograft recipients, most commonly 1 to 6 months after transplantation. Clinical disease can take place later, especially after intensification of immunosuppression or physiologic insults that increase the net state of immunosuppression (Ortiz and André, 2011). The more potent immunosuppressive therapy, the higher incidence of viral infection (Smith et al., 2010). Opportunistic viral infections make an important threat to renal transplantation recipients (RTRs), and with the use of more intense newly-developed immunosuppressive drugs, the risk of renal allograft loss due to reactivation of these viruses has increased considerably (Al-Obaidi et al., 2015). Infection rates increased in adult kidney transplant recipients of >50 years from 48% to 69% during the first year post-transplantation (Egli et al., 2007).

Some of viral infection result of community exposures (influenza, adenovirus), whereas some are commonly transmitted with the allograft (CMV, Epstein-Barr virus (EBV)) (Kotton and Fishman, 2005), also Human BK polyomavirus (BKV) (Adrian et al., 2007), JC polyomavirus (JCV), and Simian virus (Cukuranovic et al., 2012). Others are the result of more distant exposures reactivated in the setting of immune suppression (chicken pox and varicella zoster (VZV) as shingles) (Kotton and Fishman, 2005), as well as Human herpesvirus1, Human herpesvirus 6, Human herpesvirus 7, and Human herpesvirus 8 (Cukuranovic et al., 2012). Hepatitis B virus (HBV) and hepatitis C virus (HCV) infection rates increased recently (Ortiz and André, 2011).

CMV infection produces a profound suppression of a variety of host defenses, predisposing to secondary invasion by such pathogens as Pneumocystis carinii (jiroveci), Candida, and Aspergillus (Ortiz and André, 2011). Multiple simultaneous infections,
viral and non-viral, are also common, such as CMV and human herpes virus 6 or CMV and Pneumocystis (Kotton and Fishman, 2005).

Reactivation of these viruses especially CMV and BKV in these chronically immunosuppressed renal transplant recipients can lead to renal impairment and subsequently allograft loss, unless it is treated (Al-Obaidi et al., 2015).

Viral infections in donor are considered as the risk of infection to the organ recipient, and contraindicated to organ donation such as herpes simplex encephalitis, West Nile virus infection, rabies, HIV, and active hepatitis A, B and C (Hariharan, 2007).

CMV infection is one of most frequent infectious complications after renal transplantation (Requião-Moura et al., 2015). It is a significant risk factor for the development of graft failure and death after renal transplantation. (Yilmaz et al., 1996).

CMV disease, but not asymptomatic infection, is an independent risk factor for biopsy-proven acute rejection, particularly in the first 12 months following renal transplantation (Costa, 2011).

2.6. Cytomegalovirus (CMV)

2.6.1. History of Cytomegalovirus

Cytomegalic inclusion disease (CID) was known as an infectious disease recognizable by the owl's eye cytopathology in salivary gland, liver, lung, kidney, pancreas, and thyroid autopsy materials from infants by the early 1930s (Knipe and Howley, 2007).

CMV was initially called “salivary gland virus” or “salivary gland inclusion disease virus”. In 1960, Weller et al. proposed the use of the term Cytomegalovirus. Klemola and Kaarianinen firstly described CMV mononucleosis, the principal presentation of previously healthy individuals, in 1965. CMV was first isolated in a renal transplant recipient in 1965 (Brennan, 2001).

HCMV infects man, but there are other Cytomegaloviruses that are specific for other animal species (e.g. murine CMV) (Greenwood et al., 2012). The name means ‘large cell virus’ and derives from the swollen cells containing large intranuclear inclusions that characterize these infections (Collier and Oxford, 2006).

2.6.2. General characteristics

HCMV is a ubiquitous virus infection with worldwide distribution and associated with opportunistic disease that has been recognized in more highly developed areas of the
world. CMV acute disease occurs in only a small proportion of infected individuals, and it is restricted to settings where the ability to mount a cellular immune response is compromised, such as transplacental transmission during pregnancy leading to fetal damage and reactivation or primary infection of immunocompromised individuals (Murray et al., 2007).

2.6.3. Physical prosperity
CMV is a labile virus and readily inactivated by lipid solvents, pH below 5, heat (37°C for 1 h or 56°C for 30 min) (Brennan ., 2001), cycles of freezing and thawing (Murray et al., 2007), and ultraviolet light for 5 min. It can survive on environmental surfaces for several hours. CMV can be stored at 4°C for a few days without loss of infectivity. Storage at 70°C without loss of infectivity is possible for several months. It can be stored at 190°C (liquid nitrogen) indefinitely (Brennan, 2001).

2.6.4. Cytomegalovirus virology
CMV is a member of the genus Herpesvirus and belongs to the family Herpesviridae. There are 8 known human herpes viruses. The HHV are divided further into three subfamilies: the α-herpesvirinae, the β-herpesvirinae, and the γ-herpesvirinae. The α-herpesvirinae includes Human Herpes Simplex Viruses (HSV) 1 and 2 and Varicella Zoster virus. The β-herpesvirinae includes Cytomegalovirus (CMV), Human Herpes virus (HHV-6), and Human Herpes virus HHV-7. The γ-herpesvirinae includes Epstein-Barr virus (EBV) and Human Herpes virus HHV-8 (Brooks et al., 2007).

2.6.5. Morphology
Morphologically Herpes viruses are indistinguishable from one another. The complete virion is 150 to 200 nm in diameter and icosahedral in shape and consists of an inner core, a capsid, and an envelope. The inner core (genome) of the CMV virus is a 64-nm linear double-stranded DNA molecule. The capsid is 110 nm in diameter and consists of 162 protein capsomers. The envelope contains lipoproteins and at least 33 structural proteins, some of which are glycosylated (glycoproteins). The glycoproteins determine the strain of CMV (Brennan, 2001).

The central DNA-containing core is surrounded by a capsid composed of 162 capsomeres. The capsid is in turn surrounded the tegument, which is itself surrounded by a loosely-applied envelope (Zuckerman et al., 2009).
2.6.6. Cytomegalovirus structure

2.6.6.1. Nucleic acid

CMV is a DNA virus containing 230-kb double-strand DNA (Ishibashi and Suzutani, 2012). It has the largest genetic content of the human herpesviruses (Brooks et al., 2007), in that long and short unique sequences are bounded by terminally repetitive segments. The viral genome is divided into two unique components, unique long (UL) and unique short (US) regions (Heli., 2004) (Figure 2.1). A pair of inverted repeats (terminal/internal repeat long TRL/IRL and internal/terminal repeat short IRS/TRS) flanks the unique regions (Sijmons et al., 2014).

Each long and short sequence can be orientated in one of two directions, so that four DNA isomers are produced by cells in culture (Zuckerman et al., 2009). Many proteins encoded by the virus (over 200) have been characterized (Brooks et al., 2007). By international agreement, the proteins they encode are designated by p (for protein), gp (glycoprotein) or pp (phosphoprotein), followed by the gene number. This formal terminology may then be followed by a trivial name, for example gpUL75 (gH) is glycoprotein H, the product of gene number 75 in the unique long region.

Productively infected cells produce linear genomes from concatameric precursors. Cleavage is accomplished by an endonuclease (terminase) coincident with packaging some areas of the genome are homologous with regions of human chromosomal DNA, which has practical importance for the selection of CMV DNA probes. The DNA can be digested with restriction endonucleases so that, following gel electrophoresis, oligonucleotide patterns characteristic of distinct CMV strains are produced (Zuckerman et al., 2009).

2.6.6.2. Virion structure

CMV virion structure consisting of viral DNA, capsid, tegument and envelope (Ishibashi and Suzutani, 2012). The virion is 200 to 300 nm diameter. Virions contain a 125 nm icosahedral nucleocapsid composed of five herpesvirus core proteins: major capsid protein (MCP, the UL86 gene product), the minor capsid protein (TRI1, the UL46 gene product) together with the minor capsid protein binding protein (TRI2, the UL86 gene product). The smallest capsid protein (SCP, the UL48A gene product) that decorates MCP tips; and a portal protein (PORT, the UL104 gene product) that constitutes one
specialized penton used for encapsidation of viral DNA. The nucleocapsid encloses an S (oriLyt)-associated RNA. The nucleocapsid itself is embedded in a tegument (or matrix) (Knipe and Howley, 2007).

![Figure (2.1): Genome structure of HCMV](image)

2.6.6.3. Capsid structure
The CMV nucleocapsid exhibits icosahedral symmetry with 162 capsomeres and is approximately 130 nm in diameter. Capsid assembly intermediates as well as aberrant particles are formed during. Within infected cells, three distinct mature capsid forms are observed, termed A, B, and C capsids, but only DNA-containing HCMV nucleocapsids, termed C capsids, have completed maturation (Knipe and Howley, 2007).

2.6.6.4. Tegument
The nucleocapsid is embedded in a tegument (or matrix). The tegument appears is amorphous virion region located between the capsid and envelope. It contains most of the virion proteins (at least 27) as well as a selection of viral and cellular RNA and makes up about 40% of the total virion mass. Tegument proteins carry out a remarkably diverse
range of activities during infection and tend to be phosphorylated and highly immunogenic (Knipe and Howley, 2007).
The most abundant tegument proteins are the beta herpesvirus-conserved UL82 family members, including the most abundant HCMV tegument protein pp65 (lower matrix protein, UL83 gene product is the antigen that is detected in the antigenemia assay. Tegument is surrounded by a host cell endoplasmic reticulum (ER)-Golgi intermediate compartment (ERGIC)-derived lipid bilayer envelope containing at least 20 virus-encoded glycoproteins. Tegument proteins play important roles during entry or maturation (Knipe and Howley, 2007).

2.6.6.5. Envelope
HCMV encodes more than 50 proteins that are potentially glycosylated or contain predicted transmembrane domains. The most functionally critical HCMV envelope glycoproteins are members of the herpesvirus core set which form three separate complexes that have been termed gcI, gcII, and gcIII on the cell surface and on the viral envelope, but are better known by common nomenclature glycoprotein (g)B, gH:gL, and gM:gN, respectively. All of these genes are required for CMV replication, including gM:gN, which is dispensable in many other herpesviruses (Knipe and Howley, 2007).

2.6.6.6. Glycoproteins of Cytomegalovirus
At least 57 potential glycoproteins are known to be encoded by the laboratory strain of CMV AD169 (Britt and Mach, 1996). These glycoproteins associate in high molecular weight complexes and the mature complexes are referred to as glycoprotein complex I (gC-I), glycoprotein complex II (gC-II) and glycoprotein complex III (gC-III). The genes encoding glycoproteins often show genetic polymorphism (Ishibashi and Suzutani, 2012).

2.6.6.6.1 Glycoprotein H (gH)
The disulphide-bond tripartite gC-III envelope complex consists of gH, gL and gO (Huber and Compton, 1999). The gH–gL dimer, which is essential for entry into all cell types and is postulated to be important for triggering gB fusion at the plasma membrane (Griffiths et al., 2015). Glycoprotein H is one of the immunologically dominant glycoproteins in the CMV envelope, and is encoded by open reading frame (ORF) unique long (UL) region 75 (Britt and Mach, 1996). Although the UL75 is highly conserved among multiple CMV strains, sequence variations were found in the first 37 aa. Based on
the sequence analysis of UL75 from multiple strains, it was estimated that CMV gH has
two genotypes (Chou, 1992). gH mediates viral/host cell membrane fusion in the initial
step of infectivity. Anti-CMV gH antibodies show virus neutralizing activity and gH is
considered a major antigen for the humoral immune response (Urban, et al., 1996).

2.6.6.6.2. Glycoprotein B (gB)

Glycoprotein B, a component of the envelope complex gC-I, is the most abundant
glycoprotein in the CMV envelope. gB is one of the most highly conserved components
among all members of the herpesvirus family (Ishibashi and Suzutani, 2012). After
budding of the envelope through the inner nuclear membrane, the particles of the virus go
through the trans-Golgi network, where the virus particle becomes pathogenic through
proteolytic cleavage of a consensus furin site to form glycoprotein B (gB). Furin was the
first proprotein convertase to be identified. It is localized mainly in the trans-Golgi
network (Brennan, 2001). gB is encoded by UL55 and exhibits genetic polymorphism.
The 906 aa polypeptide of the AD169 strain gB is cleaved at position 460 by a cellular
endoprotease. Nucleotide and peptide sequence analysis revealed that variations were
most frequent between positions 448 and 480, which include the cleavage site (Chou and
Dennison, 1991). Restriction enzyme analysis has identified four main gB groups (gB-1,
gB-2, gB-3 and gB-4). While variations were found in gB, substantial conservation of the
peptide sequence is observed in this region. The closely regulated variations in gB may
suggest its important role in the viral life cycle (Chou and Dennison, 1991). Genetic
variations of gB have been used for epidemiologic purpose. gB has a role in binding to
cell surface receptors, and neutralizing gB-specific antibodies can inhibit the binding
(Ohizumi et al., 1992). The deletion of glycoprotein B (gB) renders HCMV incapable of
entering cells unless a chemical fusogen (ie: polyethylene glycol) is added and show that
gB is important for virus fusion (Griffiths et al., 2015). gB also serves a role in the initial
attachment to the cell via interactions with heparan sulphate glycosaminoglycans
(Griffiths et al., 2015).

The antigen domain 1 (AD1), which is located between positions 560 and 640 of gB, is a
major neutralizing epitope (Schoppel, et al. 1996) and is the most highly conserved
region among viral strains. The second antibody-binding site on gB is the antigen domain
2 (AD2), which is located between aa 28 and 84 of gB (Meyer et al., 1992). Within the
AD2 domain, two antigenic sites have been identified. Site I is located between aa 68 and 77, and this region is conserved among CMV wild-type strains and is the target of neutralizing antibodies. Site II, another binding sequence in the AD2, is located between aa 50 and 54. Site II binds non-neutralizing antibodies and is strain-specific (Meyer et al., 1992; Ishibashi and Suzutani, 2012).

2.6.6.6.3. Glycoprotein N (gN)

Glycoprotein N is a component of the envelope complex gC-II (Mach et al., 2000; Dal Monte et al., 2001). gN has been recognized as one of the major antigens together with gH and gB (Shimamura et al., 2006). It is encoded by the ORF UL73, and antibodies against gN neutralize virus infectivity. UL73 has four main genomic variants, known as gN-1, gN-2, gN-3 and gN-4 (Pignatelli et al., 2001). The gN genomic variants are related to the immunopathogenesis of CMV in immunocompromised hosts and in congenitally infected infants (Pignatelli et al., 2003a; Pignatelli et al., 2003b).

2.6.6.6.4. Other glycoproteins

The large CMV genome encodes many additional glycoproteins other than gH, gB and gN. UL100 encodes glycoprotein M (gM), which, together with gN, is a component of gC-II. gM is essential for viral replication (Hobom et al., 2000), and seems to be highly conserved (Lehner et al., 1991). It was shown that most sera failed to react with either gM or gN alone (Mach et al., 2000). Virus neutralizing antibodies were shown to be directed at the gN component of the gM–gN complex. In addition to gH, the gC-III envelope complex contains glycoprotein O (gO) and glycoprotein L (gL) (Huber and Compton 1999). gO is encoded by the UL74 ORF. The sequence analysis of UL74 showed a high degree of variability at the N-terminal end (Paterson et al., 2002). The analysis of clinical isolates identified four major phylogenetic groups, known as gO-1, gO-2, gO-3 and gO-4 (Mattick et al., 2004). gL is encoded by the UL115 ORF. Four major phylogenetic groups were identified and denoted gL-1, gL-2, gL-3 and gL-4 (Rasmussen et al., 2002). gL is essential for the transport of the gH glycoprotein to the cell surface (Kaye et al., 1992; Spaete et al., 1993).

The large number of gH-gO-gL combinations suggests that gC-III has an immunological potential, and has implications for viral tropism and spread (Rasmussen et al., 2002).
2.6.6.7. Noninfectious particles

When CMV is propagated in cell cultures, two additional morphological forms are produced from the virus-specific proteins and envelope. The first is a dense body and appears as a large amorphous structure without nucleocapsid or DNA. The second is a noninfectious enveloped particle and consists of an empty capsid surrounded by a lipid envelope (Zuckerman et al., 2009). Whereas 1% of viral progeny are infectious, 99% or more is noninfectious because of three factors: instability of HCMV virion following release from cells, production of noninfectious enveloped particles and production of dense bodies, which can constitute more than 50% of a virion preparation. Purified virion preparations are predominantly noninfectious. Dense bodies are more heterogeneous in size than other particles; they lack an organized capsid or viral DNA, which composed entirely of tegument surrounded by an envelope, and are composed predominantly of the UL83-coded pp65 tegument protein (Knipe and Howley, 2007).

2.6.7. Virus replication

CMV replication is much slower than for HSV, and CPE may not be seen for 7 to 14 days. This may facilitate the establishment of latent infection in myeloid stem cells, monocytes, lymphocytes, the stromal cells of the bone marrow, or other cells. Fusion of the virion envelope and the cell membrane occurs, after the attachment of the virus to the cell surface. The cellular receptor (s) for HCMV is still unclear, but it has been suggested to be widely distributed in cells. The virus capsid is rapidly transported to the cell nucleus and, after the proteolysis of the capsid proteins; the viral DNA is released into the nucleus (Heli, 2004).

Viral gene expression in productive replication occurs in a temporally ordered cascade (Zuckerman et al., 2009). The first proteins to be synthesized (α or immediate-early) are required for the transcription of the messenger ribonucleic acid (mRNA) for the second group of proteins (β or early). The early proteins allow DNA replication to proceed and this is followed by the appearance of the last proteins (γ or late) (Zuckerman et al., 2009). Immediate-early (IE) gene expression is first activated followed by early and late gene expression which ultimately leads to virus assembly and release from the infected cells (Heli, 2004).
IE antigens appear in the nucleus of CMV-infected cells 1 to 3 hours after infection and remain present even in latent infection. IE antigen gene products direct production of both viral and cellular genes. Early antigens appear in the cytoplasm or membrane approximately 3 hours after infection. Early antigen gene products direct viral DNA synthesis. Late antigens appear in the nucleus and cytoplasm within 6 to 24 hours after infection late antigen gene products direct production of structural nucleocapsid proteins (Bernnan, 2001), and therefore are associated with active infection (Requião-Moura et al., 2015).

The most abundantly expressed IE genes are transcribed from the major IE (MIE) locus, located in the UL region. Differential splicing of a primary transcript generates the gene products of UL122/123, IE1 and IE2. The IE proteins are transactivators of gene expression and play an important role in controlling both viral and cellular gene expression. One function of these proteins is to optimize the cellular environment for viral replication and viral gene expression. The early (E) proteins are involved in the replication of the viral DNA, whereas late (L) gene expression provides the structural proteins/glycoproteins of the viral capsid, tegument and envelope (Heli, 2004). DNA replication, capsids formation and packaging of viral DNA occur in the nucleus. Subsequently, nucleocapsids acquire a primary envelopment by budding at the nuclear membrane. They further mature through a de-envelopment/re-envelopment process in the cytoplasm before leaving the cell via an exocytic-like pathway (Mocarski and Courcelle, 2001). Enveloped virions are found within vesicles in the cytoplasm and these appear to fuse with cellular membranes to allow egress of the mature virus particles. Dense bodies also mature and are released from the infected cell in the same way as virions, so that they contain virus-specific glycoproteins (Zuckerman et al., 2009). The whole replication cycle of human CMV is slow, requiring approximately 48-72 hours. The cytopathic effect (CPE) in response to HCMV is characteristically cell enlargement with intranuclear inclusions (Heli, 2004).

2.6.8. Epidemiology

2.6.8.1. Transmission of CMV

HCMV has a worldwide distribution and infects humans of all ages, with no seasonal or epidemic patterns of transmission (Murray et al., 2007). Initial infection with HCMV
commonly occurs during childhood (Harvey et al., 2013) and often already during the first year or early in life, mainly during the first two decades (Heli, 2004).

CMV can be transmitted vertically and horizontally (Murray et al., 2007). Most infections are acquired by direct close personal contact with individuals who are shedding virus. CMV is transmitted via bodily secretions (Kudesia and Wreghitt, 2009), including saliva, urine, breast milk, tears, stool, vaginal and cervical secretions, blood, and semen, it is obvious that transmission can occur in a variety of ways (Murray et al., 2007). Thus, hygiene and virus shedding patterns remain important determinants of virus transmission patterns (Knipe and Howley, 2007). HCMV can cross the placenta and infect a fetus in utero. The virus is also present in breast milk, and neonates can be infected by this route. Child-to-child transmission has occurred especially in day care centers. Once infected, such children can transmit CMV to their parents (Murray et al., 2007). By age 18 months, up to 80% of infants in a day care center are infected and actively excreting virus in saliva and urine (Ryan and Ray, 2010).

Developing areas of the world typically exhibit widespread transmission early in life, individuals may escape infection early in life and remain susceptible during the childbearing years (Knipe and Howley, 2007).

2.6.8.2. Prevalence of CMV

Prevalence of CMV immunity, in primary or secondary infection, varies significantly by geographic region, socioeconomic status, and ethnicity (The American College of Obstetricians and Gynecologists, 2015).

Antibodies of IgG class, representing past infection, are found in approximately 60% of adults in developed countries and 100% in developing countries (Griffiths et al., 2015), with lower rates in Europe, parts of North America, and Australia, and higher rates in Africa and Asia (Kim and Kim, 2011). The seroprevalence of CMV increases with age in all populations and ranges from 40% to 100% (Murray et al., 2007), while 35% to 90% of the population have antibody against the virus by adulthood (Harvey et al., 2013). Primary CMV infection is acquired by 40–60% of persons by mid-adult life, and by more than 90% of those with multiple intimate exposures (Greenwood et al., 2012).

The seroprevalence of CMV in general population ranging from 30% to 97% according to (Paya, 2001; Preiksaitis et al., 2005). Prevalence is higher among people of low
socioeconomic status, in whom CMV prevalence can be as high as 100% (Festary et al., 2015).

In different countries the seroprevalence varies between 30% to 100%, in Scandinavian countries seroprevalence is as high as 60-80%, and in Finland even 70-80% (Heli, 2004). CMV seroprevalence is 93.8% in Japan, 86.7% in Chile (Lagasse et al., 2000), 82.5% in the United States (Fowler et al., 2003), 49.5% in France (Lepage et al., 2011).

The prevalence of CMV IgG antibodies in organ transplant recipients reflects their socioeconomic grouping. Homosexual males with HIV-positive have a very high prevalence of CMV IgG antibodies (typically 95%) (Murray et al., 2007).

2.6.8.3. Infectious period

The incubation period is about 3–6 weeks (Haaheim et al., 2002). The infectious period in immunocompetent people, the virus is present for a few weeks in saliva, blood and some other body fluids after primary infection. In immunocompromised people, prolonged infectious period may be after primary infection (Kudesia and Wreghitt, 2009).

2.6.9. Pathogenesis and clinical syndromes

CMV can cause disease by a variety of different mechanisms, including direct tissue damage and immunologic damage (Ryan and Ray, 2010).

CMV is an excellent parasite and readily establishes persistent and latent infections rather than an extensive lytic infection. CMV is highly cell associated and is spread throughout the body within infected cells, especially lymphocytes and leukocytes (Murray et al., 2013).

In any case, leukocyte-associated and endothelial cell-associated viremia appears. Viremia may last for several weeks during the primary infection. Hematogenous spreading typically results in the infection of ductal epithelial cells at the initial site. HCMV can infect a wide range of tissues including salivary glands, gastrointestinal tract, lung, liver, brain, kidney, spleen, pancreas, eye, heart, adrenals, thyroid and genital tract and then replicate in various cell types, including fibroblasts, epithelial cells, macrophages, vascular smooth muscle and endothelial cells (Heli, 2004). It can infect most renal cell types, including glomerular, tubular, and endothelial cells (Brennan, 2001).
CMV infects vascular endothelial cells and leukocytes and produces characteristic inclusions in the former. In vitro, CMV DNA can be demonstrated in monocytes showing no cytopathology, indicating a restricted growth potential in these cells. It is conjectured that these are the cells of latency for CMV (Ryan and Ray, 2010).

2.6.9.1. Primary CMV infection

Primary CMV infection is defined as the detection of CMV infection in an individual previously found to be CMV seronegative (Ljungman et al., 2002). In healthy individuals, primary HCMV infection is usually subclinical (no apparent symptoms) (Harvey et al., 2013). In older children and adults is usually asymptomatic but occasionally causes a spontaneous infectious mononucleosis syndrome (Brook et al., 2007), that identical to that caused by EBV (Harvey et al., 2013). It is characterized by malaise, myalgia, protracted fever, liver function abnormalities, lymphocytosis (Brook et al., 2007), and lymphadenopathy are characteristic infectious mononucleosis symptoms (Harvey et al., 2013). CMV mononucleosis is a mild disease, and complications are rare. Subclinical hepatitis is common. In younger children (under 7 years old), hepatosplenomegaly is frequently observed (Brook et al., 2007). About 8% of infectious mononucleosis cases are caused by HCMV (Brook et al., 2007; Harvey et al., 2013), and HCMV is cause 20–50% of heterophil-negative (non-Epstein-Barr virus) mononucleosis cases. They thought that HCMV could infect the vascular wall and play a role in the development of atherosclerosis (Bruggeman, 2000).

2.6.9.2. Congenital infection

CMV infection is the most common congenital viral infection worldwide (de Vries et al., 2011). Primary infection, reinfection and reactivation for the duration of pregnancy can all lead to in utero transmission to the developing fetus. CMV infection was relatively common among women of reproductive age, with seroprevalence ranging from 45 to 100% (Cannon et al., 2010). HCMV infection at any stage of pregnancy can give rise to congenital infection even in women who have no symptoms. The risk of transmission is greatest in the third trimester among pregnant women. Transmission rates for primary infection are 30% in the first trimester, 34–38% in the second trimester, and 40–72% in the third trimester (The American College of Obstetricians and Gynecologists, 2015). The major source of
HCMV for such women is young children, especially toddlers, whose saliva and urine contain high levels of HCMV. Forty percent of women with primary infection will transmit infection to their babies (Kudesia and Wreghitt, 2009). Cytomegalic inclusion disease ranging from varying degrees of damage to liver, spleen (hepatosplenomegaly), blood-forming organs, and components of the nervous system to fetal death. Damage to the nervous system is a common cause of hearing loss and mental retardation (Harvey et al., 2013). Other symptom includes chorioretinitis, petechial rash, (Kudesia and Wreghitt, 2009), Jaundice, thrombocytopenic purpura, myocarditis, and pneumonitis (Murray et al., 2007). Approximately 30% of severely infected infants die, and 65–80% of survivors have severe neurologic morbidity (The American College of Obstetricians and Gynecologists, 2015).

2.6.9.3. Perinatal infection

This is mainly acquired through three routes, from infected maternal genital tract secretions, from breast-feeding (Murray et al., 2013) or through transfusions of CMV seropositive blood (Ross et al., 2011). About 10% of women shed CMV in the genital tract at or near the time of delivery, and virus is transmitted to approximately 50% of the newborns. Such infants begin to excrete of the virus at 3 to 4 weeks of age, but usually remain asymptomatic (Murray et al., 2007), or a mild course. Nevertheless, sporadic cases of pneumonia or multiorgan infections are also described (Figlerowicz et al., 2011). Perinatal infection causes no clinically evident disease in healthy full-term infants (Murray et al., 2013).

2.6.9.4. Postnatal infection

This can be acquired in many ways. Saliva containing CMV is spread among young children, and, at older ages, by kissing (Greenwood et al., 2012). Semen can contain high titres of virus, and may be a source of sexual transmission or artificial insemination-associated infection. Whole blood transfusion used to be (and donated organs remain) an important source of CMV. The infections are usually subclinical, but infectious mononucleosis may occur. The disease is characterized by malaise, myalgia, protracted fever and liver function abnormalities (Haaheim et al., 2002), sore throat, swollen glands, abdomen pain and jaundice can occur (SA Health, 2012). Atypical, peripheral
lymphocytes may resemble those of EBV mononucleosis. Lymphadenopathy is usually not prominent, and heterophile antibodies are not present (Haaheim et al., 2002).

2.6.9.5. CMV latency
After primary infection, CMV can establish latency. The virus may persist at specific sites in the host without any detectable viral infection (Sinclair and Sissons, 2006), in which the genome of the virus is present in cells, but infectious virus is not recovered. During latent infection of cells, viral DNA is maintained as an episome (not integrated), with limited expression of specific virus genes required for the maintenance of latency (Ryan and Ray, 2010).

Latently infected blood leukocytes are an important reservoir for transmission of the virus in organ transplantation and blood transfusion mainly peripheral blood mononuclear cells (Heli, 2004). HCMV genome establishes a latent infection in the nucleus of these infected CD34+ haematopoietic cell population in the bone marrow (Sinclair and Reeves, 2013), and CD14+ monocytes. Viral DNA has also been detected in the common precursors of dendritic and myeloid cells (CD33+/CD14+ and CD33+/ CD15+, along with the dendritic cell markers CD1a and CD10) (Heli, 2004). It can also persist in kidneys for years (Levinson, 2010).

The major immediate early promoter (MIEP) is extremely suppressed in these cells and that this is achieved through cellular transcriptional repressors directing histone-modifying enzymes to impart repressive post-translational modifications of MIEP-associated histones (Sinclair and Reeves, 2013). CMV can be reactivated when cell-mediated immunity is decreased (Brennan, 2001).

2.6.9.6. Transmission via blood transfusion
CMV can be transmitted by blood, only 1–5% of blood units taken from seropositive donors lead to infection of seronegative recipients (Zuckerman et al., 2009), but if occur it often results in an asymptomatic infection (Murray et al., 2013). Transmission via blood products has been virtually eliminated by the routine use of filters to remove leukocytes during transfusion (Zuckerman et al., 2009).

2.6.9.7. CMV and malignancy
The detection of CMV-DNA, mRNA, or antigens in tumor tissues in some studies suggests a possible role of CMV infection in the pathogenesis of several human
malignancies. HCMV nucleic acids and proteins have been discovered in a high percentage of low- and high-grade malignant gliomas. CMV is found within the breast epithelial cells, which suggests that it may play a role in the neoplastic process. US28 is a costimulatory chemokine receptor encoded by CMV. Transgenic coexpression of the US28 ligand, CCL2, which is an inflammatory chemokine, increases intestinal endothelial cell proliferation and the development of intestinal neoplasia (Ardalan, 2012).

2.6.9.8. CMV infections in immunosuppressed and immunodeficient patients

The group of patients potentially affected by CMV infection includes patient with solid organ transplantation, hematopoetic stem cell transplant recipients and patient with HIV/AIDS. The latter two groups can exhibit the most severe CMV disease due to severely impaired cellular immunity (Ross et al., 2011). Another group is cancer patients (particularly those with leukemia and lymphoma receiving chemotherapy) (Murray et al., 2007).

2.6.9.8.1. CMV Infection and AIDS patients

CMV remains among the main causes of secondary infections to AIDS because it seems that acts as an inducer or co-factor in the progression of HIV infection pathogenesis (Silva et al., 2015). Most infections are caused by a reactivation of latent CMV infection (Kudesia and Wreghitt, 2009). Before the use of highly active antiretroviral therapy (HAART), approximately 20% to 40% of adults with AIDS developed CMV disease. The risk of CMV disease among persons with HIV infection, is linked closely with immune impairment as reflected by low CD4 T-cell count (Knipe and Howley, 2007) declined below 100/µL. In 85% of HIV with coinfection with CMV, the end-organ disease was retinitis (Griffiths et al., 2015). CMV disease is associated with colitis, encephalitis, falling white blood cell counts (Kudesia and Wreghitt, 2009), fever, polyradiculomyelopathy, gastritis, ulcerative colitis (Murray et al., 2007), oesophagitis (Greenwood et al., 2012), viremia and high CMV virus load (Zuckerman et al., 2009).

Transactivation of the promoter in the HIV-1 is the proposed mechanisms through which CMV could enhance replication or reactivation from latently infected cells (Knipe and Howley, 2007). CMV can also down regulate HIV replication, but CMV is more likely to stimulate HIV. HIV has an integrated provirus and when CMV is not actively replicating
(Zuckerman et al., 2009), when CMV early genes transactivate other viral and cellular genes leading to the production of HIV from latently infected cells (Greenwood et al., 2012).

2.6.9.8.2. CMV infection in solid organ transplantation

I. Risk factors

The specific risk factors for CMV infection after transplant include, CMV donor-recipient mismatching and the use of lymphocyte-depleting preparations induction for rejection therapy, episodes of allograft rejection, comorbid illnesses, potentially coinfection with HHV-6 and -7 (Hartmann et al., 2006; Karuthu, and Blumberg, 2012), neutropenia (Hartmann et al., 2006) and older donors (>60 years) (Karuthu, and Blumberg., 2012).

In SOT recipients, the risk of CMV disease is the result of the balance between the degree of viral replication and the recipient’s level of cellular competence and humoral immune response (Aguado et al., 2012).

The effect on CMV replication is especially intense when using high doses of methyl prednisolone or agents (such as antilymphocyte globulin (ALG) and antithymocyte globulin (ATG)), antilymphocyte antibodies (such as OKT3), mycophenolate mofetil and azathioprine (Aguado et al., 2012). Mycophenolate mofetil (MMF) has also been variably reported to be associated with an increased incidence of CMV viremia and CMV disease (late CMV) as reported by (Pereyra and Rubin, 2004).

II. Impact of CMV infection in solid organ transplant

CMV infection in transplant recipients is a significant cause of direct (caused by the virus) and indirect (caused by virus interactions with the immune system) (Greenwood et al., 2012).

The frequency and severity of CMV infection in organ transplant recipients will vary, depending on; the type of transplant, the source of the donated organ, the immune status of the recipient, and the duration of the immunosuppressive therapy. The major symptoms in these patients usually include fever, malaise, lethargy, myalgia or arthralgia, leukopenia, thrombocytopenia, and hepatitis. Specific organ damage may lead to pneumonitis in recipients of lung or heart-lung transplants; the development of myocarditis, retinitis, or accelerated vascular damage and atherosclerosis after cardiac
transplantation; hepatitis and pancreatitis in liver and pancreas transplant recipients, respectively; and gastrointestinal disease. These lead to an increased risk of graft rejection in solid-organ transplants. CMV is also responsible for the failure of many kidney transplants. This may be the result of virus replication in the graft after reactivation in the transplanted kidney or infection from the host (Murray et al., 2013). Death may occur as a result of various complications, including bacterial and fungal superinfections (Murray et al., 2007).

2.6.10. Immunity to CMV

The protective immunity against the virus to primary CMV includes both humoral (IgM, IgG) and cellular (T lymphocyte) responses (Greenwood et al., 2012). The responses in people with normal immunity keep CMV suppressed into a latent state in most individuals for most of the time (Zuckerman et al., 2009). Natural maternal immunity present prior to conception provides 69% protection against delivering a subsequent neonate with congenital CMV infection (Zuckerman et al., 2009).

2.6.10.1. Humoral immunity

Antibodies to CMV occur in most human sera. CMV-specific antibodies of the IgM, IgA, and IgG classes have all been detected (Brook et al., 2007). Antibodies of IgG class are produced at the time of primary infection and persist for life. IgM-class antibodies are produced on primary but not recurrent infection of immunocompetent individuals and persist for three to four months (Zuckerman et al., 2009). The range of outstanding antibodies are those against surface glycoproteins (glycoproteins gB and gH) that almost certainly participate in the blockage of cell infection (Caltenco-Serrano et al., 2001).

Many HCMV proteins are immunogenic and nearly all immune human sera have antibody to envelope glycoproteins gB (UL55), gH (UL75), and the tegument phosphoprotein pp150 (UL32) as well as to a nonstructural DNA binding phosphoprotein, pp52 (UL44). Neutralizing antibody has been detected against principal envelope glycoproteins gB, gM:gN, and gH:gL, and may be associated with protection from infection and disease. Neutralizing antibody may play important roles in controlling transmission (Knipe and Howley, 2007).
Immunocompromised patients may fail to produce IgM antibodies with primary infection and one third of them have IgM detectable with recurrent infections. With intrauterine infection, IgM antibodies are produced by the fetus, together with an IgG-class response (Zuckerman et al., 2009).

2.6.10.2. Cell mediated immunity
Cell-mediated immunity is essential for resolving and controlling the outgrowth of CMV infection (Murray et al., 2013) especially disseminated infection in the immunocompromised host (Greenwood et al., 2012). CMV infection alters the function of lymphocytes and leukocytes. Because CMV infects mononuclear cells, there is a degree of immunosuppression associated with the acute infection (Greenwood et al., 2012). The virus prevents antigen presentation to both CD8 cytotoxic T cells and CD4 T cells by preventing the expression of MHC I molecules on the cell surface and by interfering with cytokine-induced expression of MHC II molecules on antigen-presenting cells (including the infected cells) (Murray et al., 2013).

Antigens induce the response of the CD8+ memory lymphocytes during the primary infection in about 90% of immunocompetent individuals (Caltenco-Serrano et al., 2001). A viral protein also blocks natural-killer-cell attack of CMV-infected cells. CMV also encodes an interleukin-10 analogue that would inhibit TH1 protective immuneresponses (Murray et al., 2013).

2.6.11. CMV Infection in renal transplant recipients
CMV infection is the most common opportunistic infection in kidney transplant recipients (Karuthu, and Blumberg, 2012). CMV activation and CMV disease in the early period after renal transplantation are independent risk factors for chronic allograft nephropathy and kidney allograft loss in the late post transplant period. CMV disease is an additional factor in the pathogenesis of chronic allograft nephropathy in the presence of acute rejection (Bernarde et al., 2007).

CMV infection can be classified as primo-infection, when the transmission occurs through the graft, or reactivation, when the recipient is CMV seropositive (Requião-Moura et al., 2015).
After transplantation, CMV can appear as an infection, when the patient presents with evidence of viral replication without symptoms or disease, which has two clinical spectra,
typical viral syndrome or invasive disease, which is a less common form. Their effects can be classified as direct, while the disease is developed, or indirect, with an increase of acute rejection and chronic allograft dysfunction risks (Requião-Moura et al., 2015).

A symptomatic infection with CMV is associated with renal allograft dysfunction, mortality, and graft loss, in spite of early detection and treatment of asymptomatic infections. Poor outcomes are associated with poor HLA matching and may reflect increased immunogenicity, inflammatory response, or failure to treat CMV (Bohl et al., 2007). Finding of McLaughlin and Wu (2002), proposed that the D+/R- group have an increased risk of early allograft loss due to acute rejection, which have poorer allograft function 3 years post-transplant.

2.6.11.1. Direct effects of CMV infection after kidney transplantation

The effects of CMV are classified as direct and indirect. This classification serves to separate the effects of invasive viral infection (cellular and tissue injury) from effects mediated by inflammatory responses (e.g., cytokines) or by alterations in host immune and inflammatory responses (Kotton and Fishman, 2005). The direct clinical effects of CMV include CMV infection, CMV disease and end-organ diseases (Ljungman et al., 2002). Symptoms of CMV disease are largely nonspecific, such as fever, fatigue, body aches, and myelosuppression. CMV disease in some patients is manifested as tissue-invasive disease. The gastrointestinal tract is the most common site for tissue-invasive CMV disease, independent of the type of allograft transplant (Corte et al., 2010), which can cause abdominal pain and diarrhea. In severe cases, CMV ulceration of the gastrointestinal tract can lead to hemorrhage and perforation. Other organs that may manifest tissue invasive disease include the liver, lungs, heart, pancreas, and kidneys, and may present with allograft dysfunction easily misdiagnosed as acute or chronic rejection (Couzi et al., 2010).

2.6.11.1.1. CMV infection

CMV infection is defined as isolation of the CMV virus or detection of viral proteins or nucleic acid in any body fluid or tissue specimen. It is recommended that both the source of the specimens tested (e.g., plasma, serum, whole blood, peripheral blood leukocytes, CSF, urine, or tissue) (Ljungman et al., 2002) and there is evidence of CMV replication regardless of symptoms (Kotton et al., 2010; Kim and Kim, 2011).
2.6.11.1.2. CMV recurrent infection, reinfection and reactivation

The latent virus has been detected in most tissues in the body. It can infect most renal cell types, including glomerular, tubular, and endothelial cells (Brennan, 2001). Recurrent infection is defined as new detection of CMV infection in a patient who has had previously documented infection and who has not had virus detected for an interval of at least 4 weeks during active surveillance. Recurrent infection may result from reactivation of latent virus (endogenous) or reinfection (exogenous) (Ljungman et al., 2002).

Reinfection is defined as detection of CMV strain that is distinct from the strain that was the cause of the patient’s original infection. Reinfection may be documented by sequencing specific regions of the viral genome or by using a variety of molecular techniques that examine genes known to be polymorphic. Reinfection with exogenous virus may introduced by the transplanted organ. It diagnosed if the tow strains are distinct (Ljungman et al., 2002). Symptoms have a tendency to be most severe after primary infection; however, reactivation infection or reinfection in a severely immunocompromised host may also cause serious illness (Murray et al., 2007).

Sporadic reactivation events may occur, but they are generally controlled by cell mediated immunity, cytotoxic T-cells, and NK cells (Kim and Kim, 2011). Reactivation is common, and virus is shed in body secretions. Mononuclear cells carry the latent virus genome and viral RNA transcripts of early genes have been detected in such cells. Bone marrow progenitor cells of the myeloid line once their descendants have been activated to differentiate into tissue macrophages, the virus can enter the replication cycle (Greenwood et al., 2012).

Reactivation of CMV is usually associated with less severe disease, but can be fatal in severely immunocompromised patients (Kudesia and Wreghitt, 2009), especially those with altered cellular immunity, have frequent reactivations of herpesviruses that can lead to clinically severe disease (Ryan and Ray, 2010).

The virus is reactivated by immunosuppression (e.g., corticosteroids, infection with HIV) and possibly by allogeneic stimulation (i.e., the host response to transfused or transplanted cells) (Murray et al., 2013).
In renal transplant recipients, viral reactivation, and the primary risk factors identified are the use of anti-lymphocyte antibodies (ALA), type of immunosuppression protocol used (type of drug, dose and duration), and treatment of acute rejection. A few factors related to the recipient, such as age, co-morbidities, and the development of neutropenia. Reactivation is related to reduction of cellular immune activity, especially of CD8+ cells, as result of the immunosuppressed state, also due to activity of cytokines that induce the virus to move from the state of latency, especially tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β). The use of ALA, besides causing intense and prolonged lymphopenia, is related to the release of cytokines, especially TNF-α. Acute rejection, in addition to requiring increase of immunosuppression, causes an increased expression of IL-1β, which is a cytokine that stimulates viral replication (Requião-Moura et al., 2015).

2.6.11.1.3. CMV disease and CMV syndrome

The CMV disease is defined as detection of CMV in a clinical specimen, accompanied by either CMV syndrome with fever (Chakravarti et al., 2009), the temperature >38°C for at least 2 days within a 4-day period (Ljungman et al, 2002). Muscle pain (Chakravarti et al., 2009), leucopoenia (white blood cell count (WBC) < 3 x 10³/cmm) (Liang et al., 2013) and/or thrombocytopaenia. Moreover, organs involvement, such as hepatitis, nephritis, myocarditis, retinitis, cystitis, or pancreatitis (Chakravarti et al., 2009) as well as gastrointestinal invasion with colitis, gastritis, ulcers, bleeding, or perforation (Ortiz and André, 2011). When a CMV syndrome is complicated by specific organ involvement, the term CMV disease is used (Dirk et al., 1998).

2.6.11.1.4. CMV end-organ disease

Patients receiving immunosuppressive drugs to allow allo-transplantation are, therefore at risk of developing CMV end-organ disease (Griffiths, and Lumley, 2015). CMV causes direct damage in the form of viral syndrome or end-organ disease (Aguado et al., 2012). In solid organ transplant recipients, CMV end-organ diseases can present as lung, liver, gastrointestinal, renal or retinal disease and is considered the most important infectious complication in those patients (Kalpoe, 2007). A high viral load is required for CMV end-organ disease to develop (Griffiths, and Lumley, 2015).
2.6.11.2. Indirect effects of CMV infection after kidney transplantation

CMV is associated with a variety of indirect effects due to the virus’ ability to modulate the immune system (Couzi et al., 2010). Indirect effects of viral infections include responses to viral infections such as release of cytokines, chemokines, or growth factors. These effectors are immunomodulatory, results in further immune suppression and increasing the risk of other opportunistic infections from other viruses, such as CMV and hepatitis C in a form of viral “cross-talk.” (Kotton and Fishman, 2005). Secondary infections might develop through different mechanisms in which CMV could disrupt mucosal surfaces, predisposing the patient to superinfection, or it could cause alterations in humoral and cell-mediated immunity (Ljungman et al., 2002).

CMV infection may trigger HHV-6 and HHV-7 reactivation (Kotton and Fishman., 2005; Cukuranovic et al., 2012) and Epstein-Barr virus-related post-transplant lymphoproliferative disease (Razonable et al., 2003). As well as secondary bacterial infections (e.g., Nocardia spp.) (Peleg et al., 2007) and fungal infection (e.g., Aspergillus spp) (Husni et al., 1998). A higher rate of allograft failure and death in renal transplant recipients considerably associated with CMV infection, in part due to increased opportunistic infections as well as acute and chronic allograft rejection and reduced allograft function and survival after renal transplantation was associated with CMV persistence in the allograft (Sagedal et al., 2007).

Co-infection of BKV and CMV has been reported in renal transplant recipients, CMV has been shown to induce polyomaviruses amplification and DNA replication in vitro (Al-Obaidi et al., 2015).

In addition, CMV infection may alter expression of surface antigens (e.g., histocompatibility antigens), provoking graft rejection, the patients with CMV infection are more likely to experience acute and chronic rejection (Cukuranovic et al., 2012). In addition, new-onset diabetes mellitus has been reported in patients with CMV infection or disease after renal transplantation (Hartmann et al., 2006; Rodrigo et al., 2006).

2.6.11.3. Prevalence and incidence of CMV infection in renal transplant recipients

Incidence of CMV infection and disease during the first 100 days post-transplantation being 60% and 25%, respectively, when no CMV prophylaxis or preemptive therapy is
given (Chakravarti et al., 2009). Lower incidence rates of CMV is estimated in the renal transplant population between 8% and 32% (Hartmann et al., 2006). Dar (2007), found that although 60-100% of renal transplant recipients develop HCMV infection, only 20-30% has symptomatic infection.

In a study of Requião-Moura et al., (2015) conducted on 477 renal transplant patients. The prevalence of acute rejection is 38% confirmed by biopsy, 64% of infection by CMV, and 24% of disease, the study observed that infection and the disease by CMV increased the risk of acute rejection by 1.6- and 2.5-fold, respectively.

Sixty to 90% of all renal transplant candidates have latent CMV infections, but symptomatic infection occurs only in 20-60% of them. CMV is a significant cause of increased morbidity and mortality in this population (Chakravarti et al., 2009). CMV infection occurs in 35%–55% in renal transplant recipients (Sia and Paya, 1998). Sagedal and his collage (2002), were found that among 451 patients, 290 (64%) had CMV infection and among these 108 (24%) had CMV disease.

CMV infections have been reported to occur in 65–88% of recipients, in which 48–60% of them develop CMV disease (Hartmann et al., 2006).

Finding of Cordero et al., (2012), showed that incidence of CMV disease was 5.8% with fever as the most common presenting symptom and 55% developed CMV disease ≤ 3 months post transplantation. Coinfection occurred in 53% and case fatality rate in 11%. Risk factors for developing CMV disease included CMV donor+/recipient-.

2.6.11.4. CMV serostatus in donors and recipients

The serological status should be conducted by means of identifying IgG class antibodies (Requião-Moura et al., 2015).

Primary infection is defined as a new-onset infection in seronegative recipients (D+/R-), in whom the viral infection is transmitted by the transplanted organ. This led to the highest risk without prophylaxis (Requião-Moura et al., 2015). The latent virus can be easily transmitted from a transplant donor to recipient by either the leukocytes, or possibly even tissue cells, of the kidney. Transplant patients’ cell mediated immunity is impaired and cannot control the virus, resulting in reactivation without immunity to CMV (D+/R-), as well as in CMV-seropositive (R+). Other recipients undergo reactivation of their own latent virus (Kim and Kim, 2011).
In D+/R+ cases, approximately 20% of recipients experienced CMV disease in the absence of any prophylaxis (Sagedal et al., 2000). The incidence of CMV infection is 50-75% in D+/R- groups in the study of Chakravarti et al., (2009). The D+/R+ not the D+/R- groups had the worst graft and patient survival by 3 years. This may reflect the prevalence multiple CMV virotypes, and that D+/R+ recipients may have double exposure to different CMV strains (Kim and Kim, 2011).

In a study of Emery et al., (2012), showed that CMV syndrome/disease in SOT occurred in 20.5% of the D+/R− patients, whereas the incidence was only 8.1% and 9% in the D+/R+ and D−/R+ group, respectively (p < 0.001 compared to the D+/R− group). CMV viremia in the D+/R− group was associated with a high probability (65%) of CMV syndrome/disease in renal transplant recipients.

The incidence of CMV disease in D−/R− is <5% in Karuthu, and Blumberg, (2012) studies.

In study of Requião-Moura et al., (2015) conducted on 20,000 transplanted patients found the distribution of serological matchings to IgG status, D+/R+=47.7%, D-/R+=24.1%, D+/R-=18.2%, and D-/R-= 10.3%. When D+/R- are compared with D-/R-, there is a 28% increase in risk of graft loss, 36% in the risk of death due to all causes, and eight-fold the risk of dying by a viral infection.

2.6.12. Laboratory diagnosis of CMV

There are two potential strategies for providing a diagnosis of CMV: the detection of virus or the demonstration of a specific immune response (Zuckerman et al., 2009). Variety of methods is available for use in the diagnosis and management of patients infected with CMV include isolation of the virus in cell culture (Murray et al., 2007), shell vial culture, pp65 antigenemia test, and qualitative and quantitative nucleic acid (Khoury and Brennan, 2005), or viral proteins detection assays (Murray et al., 2007).

2.6.12.1. Specimens collection for direct detection

Tissue specimens, respiratory secretions, urine sediment, cerebrospinal fluid (CSF), amniotic fluid, and peripheral blood leukocytes have been used for the direct detection of CMV antigens or nucleic acids (Murray et al., 2007).

For PCR, from blood acid-citrate-dextrose or EDTA (Zuckerman et al., 2009), plasma obtained from anticoagulated whole blood, serum obtained from clotted blood, or
purified peripheral blood leukocytes have all been used to quantitate CMV DNA in molecular amplification assays (Mengelle et al., 2003). Delays in preparation of whole blood samples, purified leukocytes, or plasma samples after blood collection, can result in lysis of leukocytes, which may result in inaccurate quantitation of CMV DNA (Sanche and Storch, 2002). Plasma or serum may be preferable in neutropenic patients who may have inadequate numbers of leukocytes for testing (Murray et al., 2007).

Immunocompromised patients should be investigated by means of surveillance samples, taken preferably twice weekly, of blood and possibly urine or saliva must be done as routine on all patients, rather than waiting for symptoms to develop. CMV excretion from urine and saliva is very common in allograft recipients so the relative risk for future disease is typically (Zuckerman et al., 2009).

**2.6.12.2. Specimens for serologic testing**

Serum specimens are useful in screening of CMV IgG for evidence of past infection and for identifying individuals at risk for CMV infection. Detection of IgM in a single serum specimen may be beneficial. In patients with CMV neurologic disease, CSF may be tested for viral antibody if paired with a serum specimen collected on or close to the same date (Murray et al., 2007).

**2.6.12.3. Direct examination**

Several specific definitions for CMV detection in blood are recommended. Viremia is defined as the isolation of CMV by culture that involves the use of either standard or shell vial techniques. Antigenemia is defined as the detection of lower matrix phosphoprotein pp65 in leukocytes and nucleic acid testing including CMV-PCR and hybrid-capture DNA (Weikert and Blumberg, 2008). DNAemia is defined as the detection of DNA in samples of plasma, whole blood, and isolated peripheral blood leukocytes or in buffy-coat specimens. RNAemia is defined as the detection of RNA (e.g., by nucleic acid sequence–based amplification or noncommercial reverse transcriptase–PCR) in samples of plasma, whole blood, or isolated peripheral blood leukocytes or in buffy-coat specimens (Ljungman et al., 2002).

**2.6.12.3.1. Histopathologic testing**

The demonstration of CMV inclusions in tissues in the setting of a compatible clinical presentation is the “gold standard” for diagnosis (Ortiz and André, 2011). It can be
identified by the characteristic of cytomegalic cell, which is an enlarged cell (25 to 35 mm in diameter) containing a dense, central, “owl’s eye,” basophilic intranuclear inclusion body (Murray et al., 2013). These intranuclear inclusions have a surrounding halo and marginated chromatin (Zuckerman et al., 2009). These cytomegalic cells are present in the epithelial or endothelial cells of most viscera (Murray et al., 2013). They can be found in kidney tubules, bile ducts, lung and liver parenchyma, gut, inner ear and salivary gland but are less prominent in brain tissue (Zuckerman et al., 2009). The presence of characteristic cytologic changes suggests CMV infection and correlates with active disease in most cases. The inclusions are readily seen with Papanicolaou, hematoxylin-eosin staining (Murray et al., 2013) or Wright-Giemsa (Murray et al., 2007). The sensitivity of the histological examination has been enhanced by using immunostaining or in situ hybridization (ISH). However, these techniques have limited usefulness with transplant patients (Heli., 2004), because the secretion of CMV into lung fluids or urine is common in immunosuppressed individuals in the absence of invasive disease (Kotton and Fishman, 2005), and they are mainly used for diagnosing HCMV in an organ involvement (Heli, 2004).

2.6.12.3.2. Electron microscopy

Samples of urine from infants infected congenitally or perinatally contain high titres ($10^3$– $10^6$ TCID$_{50}$/ml) of CMV. Using the pseudoreplica electron microscopy technique has been possible to demonstrate this viruria. The viral specificity of the technique has been reported at $100\%$. Electron microscopy cannot be used in immunocompromised patients because the titre of CMV found in clinical samples from adults is generally lower than that found in infants, also human herpesviruses frequently infect immunocompromised patients and cannot be distinguished from each other by electron microscopy (Zuckerman et al., 2009).

2.6.12.3.3. Cell culture (conventional tube method)

Human fibroblasts best support the growth of CMV and serially passaged diploid human fetal lung strains such as WI-38, MRC-5, or IMR-90 (Murray et al., 2007). CMV can be propagated only in human fibroblasts, in which it gives rise to foci of swollen multinucleate cells with characteristic intranuclear inclusion (Collier and Oxford, 2006). The virus stays cell-associated (Brooks et al., 2007), such changes may take at least 4 to
6 weeks to appear, because the characteristic CPE develops very slowly in specimens with very low titers of the virus. The time to appearance of cytopathic effect in vitro is long. CMV replication in vivo is a highly dynamic the doubling time/half-life of CMV in blood is \( \sim 1 \) day (Emery et al., 1999). Viral culture is a qualitative method with a limited role in the diagnosis of HCMV infection (Heli, 2004). Isolation of CMV is especially reliable in immunocompromised patients, who often have high titers of virus in their secretions (Murray et al., 2013).

Tubes are examined for CPE daily for the first 5 days and then twice a week for at least 4 weeks for most specimens (6 weeks for leukocyte specimens). More commonly, foci of CPE, consisting of enlarged, rounded, refractile cells, appear during the first week. In cultures inoculated with urine or respiratory specimens from older individuals, CPE usually appears within 2 weeks. Leukocyte cultures may not become positive until after 3 to 6 week (Murray et al., 2007).

Identification of CMV isolates is made by observing of characteristic CPE. Furthermore, the suspected CMV isolates are best confirmed by an immune-fluorescence assay (IFA) using monoclonal or polyclonal antibodies (Murray et al., 2007).

2.6.12.3.4. Spin-amplification shell vial assay

The technique of detection of early antigen fluorescent foci was developed as a means of retaining the specificity and sensitivity of cell culture without having to wait for the production of CPE as a diagnostic end point (Zuckerman et al., 2009). More rapid results are achieved by centrifuging a patient’s sample at a low speed (Akhter and Bronze, 2015), inoculated onto confluency grown MRC-5 fibroblast cells on 12-mm round coverslips in 1-dram (3.7-ml) shell vials (Murray et al., 2007). Following inoculation CMV is absorbed into the cell within minutes and rapidly starts to produce \( \alpha \)- and \( \beta \)-proteins but CMV DNA synthesis is delayed and protracted until several days after infection. This explains the long delay seen in conventional cell cultures, since CMV needs to replicate, produce daughter virions and infect neighboring cells in order to produce the CPE. Infection can be diagnosed as early as 24 hours after inoculation by indirect immunofluorescent staining with monoclonal antibody (Collier and Oxford, 2006). After 24 and 48 hours, the tissue culture medium is removed and the cells are stained using a fluorescein-labeled anti-CMV antibody. The cells are read using a
fluorescent microscope (Akhter and Bronze, 2015) for the presence of one or more of the immediate early viral antigens (Murray et al., 2013).

2.6.12.4. The antigenemia assay

The antigenemia assay is the detection of lower matrix phosphoprotein pp65 (which is encoded by UL83 gene) in leukocyte (peripheral blood mononuclear cells as targets) (Cukuranovic et al., 2012). It is a semi-quantitative immunofluorescent assay based on detection of infected cells in the peripheral blood (Kim and Kim, 2011) and to estimate of the systemic viral load (Aguado et al., 2012). This assay has far higher sensitivity and specificity than culture-based methods, and is comparable in sensitivity to CMV PCR (Kim and Kim, 2011). A pp65 is a protein located in the nucleocapsid of CMV and can be identified within infected leukocytes using fluorescein-labeled monoclonal antibody specific for pp65 (Levinson, 2010).

2.6.12.5. Molecular methods

2.6.12.5.1. Polymerase chain reaction (PCR assay)

The molecular assays by direct DNA polymerase chain reaction, hybrid capture, or amplification assays are highly specific and sensitive for the diagnosis of CMV disease associated with viremia and to monitor response to antiviral therapy (Ortiz and André, 2011). PCR successfully used in a variety of clinical specimens from organ transplant recipients, patients with AIDS, and infants with congenital infection. It has replaced virus isolation for routine detection of CMV infections, especially, in immunosuppressed patients. It designed to detect replicating virus, not latent viral genomes (Brooks et al., 2007).

The sensitivity of the assay was increased by amplifying genomic regions from both the immediate-early and the late CMV genes or by using nested primers to a single gene fragment (Murray et al., 2007).

2.6.12.5.2. Other nucleic acid-based techniques

Successfully been used in the detection of viral mRNA is nucleic acid sequence based amplification (NASBA), nucleic acid sequence based amplification have been developed, and the diagnostic value of monitoring the expression of CMV immediate-early and late pp67 mRNA has been evaluated for blood leukocytes and CSF specimens. Assays used to detect CMV mRNA are usually less sensitive than other methods, but they have a high
specificity for diagnosing CMV disease. Reverse transcriptase PCR (RT-PCR) assays have been used instead of quantitative assay to demonstrate that qualitative amplification of specific CMV mRNA transcripts that are expressed only during active infection may make it possible to identify the patients at greatest risk for developing symptomatic infection (Murray et al., 2007). In addition, signal amplification hybrid capture assay uses RNA probes to detect and quantify viral DNA in an ELISA-type format where the resulting signal is measured (Ross et al., 2011).

2.6.12.5.3. Qualitative PCR assay for detection of HCMV in renal transplant recipients
Detection of the virus from the blood of immunocompromised patient is a sign of viremia and is always considered as a serious finding. Additionally, positive virus isolation from any site of a seronegative recipient is a clear indication of primary infection (Heli, 2004). CMV DNA has been successfully detected in whole blood, purified peripheral blood leukocytes, plasma, and serum by PCR (Murray et al., 2007), but cannot differentiate active disease or latent infection (Ortiz and André, 2011) especially, when peripheral blood leukocytes, whole blood are used. This is due to the capability of these tests to detect HCMV DNA even in the case of latent infection and in monitoring the success of antiviral therapy in immunocompromise patients (Murray et al., 2007). The most common assay for the detection of HCMV DNA is plasma specimen, because the results correlate more closely with disease than that in leukocytes or whole blood (Heli, 2004). The results provided by qualitative PCR is appears to be useful in detection of CMV in urine, tissue, amniotic fluid, or fetal blood for diagnosis of congenital CMV infection. In addition to the aqueous or vitreous humor in patients with CMV retinitis, and in the blood of patients at high risk of severe infection, such as CMV D+/R- transplant patients (Murray et al., 2007). Finding of Olyaeet al., (2005), showed that 12/37 renal transplant recipients had positive samples containing HCMV DNA in PBLs (32.4%), whereas, 5 of them showed symptomatic CMV disease (13.5%) and 7 of them did not show symptomatic CMV disease, 25 patients had negative PCR results, and all of them did not have symptomatic CMV disease.
Lashini and his collage (2011), reported that CMV DNA was detected in 33(25.9%) samples of 127 renal transplant patients using PCR. From 33 patients with positive PCR test, 20 patients had clinical symptoms and 13 (33.4%) of the patients had no clinical symptoms of disease.

The prevalence of CMV was detected in plasma and/or urine by PCR in renal transplant recipient in Serbia. CMV DNA was detected in 14/112 (12.5%), EBV DNA in 4/49 (8.16%), BKV DNA in 10/31 (32.26%) and JCV DNA in 3/31 (9.68%) renal transplant recipients. These results show that CMV infection is more often present in renal transplant recipients compared to other investigated viral infections (Ćupic et al., 2012). CMV viremia was detected by Khalafkhany et al., (2016) in Iran, in 68 (15.9%) of renal transplant recipients in Iran. The mean post-transplantation time in recipients was 50 months, ranging from 1 to 354 months. Viremia was detected in 31.2%, 30.7%, 17.5%, 10.2%, and 6.4% of the recipients in 0-3, 4-6, 7-12, 13-24, and more than 24 months post-transplantation, respectively.

2.6.12.5.4. Quantitative Real Time PCR (qRT-PCR) for detection and quantitation of CMV

The gold standard for diagnosis of CMV is the quantitative nucleic acid testing (QNAT). QNAT-CMV has been performed preferentially by real time PCR (RT-PCR), using plasma or total blood (Requião-Moura et al., 2015). Whole blood assays often have higher viral loads than plasma assays. The highest viral loads are associated with tissue-invasive disease, whilst the lowest are seen with asymptomatic CMV infection (Kim et al., 2011; Kim and Kim, 2011).

Real-time PCR is widely considered as an efficient and highly sensitive technique for the evaluation of CMV DNA kinetics (Habbal et al., 2009), and are currently the primary choice for the surveillance of active CMV infection in the SOT setting. Because of their extreme sensitivity, simplicity, accuracy, superior reproducibility, and dynamic linear measuring range, and short turnaround time (Aguado et al., 2011).

The qualitative standard indicates CMV in activity, but has no direct relation with the presence of the disease, which requires quantification. For that reason, the viral load detected by PCR has high predictive power for the disease (Requião-Moura et al., 2015), at the same time understanding pathogenesis of the disease (Zuckerman et al., 2009).
Opposing to antigenemia, sensitivity is not altered by blood storage, and can be transported for its use in distant centers (Requião-Moura et al., 2015).

Different type of quantitative PCR assays both in-house and commercial ones have been employed. Commonly used assays include PCR testing of plasma or whole blood, which is commercially available. CMV viral load testing using PCR techniques (including real-time PCR) or hybrid capture assays can detect and quantify CMV DNA or DNA-RNA hybrids in clinical specimens, including the CSF (Cobo, 2012).

I. Principle of quantitative Real Time PCR

In real-time PCR (kinetic PCR), the accumulation of the PCR products is monitored continuously during the PCR run, compared with the end-point measurements that quantitate the final PCR product. Chemistries for the detection of PCR products during real-time PCR can be classified into amplicon sequence specific or non-specific detection methods. The most commonly used detection methods in the virus diagnostic assays are based on the use of specific fluorogenic oligoprobes. These methods depend on fluorescence resonance energy transfer (FRET), which is the interaction of two fluorescent dyes. TaqMan probes, also called 5´ nuclease or hydrolysis oligoprobes, were the first ones used in special real-time instruments (Heli, 2004).

For target amplification, oligonucleotide primers and probes for amplification and detection, respectively, of nucleic acid are selected from conserved nucleotide sequences within a viral gene; these products represent the first level of sensitivity and specificity for quantitative real-time PCR. The assay is subsequently adjusted to allow the polymerase enzyme to function optimally and to produce sensitive and specific signals from labeled probes that are proportional to the amount of target DNA present in the blood sample. Three to 5 commercial quantitative standards are included in the quantitative test. The software for the real-time PCR instrument generates a standard curve with use of these quantitative standards. This plot relates the cycle number in which the amplified nucleic acid target from the standards is detected (by measuring fluorescence) to the amount of target present in the standards. The quantitative level of viral nucleic acid in a test specimen is then determined by comparing the cycle number (crossover point) of the specimen with the standard curve generated with the known levels of the target nucleic acid (Smith et al., 2007).
2.6.12.5.5. Quantitation of CMV from renal transplant recipients

In qRT-PCR assays both plasma as well as whole blood allows a good idea of the viral expansion processes due to de novo infection or reactivation that a transplant patient is experiencing (Reyes-Pérez et al., 2016). Concentrated leukocytes may be more productive for detecting CMV DNA than cell free specimens. Reactivated CMV infection in the transplant recipient may result in predominately cell-free (e.g., plasma) virus (Smith et al., 2007). There is a very close correlation between viral load present in whole blood and in plasma, at least in immunosuppressive patients (Ortiz and André, 2011).

According to the study of Kalpoe et al (2004), correlation between CMV DNA loads in plasma and whole blood showed correlation coefficient (r) of 0.962 indicated is a high correlation between CMV DNA loads in plasma and whole blood. In other prospective study, CMV DNA was quantified in blood samples of 255 kidney recipients with and without CMV-related symptoms in Kuwait. CMV DNA was detected in 54/255 (24%) patients; of these, 17 (31.5%) were asymptomatic, and 37 patients (68.5%) had symptomatic CMV infection (Madi et al., 2007).

In study of Zhang et al (2012), which uses qRT-PCR, CMV-DNA was detected in plasma of 29/77 recipients, yielding a positive rate of detection of 37.7%. Twelve of 21 recipients (57.1%) who suffered acute rejection had positive CMV-DNA. Among the 56 recipients suffered from chronic or mild rejection, 17(30.4%) had positive CMV-DNA plasma. Moreover, of the 29 recipients who had detectable CMV-DNA after transplant, 12 (41.4%) suffered from acute rejection; of the 48 recipients with undetectable CMV-DNA, only nine (18.8%) developed acute rejection. Post-transplant patients with acute rejection had a higher rate (57.1% vs. 30.4%, P = 0.03) of post-transplant CMV infection than those with chronic or mild rejection.

2.6.12.5.6. Plasma viral load

Plasma viral load monitoring is of modest clinical utility for prediction of CMV disease (Kotton and Fishman, 2005; Garrigue et al., 2008), because CMV is highly cell associated, samples of whole blood or leukocyte preparations provide for more sensitive detection of virus than assays that exclude leukocytes (Sanchez and Storch, 2002). However, the presence of CMV DNA in plasma suggests active viral replication due to spread of the virus from the leukocyte into the plasma (Rangbar-Kermani et al., 2011).
One the other hand, virus release into plasma from multiple pools, including endothelial cells and the reticuloendothelial system (Garrigue et al., 2008), and thus could be more associated with clinically significant disease (Sanchez and Storch, 2002). In addition, plasma viral load and its incremental rate could be used as suitable diagnostic tools in R+ recipients (William et al., 2000).

Quantification of CMV by real-time PCR showed that $3 \times 10^3$ genome equivalents per milliliter of whole blood or $1 \times 10^3$ genome equivalents per milliliter of plasma correlated with the presence of CMV disease (Knipe and Howley, 2007).

Huurman et al., (2006) adopted another opinion of view that viremia is only marker of active CMV infection. CMV viremia was defined as detection of two consecutive CMV DNA loads of more than $2.7 \log_{10} (= 500)$ copies/ml plasma.

In a study of Enan et al (2011) HCMV DNA was detected in 32/98 (32.7%) from plasma samples of Sudanese renal transplant recipients, with viral loads ranging from <200 to 42932 copies/ml.

Another study of Rangbar-Kermani et al., (2011) aimed to investigate CMV-DNA plasma viral load in active CMV infected renal transplant patients in Iran using Real-Time CMV PCR technique. The average of CMV DNA viral load was $7.61 \times 10^7$ copies/ml (min. load: $1.38 \times 10^2$ copies/ml and max load: $1.9 \times 10^9$ copies/ml).

In a prospective study of Helanterä et al., (2009), showed that CMV infection developed in 12/25 patients a mean of 107 days (range 26-330 days) after prophylaxis ended. Two were asymptomatic. In 10 patients symptoms include fever (N = 7), gastrointestinal (N = 5), upper respiratory tract (N = 3) and hepatopathy (N = 2). One patient with infection had prophylaxis terminated after 5 months (leukopenia). The mean viral load at diagnosis was 49 517 (range 490 -325 300)

In observational prospective cohort study of Silva Junior (2015), was conducted to assess CMV replication antigenemia and viral load in 200 renal transplant recipients receiving preemptive therapy. Antigenemia reference: zero Positive Cells /200.000 cells. Viral load using Real Time PCR - TaqMan Result: < 50 copies/mL Log: < 1.70. Detection Limit: 50 copies/mL. The viral loads above 100 copies /mL should be considered as active replication. The range of viral load from 50 to 109 copies/ml.
The prediction of HCMV plasma load from evaluation of CMV whole-blood load in samples from renal transplant recipients. According to the model, the plasma viral load was >500 copies/ml when the whole-blood load was >3,170 or >4,000 copies/ml with (95% confidence intervals, 73.5 and 80.5%) which is positive in patients with or without treatment, respectively (Garrigue et al., 2008).

2.6.12.6. Tissue immunofluorescence assay (IFA)
In tissue immunofluorescence assay an immunostaining with specific polyclonal or monoclonal antibodies may be used for the detection of HCMV (Heli, 2004) in biopsy material (e.g liver, lung) (Zuckerman et al., 2009) and blood leucocytes or cell obtained by bronchoalveolar lavage (Haaheim et al., 2002). These biopsy samples may contain cells infected with HCMV, which can be visible by staining frozen sections with antisera to HCMV. Alternatively, the tissue can be disrupted and the cells fixed to glass slides before staining. Tissues should be brought to the laboratory in transport medium or fixed in ethanol (Haaheim et al., 2002).

2.6.12.7. In situ hybridization (ISH)
In situ hybridization (ISH) methods have been employed to improve the histological diagnosis of infection. HCMV specific probe, usually labeled by biotin, is used in these assays (Heli, 2004). The concentration of CMV infected cells per ten high-power fields (HPF) was assessed by counting the total number of cells showing definite CMV staining in each biopsy fragment or representative section dividing this number by the total number of HPFs present in the slide (Moreira et al., 2010).

2.6.12.8. Serological diagnosis (serological tests)
Humoral response to primary HCMV infection is manifested by the production of IgG and IgM antibodies (Heli, 2004). Detection of viral IgM antibodies suggests a current infection (Requião-Moura et al., 2015) and IgM antibodies can also persist for a long time after infection in some healthy individuals (Heli, 2004).

Serologic tests are useful before transplantation to predict risk for disease (Requião-Moura et al., 2015). HCMV IgG antibody testing has a role in the evaluation of organ and recipient prior to transplantation.

Seroconversion may not occur until well after the resolution of symptoms and after renal transplantation occurs in the majority of seronegative recipients of seropositive donors
within 6 to 12 month. This may correlate with some degree of immunological protection against CMV (Kotton and Fishman, 2005). Specific IgM antibody may not be present especially during reactivation of virus (Ryan and Ray, 2010). Seroconversion is diagnostic but rarely occurs, especially in AIDS patients, because more than 95% of these patients are seropositive for CMV before infection with human immunodeficiency virus (HIV). Interpretation of the results may be confounded by the presence of passive antibody that may have been acquired from a blood or body-fluid contamination (KDIGO, 2009; Ortiz and André, 2011), and by antibody based therapy. In the other hand, increase or decrease in antibody levels, in general, does not provide an actual diagnosis of HCMV infection in the immunosuppressed patient population, due to frequent reactivations of the virus (Heli, 2004).

Detection of IgM antibodies may be helpful in cases of suspected CMV mononucleosis or in pregnant women (Zuckerman et al., 2009).

2.6.12.8.1. IgG Antibody as a marker of past infection
The detection of IgG antibodies against CMV is hallmark of infection sometime in the past. The individual is said to be seropositive and is liable to experience reactivations of their latent infection. The presence of IgG antibodies against CMV is thus a marker of potential infectivity; although a seropositive individual is ‘immune’ in the immunological sense, this term doesn’t means protection from endogenous or exogenous infection (Zuckerman et al., 2009).

2.6.12.8.2. Enzyme-linked Immunosorbent Assay (ELISA)
ELISA (enzyme-linked immunosorbent assay)-based methods can be easily automated; they are fast and convenient to perform. They usually have good sensitivity and they are also commercially available. Sensitive ELISA allows the detection of specific IgG and IgM antibodies (Haaheim et al., 2002). The serologic diagnosis using ELISA techniques can distinguish primary from recurrent infection by demonstrating IgG seroconversion or the presence of HCMV-specific IgM (Harvey et al., 2013). IgM antibodies peak early in the infection and are usually undetectable 12–16 weeks after the onset of subclinical infections. IgM persists for longer periods in symptomatic infections and especially in congenital infections. Low levels of IgM antibodies may be detected in recurrent CMV infections. Specific CMV serum IgG antibodies last for decades. A variable rise in the
IgG titre is seen in recurrent CMV infections. A pretreatment serological status is of great value in transplant patients and cancer patients receiving chemotherapy (Haaheim et al., 2002).

However, the rise in serum antibody levels is an insensitive sign of actual HCMV infection in transplant patients. The seroprevalence is high and the presence of IgG antibodies is only informative of the patient’s past history regarding HCMV infection. Furthermore, there is a time lag between primary infection and IgM antibody production (IgM level can remain undetectable because of delayed seroconversion owing to immunosuppressive agents). Therefore, serology has a limited diagnostic value in the transplant patient group (Halling et al., 2001).

In a study of Enan et al., (2011), the sero-reactivity against HCMV in renal transplant patients in Khartoum state – Sudan using ELISA were 100% for IgG, while only 6% of them showed IgM antibodies against CMV. Another report in Sudan by Awad Alkareem et al., (2013) found that the seropositivity of IgG were 98% and 95% in renal transplant and haemodialysis patients respectively, while 6% of renal transplant had IgM using ELISA technique.

Khairi et al., (2013), conducted another study among pregnant women in Sudan, aimed to determine the seroprevalence of HCMV using ELISA technique. Out of the 200 pregnant women tested, 195 (97.5%) and 12 (6.0%) were CMV IgG and CMV IgM positive, respectively.

In study of Abd Alla et al., (2015), the seroprevalence of IgM and IgG of CMV among hemodialysis patients in Gezira state, central Sudan using ELISA technique were 45.2% (42/93) and 95.7% (89/93), respectively.

Al- Khaweledy et al., (2014) carried out a study in Iraq aimed to detect the HCMV among patients suffering from acute or chronic renal failure. The obtained results showed that HCMV IgG was detected by ELISA in (100%) of renal failure patients while IgM were (18.66%).

2.6.12.8.3. Indirect immunofluorescence assay (IFA)

Indirect immunofluorescence assay is commonly used method for detecting CMV antibodies, in the indirect IFA dilutions of test serum are incubated with virus-infected cells that have been fixed to a glass microscope slide. Specific antibody-antigen
complexes are detected using an anti-human antibody conjugated with fluorescein isothiocyanate and fluorescence microscopy. IFAs are useful for the qualitative and quantitative detection of CMV antibodies (Murray et al., 2007).

2.6.12.8.4. Other serological tests

Varieties of laboratory tests with different degrees of sensitivity have been described for the measurement of HCMV antibodies in human sera. The methods include complement fixation (CFT), indirect hemagglutination, latex agglutination, radioimmunoassay (RIA) (Sia and Patel, 2000) and neutralization test are other serologic tests used to measure CMV antibody (Murray et al., 2007).

2.6.13. Genotyping of CMV

The large CMV genome encodes several hyper variable loci. Many genetically different strains of CMV circulate in the human population. It has been suggested that difference in virulence, pathogenicity, progression and severity of disease in immunocompromised individuals, including transplant recipient may be attributed to variation between HCMV strains (Gandhoke et al., 2013). They thought that the genetic variations not only affecting the viral pathogenicity but also clinical outcomes in immunocompromised patients (Madi et al., 2011b).

Glycoprotein B (gB), gM, gN, gH, gL and gO are not only key targets for neutralizing antibodies but also are believed to be involved in viral egress and entry into cells (Yan et al., 2008). The gH component induces virus-neutralizing antibodies and facilitates the penetration of the virus into the host cell. gL is necessary for transport of gH to the cell surface, whereas gO is dispensable for viral replication. It has recently been proposed that gH/gL binds cellular receptors before triggering gB, which is not required for binding ligands, and instead acts as the fusion protein (Paradowska et al., 2014).

The HCMV gB, gH and gN are the most abundant. The gB gene, the gH gene and the gN gene have all been utilized for genotyping HCMV most frequently (Dar, 2007). Extensive attention has recently been focused on the analysis of strain variation among HCMV isolates. Some 20 different strains have been isolated and differentiated by restriction fragment length polymorphism (RFLP) (Gandhoke et al., 2013).
2.6.13.1. Glycoprotein B genotyping

Glycoprotein B the most widely characterized polymorphic gene and it is encoded by UL55 (Sowmya et al., 2007) is considered to be an important multifunctional envelope component, responsible of virus entry, cell-to-cell spread, and the fusion of infected cells (Jun et al., 2012). It is a critical factor in tissue tropism, viral pathogenesis (Sowmya et al., 2007) and specific cytotoxic T-cell immune responses (Arista et al., 2003). gB antibodies have been of interest because of their therapeutic potential for neutralization (Jun et al., 2012).

CMV gB is expressed as a precursor molecule that is glycosylated and then cleaved at codon 461 to form a disulfide-linked complex of gp55 and gp116 (Coquelle et al., 2004). HCMV gB genotyping is based on the highly variable region around the proteolytic cleavage site (Bhattarakosol and Chantaraarphonkun, 2007). Wild type CMV strains can be classified into four major gB genotypic variants (gB 1-4) (Cunha et al., 2011) based on gB nucleotide sequence, which encodes a variable region that encompasses the protease cleavage site (Coquelle et al., 2004). A fifth gB genotype (gB 5) was detected in several AIDS patients (Deckers et al., 2009). Each of them has tropism for distinct cell lines, leading to different pathogenesis and severity of disease (Cunha et al., 2011). It has been suggested that differences in virulence, pathogenicity, progression and severity of disease in immunocompromised individuals, including transplant recipients, may be attributed to variations between HCMV strains (Dar, 2007).

Several reports find a correlation between gB genotype and the occurrence of CMV infection or disease in organ transplant recipients (Ishibashi and Suzutani, 2012). Furthermore detecting gB antigen in patients with HCMV infection may facilitate the monitoring of the infection (Jun et al., 2012). According to analysis of two envelope glycoproteins (gB and gH), clinical HCMV isolates have been shown to adopt one of four gB and two gH sequence configurations at certain variable loci (Madi et al., 2011b). Reported data indicate that gB type 1 HCMV strains are less virulent than other gB genotypes strains. gB types 2 and 3 in bone marrow-transplant (BMT) recipients and AIDS patients are associated with greater virulence, at the same time as the gB type 4 virus is rarely found in transplant recipients. In congenitally infected infants, strains with the gB genotype 1 are frequently encountered (Arista et al., 2003). Transplantation in a
D+/R+ setting is usually accompanied by multiple CMV strains in recipients after transplantation, with mixtures of gB and gH genotypes were commonly observed in organ transplant recipients (Ishibashi and Suzutani, 2012). Presence of multiple gB genotypes in immunocompromised patients is associated with a higher viral load, higher prevalence of HCMV disease and concomitant infection with other herpesviruses such as EBV (Sowmya et al., 2007).

The genotyping of HCMV strains based on the gB gene have been determined by PCR-based RFLP, Single-stranded conformation polymorphism, heteroduplex mobility analysis, DNA sequencing (Renzette et al., 2014). Genotyping of HCMV among renal transplant recipients in study of Madi et al., (2011b), revealed that HCMV gB1 and gH1 genotypes were the most the predominant HCMV genotypes ($P < .05$, $P < .05$, respectively). Both HCMV gB1 and gH1 genotype were significantly more often associated with the development of fever with leucopenia and severe HCMV disease than other gB or gH2 genotypes.

Another study conducted by Bhattarakosol and Chantaraarphonkun (2007), in which a total of 128 patients including renal transplant were undergoing CMV genotyping directly by using nested PCR and RFLP with restriction enzyme Hinfl and Rsal. HCMV gB genotyping was successful in 113 (70%) samples. Mixed gB genotype was most frequently found (39 samples, 35%), followed by gB1 (37, 33%), gB3 (17, 15%), gB2 (12, 11%), and non-typed (8, 7%). No gB4 was observed.

In a large prospective cohort study of Manuel et al., (2009) observed that organ transplantation have shown mixed infection to be associated with higher viral loads and delayed virological clearance according on the basis of gB distribution analysis. Mixed genotype infection was more likely shown in D+/R.

A prospective analysis of active HCMV infection was conducted on 33 pediatric renal or hematopoietic stem cell post-transplant patients in Brazil. The study evaluated the prevalence of different gB and correlation with clinical signs. Twenty (60.6%) patients demonstrated active HCMV infection. gB1 and gB2 genotypes were more frequent in this population. they observed that gB2 had correlation with reactivation of HCMV infection and that patients with mixture of genotypes did not show any symptoms of HCMV disease (Dieamant et al., 2010).
Finding of study of Coaquette et al., (2004) showed that patients infected with a single gB genotype, patients infected with multiple gB genotypes developed progression to CMV disease, had an increased rate of graft rejection, higher CMV loads. The presence of multiple gB genotypes, rather than the presence of a single gB genotype, could be a critical factor associated with severe clinical manifestations in immunocompromised patients.

Arista et al., (2003) showed that predominant circulation of HCMV strains with gB type 2 and 3 was detected in both the immunocompetent host with a primary HCMV infection and the immunocompromised host (including renal transplant) with or without HCMV disease. No association between gB types and subjects with different risk of developing HCMV disease was found.

In study of Khalafkhany et al., (2016), the distribution of gB1, gB2, gB3, and gB4 genotypes in renal transplant recipients in Iran was detected as 26.5%, 20.5%, 17.6%, and 5.9%, respectively. Mixed genotype infection was observed in 29.4% of the recipients.

In study of Gandhoke et al., (2012) in India, both RFLP and sequencing of gB gene fragment showed that gB 1, 2 and 3 genotypes were in circulation. gB 3 was the most prevalent genotype in symptomatic infants. Hepatosplenomegaly was the most common feature in gB-3 genotype of CMV. gB2 congenital CMV infection was more commonly associated with long term sequelae.

2.6.14. CMV sequencing

HCMV is highly diverse within humans (Renzette et al., 2014). The complete genome of HCMV was elucidated almost 25 years ago using a traditional cloning and Sanger sequencing approach. Analysis of the genetic content of additional laboratory and clinical isolates has lead to a better, albeit still incomplete, definition of the coding potential and diversity of wild-type HCMV strains (Sijmons et al., 2014).

Extensive sequence variation is found in the gB and gH genes (5–10 %), a greater level is found in the gN and gO genes (40–50 %); the gL and gM genes are highly conserved among clinical strains (Yan et al., 2008).

The most commonly used HCMV genotyping methods, such as traditional Sanger DNA sequencing, restriction fragment length polymorphism of PCR products, and genotype-specific PCR assays, lack sensitivity and are not quantitative. Even cloning of PCR
products and subsequent Sanger sequencing do not allow a sensitive assessment of the genotypes present unless a very large number of individual clones is sequenced. Improved genotype-specific real-time-PCR-based assays have been established recently for gB and gH genotyping, and these allow simultaneous detection and quantitation of distinct genotypes in mixed infections down to a level of 5% or even less (Görzer et al., 2010).

Ultra-deep pyrosequencing, is the most sensitive method for quantitative HCMV genotypes, with which even low-abundance genotypes at a frequency of less than 1% of the population can be detected. It detects sequence variations over the entire amplified region, it is especially useful for genotyping highly variable regions that show a large number of distinct genotypes, as is the case for gO and gN. Novel genotypes or variants of genotypes can also be discovered with ultra-deep pyrosequencing, even when they are present in the mixture at low abundance (Puchhammer-Stöckl and Görzer, 2011). Ultra-deep pyrosequencing to study the gO, gN and gH loci from HCMV-infected transplant recipients. They found that all patients studied had mixed infections, with as many as six genotypes observed in a single patient (Renzette et al., 2014). On the other hand, deep sequencing enables novel diagnostic applications for sensitive in detecting drug resistance mutation (Sijmons et al., 2014).

2.6.15. Treatment and prevention of CMV

Treatment of established CMV disease requires a multifactorial approach, including reduction of immunosuppressive agents, antiviral agents, and in some cases adjuvant therapy. Intravenous ganciclovir has been considered the mainstay of therapy (Karuthu, and Blumberg., 2012). Both of these strategies effectively control CMV end-organ disease in transplant patients (Griffiths, and Lumley, 2015). There are two main methods for CMV prevention: universal prophylaxis and preemptive therapy (Helanterä et al., 2010).

2.6.15.1. Universal prophylaxis

Universal prophylaxis involves giving antiviral medication at prophylaxis dose for a defined time to a cohort (i.e. when either donor and/or recipient are seropositive for CMV) or defined subset of a cohort (i.e. given only to the highest risk subset, D+/R−). D+/R− are at greatest risk of CMV infection, and without prophylaxis more than 50% of
these high-risk patients will develop symptomatic infection. Therefore, prophylaxis is recommended for D+/R– for at least 3 months after transplantation (Helanterä et al., 2010). Primary antiviral prophylaxis appears to be more effective in preventing the indirect effects of CMV than pre-emptive therapy (Hodson et al., 2005).

2.6.15.2. Preemptive therapy

Preemptive therapy is defined as serial testing done weekly or biweekly for the first few months after transplant or after treatment of rejection, with treatment dose antiviral therapy initiated once a certain defined positive threshold is reached (Kotton, 2013), with absence of signs and symptoms (Costa, 2011). Pre-emptive therapy is very effective at preventing HCMV end-organ disease, such as gastrointestinal ulceration, hepatitis, pneumonitis or retinitis, which are caused by viremic spread of virus (Griffiths et al., 2015). Aguado et al (2011), proposed for initiation of pre-emptive therapy in SOT recipients at DNAemia levels, ranging from 1,000 to 5,000 copies/ml of plasma or from 1,000 to 300,000 copies/ml of whole blood, at low or intermediate risk for CMV end-organ disease.

Ganciclovir is a potent inhibitor of CMV replication and can be used either for prophylaxis or for pre-emptive therapy (Griffiths, and Lumley., 2015). Ganciclovir GCV is a synthetic acyclic nucleoside analogue, structurally similar to guanine. GCV requires phosphorylation to achieve antiviral activity. The enzyme responsible for its phosphorylation is the product of the CMV UL97 gene, a protein that functions as a protein kinase and phosphotransferase which generate the triphosphate form. GCV triphosphate competitively inhibits DNA synthesis catalyzed by inhibition of the CMV polymerase (encoded by the UL54 gene) (Karuthu, and Blumberg, 2012). Ganciclovir lead to improved graft function in patients with CMV-associated late acute rejection (Yilmaz et al., 1996).

Valganciclovir (Val-GCV) is a mono-valyl ester pro-drug of GCV (Buonsenso et al., 2012). Valcyte in CMV-Disease Treatment of Solid Organ Recipients (VICTOR) trial found that valganciclovir was as effective as intravenous ganciclovir at least, in some SOT recipients with mild to moderate disease. For patients with life-threatening CMV disease, high viral loads, leukopenia, and impaired absorption, intravenous ganciclovir is preferable and maintenance. Immunosuppression should be decreased despite the
potential risk of rejection (Karuthu, and Blumberg, 2012). Acyclovir antiviral also used for universal prophylaxis of CMV infection (Kotton et al., 2013).

Number of factors are contributes to emerging of drug resistance including; treatment with GCV for prolonged periods, lack of specific CMV immunity, type of graft, potent immunosuppression, suboptimal antiviral drug levels, and delayed viral clearance during treatment (Menghi et al., 2016). Resistance can be identified by genotype testing for mutations of the genes encoding UL97 and UL54 (Karuthu, and Blumberg., 2012) or sequencing of both genes (Madi et al., 2011a). An alternate therapy for drug-resistant CMV; high-dose ganciclovir (for specific genetic mutations), foscarnet, and cidofovir have been used effectively (Karuthu, and Blumberg., 2012). In addition, three new drugs have recently completed phase II clinical trials (maribavir, brincidofovir and letermovir) (Griffiths and Lumley, 2015).

2.6.15.3. Vaccination of CMV

Several CMV vaccines are under development; none is currently available for routine clinical use. Types of vaccines includes live attenuated, DNA, subunit vaccine proteins with an adjuvant, and recombinant viral vaccines (Kotton et al., 2013). A Towne strain vaccine study was also conducted in post-renal transplantation patients with high risk of CMV infection. After vaccination, cellular and humoral immune response was observed. A recombinant gB vaccine with MF59 adjuvant was shown to induce neutralizing antibodies and prevent infection (Kotton et al., 2013). Passive immunoprophylaxis is still on trail state (British Transplantation Society Guidelines, 2011).
CHAPTER THREE
MATERIALS AND METHODS

3.1. Materials
Blood specimens were collected from renal transplant recipients.

3.2. Methods
3.2.1. Study design
This is an analytical cross sectional study, aimed to detect CMV infection among renal transplant recipients, in addition to genotype the CMV detected.

3.2.2. Study area and duration
This study was conducted in Kidney transplanted association hospital and Ahmed Gassim teaching hospital in Khartoum state from June 2013 to June 2015.

3.2.4. Study population
Renal transplant recipients in two hospitals in Khartoum State.

3.2.5. Inclusion criteria
Renal transplant recipients, who agreed to participate in this study, adults and children, of both sexes with or without signs and symptoms of CMV infection were included in this study.

3.2.6. Exclusion criteria
Other organ transplant recipients were excluded.

3.2.7. Sample size
One-hundred and four (n=104) renal transplant recipients were selected for this study.

3.2.8. Sampling technique
This study is based on non-probability convenience sampling technique. Samples were taken from attended agreed recipients during their regular medical checkup.

3.2.9. Method of data collection
Data were collected through non self-administrated questionnaire from renal transplant recipients. The interview questionnaire consists of 10 opened and
closed-end questions. Some information were taken from patient’s clinical reports.

3.2.10. Specimen collection

Five ml of blood specimens were collected in EDTA (for TWBCs, platelets count and DNA extraction) and plain container (for detection of IgG and IgM) from each individual. TWBCs, platelets count were done from EDTA samples then blood samples from EDTA and plain containers were centrifuged at 3000 rpm for 5 minutes. Plasma and sera were collected in aliquots of 3 plain sterile containers for each one and stored at -20°C until tested. EDTA sample for (TWBCs, platelets count) was tested immediately.

3.2.11. Total white blood cells count (TWBCs) and platelets count

Blood samples were aspirated and TWBCs, platelets count were measured by (Sysmex Corporation 2012-2014) Leucopenia was defined as total white cell count of < 4000 TWBCs/cmm. Persistent thrombocytopenia was defined as a platelet count <150,000 platelet/cmm.

3.2.12. Enzyme-Linked Immunosorbent Assay (ELISA) for detection of CMV IgG antibodies

The Enzyme-Linked Immunosorbent Assay (ELISA) was used to detect the specific HCMV IgG and IgM antibodies. Commercial ELISA Kits (G.E.N.E.S.I.S diagnostics, Omega diagnostics group PLC, Cambridge shire, UK) were used as described by the manufactures. The sample diluent was diluted 1:14 in distilled water. A 100 μl of each of the following: the negative control, 3 U/ml standard, positive control and diluted samples (1:100) (all were in duplicate) were incubated in microplate well coated with CMV antigen at room temperature for 20 minutes. The wells were washed three times manually by washing buffer. Then 100 μl of conjugate reagents were added to each well and incubated at room temperature for 20 minutes. After four times washing, substrate solution (TMB Substrate) was added (100 μl /well) and the plate was incubated for 10 minutes then 100 μl of the stop solution were added. Optical densities (O.D) of controls, the standard and samples are measured using a microplate reader within 10 minutes at 450 – 630 nm.
3.2.12.2. Calculation and interpretation of the results
To determine the presence or absence of CMV-IgG, the measured O.D is compared to O.D mean of 3 U/ml standard as follow: Negative samples: O.D < O.D of 3 U/ml standard and positive samples: O.D ≥ O.D of 3 U/ml standard.

3.2.13. ELISA for detection of CMV IgM antibodies
Commercial ELISA Kits (G.E.N.E.S.I.S diagnostics, Omega diagnostics group PLC, Cambridge shire, UK) were used as described by the manufactures. IgG-absorbent is prepared by addition of 40 ml of sample diluent to 10 ml IgG absorbent (1:4) and all samples were diluted 1:100. A100 μl of the negative control, 10 U/ml standard, positive control and diluted samples were incubated in microplate well coated with CMV antigen at room temperature for 20 minutes. The wells were washed three times manually by washing buffer (which diluted firstly by distilled water 1:9). Then 100 μl of conjugate reagent were added to each well and incubated at room temperature for 20 minutes. After another washing step to eliminate unbound material, substrate solution (TMB Substrate) was added (100 μl / well) and the plate was incubated for 10 minutes then the stop solution (100 μl) were added. The optical densities (O.D) of controls, 10 U/ml standard and samples are measured in a microplate reader within 10 minutes at 450 – 630 nm.

3.2.13.2. Calculation and interpretation of the results
To determine the presence or absence of CMV-IgM, the measured O.D is compared to O.D mean of 10 U/ml standard as follow: Negative samples: O.D < O.D of 10 U/ml standard and positive samples: O.D ≥ O.D of 10 U/ml standard.

3.2.14. Quantitative Real Time PCR
3.2.14.1. DNA extraction from plasma samples
DNA is extracted from peripheral blood plasma using QIAamp DNA mini kit (Qiagen-Germany). Internal control (IC Glob- ß-globin gene DNA), should be added to each sample during extraction. The preparation for extraction as follow: twenty (20 μl) Qiagen protease (proteinase K) were pipette into the bottom of a 1.5 ml microcentrifuge tubes labeled by number of samples, (C-) and (Pos) (for negative control and positive control). Negative control and
positive control were included in each set of DNA extraction. A 10 μl of Internal Control (IC) were added to each tested tubes. Then 200 μl plasma samples were added to the corresponding microcentrifuge tubes, then 100 μl of negative control was added to tube labeled (C-), 90 μl of negative control, and 10 μl of positive DNA CMV/human (CMV Real –RT Quant - Sacace- Italy) was added to tube labeled (Pos). After this step 200 μl of AL buffer were added to all tubes, mixed by pulse vortexing for 15 second and incubated at 56°C for 10 minutes. Briefly, the tubes were centrifuged to remove the drops from inside of the lid. Then 200 μl of (98-100%) ethanol were added to all tubes and were mixed again by pulse vortexing for 15 second then briefly centrifuged. Carefully the mixture were applied to the QIAamp mini spin column (in 2 ml collection tube) then all tubes were centrifuge at 8000 rpm for 1 minute. Then spin column were placed in a clean 2 ml tubes, and the tubes containing filtrate were discarded. Carefully QIAamp mini spin column were opened and 500 μl Buffer AW1 were added then all tubes were centrifuge at 8000 rpm for 1 minute. Then spin column were placed in a clean 2 ml tubes, and the tubes containing filtrate were discarded. Carefully mini spin column were opened and 500 μl Buffer AW2 were then all tubes were centrifuge at full speed 14000 rpm for 3 minute. Then spin column were placed in a clean 2 ml tubes, and the tubes containing filtrate were discarded. Then centrifuged again at full speed for 1 minute. The spin column were placed in a clean 1.5 ml tubes and, tubes containing filtrate were discarded again. Carefully spin column were opened and 100 μl Buffer AE were added and incubated at room temperature (15-25°C) for 1 minute and then centrifuged at 8000 rpm for 1 minute. DNA yield were stored at -20°C for one month or at -70°C for one year.

3.2.14.2. qRT- PCR (detection and viral load estimation)

CMV amplification and quantification (estimation of viral load) was done using CMV Real – RT Quant kits (Sacace- Italy) (Appendix I) and Rotor –Gene Q series software 2.0.3 (build 2) Real time PCR machine (Qiagen-Germany). For reproducibility of qRT- PCR in measuring CMV viral load accurately in clinical
specimens, intraassay variability was included using a duplicates of CMV calibrator standard containing $10^2$ and $10^4$.

The total reaction volume was 25 μl. PCR–mix -2- FRT and polymerase (TaqF) mixture was prepared by transfer the content of the tube with polymerase (TaqF) (30 μl) into the tube with PCR – mix -2-FRT (300 μl). Test preparation for quantitative PCR amplification is described in table (3.1).

**Table (3.1) Tested samples procedure for qPCR.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>PCR–mix- FRT CMV</th>
<th>PCR–mix-2-FRT and Polymerase (TaqF)</th>
<th>QS1</th>
<th>QS2</th>
<th>Pos</th>
<th>C- RNA-buffer</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>QS1*</td>
<td>10 μl</td>
<td>5 μl</td>
<td>10 μl</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>QS2*</td>
<td>10 μl</td>
<td>5 μl</td>
<td>-</td>
<td>10 μl</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PCE</td>
<td>10 μl</td>
<td>5 μl</td>
<td>-</td>
<td>-</td>
<td>10 μl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NCE</td>
<td>10 μl</td>
<td>5 μl</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10 μl</td>
<td>-</td>
</tr>
<tr>
<td>NCA</td>
<td>10 μl</td>
<td>5 μl</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10 μl</td>
</tr>
<tr>
<td>Sample</td>
<td>10 μl</td>
<td>5 μl</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

Key: - QS1: DNA calibrator 1, QS2: DNA calibrator 2, *: Each calibrator in duplicates, PCE: positive control of extraction, NCE: negative control of extraction, NCA: negative control of amplification.

**3.2.14.3. Amplification**

For rotor-type thermocycler instruments, the temperature profile program was created as described in table (3.2).
Table (3.2): Amplification profile program

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature °C</th>
<th>Time</th>
<th>Fluorescent detection</th>
<th>Cycle repeats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold</td>
<td>95</td>
<td>15 min</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>5 sec</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>20 sec</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>15 sec</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>95</td>
<td>5 sec</td>
<td>FAM, HEX/JOE/Cy3, ROX/ Texas Red</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>20 sec</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>15 sec</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Fluorescent was detected on the second step (60°C) in FAM/Green, HEX/JOE/Cy3/Yellow and ROX/Orange/Texas Red fluorometer channels.

3.2.14.4. Calculation of the results

The real-time monitoring of the fluorescent intensities during the real-time PCR allows the detection of accumulating product. The fluorescent were detected at the annealing step (60°C) in cycling 2 of each cycle, and cycle threshold (Ct) for each sample was calculated by determining point at which the fluorescence exceeded threshold limit. CMV DNA (Positive control and human DNA) was detected in the HEX/JOE/Cy3/Yellow channel. Internal Control STI-87(IC) was detected in the ROX/Orange channel. Fluorescent intensities detected in two channels: CMV DNA in the HEX/Yellow/JOE/Cy3 channel, Internal Control (IC) DNA in ROX/Orange/Texas Red channel. Interpretation of the results of (Pos) and (NC) and (NCA) according to guiding sheet.

3.2.14.5. Estimation of viral load

For quantitative analysis (viral load copies for samples and positive control), the concentration of CMV DNA \( ^{kP} \text{CMV DNA} \) per ml of sample for peripheral blood plasma samples was calculated by the following formula:
CMV DNA = the number of the CMV DNA copies in DNA sample.

IC = coefficient corresponds to the number of IC DNA copies in DNA samples. Coefficient was specific to each lot in data sheet (1.25x 10^5) (Appendix II and III, IV).

3.2.15. Glycoprotein B genotyping

Genotyping of HCMV gB was carried out by nested PCR and sequencing of highly diverse region of glycoprotein B.

3.2.15.1. Nested PCR glycoprotein B genotyping

3.2.15.1.1. Primers

PCR amplification was performed using published primer pairs according to (Gandhoke et al., 2012), which are shown in table (3.4).

3.2.15.1.2. Preparation of primers

A10 µl form 100 pmol/ml from each primer was dissolved into 90 µl DW for preparing 10 pmol/ml as working primer.

Table (3.3): The selected primers for amplification of gB CMV genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>gB-1</td>
<td>5,CAAGARGTGAACATGTCCGA 3,</td>
</tr>
<tr>
<td>gB-2</td>
<td>5, GTCACGCAGCTGGCCAG,3</td>
</tr>
<tr>
<td>gB-3</td>
<td>5,TGGAAACTGGAACGTTTGGC,3</td>
</tr>
<tr>
<td>gB-4</td>
<td>5,GAAACGCGCGGC AATCGG ,3</td>
</tr>
</tbody>
</table>

3.2.15.1.3. Preparation of reaction mixture for outer nested PCR

Reagents were used for each gene in the following volumes (total reaction volume was 25 µl) in 0.2 ml eppendorff tube. A 10 µl deionized sterile water and 5 µl Master mix (iNtRON, Seongnam, Korea), then 1 µl forward primer (Metabion, Germany) followed by 1 µl reverse primer (Metabion, Germany) finally 8 µl DNA (template DNA).
3.2.15.1.4. Protocol for outer nested PCR of gB CMV gene

The amplification was performed by using primus 96 thermal cycle (BIO-RAD). The PCR mixture was subjected to initial denaturation step at 95 °C for 10 minutes. DNA was amplified for 35 cycles as followed: denaturation at 95 °C for 1 minute, primer annealing at 55 °C for 1 minute, followed by a step of elongation at 72 for °C 1 minute, the final elongation was at 72 °C for 7 minute, according to (Gandhoke et al., 2012).

3.2.15.1.5. Preparation of reaction mixture for inner nested PCR

Reagents were used for each gene in the following volumes (total reaction volume was 25 µl) in 0.2 ml eppendorff tube. Volume of 17 µl deionized sterile water and 5 µl Master mix (iNtRON, Seongnam, Korea) then 1 µl forward primer (Metabion, Germany) followed by 1 µl reverse primer (Metabion, Germany) finally 1 µl product from outer nested PCR (template DNA).

3.2.15.1.6. Protocol for inner nested PCR of gB CMV gene

The PCR mixture was subjected to initial denaturation step at 95 °C for 10 minutes. DNA was amplified for 35 cycles as followed: Denaturation at 95 °C for 30 seconds, primer annealing at 54 °C for 45 seconds, followed by a step of elongation at 72 for °C 30 seconds, the final elongation was at 72 °C for 7 minute, according to (Gandhoke et al., 2012).

3.2.15.1.7. Preparation of agarose gel

The PCR products (520bp, 305bp) were subjected to gel electrophoresis on 1.5% agarose. For preparation of 75 ml of 1.5% agroase gel, 1.12 gram of agarose powder were dissolved in 67.5 ml DW and 7.5 ml of 10X Tris base EDTA (TBE) buffer (Appendix V) and heated until became clear. Then the mixture was cool to 55 °C, 2.5 µl of (20mg/ml) ethidium bromide were added, mixed well and poured in a casting tray, any bubbles were removed and left to solidify at room temperature.

3.2.15.1.8. Visualization of PCR products

The gel casting tray was flooded by 1X TBE buffer (Appendix VI) near the gel cover surface, then 5 µl of PCR products of each sample was loaded into each well, in addition to control positive and negative. Then to the first well of
casting tray 5 µl of DNA ladder 50 bp (marker) was injected for each run. The gel electrophoresis apparatus was connected to the power supply (CONSORT E865, Belgium). Then the electrophoresis was run at 80 volte for 40 minute. Gel was removed by gel holder and visualized by U.V transilluminater (Upland, USA). Gel results were photographed using computer software.

3.2.16. DNA sequencing
Sequencing was carried out from the inner (305bp) PCR product. DNA sequencing was performed for 10 PCR product of CMV gB gene. DNA purification and standard sequencing was performed for both strands of gB genes by Macrogen Company (Seoul, Korea).

3.2.17. Bioinformatics Analysis

3.2.17.1. Sequences similarity and alignment
PCR product purification, direct sequencing for strands, as well as nucleotide and translated amino acid sequence analysis were performed. Before uploading the sequences to NCBI we proofread the nucleotides chromatogram to ensure that all ambiguous sites are correctly called and determined the overall quality of it. Then nucleotides sequences of gB CMV genes achieved were searched for sequence similarity using nucleotide BLAST (Atschul et al., 1997) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Highly similar sequences were retrieved from NCBI and subjected to multiple sequence alignment using the BioEdit software (Hall, 1999). In Gene Mark S version 4.25 (http://exon.gatech.edu/genemark/genemarks.cgi), the gene sequences were translated into amino acid sequence (John et al., 2001). Sequences similarities were searched with BLASTp (http://blast.ncbi.nlm.nih.gov/Blast/cgiCMD =Web and PAGE_TYPE=WEB last Docs), highly similar sequences were achieved from NCBI and subjected to multiple sequence alignment and evolutionary analysis using BioEdit software.

3.2.17.2. Mutant genes analysis
The mutant nucleotides were confirmed by their reverse strands. I-mutant version 3 (Capriotti et al., 2008), was used to study stability of mutant protein.
Chimera software version 1.9 was used to predict the tertiary model of protein (Huang et al., 2014).

3.2.17.3. Phylogenetic tree

Phylogenetic tree of CMV gB genes and their evolutionary relationship with well-characterized reference strains obtained from NCBI database (M60926.2, KR992839.1, AY18611.1, KR992940.1, AY186112.1 and KR992932.1) (Nogueira et al., 2002; Chou, 2014; Barans et al., 2015) was constructed by the neighbor-joining method with the Bootstrap test of phylogeny in Molecular Evolutionary Genetics Analysis (MEGA) program, version 6 (Tamura et al., 2013). Bootstrap resembling strategy and reconstruction were carried out 100 times to confirm the reliability of the phylogenetic tree.

3.2.19. Data analysis

Data were analyzed using statistical package for social science software (SPSS v.11.5) Data was presented in form of tables and figures. Frequencies and mean were calculated. Chi square test were performed between qualitative variables. Correlation were performed between two quantitative variables. A $P.value$ of $< 0.05$ was considered as significant for all statistical tests in the present study.

3.2.20. Ethical consideration

The study proposal was approved by Ethical Board of Sudan University of Science and Technology and approved from two hospitals administration. Verbal consent was taken from each renal transplant recipients prior to enrolment into the study. Data and samples were collected after informing and agreement of renal transplant recipients about the purposes and importance of the study.
CHAPTER FOUR
RESULTS

A total of 104 renal transplant recipients were participated in this study, their age ranged from 11 to 72 years and mean age of 37 years ±14.37 (SD). Out of them, males recipients were 72 (69.2%), while 32 (30.8%) were females, as shown in figure (4.1).

In this study, 50 (48%) of renal transplant recipients had received their organs in localized hospitals, while 54 (52%) received their organs in abroad as shown in figure (4.2).

Most of the renal transplant recipients received organs form relative donors 79 (76%) and only 25 (24%) from non-relative donors as shown in table (4.1).

The mean post-transplantation time in renal transplant recipients was 54 months, ranged from < than one to 204 months. The majority of post-transplantation duration in our study group were < than 1 to 12 month which represents 53(51%), 13 to 24 month 18(17.3%), 25 to 36 month 9(8.8%), 37 to 48 were 8(7.7%), and more than 48 months 16(15.4%) of recipients (as shown in figure 4.3).

Maintenance immunosuppression is necessary to prevent immunologic rejection of the allograft. The given immunosuppressive drugs among the study group are as follows: 28(26.9%) of recipients received tacrolimus, prednisolone and imuran, 15(14.4%) received tacrolimus, prednisolone and cellcept. About 10(9.8%) received prednisolone, cyclosporine and cellcept, while 8(7.7%) received each of (cyclosporine, prednisolone and imuran) and (tacrolimus, cyclosporine and imuran). Seven (6.7%) received tacrolimus, cyclosporine and cellcept. Five (4.8%) received by each of (tacrolimus and prednisolone), (cyclosporine and cellcept), and (tacrolimus and cellcept). Each of (cyclosporine and imuran) and (tacrolimus and cyclosporine) were given to 3(2.9%) of recipients, 2(1.9%) given each of (tacrolimus and imuran), and (cyclosporine, cellcept and Imuran). One recipient 1(1%) received (tracrolimus, cellcept, and imuran), (prednisolone and immuran) and cellcept respectively. This reflects that the majority of population 79(75.9%) received triple therapy while
24(23.1%) received two drugs and only one (1%) received one drug as shown in table (4.2).

The mean total white blood cells count among renal transplant recipients was 7100 WBCs/cmm ±2586.669 (std) with minimum count of 3200 WBCs/cmm and maximum count of 18600 TWBCs/cmm. All the study group showed adequate platelet counts and no thrombocytopenia was observed.

One hundred and four serum samples of renal transplant recipients were examined by direct ELISA for detection of CMV IgM and IgG antibodies. Twenty seven 27(26%) were positive for CMV IgM and 77(74%) of them showed negative results, while 103(99%) had CMV IgG in their serum and only 1(1%) sample was negative as shown in figure (4.4), (4.5), respectively. All positive CMV IgM samples were also positive for IgG, while 76 of 77 (98.7%) negative CMV IgM were positive for CMV IgG and only one of 77 (1.3%) samples showed negative results for both IgM and IgG, as shown in table (4.3).

All plasma specimens (n=104) were investigated for the presence of CMV DNA and viral load were estimated using hot start quantitative real time PCR kits. In real Time -PCR a data curve was constructed in which PCR cycles (x axis) are plotted against the fluorescence intensity (y axis) for both DNA samples and internal control. Each sample regarded as positive for tested DNA and internal control if exceeded cycle thresh old (Ct) as shown in figure (4.6).

Based on the constructed standard curve the correlation coefficient was at least 0.999, the amplification efficiency were varies between 97% to 100% and coefficient of variation (CV%) were from 0.00% to 8.5% for tested DNA and internal control for all trails, as shown in figure (4.7).

CMV DNA (viremia) was detected in 40/104 (38.5%) of renal transplant recipients, while 64/104 (61.5%) showed negative results, (figure 4.8).

The average of CMV DNA viral load was 358 x10^4 copies/ml (6.5 log_{10}) with minimum viral load 62 copies/ml (1.8 log_{10}) and maximum viral load 1.43x10^8 copies/ml (9 log_{10}) .

Table (4.4) displays that CMV DNA were detected in 26/104 (25%) males and 14/104(13.5%) females, while 46(44.2%) of male and 18(17.3%) of females
were negative for CMV DNA which revealed that there was no significant difference ($P_{value} > 0.05$) between sex and CMV viremia. Although most of recipients with positive CMV DNA 25/40 (65%) were males and 14/40 (35%) were females.

The sensitivity of ELISA was estimated to be 67.5% and specificity 100% in comparison to real time PCR as gold standard method.

Figure (4.9) demonstrated that CMV viremia was detected in (60%), (17%), (10%), (5%) and (8%) of the recipients in < than 1-12, 13-24, 25-36 37-48, and more than 48 months post-transplantation respectively, and there was no significant difference ($P_{value} = 0.296$) between CMV viremia and post renal transplantation time as shown in table (4.5).

Table (4.6) exhibits the association between immunosuppressive drugs and positive CMV viremia as follow: 10(66.7%) with positive CMV viremia and 5(33.5%) negative CMV received tacrolimus, prednisolone and cellcept, 6(21.4%) with positive and 22(78.6%) negative were given tacrolimus, prednisolone and imuran. Four (57.1%) with positive and 3(42.9%) with negative CMV viremia received tracrolimus, cyclosporine and cellcept. Four (40%) with positive and 6 (60%) with negative given prednisolone, cyclosporine and cellcept. While 3(37.5%) with positive and 5(62.5%) negative were received (tracrolimus, cyclosporine and imuran), and (cyclosporine, prednisolone and imuran). Only one recipient (100%) with negative CMV viremia received tacrolimus, imuran and cellcept, while tacrolimus and cyclosporine were taken by 3(100%) recipients with negative CMV viremia. Tacrolimus and prednisolone taken by 3(60%) with positive and 2(40%) with negative CMV viremia. Only 1(20%) recipient with positive and 4(80%) with negative CMV viremia taken tracrolimus and cellcept. While (tracrolimus and imuran) and (cyclosporine, prednisolone, and imuran) received by 1(50%) of recipient with both positive and negative CMV viremia. Cyclosporine and cellcept were taken by 3(60%) and 2(40%) with positive and negative CMV viremia respectively. One recipient (33.3%) with positive and 2(66.7%) with negative CMV viremia received cyclosporine and imuran. Only 1(100%) recipient with negative CMV
viremia taken each of (prednisolone and imuran) and cellcept respectively. This finding showed no significant difference ($P\text{. value} = 0.386$) between immunosuppressive drugs and positive CMV viremia.

According to Knipe and Howley, (2007) viral load of 1000 copies/ml from plasma, which is equivalent to 3000 copies/ml of whole blood, was selected to be the positive predictive value of the qRT-PCR for a group of symptomatic patients and correlate with presence of CMV disease.

Results of table (4.7) showed significant difference ($P\text{. value} = 0.05$) between the type of received immunosuppressive drug and high viral loads (>1000 copies /ml) which indicates CMV disease and < 1000 copies /ml that indicates CMV infection. Patients received tracrolimus, prednisolone and cellcept with viral loads of >1000 copies /ml were 9 (81.8%) and <1000 copies /ml were 2 (18.2%). Patients received tacrolimus, prednisolone and imuran with viral loads of > 1000 copies /ml were 2 (40%) and < 1000 were 3 (60%). Triple therapy (tacrolimus, cyclosporine and cellcept) and (cyclosporine, prednisolone and cellcept) were received by 2 (50%) recipients with >1000 and < 1000 copies /ml.

One of recipients (33%) with >1000 copies /ml and 2 (67 %) with < 1000 copies /ml were received (tacrolimus, cyclosporine and imuran) and (cyclosporine and cellcept) respectively. Two (67 %) and 1(33%) of recipients received tacrolimus and prednisolone with >1000 and <1000 copies /ml respectively. Two (100%) of recipients received cyclosporine, prednisolone and imuran with >1000 copies /ml. One (50%) of recipients with >1000 and <1000 copies /ml respectively received cyclosporine and imuran. Only one recipient (100%) of >1000 copies /ml were received (tracrolimus and cellcept), (tracrolimus and imuran) and (cyclosporine, cellcept and imuran) respectively.

Figure (4.10) and table (4.8) displayed that recipients with positive CMV viremia and had symptoms of CMV disease were 17/104 (16.3%), while 23/104 (22.1%) were asymptomatic. Furthermore, 14/17(82.4%) patients with positive CMV viremia had clinical symptoms of CMV disease with viral loads >1000 copies/ml and 3/17(17.6%) had clinical symptoms of CMV disease with viral loads <1000 copies/ml. While 1/23(4.3%) had no clinical symptoms of CMV
disease with viral loads >1000 copies/ml and 22/23 (95.7%) had no clinical symptoms of CMV disease with viral loads <1000 copies/ml. The results revealed that the correlation between CMV loads of >1000 copies/ml and presence of symptoms of CMV disease were highly significant (P.value =0.000). The medium CMV DNA viral loads copies/ml among symptomatic patients was (8.4 x10^6 copies/ml= 6.9 log{10}) and in asymptomatic patients was (316 copies/ml = 2.5 log{10}). Individual DNA values for asymptomatic patients ranged between 62-1016 copies/ml (1.8 to 3 log{10}), whereas for symptomatic patients they ranged from 537–1.43x10^8 copies/ml (2.7 to 9 log{10}). The discrepancies in three symptomatic patients showed viral loads of (537, 711, 690 copies/ml) which is relatively low and one patient was asymptomatic with viral loads of 1016 copies/ml. The findings of this study indicated that fever 7(41%), fever and leucopenia 6(35%) and gastrointestinal disease 4(24%) were the most common presenting symptoms of CMV disease as shown in figure (4.11).

Successful sequencing of CMV encoding gB was determined for 10 samples of symptomatic Sudanese renal transplant recipients after performing nested PCR, with gB gene (UL55) product of 305bp as shown in figure (4.12). The nucleotide sequences of 10 isolates and their accession numbers were deposited in the GenBank database shown in table (4. 9). The result of CMV-genotyping by sequencing based on MEGA software revealed 8 cases (80%) for gB3, and 2 cases (20%) for gB4 genotypes among Sudanese renal transplant recipients. The most frequent genotype in HCMV-positive Sudanese renal transplant recipients was gB3 and no mixed genotypes observed as shown on table (4.10). BLAST nucleotide search showed that isolate 24 and 149 were 99% identity with CMV gB genotype 4 (Genbank accession number M60926.2) from United State of America, Spain (Genbank accession number KR992839.1. and KR992940.1), Brazil (Genebank accession number AY186111.1 and AY186112.1). Isolates 230, 189, 164, 147, 135, 118, 10, 11 were 100% identity
with CMV gB genotype 3 (Genbank accession number KR992932.1) from Spain as shown in figure (4.13) (Appendix VII, VIII).

Multiple sequence alignment of obtained CMV gB sequences compared with reference sequences previously published in data base exhibited transversions mutation in isolates 230, 189, 164, 147, 135, 118, 10, 11. In which C was replaced by A at position 253 from reference CMV gB 3 (KR992932.1) as shown in figure (4.13). That resulted in substitution of the codon CGT Arginine (R), to AGT Serine (S) as shown in figure (4.14) and (4.15) (Appendix IX).

Resultant of substitution protein shown by tertiary protein structure of wild type (R), and mutant type (S) at position 85, figure (4.16) and (4.17). This substitution resulted in decrease of protein stability as illustrated by 1-mutant software, table (4.11).

The Phylogenetic tree analysis was performed to compare the genetic distances and evolutionary lineage for all 10 isolates with well-characterized reference isolates from Genbank as shown in figure (4.18).
Figure (4.1): Frequency of sex among renal transplant recipients

- Male: 69.2%
- Female: 30.8%
### Table (4.1): Frequency of renal transplant donors and recipients relationship

<table>
<thead>
<tr>
<th>Donor recipient relationship</th>
<th>Frequency</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative</td>
<td>79</td>
<td>76%</td>
</tr>
<tr>
<td>Non-relative</td>
<td>25</td>
<td>24%</td>
</tr>
<tr>
<td>Total</td>
<td>104</td>
<td>100%</td>
</tr>
</tbody>
</table>
Figure (4.2): The distribution of transplantation place
Figure (4.3): Post-renal transplantation time per month among the study group.
Table (4.2): Distribution of immunosuppressive drugs among renal transplant recipients

<table>
<thead>
<tr>
<th>Immunosuppressive drug</th>
<th>Frequency</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tacrolimus+ Prednisolone + Imuran</td>
<td>28</td>
<td>26.9%</td>
</tr>
<tr>
<td>Tacrolimus+ Prednisolone+ Cellcept</td>
<td>15</td>
<td>14.4%</td>
</tr>
<tr>
<td>Cyclosporine + Prednisolone + Cellcept</td>
<td>10</td>
<td>9.8%</td>
</tr>
<tr>
<td>Cyclosporine + Prednisolone+ Imuran</td>
<td>8</td>
<td>7.7%</td>
</tr>
<tr>
<td>Tracrolimus + Cyclosporine + Imuran</td>
<td>8</td>
<td>7.7%</td>
</tr>
<tr>
<td>Tracrolimus + Cyclosporine + Cellcept</td>
<td>7</td>
<td>6.7%</td>
</tr>
<tr>
<td>Cyclosporine + Cellcept</td>
<td>5</td>
<td>4.8%</td>
</tr>
<tr>
<td>Tracrolimus + Cellcept</td>
<td>5</td>
<td>4.8%</td>
</tr>
<tr>
<td>Tracrolimus+ Prednisolone</td>
<td>5</td>
<td>4.8%</td>
</tr>
<tr>
<td>Cyclosporine + Imuran</td>
<td>3</td>
<td>2.9%</td>
</tr>
<tr>
<td>Tracrolimus + Cyclosporine</td>
<td>3</td>
<td>2.9%</td>
</tr>
<tr>
<td>Cyclosporine + Cellcept + Imuran</td>
<td>2</td>
<td>1.9%</td>
</tr>
<tr>
<td>Tracrolimus + Imuran</td>
<td>2</td>
<td>1.9%</td>
</tr>
<tr>
<td>Tracrolimus + Cellcept + Imuran</td>
<td>1</td>
<td>1%</td>
</tr>
<tr>
<td>Prednisolone + Imuran</td>
<td>1</td>
<td>1%</td>
</tr>
<tr>
<td>Cellcept</td>
<td>1</td>
<td>1%</td>
</tr>
<tr>
<td>Total</td>
<td>104</td>
<td>100%</td>
</tr>
</tbody>
</table>
Figure (4.4): Frequency of CMV IgM in renal transplant recipients
Figure (4.5): Frequency of CMV IgG in renal transplant recipients

- Positive: 99%
- Negative: 1%
Table (4.3): The relation between CMV IgM and IgG among renal transplant recipients

<table>
<thead>
<tr>
<th>CMV IgM</th>
<th>CMV IgG</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive %</td>
<td>Negative %</td>
</tr>
<tr>
<td>Positive</td>
<td>27 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Negative</td>
<td>76 (98%)</td>
<td>1 (1.3%)</td>
</tr>
<tr>
<td>Total</td>
<td>103 (99%)</td>
<td>1 (1%)</td>
</tr>
</tbody>
</table>
Figure (4.6): (A) Quantitation curve for cycling yellow (tested DNA); (B) for cycling orange (internal control).
Figure (4.7): The standard curve (A): for cycling yellow (tested DNA), (B): for cycling orange (internal control)

$r = 0.99999. r^2 = 0.99999. \text{Efficiency} = 1.00$
Figure (4.8): Frequency of positive CMV (viremia) in renal transplant recipients by qRT-PCR.
Table (4.4): The correlation between sex and CMV viremia.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Real time PCR</th>
<th>Total</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (%)</td>
<td>Negative (%)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>26 (25%)</td>
<td>46 (44.2%)</td>
<td>72 (69.2%)</td>
</tr>
<tr>
<td>Female</td>
<td>14 (13.5%)</td>
<td>18 (17.3%)</td>
<td>32 (30.8%)</td>
</tr>
<tr>
<td>Total</td>
<td>40 (38.5%)</td>
<td>64 (61.5%)</td>
<td>104 (100%)</td>
</tr>
</tbody>
</table>
Table (4.5): The association between positive CMV and Post-transplantation time / month.

<table>
<thead>
<tr>
<th>Post-transplantation time / month</th>
<th>Real time PCR</th>
<th></th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive N (%)</td>
<td>Negative N (%)</td>
<td></td>
</tr>
<tr>
<td>&lt; than 1-12</td>
<td>24 (23.1%)</td>
<td>29 (27.9%)</td>
<td>53 (51%)</td>
</tr>
<tr>
<td>13-24</td>
<td>7 (6.7%)</td>
<td>11 (10.6%)</td>
<td>18 (17.3%)</td>
</tr>
<tr>
<td>25-36</td>
<td>4 (3.8)</td>
<td>5 (4.8%)</td>
<td>9 (8.7%)</td>
</tr>
<tr>
<td>37-48</td>
<td>2 (1.9%)</td>
<td>6 (5.8%)</td>
<td>8 (7.7%)</td>
</tr>
<tr>
<td>More than 48</td>
<td>3 (2.9%)</td>
<td>13 (12.5%)</td>
<td>16 (15.4%)</td>
</tr>
<tr>
<td>Total</td>
<td>40 (38.5%)</td>
<td>64 (61.5%)</td>
<td>104 (100%)</td>
</tr>
</tbody>
</table>
Figure (4.9): The relation between positive CMV viremia and post-transplantation time/month.
Table (4.6): The association between immunosuppressive drugs and positive CMV viremia.

<table>
<thead>
<tr>
<th>Immunosuppressive Drugs</th>
<th>RT-PCR results</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>positive CMV</td>
<td>Negative CMV</td>
</tr>
<tr>
<td>Tracrolimus + Prednisolone + Cellcept</td>
<td>10 (66.7%)</td>
<td>5 (33.3%)</td>
</tr>
<tr>
<td>Tracrolimus + Prednisolone + Imuran</td>
<td>6 (66.7%)</td>
<td>22 (72.6%)</td>
</tr>
<tr>
<td>Tracrolimus + Cyclosporine + Cellcept</td>
<td>4 (57.1%)</td>
<td>3 (42.9%)</td>
</tr>
<tr>
<td>Cyclosporine + Prednisolone + Cellcept</td>
<td>4 (40%)</td>
<td>6 (60%)</td>
</tr>
<tr>
<td>Tracrolimus + Cyclosporine + Imuran</td>
<td>3 (37.5%)</td>
<td>5 (62.5%)</td>
</tr>
<tr>
<td>Cyclosporine + Cellcept</td>
<td>3 (60%)</td>
<td>2 (40%)</td>
</tr>
<tr>
<td>Tracrolimus + Prednisolone</td>
<td>3 (60%)</td>
<td>2 (40%)</td>
</tr>
<tr>
<td>Cyclosporine + Prednisolone + Imuran</td>
<td>3 (37.5%)</td>
<td>5 (62.5%)</td>
</tr>
<tr>
<td>Cyclosporine + Imuran</td>
<td>1 (33.3%)</td>
<td>2 (66.7%)</td>
</tr>
<tr>
<td>Tracrolimus + Cellcept</td>
<td>1 (20%)</td>
<td>4 (80%)</td>
</tr>
<tr>
<td>Tracrolimus + Imuran</td>
<td>1 (50%)</td>
<td>1 (50%)</td>
</tr>
<tr>
<td>Cyclosporine + Cellcept + Imuran</td>
<td>1 (50%)</td>
<td>1 (50%)</td>
</tr>
<tr>
<td>Tracrolimus + Cyclosporine</td>
<td>0 (0%)</td>
<td>3 (100%)</td>
</tr>
<tr>
<td>Cyclosporine + Imuran</td>
<td>1 (33.3%)</td>
<td>2 (66.7%)</td>
</tr>
<tr>
<td>Tracrolimus + Cellcept+ Imuran</td>
<td>0 (0%)</td>
<td>1 (100%)</td>
</tr>
<tr>
<td>Prednisolone + Imuran</td>
<td>0 (0%)</td>
<td>1 (100%)</td>
</tr>
<tr>
<td>Cellcept</td>
<td>0 (0%)</td>
<td>1 (100%)</td>
</tr>
<tr>
<td>Total</td>
<td>40 (38.5%)</td>
<td>64 (61.5%)</td>
</tr>
</tbody>
</table>
Table (4.7): The association between immunosuppressive drugs and CMV infection and disease.

<table>
<thead>
<tr>
<th>Immunosuppressive drugs</th>
<th>Viral load copies/ml</th>
<th>Total</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;1000</td>
<td>&gt;1000</td>
<td></td>
</tr>
<tr>
<td>Tracrolimus + Prednisolone+ Cellcept</td>
<td>2(18.2%)</td>
<td>9(81.8%)</td>
<td>11(100%)</td>
</tr>
<tr>
<td>Tracrolimus + Prednisolone + Imuran</td>
<td>2 (40%)</td>
<td>3 (60%)</td>
<td>5(100%)</td>
</tr>
<tr>
<td>Tracrolimus + Cyclosporine + Cellcept</td>
<td>2(50%)</td>
<td>2(50%)</td>
<td>4(100%)</td>
</tr>
<tr>
<td>Cyclosporine + Prednisolone+ Cellcept</td>
<td>2 (50%)</td>
<td>2 (50%)</td>
<td>4(100%)</td>
</tr>
<tr>
<td>Tracrolimus + Cyclosporine + Imuran</td>
<td>1(33.3%)</td>
<td>2(66.7%)</td>
<td>3(100%)</td>
</tr>
<tr>
<td>Cyclosporine + Cellcept</td>
<td>1(33.3%)</td>
<td>2(66.7%)</td>
<td>3 (100%)</td>
</tr>
<tr>
<td>Tracrolimus + Prednisolone</td>
<td>2(66.7%)</td>
<td>1(33.3%)</td>
<td>3(100%)</td>
</tr>
<tr>
<td>Cyclosporine + Prednisolone + Imuran</td>
<td>2(100%)</td>
<td>0(0%)</td>
<td>2 (100%)</td>
</tr>
<tr>
<td>Cyclosporine + Imuran</td>
<td>1 (50%)</td>
<td>1 (50%)</td>
<td>2 (100%)</td>
</tr>
<tr>
<td>Tracrolimus + Cellcept</td>
<td>0 (0%)</td>
<td>1 (100%)</td>
<td>1 (100%)</td>
</tr>
<tr>
<td>Tracrolimus + Imuran</td>
<td>0 (0%)</td>
<td>1 (100%)</td>
<td>1 (100%)</td>
</tr>
<tr>
<td>Cyclosporine + Cellcept + Imuran</td>
<td>0 (0%)</td>
<td>1 (100%)</td>
<td>1 (100%)</td>
</tr>
<tr>
<td>Total</td>
<td>15(37.5%)</td>
<td>25(62.5%)</td>
<td>40 (100%)</td>
</tr>
</tbody>
</table>
Figure (4.10): Frequency of CMV infection and CMV disease among renal transplant recipients
Table (4.8): The association between presence of symptoms and viral load copies/ml.

<table>
<thead>
<tr>
<th>Presence of CMV Symptoms</th>
<th>Viral load copies/ml</th>
<th>Total</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;1000</td>
<td>&gt;1000</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>3 (17.6%)</td>
<td>14 (82.4%)</td>
<td>17 (100%)</td>
</tr>
<tr>
<td>No</td>
<td>22 (95.7%)</td>
<td>1 (4.3%)</td>
<td>23 (100%)</td>
</tr>
<tr>
<td>Total</td>
<td>25 (62.5%)</td>
<td>15 (37.5%)</td>
<td>40 (100%)</td>
</tr>
</tbody>
</table>
Figure (4.11): Distribution of CMV symptoms in recipients with CMV disease.
Figure (4.12): Gel electrophoresis of CMV UL55 gene PCR product (305 bp), M= Marker (50bp). Lane 1=Negative control, Lane 2, 3, 4, 5, 7, 8, 9, 11, 12, 13 tested samples.
Table (4.9): CMV isolates and its gB genotypes and accession number according to Genbank.

<table>
<thead>
<tr>
<th>CMV Isolates</th>
<th>CMV Genotype</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>BankIt2021642 Isolate-24</td>
<td>gB-4</td>
<td>MF179785</td>
</tr>
<tr>
<td>BankIt2021642 Isolate-149</td>
<td>gB-4</td>
<td>MF179786</td>
</tr>
<tr>
<td>BankIt2021642 Isolate-230</td>
<td>gB-3</td>
<td>MF179787</td>
</tr>
<tr>
<td>BankIt2021642 Isolate-189</td>
<td>gB-3</td>
<td>MF179788</td>
</tr>
<tr>
<td>BankIt2021642 Isolate-164</td>
<td>gB-3</td>
<td>MF179789</td>
</tr>
<tr>
<td>BankIt2021642 Isolate-147</td>
<td>gB-3</td>
<td>MF179790</td>
</tr>
<tr>
<td>BankIt2021642 Isolate-135</td>
<td>gB-3</td>
<td>MF179791</td>
</tr>
<tr>
<td>BankIt2021642 Isolate-118</td>
<td>gB-3</td>
<td>MF179792</td>
</tr>
<tr>
<td>BankIt2021642 Isolate-10</td>
<td>gB-3</td>
<td>MF179793</td>
</tr>
<tr>
<td>BankIt2021642 Isolate-11</td>
<td>gB-3</td>
<td>MF179794</td>
</tr>
</tbody>
</table>
Table (4.10): Distribution of CMV gB genotypes among Sudanese renal transplant recipients.

<table>
<thead>
<tr>
<th>CMV gB genotype</th>
<th>Frequency</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gB3</td>
<td>8</td>
<td>80%</td>
</tr>
<tr>
<td>gB4</td>
<td>2</td>
<td>20%</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>100%</td>
</tr>
</tbody>
</table>
Figure (4.13): Bio-Edit multiple sequence alignment of CMV gB gene compared to other CMV gB gene from Genbank. The transversion mutations in isolates 230, 189, 147, 164, 135, 118, 10 and 11 are indicated by black arrow.
Figure (4.14): Normal codon and protein sequence of CMV wild type from Genbank (A) versus mutant codon and protein resulted (B) as indicated by blue color.
Figure (3.15): Amino acid multiple sequence alignment of Sudanese mutant gB gene compared to other gB genes from database. The transversion mutations in isolates 230, 189, 147, 164, 135, 118, 10, and 11. Substitution of the amino acid Arginine (R) to Serine (S) as indicated by black color. The alignment was performed using BioEdit.
Figure (4.16): Tertiary protein structure of wild gB-3 gene of isolates 230, 189, 147, 164, 135, 118, 10 and 11. The predicted amino acid Arginine at position 85 from Genbank that predicted by Chimera software version 1.9.
Figure (4.17): Tertiary protein Structure of Mutant gB-3 gene of isolates 230,189, 147,164, 135, 118, 10, and 11. The predicted amino acid Serine at positin 85 that drawn by Chimera software version 1.9.
## Table (4.11): Predictor of protein stability

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position of protein</td>
<td>85</td>
</tr>
<tr>
<td>Amino acid in wild type protein (WT)</td>
<td>R</td>
</tr>
<tr>
<td>New amino acid after mutation</td>
<td>S</td>
</tr>
<tr>
<td>Stability</td>
<td>Decrease</td>
</tr>
<tr>
<td>Reliability index (RI)</td>
<td>9</td>
</tr>
<tr>
<td>Temperature in Celsius degrees</td>
<td>25</td>
</tr>
<tr>
<td>Ph= -log(H+)</td>
<td>7</td>
</tr>
</tbody>
</table>
Figure (4.18): Phylogenetic tree based on gB gene sequences of 10 CMV isolates from renal transplant recipients. The phylogenetic tree analysis was constructed using the neighbor-joining method in MEGA 6.
CHAPTER FIVE
DISCUSSION

5.1. Discussion

Cytomegalovirus infection is one of the most frequently encountered opportunistic viral pathogens in renal transplantation. CMV disease is a risk factor for acute allograft rejection in patients with kidney transplantation (Hasanzamani et al., 2016). It is considered as a potential contributor to graft loss and a cause of severe mortality and morbidity. Without intervention and preventative therapy, symptomatic CMV infection can develop in 20% to 60% of kidney transplant recipients (Nafar et al., 2014) of a mortality rate of 5% (Madi et al., 2011a).

This study was designed to determine the frequency of CMV infection and its gB genotypes distribution among Sudanese renal transplant recipients. The study population was 104 renal transplant recipients (69.2%) were males and (30.8%) were females. The study found that most of recipients were males with male/female ratio about 2:1. This finding in agreement with Hasanzamani et al (2016) in Iran, who reported that 41(62.1%) of population were males and 25(37.9%) were females. Similar results were observed by Khameneh et al (2013) in Iran, his finding showed that most of populations under study were males 22 (61.1%), and females were 14 (38.9%).

In the present study 79 (76%) of population received organs from relative donors and only 25 (24%) were received organs from non-relative donors. This results is relatively differ from those obtained by several studies. Pourmand et al (2006) in Iran, 85% of his population received living non-relative organs, 8% living-relative organs and 7% cadaveric organs, Nafar et al (2014) in Iran found that most of his population received Living unrelative organs (71%), Living relative were (4%), and cadaveric were (25%). van Ree et al., (2011) in Italy also found that (83%) of his population received cadaveric organs.

Most recipients in the present study received triple immunosuppressive therapy that make them more liable to CMV infection as reported by Nafar et al (2014) which indicate that high immunosuppressive regimen is associated with a higher
risk for CMV infection. Al-Alousy et al (2011) observed that the type, intensity of immunosuppressive therapy, and the level of immunosuppression act as a critical exogenous factor influencing the HCMV reactivation following transplantation such as cyclosporine.

The present results revealed high serofrequency of CMV IgG (99%), among this population which indicate that this population may experience CMV early in their life. This result in agreement with that obtained by Enan et al (2011)(100%), and Awad Alkareem et al (2013) (98%) in Sudan in the same population. Khairi et al (2013) also observed high seroprevalence of CMV in Sudan among pregnant women (97.5%). High prevalence of CMV in Sudan observed by Abd Alla et al (2015) among hemodialysis patients (95.7%), which indicate an earlier acquisition of the infection. The results in this study in agreement with Bates and Brantsaeter, (2016) who reported that seroprevalence of CMV IgG in Africa is close to 100% in most studies of adults, that could possibly attributed to lower socioeconomic status, broadly neglected diagnosis and treatment of CMV-related disease, and no randomized clinical trials of anti-CMV drugs have been conducted to date.

On the other hand the serofrequency of IgM antibodies among population was (26%), which indicates reactivation of CMV infection, this finding is relatively higher than those obtained by Enan et al (2011) and Awad Alkareem et al (2013) in Sudan, who reported that IgM was detected in (6%) of renal transplant recipients. In contrast, Khameneh et al (2013) in Iran obtained higher result of anti-CMV IgM seropositivity (37.5%) among transplant recipients. Lower finding was observed in another Iranian study of Tarabadi et al (2011), on renal transplant recipients, 16.1% were seropositive for anti-CMV IgM. Although serofrequency of IgM is high in this study in contrast to other previous studies in Sudan, but serological methods remain has a limited diagnostic value in the transplant patient groups. This might be to a time lag between active infection and IgM production and delayed seroconversion due to immunosuppressive therapy. Results can also be confounded by antibody based therapy (Heli, 2004), and blood transplantation (Ortiz and André, 2011).
The current study showed that CMV DNA (viremia) was detected in (38.5%) of renal transplant recipients using quantitative real time PCR. These results are relatively higher than those observed by William et al (2000) (22%), Madi et al (2007) in Kuwait (24%), Enan et al (2011) in Sudan (32.7%), and Lashini et al (2011) in Iran (25.9%). In addition, parallel finding to this results observed by Garrigue et al (2008) (36.6%) and Zhang et al (2012) in China (37.7%). In contrast, lower result was obtained by Cordero et al (2012) in Philippines (5.8%), Cupic et al (2012) in Serbia (12.5%), and Khalafkhany et al (2016) in Iran (15.9%). No antiviral prophylactic or preemptive therapy may explain the higher frequency of CMV among this study group.

It is of interest to observe that the average of CMV DNA viral load was 358 x10^4 copies/ml (6.5 log10) with minimum viral load 62 copies /ml (1.8 log 10) and maximum viral load 1.43x10^8 copies/ml (8.2 log10). The lack of screening in most patients probably explains the high viral loads at diagnosis and the large variation in viral loads.

In the present study (51%) of population had post-transplantation time from < than 0ne - 12 months. This finding increases the possibility of primary CMV infection or reactivation. The frequency of CMV viremia from the total positive was higher in the first 12 month of transplantation (60%) compared with the later onset. Similar results were obtained by Khalafkhany et al (2016) in Iran who detected CMV viremia in 31.2% of 0-3 months, 30.7% of 4-6 months, 17.5% of 7-12 months, 10.2% of 13-24 months, and 6.4% of the recipients of more than 24 months post-transplantation.

Although Pereyra and Rubin, (2004), and Aguado et al (2012), reported that high doses of methyl prednisolone or agents such as ALG, ATG, mycophenolate mofetil (cellcept) and azathioprine (imuran) were associated with increased incidence of CMV viremia. The present results showed no significant difference (P.value > 0.05) between the types of immunosuppressive therapy and CMV viremia. On the other hand, there was significant difference (P.value = 0.05) between the types of immunosuppressive therapy and high viral loads which correlate with CMV disease, may be due to triple immunosuppressive therapy used . In addition to intensive immunosuppressive regimen,
such as using tacrolimus, and cyclosporine that associated with a higher risk of CMV infection and disease.

In this study higher viral load correlate precisely with development of CMV-related symptoms and viral loads were slightly lower with asymptomatic patients (high significant difference $P_{value} = 0.00$), in which (82.4%) of patients had clinical symptoms of CMV disease with viral loads $>1000$ copies/ml. Moreover (95.7%) of patients had no clinical symptoms of CMV disease with viral loads $<1000$ copies/ml confirming previous reports by Hadaya et al., (2003), Knipe and Howley (2007), Madi et al., (2007), Helanter et al., (2010) and Rangbar-Kermani et al., (2011)

Furthermore, the median level of viral load was higher in patients with symptomatic CMV disease than those without symptoms. The discrepancy only in three patients (17.6%) had clinical symptoms of CMV disease with viral load $<1000$ copies/ml, and one patient (4.3%) had no obvious symptoms of CMV disease with viral load $>1000$ copies/ml. These discrepancy could be explained by several factors such as the source of donor kidney, nature of immunosuppressive, and genotypes of the virus.

Findings of the present study indicated that fever, fever leucopenia and gastrointestinal disease with abdominal pain and diarrhea were the most common presenting symptoms of CMV disease. This finding is similar to that obtained by Ardalan, (2012) who reported that most symptomatic CMV infections manifest as fever, fatigue, cytopenia and gastrointestinal tract is the most common site of tissue-invasive CMV infection. Another opinion was adopted by Helanter et al., (2010), which found that most of population under study suffering from gastrointestinal symptoms, suggestive of gastrointestinal CMV disease. Lashini et al., (2011) observed that fever, leucopenia, interstitial pneumonia and joint inflammation were the most prominent clinical symptoms of CMV disease.

The result of sequencing and genotyping of HCMV gB gene (UL 55) for 10 CMV isolate that were confirmed to be positive by PCR were taken from symptomatic transplant recipients revealed that gB3 (80%) was the most
frequent genotype among Sudanese renal transplant recipients whereas gB4 was (20%) and no mixed genotypes were observed to our knowledge. No data is available in Sudan on gB genotyping in renal transplant recipients nor immunocompetent host with CMV infection. The CMV sequences in this study considered the first Sudanese CMV sequences submitted and published in GenBank. These results in agreement with previous reports in Italy by Arista et al., (2003) in which the predominant circulation of HCMV strains were gB type 2 and 3 among both the immunocompetent host with a primary HCMV and renal transplant recipients with or without HCMV disease. Rather similar results were obtained by de Vries et al., (2011), in Netherland involved renal transplant recipients and congenitally infected newborns in which gB1 and gB3 being the most prevalent genotypes. Gandhoke et al., (2012) in India found that gB 3 was the most prevalent genotype in symptomatic infants.

The results of this study differ from previous studies undertaken in other parts of the world. WOO et al., (1997) in Hong Kong in which the most prevalent gB genotype in renal-transplant recipients was gB type 1, whether the patient manifested CMV disease or not. In contrast, Pasca et al., (2003) in Kuwait reported that gB1 (27%) was the most frequent genotype followed by gB2 (25%), gB3 (19%), gB4 (1%) and mixed genotypes were (27%). Coaquette et al., (2004) in France indicated that the distribution of gB genotypes among renal transplant was gB1 (28.9%) of patients; gB2 (19.6%); gB3 (23.7%) gB4 (2.0%); and mixed infection (25.8%). Dieamant et al (2009), reported different results, that gB1 and gB2 were the most frequent genotypes among Brazilian pediatric renal transplant patients. Differ results also was observed by Khalafkhany et al., (2016) in Iran, which mentioned that gB1 (26.5%), gB2 (20.5%), gB3 (17.6%), and gB4 (5.9%) genotypes was detected. Mixed genotype infection was observed in 29.4% of the recipients.

The substantial differences in genotype frequencies in this study compared to previous reports and no gB1 and gB2 were detected might in part, be due to variation in the geographical distribution of the CMV genotypes.
In the current study, no mixed genotypes were observed this might be due, the fact that, limitation of the available technologies or PCR protocol, and or low number of individual clone is sequenced down to the level of 5%. In addition, mixed infections accounted for roughly one quarter to one-half of HCMV infections over a wide range of human populations (Renzette et al., 2014).

The results of genotyping and sequencing in this study represent the first genetic characterization of HCMV mutations in Sudan. Transversion mutations in gB gene was identified in 8 of the Sudanese gB3 genotypes suggesting that a high gB3 gene nucleotide sequence variability correlated with an elevated amino acid substitution rate. This substitution mutation resulted in decrease of protein stability.

The obtained results of protein tertiary structure showed difference in size between wild type and mutant type. Wild type residue is bigger. This is probably altering or particularly increasing viral pathogenicity, as, gB gene is one of the most important envelope glycoproteins of HCMV, is implicated in virus entry, cell-to cell spread, and the fusion of infected cells (Jun et al., 2012). The variability and mutations particularly in gB that arise can be advantageous to the virus resulting in increase in viral fitness and adaptation (Stangherlin et al., 2017).

Findings of phylogenetics analysis in this study indicated that the HCMV was related to several strains worldwide that are far from Sudan (USA, Spain and Brazil). This is believed that their presence reflects the wider circulation of these strains in our geographical area and worldwide for both renal transplant recipients as well as immunocompetent with primary HCMV infection or disease.
5.2. Conclusion

This study concluded that qRT-PCR from plasma is a very useful and sensitive method, allowed an early diagnosis of CMV replication after transplantation and can help monitoring patients during CMV infection and disease.

CMV viremia viral loads were slightly lower in asymptomatic patients.

The present study documented the association of CMV disease with intensive immunosuppressive regimen, such as using triple therapy, tacrolimus, cyclosporine and as a higher risk factors for CMV infection and disease.

In this study, CMV gB3 is considered the most predominant glycoprotein B genotype in Sudanese renal transplant recipients.

The CMV sequences in this study considered the first Sudanese CMV sequences submitted and published in GenBank.
5.3. Recommendations

Early monitoring of CMV using sensitive method such as qRT-PCR that exactly detect viral replication can provide guiding information help the clinician to starting preemptive antiviral therapy that might have the advantages of reducing the occurrence of CMV disease.

Additional research is suggested to investigating other distinct glycoprotein genotyping (gH, gO, gN) to detect the association of gB genotypes with specific clinical features that might eventually shed light on the relevance of gB to the development of CMV-associated disease. Improved genotype-specific real-time-PCR-based assays or Ultra-deep pyrosequencing for gB genotyping, allowing detection and quantitation of distinct genotypes in mixed infections even less than 1%.
REFERENCES


119


123


**South Australia Health.** (2012). ABN: 97 643 356 590.


APPENDICES

Appendix (I):
CMV Real – RT Quant Reagents (Sacace- Italy)
PCR – mix -1-FRT CMV, PCR – mix -2-FRT, Polymerase (TaqF), RNA –
buffer, DNA calibrator (QS1), DNA calibrator (QS2). Negative Control (C-),
Positive Control DNA CMV/human DNA. Internal Control (IC).

Appendix (II):
Results of controls DNA samples together with the Internal Control.

<table>
<thead>
<tr>
<th>Control</th>
<th>Stag of control</th>
<th>Ct in channel</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HEX/JOE/Yellow /Cy3</td>
<td>ROX/ Orange/ Texas Red</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Qualitative format</td>
<td>Quantitative format</td>
</tr>
<tr>
<td>NCE</td>
<td>DNA extraction amplification</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Positive (&lt;boundary value)</td>
<td>Ct value is in the range indicated in data card</td>
</tr>
<tr>
<td>PCE</td>
<td>DNA extraction amplification</td>
<td>Positive (&lt;boundary value)</td>
<td>Ct value is in the range indicated in data card</td>
</tr>
<tr>
<td>NCA</td>
<td>Amplification</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>QS1 and QS2</td>
<td>Amplification</td>
<td>-</td>
<td>Ct value and calculated, concentration determined</td>
</tr>
</tbody>
</table>

If the controls are out of the expected range (results and control), all of the
specimens and controls from that run must be processed beginning from the
sample preparation step.
Appendix (III):
The results of tested samples, negative control, positive control, negative
control of amplification and quality control standards (calibrators) for
cycling yellow(Tested DNA).

<table>
<thead>
<tr>
<th>No.</th>
<th>Color</th>
<th>Name</th>
<th>Type</th>
<th>Ct</th>
<th>Given Conc (copies/ml)</th>
<th>Calc Conc (copies/ml)</th>
<th>% Var</th>
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</thead>
<tbody>
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</tr>
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<tr>
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<td></td>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
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<td>Positive Control</td>
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</tr>
<tr>
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<td>Tan</td>
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<td>NTC</td>
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<tr>
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</tr>
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</table>
Appendix (IV):
The results of tested samples, negative control, positive control, negative control of amplification and quality control standards (calibrators) for cycling orange (internal control).

<table>
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<tr>
<th>No.</th>
<th>Color</th>
<th>Name</th>
<th>Type</th>
<th>Ct</th>
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<th>Calc Conc (copies/ml)</th>
<th>% Var</th>
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<tr>
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<tr>
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<td></td>
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<tr>
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<td></td>
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<td>9977.52547248266</td>
<td>0.2%</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>QS1</td>
<td>Standard</td>
<td>19.89</td>
<td>10000</td>
<td>10022.5251517316</td>
<td>0.2%</td>
</tr>
<tr>
<td>16</td>
<td></td>
<td>QS2</td>
<td>Standard</td>
<td>26.54</td>
<td>100</td>
<td>98.80462543322</td>
<td>1.2%</td>
</tr>
<tr>
<td>17</td>
<td></td>
<td>QS2</td>
<td>Standard</td>
<td>26.50</td>
<td>100</td>
<td>101.20983664635</td>
<td>1.2%</td>
</tr>
</tbody>
</table>
Appendix (V):

10 X TBE Buffer

Tris EDTA  48.4 gram
Boric acid  55.9 gram
EDTA       7.44 gram
D.W        500 ml

Appendix (VI):

1 X TBE Buffer

1 ml of 10 X TBE Buffer + 9 ml of D. W.
Appendix (VII):

Sequencing of comparing CMV reference GenBank Genotype.

<table>
<thead>
<tr>
<th>Reference Genotype</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;M60962.2 HCMV glycoprotein B (UL55) Genotype 4</td>
<td>ATGGAGACATCAAGGAATCGTGGGTGCTCATGCAAGTTGCTTTTCGGCTTCTCTCATCTGTCTGACACAGAGTGACTATCAGACAA</td>
</tr>
<tr>
<td>&gt;KR998260.1 HCMV glycoprotein B (UL55) Genotype 4</td>
<td>GCCCAACCTGCTAGACCGACTGCGACACCGCAAAAACGGCTACCGACACTTGAAAGACTCCGACGAAGAAGAGAACGTCTGAACAGATGCTTCTGGCCCTGGTCCGTCTGG</td>
</tr>
<tr>
<td>&gt;AY186111.1 HCMV glycoprotein B (UL55) Genotype 4</td>
<td>TCAACCTGACATCGGTGGGTCACTACACCGGGGTTGAACCAATATCAGCAAGCCTGCTTGTTGGAACGCCGACCCTTAGAAGCTGAAGAA</td>
</tr>
<tr>
<td>&gt;KR992920.1 HCMV glycoprotein B (UL55) Genotype 3</td>
<td>GCCCAAGACCTGTGACACTCCTTCTCGCATGGAACAGATCAGGAGGCTGGATATGTCGCGGATGAGGTCTTCAAGGAACTCAGCAAGATCAATCCATCAGCCATTCTCTCGGCCATCTACAACAAACCGACCACCACCCTGTCGCTTGAAAGCGAATCTGTACGAAATGTGCTCTACGCTCAGCTGCAGTTCACCTATGATACGTTGCGCAGCTACATCAATCGGGCGTTGGCGCAGTTTGGCAAGGTATCAAGCAAAAATCTTTGTTGGAACTGGAACGTTTGGCCAATAGCTCCGGTGTGAAGGCTCTAAATAAGCTACAACAGATCTTCAACGCCTCGTACAATCAAACATACGAAAAGTACGGTAACGTGTCGGTTTTTGAAACCACAGGCGGTCTGGTGGTGTCGCCGAAGACTCGTACCACTTT</td>
</tr>
</tbody>
</table>

Sequencing of comparing CMV reference GenBank Genotype.
Appendix (VIII):
Nucleotide sequencing of CMV isolates gB gene from (1306-1611).

Isolate-24
AACTGGAACGTGGCAACGGCTCAGTCTGAACTTACTACTATAGAAACTACAACGATGCAATAGGACAACTTGCATATCAGCTATGCTATATCAT
CTATACTGGAAGCTGTAACCACTTTGCTACACACAGCTGAGCTGTTAGACCGTCTACCATCTACACGTGACTTGCAGCCGTTGACACAC
AAATCGACAAAGGCTGATGTTGATGACTACCGGTGACCACCTTACAGGGTCTTCAAGGAACTTACAGAAGTACACCGTACGCTATTCTCCG
CCACCTACAAAACCGATTGCCGCGGTTTCC

Isolate-149
AACTGGAACGTGGCAACGGCTCAGTCTGAACTTACTACTATAGAAACTACAACGATGCAATAGGACAACTTGCATATCAGCTATGCTATATCAT
CTATACTGGAAGCTGTAACCACTTTGCTACACACAGCTGAGCTGTTAGACCGTCTACCATCTACACGTGACTTGCAGCCGTTGACACAC
AAATCGACAAAGGCTGATGTTGATGACTACCGGTGACCACCTTACAGGGTCTTCAAGGAACTTACAGAAGTACACCGTACGCTATTCTCCG
CCACCTACAAAACCGATTGCCGCGGTTTCC

Isolate-230
TTGGAAACTGGGAATGCTGCTCCGTTGAACTCACCACGC2TAGAACCAAGGAGATACGCCAAATACGACACCCCTGCGTCTGTGAAAGCGAGATCC
GCAATACGACCACGTCTAGTACGAAATGTGCTCTAATGCCTCGTTCAAGGAACTTACAGAAGTACACCGTACGCTATTCTCCG
CCATCTACAAAACCGATTGCCGCGGTTTCC

Isolate-189
TTGGAAACTGGGAATGCTGCTCCGTTGAACTCACCACGC2TAGAACCAAGGAGATACGCCAAATACGACACCCCTGCGTCTGTGAAAGCGAGATCC
GCAATACGACCACGTCTAGTACGAAATGTGCTCTAATGCCTCGTTCAAGGAACTTACAGAAGTACACCGTACGCTATTCTCCG
CCATCTACAAAACCGATTGCCGCGGTTTCC

Isolate-147
TTGGAAACTGGGAATGCTGCTCCGTTGAACTCACCACGC2TAGAACCAAGGAGATACGCCAAATACGACACCCCTGCGTCTGTGAAAGCGAGATCC
GCAATACGACCACGTCTAGTACGAAATGTGCTCTAATGCCTCGTTCAAGGAACTTACAGAAGTACACCGTACGCTATTCTCCG
CCATCTACAAAACCGATTGCCGCGGTTTCC

Isolate-135
TTGGAAACTGGGAATGCTGCTCCGTTGAACTCACCACGC2TAGAACCAAGGAGATACGCCAAATACGACACCCCTGCGTCTGTGAAAGCGAGATCC
GCAATACGACCACGTCTAGTACGAAATGTGCTCTAATGCCTCGTTCAAGGAACTTACAGAAGTACACCGTACGCTATTCTCCG
CCATCTACAAAACCGATTGCCGCGGTTTCC

Isolate-118
TTGGAAACTGGGAATGCTGCTCCGTTGAACTCACCACGC2TAGAACCAAGGAGATACGCCAAATACGACACCCCTGCGTCTGTGAAAGCGAGATCC
GCAATACGACCACGTCTAGTACGAAATGTGCTCTAATGCCTCGTTCAAGGAACTTACAGAAGTACACCGTACGCTATTCTCCG
CCATCTACAAAACCGATTGCCGCGGTTTCC

Isolate-10
TTGGAAACTGGGAATGCTGCTCCGTTGAACTCACCACGC2TAGAACCAAGGAGATACGCCAAATACGACACCCCTGCGTCTGTGAAAGCGAGATCC
GCAATACGACCACGTCTAGTACGAAATGTGCTCTAATGCCTCGTTCAAGGAACTTACAGAAGTACACCGTACGCTATTCTCCG
CCATCTACAAAACCGATTGCCGCGGTTTCC

Isolate-11
TTGGAAACTGGGAATGCTGCTCCGTTGAACTCACCACGC2TAGAACCAAGGAGATACGCCAAATACGACACCCCTGCGTCTGTGAAAGCGAGATCC
GCAATACGACCACGTCTAGTACGAAATGTGCTCTAATGCCTCGTTCAAGGAACTTACAGAAGTACACCGTACGCTATTCTCCG
CCATCTACAAAACCGATTGCCGCGGTTTCC
Appendix (IX):
BioEdit multiple sequence alignment of CMV gB gene compared to other CMV gB Gene from Genbank. The transversion mutations in isolates 230, 189, 147, 164, 135, 118, 10 and 11 are indicated by black arrow.
Appendix (X):

Sudan University of Science and Technology
College of Graduate Studies
Microbiology Program
Questionnaire

Serological Detection and Molecular Characterization of CMV and its Glycoprotein B (UL55) among Sudanese renal transplant recipients

1. Name----------------------------------
2. Age-------------------------------------
3. Sex--------------------------------------
4. Date of collection------------------------
5. Date of transplantation-------------------------
6. Place of transplantation:

Ubnsina □  Ahmed Gassim □  Outside □  Other □

7. Symptoms if found----------------------------------------

8. Donor relationship:
Relative □  Non- relative □

9. Received treatment
Tacroliums. □  Cyclosporine □  Myfortic. □  Siroliums. □  Cellcept □

10. Immuno-status of donor and recipient for CMV
Donor--------------- Recipient---------------

Results:
1- WBCS ................... 2- Platelets count ...................
3- IgM....................... 4- IgG..............................
5- CMV viremia..................... 6. Viral load copies/ml......................
7-gB genotype ......................