Detection of Antibody against Cytomegalovirus in Rejected Renal Transplant Recipients and Hemodialysis Patients in Khartoum State, 2018

Dissertation Submitted in Partial fulfillment of the Requirement for the M.Sc. Degree in Medical Laboratory Science, (Microbiology)

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

الْحَمْدُ لِلَّهِ رَبِّ الْعَالَمِينَ

مَالِكِ يَوْمِ الدِّينِ

إِيَّاكَ نَعْبُدُ وَايَّاكَ نَسْتَعِينُ

صِرَاطَ الَّذِينَ أَنْعَمْتَ عَلَيْهِمْ غَيْرِ الْمَغْضُوبِ عَلَيْهِمْ وَلاَ الْضَّالِّينَ

صدق الله العظيم
DEDICATION

To my family

To my dear friends and colleagues

With love and respect
ACKNOWLEDGEMENTS

I would like to express my all sincere gratitude to my supervisor Dr. Ahmed Mohamed Ahmed Ibrahim, Department of Microbiology, Faculty of medical laboratory, University of Shendi for his patience, guidance, advice and support to accomplish this work. Also, I wish to extend my special gratitude to the Shendi University. My thanks are also due to the Faculty of Pure and Applied Science, and I would like to acknowledge the Doctor Salma for Kidney Disease, for giving me permeation to take the samples. In addition, I greatly acknowledge the assistance of the staff of Department Microbiology, Faculty of Pure and Applied Science. Last but not least, my appreciation and heartily thanks to my relatives, friends, colleagues and everyone who helped me.
### ABBREVIATIONS

<table>
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<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<td>CPE</td>
<td>Cytopathic effect</td>
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<td>HCMV</td>
<td>Human Cytomegalovirus</td>
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<td>IR</td>
<td>Inverted repeat</td>
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<td>IRL</td>
<td>Inverted repeat sequence</td>
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<td>TR</td>
<td>Terminal repeat</td>
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<td>TRS</td>
<td>Terminal repeat sequence</td>
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<tr>
<td>U_L</td>
<td>Unique long region at CMV genome</td>
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المملصّح

اجريت هذه الدراسة بغرض تحديد مدى انتشار فيروس مضخم الخلايا في مرضى عمليات زراعة الكلى المنقولة من الجسم ومريضي غسيل الكلى في الفترة من مارس إلى يونيو 2018.

تم جمع عينات الدم من مجموعات مختلفة من 9 عيّنات من زارعي الكلى المنقولة وبخضعون للغسيل الدموي، 90 عينة من مرضى الفشل الكلوي المزمن ويعالجون بالغسيل الدموي، وكان عدد المرضى المنضوين في الدراسة (56%) من الذكور و (44%) من الإناث الذين يعالجون بالغسيل الدموي، وتم تقسيم هذه الفئات على حسب العمر إلى أربع مجموعات، المجموعة الأولى من عمر 20-30 عام، ومن 31-40 عام، ومن 41-50 عام والمجموعة الأخيرة من عمر أكثر من 50 عامًا، متوسط الأعمار 46 سنة، ووجدت الأغلب من المتزوجون بنسبة 80% و 20% من غير المتزوجين.

تم تشخيص العينات باستخدام فحص الانزيم المرتبط المناعي (الإليزا) وذلك لقياس الأجسام المضادة (IgM) للفيروس من النوع (IgG) للاصابات الحادة والإصابة المزمنة من النوع. ووجدت الدراسات أن العدد الكلي للمصابين بفيروس مضخم الخلايا (74/100)% وكانت الإصابات الحادة (74/100)% و الإصابات المزمنة (74/100)%، 24.3% من الإصابات المزمنة (74/100)%، 75.7% و (26/100)%، 26% لم يصابو بفيروس مضخم الخلايا.

وكان العدد الكلي للأصابات لدى مرضى زارعي الكلى (100/100)%، (5/100)% (2/100)% للاصابات المزمنة، و (3/100)% للاصابات الحادة.

معدل انتشار فيروس مضخم الخلايا في مرضى الفشل الكلوي المزمن المعالجون بالغسيل الدموي معدل عالي جداً خصوصاً في المرضى الذين أجريت لهم عملية زراعة الكلي التي رفضت بواسطة الأجسام المضادة لديهم.
Abstract

The aim of this study was to find out the frequency of human cytomegalovirus (HCMV) infection in rejected renal transplant and haemodialysis patients in Khartoum state, from March to June 2018. Blood samples were selected randomly, 100 haemodialysis patients included in the study 9 of them had past history of rejected renal transplant. 56 were males and 44 females, the patient's age range from 20 to 60 years, the mean age was 46 years (SD±13), the study showed 80% of patients were married and 20% were not. The blood samples were investigated with the Enzyme Linked Immunosorbant Assay (ELISA) to detect HCMV IgG and IgM antibodies. The study showed total HCMV sero-positivity were 74% (74\100) and seronegativity were 26% (26\100), 24.3% (18\74) were acute infection, and 75.7% (56\74) revealed chronic sero-positivity for HCMV on haemodialysis patients, and total sero-positivity on rejected renal transplant were 55\(\%\)\(\text{(5\100)}\), 22\%\(\text{(2\100)}\) were chronic infection, and 33\%(3\100) revealed acute sero-positivity for HCMV. The frequency of HCMV in haemodialysis patients was high especially in rejected renal transplant patients.
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CHAPTER ONE

INTRODUCTION AND OBJECTIVE
1. Introduction

Human cytomegalovirus (HCMV) is a member of the genus Herpes virus and belongs to the family Herpesviridae. As with all members of the family, the virus has the ability to persist in the host in a latent state after primary infection (Emery, 2001). It is a ubiquitous virus, the seroprevalence of which varies between 30 to 100% in different countries. Acquisition of the virus in the general population mainly occurs early in life. Transmission of the virus can occur vertically or horizontally via direct contact with infectious body fluids or blood. The virus can also be transmitted by blood products or transplanted organs. After the primary infection, the virus will remain in a latent state in the host life-long but may reactivate later. Although rarely pathogenic in immunocompetent individuals, the virus posses a significant health threat to immunocompromised individuals and is a significant cause of morbidity and mortality especially in organ allograft and bone marrow transplant patients (Partel et al., 1992; Benz, 2000).

In the immunocompetent individual, the virus and host exist in a symbiotic equilibrium, such that disease manifestations are rarely encountered. However, when the host immune system is compromised, either through infection—for example, by human immunodeficiency virus (HIV), immaturity (neonate), or through iatrogenic means following organ transplantation, the virus is able to exert its full pathogenic potential (Emery, 2001). In immunocompromised patients, severe HCMV infections may occur. HCMV is a significant pathogen in organ transplanted patient causing symptomatic infections and end-organ disease. Many risk factors for the development of symptomatic infection have been suggested. Viral load has been shown to be a major factor in the development of HCMV disease. In most developed countries Human cytomegalovirus (HCMV), seroprevalence steadily increases after infancy, and
10-20% of children are usually infected before puberty. In adults the prevalence of antibodies ranges from 40-100%.

Infection with HCMV being more common in developing countries and in areas with low socioeconomic condition which is predominantly related to closeness of contacts within these population (De Jong et al., 1998). HCMV causes 8% of mononucleosis syndromes (Britt and Alford, 1996) atherosclerotic coronary artery, congenital infections (Boppana et al., 1992), and clinical syndromes associated with HCMV infections in AIDS patients (Britt and Alford, 1996). Prepheral blood mononuclear cells expressing CD13+ antigen as monocytes and certain CD8+ lymphocytes are permissive to HCMV (Soderberg et al.,1993). Monocytes have been identified as the major site of latency of HCMV in peripheral blood of healthy carriers and are more likely to transform latent infection. Allogeneic stimulation of peripheral blood mononuclear cells by T cells provides an immunologic stimulus that facilitates reactivation of latent HCMV (Soderbergy et al., 1997). Thus HCMV may be reactivated from latently infected cells after blood transfusion. In general; transfusion of unscreened cellular components leads to TT- HCMV incidence of approximately 30% in seronegative recipients (Hillyer et al., 1994). The first surveillance of HCMV infection in Sudan was done in 2004, in blood donors the second surveillance was done in 2006, in candidate recipients, kidney candidate donors and blood donors (Bushera, 2006). Diagnosis of HCMV was made by clinical symptoms, laboratory conformation was achieved by serological methods Enzyme linked Immunosorbent assay (ELISA).
Rationale
Investigate the prevalence of HCMV IgG and IgM antibodies among rejected renal transplant recipient and hemodialysis patients'. HCMV is significant cause of increased morbidity and mortality in population, CMV can predispose to rejection of kidney graft.

Objective
General Objective
To detect the antibodies against cytomegalovirus infection in rejected renal transplant and hemodialysis patients.

Specific Objectives
1. To screen hemodialysis patients for acute and chronic CMV infections.
2. To determine the frequency of CMV infections among rejected renal transplant patients.
3. To find out the risk factors which increase the incidence of CMV infection such as diabetes mellitus and HBV and HCV infections.
CHAPTER TWO

LITERATURE REVIEW
2. LITERATURE REVIEW

Definition
Cytomegalovirus (CMV) infections are common and usually asymptomatic infection; however, the incidence and spectrum of disease in newborns and in immunocompromised hosts establish this virus as an important human pathogen. CMV infection can be classified as those acquired before birth (congenital), at the time of delivery (prenatal), or later in life (postnatal) (Hodinka et al., 1991).

History of Cytomegalovirus
Cytomegalovirus (CMV) was first isolated from the salivary gland and kidney of two dying infant with cytomegalic inclusion bodies and reported in 1956 (Smith, 1956). Two other laboratories isolated CMV at approximately the same time. Thus CMV was initially called "Salivary gland virus" or "Salivary gland inclusion disease virus".

In 1960, Weller et al (1957) proposed the use of the term cytomegalovirus. Klemola and Kaarianinen first described CMV mononucleosis CMV was first isolated in renal transplant recipients in 1956.

The Genus Cytomegalovirus
Human cytomegalovirus (HCMV) is member of the Betaherpesvirinae subfamily, of the family Herpesviridae. Its replication cycle is significantly longer and infected cells typically are greatly enlarged and multinucleated thus the name cytomegalo (William et al., 2001 ).

Structure and biology of human cytomegalovirus
Human cytomegalovirus is a herpesvirus, with enveloped virion, about 150 nm in diameter, and consists of an icosahedral nucleocapsid about 100 nm in diameter, composed of 162 hollow capsomers -150 hexamers and 12 pentamers. The viral genome is wrapped around a fibrous spool –like core, which has the shape of a torus and appears to be suspended by fibrils that are anchored to the
inner side of the surrounding capsid and pass through the hol of the torus (Murphy et al., 1999). The genome of CMV is also characterized by linear DNA molecules ranging in size from 200-248 kbp. This is significantly larger than those of the other a herpesvirus.

Human cytomegalovirus genome also exhibits pattern of terminal and inverted repeats that vary in size depending on the virus strain and passage history (Knapke et al., 2001). Surrounding the capsid is a layer of globular material, known as the tegument which is enclosed by atypical lipoprotein envelope with numerous small glycoprotein peplomers at least 25 proteins of which are phosphorylated and also several transcriptional transactivator protein have also been localized in the tegument layer between the virion capsid and envelope (Knapke et al., 2001).

**Classification of human cytomegalovirus**

The family herpesviridae is divided into four subfamilies: Alphaherpesvirinae, Betaherpesvirinae, Gammaherpesvirinae, and Unnamed subfamily. This division was originally based on biological properties, but in general it has accorded well with subsequent molecular characterization, including nucleotide sequence and phylogenetic analyses (Murphy et al., 1999). The same study group classified a number of herpes virus into genera based on DNA sequence homology. The similarities in genome sequence arrangement and relatedness of important viral protein demonstrable by immunological methods (Knipe et al., 2001). Subfamily Alphaherpesvirinae, these viruses have a relatively rapid, cytocidal growth cycle the prototypic viruses of the genus of this subfamily is human herpesvirus I (herpes Simplex virus I: genus simplexvirus) and human herpesvirus III (Varicella-zostervirus; genus Varicellavirus). Gallid herpesvirus I (infectious laryngotracheitis virus) and maret's disease virus.

Subfamily Betaherpesvirinae these viruses have a relatively slow replication cycle. The protype of the subfamily is human herpesvirus
VI (human cytomegalovirus). Subfamily Gammaherpesvirinae, these viruses replicate in mucosal epithelium where they also establish latent infection and this subfamily comprises the herpesviruses that are lymphotropic. The prototype is human herpesvirus - V (Epstein- Barrvirus; genus lympocryptovirus). Unnamed subfamily, at present this subfamily comprises only channel catfish herpes viruses (William et al., 2001; Murphy et al., 1999).

**Genome structure**

The genome of characterized CMV is all linear DNA molecule ranging in size from 200 to 240 kbp encoded more than 200 proteins. This is significantly larger than those of other herpesviruses. Human cytomegalovirus genome also exhibits a pattern of terminal and inverted repeats that vary in size depending on the virus strain and passage history.

The human CMV genome has two unique components designated unique larger region (UL) and unique short region (Us). Flanked by inverted repeats b (TRL / IRL) and c (IRS / TRS) (Figure. 2). A directly repeated sequence is found at the genome termini and also present in inverted orientation at the L-S junction. The arrangement of a sequence promotes genome inversion. The sequence carries Cis-signal called pac-1 and pac-2 for cleavage and Packaging of viral genome.

The human CMV genome is G +C rich and contains a significant number of direct and inverted repeats (Murphy et al.,1999). The regions show an extremely high density closely spliced inverted and direct repeats. Human CMV generates defective viruses carrying DNA molecules that are shorter than the genome length. The novel restriction fragments are found in the defective CMV DNA, suggesting that specific deletions or rearrangement occur relative to standard viral (Knipe et al., 2001).
Figure 1. Schematic diagram of cytomegalovirus (CMV). The CMV virus shows a similar composition to all human herpes virus.
Epidemiology of HCMV

Cytomegalovirus is endemic in all parts of the world and occurs all over the year, with non seasonal variation. The prevalence of infection varies with socioeconomic status, living condition, and hygienic practices, antibody prevalence may be moderate (40-70%) in adults, in high socioeconomic group in developed countries in contrast to prevalence of 90% in children and adults in developing nations and in low socioeconomic group in developed countries. Human are only known host for cytomegalovirus (Jawetz et al., 2004). And infection rate are higher in non whites than in whites Human (knipe et al.,2001).

Transmission and spread of HCMV

CMV is not high contagious agent and transmission appears to be requiring direct contact with infectious material. Following initial acquisition of CMV, infectious virus is present in urine, saliva, tears, semen and cervical secretion from month to years. High rates of CMV infection occur in settings where close contact with body fluid is expected, Such as between sex partners, among
children in day care centers, and between preschool-aged children. But transmission of CMV through the sexual contact is an important means of spread, and the prevalence of CMV infection is higher among populations with other markers of sexual activity; such as greater number of partner and sexually transmitted diseases and among sexually active adolescent (knipe et al., 2001). CMV virus is unique among the human herpesviruses in the transmission from mother to fetus or newborn so that it plays an important role in maintaining CMV in population. Human CMV spread from mother to baby by three major routes; transplacental, intrapartum and human milk (knipe et al., 2001). The virus can also be transmitted by organ transplants and by blood transfusion (William et al., 2001).

**Cytomegalovirus and immunocompromised patient**

Individuals at greatest risk for cytomegalovirus disease are those receiving organ transplants, those with malignant tumors who are receiving chemotherapy and those with AIDS. Both morbidity and mortality rate increased with primary and recurrent cytomegalovirus infection in immunocompromised individual. Interstitial pneumonitis caused by cytomegalovirus occurs in 10 to 20 % of bone marrow transplant recipients. Virus associated leucopenia is common in solid organ transplant recipient, graft atherosclerosis after heart transplantation and cytomegalovirus related rejection of renal allograft. Cytomegalovirus often causes disseminated disease in untreated AIDS patient; gastroenteritis and chorioretintis are common problem (Jawetz et al., 2004).

**Human cytomegalovirus and renal transplant patients**

Symptomatic CMV infection occurs in 20 to 60% of all transplant recipients and is a significant cause of increased morbidity and mortality in this population (Brennan and Singer, 1999; Sai and Patel, 2000). The incidence may be somewhat lower in kidney transplant recipients who do not receive
antilymphocyte therapy, highdose mycophenolate, or the potent combination of mycophenolate and tacrolimus without adequate antiviral prophylaxis. Historically, concern has focused mainly on avoiding CMV infection in the CMV D+/R- group because this group has been at greatest risk for severe “primary” infection during the first 3 months after transplantation. However, the indirect effects of CMV infection on graft and patient survival have been increasingly recognized in recent years. The analyses of data from the United States Renal Data System and United Network of Organ Sharing revealed that by 3 years, it is the D+/R+ group and not the D+/R- group that have the worst graft and patient survival (Schnitzler et al., 1997). The reason for this is not entirely clear but may reflect the prevalence of multiple CMV virotypes and that the D+/R+ patients may have a double CMV exposure with reactivation of differing latent donor and recipient CMV. CMV can predispose to rejection as well. Subclinical CMV may also mimic and or predispose to late acute rejection. Late subclinical infections are common and associated with relatively rapid graft loss (Kern et al., 1996). CMV has been associated with both atherosclerosis and chronic rejection, and the two most common causes of late graft loss are cardiovascular death and chronic rejection. Chronic rejection is also known as chronic allograft nephropathy and is characterized by myointimal thickening, which is a form of atherosclerosis. Latent CMV infection has been associated with a markedly increased rate of restenosis (1200% higher) after coronary angioplasty in non-transplant seropositive individuals compared with seronegative individuals (Zhou et al., 1996). Furthermore, the histologic lesion of coronary restenosis is diffuse atherosclerosis characterized by myointimal hyperplasia resembling chronic rejection. effects of p53 (Speir et al., 1994). However, more recent evidence suggests that the myointimal increase is not from proliferation but rather from migration of smooth muscle cells (SMC) mediated by virally encoded chemokine receptors that require tryosine kinase for expressions (Streblow et al., 1999). SMC migration could be blocked by
tyrosine kinase inhibitors and suggests another potential future therapeutic strategy. Finally, human CMV infection may also cause atherosclerosis and chronic rejection by increasing oxidized low density lipoprotein uptake by vascular SMC (Zhou et al., 1996). This process is mediated by the IE gene IE72 and does not require viral replication, which may explain why angioplasty patients who were infected latently with CMV had higher rates of restenosis after angioplasty. CMV has been associated with several other vascular injuries that may explain why the D+/R+ group has the worst overall survival. One such vascular injury associated with CMV is transplant glomerulopathy. However, the frequency and clinical significance of this lesion are uncertain (Birk and Chavers, 1997). The lesion of the hemolytic uremic syndrome/thrombotic microangiopathy is one of the more common vascular pathologies associated with CMV and may be confused with or present with cyclosporine or tacrolimus toxicity (Miller et al., 1997; Hochstetler et al., 1994). CMV-associated hemolytic uremic syndrome/thrombotic microangiopathy may respond to Ig infusion (Hochstetler et al., 1994). CMV has been implicated as an important contributor to restenosis of coronary arteries after angioplasty in nontransplant patients (Zhou et al., 1996). CMV has also been associated with transplant renal artery stenosis. In a study of more than 900 renal transplant recipients, 75 were diagnosed with renal artery stenosis via angiography (Pouria et al., 1998).

**Congenital Human cytomegalovirus**

Fetal and newborn infection with cytomegalovirus may be severe. About 1% of live birth annually in the United States has congenital Cytomegalovirus infection and about 5-10% of those will suffer Cytomegalic inclusion diseases. A high percentage of babies with These diseases will exhibit developmental defects and mental Retardation. The virus can be transmitted with both primary and Reactivated maternal infection. About one –third of pregnant women With primary infection transmit the virus congenital infection may result in death of fetus in utero.
Cytomegalic inclusion disease of newborns is characterized by involvement of central nervous system and the reticuloendothelial system. Clinical features include intrauterine growth retardation, jaundice, heptosplenomegaly, thrombocytopenia, microcephaly, and retinitis. Mortality rates can approach 30%. The majority of survivors will develop significant central nervous system defect within 2 years; severe hearing loss, ocular abnormalities, and mental retardation is common. About 10% of subclinical congenital cytomegalovirus infection will develop deafness (Jawetz et al., 2004). Ten percent of these infants are born symptomatic, manifesting various neurologic, hematologic, and developmental abnormalities (Demmler, 1991).

**Stability of Human cytomegalovirus**

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), 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. CMV can be stored at −190°C (liquid nitrogen) indefinitely (Daniel, 2001).

**Persistence of Human cytomegalovirus**

Human CMV persists within its host indefinitely; there is growing evidence that cells in bone marrow and peripheral blood are a key reservoir for CMV through latent infection. A small percentage of peripheral blood monocytes from subjects with the past infection harbor CMV DNA. But viral gene expression is limited to early genes. However, tissue macrophage early and late CMV antigens. Kondo et al (1994). showed that granulocyte macrophage progenitors from human fetal liver and bone marrow could be latently infected in vitro; the cells have a CD14-, CD15-, CD33-, phenotypic and expressed {E} early transcripts. Soberberg – naucler et al (1997) reported reactivation of CMV in vitro by allogenic stimulation from peripheral blood mononuclear cells. The experimental evidence
supports a model of CMV persistence which myelomonocytes stem cells resident in bone marrow maintain latent infection, and latently infected CD14+ monocytes circulate in peripheral blood, differentiation latently infected monocytes into tissue macrophages result of allogenic stimulation (transciplantation) or triggering of proinflammatotrycytokines as intercurrent infection leads to productive infection (knipe et al., 2001).

** Reactivation of HCMV infection**

Reactivation is believed to be more important than primary infection as a virus causing disease in immunocompromized host and is though to occur as a result of at least three collaborating events:

a. Cytokine stimulation of latently infected cells causing differentiation into a permissive cell type that supports viral replication (Hahn et al., 1998).

b. Viral amplification caused by reduced immune surveillance that leads to wide spread systemic infection with readily detectable viraemia (Farrel et al., 1999) and virus in many tissues.

c. Disease state develops as a result of poorly understood collaboration of immune-organ and virus species determinants.

The three stages of reactivation help illuminate on important interplay of the immune response in reactivation and pathogenesis (Farrel et al., 1999). Immunocompromized individuals who support persistent infection or who frequently reactivate and shed viruses may be predisposed to more rapid amplification and clinical disease because the first event is unnecessary. Most clinical manifestation of reactivation results from second events, after virus replication reaches high systemic levels prior to and the absence of disease is the basis of preemptive antiviral or adoptive CMV specific CTL therapies (Riddel and Greenberg, 1997). As well as being the basis for the dramatic reduction in incidence of CMV retinitis and gastroenteritis resulting from immuno reconstitution that follows highly active retroviral therapy in AIDS patients (Donniger et al., 1999). The earliest event in reactivation is fascinating area
virus biology. Cytokine mediated differentiation of peripheral blood monocytes into macrophages under strongly proinflammatory conditions is undoubtedly key to this process (Meier and Stinski, 1996).

**Diagnosis of Human cytomegalovirus**

There are several methods for diagnosing CMV. Until fairly recently, the available techniques for diagnosing CMV were limited to histologic identification of CMV inclusion bodies, viral culture, and serology. These techniques are labor intensive and not completely sensitive, and the time from primary infection or reactivation to detection of CMV is protracted, allowing for undetected and untreated disease progression. In addition, transplant recipients may fail to produce an antibody response despite other evidence for viremia, making the newer techniques preferable to serology for detection and monitoring. Newer techniques include shell vial culture, pp65 antigenemia assay, PCR, and the hybrid-capture RNA-DNA hybridization assay, which has recently received FDA clearance.

**Histopathology**

Characteristic large cells with intranuclear and, on occasion cytoplasmic inclusion can be seen in routine section of biopsy or autopsy material. Wright –Giemsa stained touch imprints of lung or other biopsy specimen may demonstrate such cells. Although the presence of characteristic cytological changes suggests CMV infection. Virological serological conformation is suggested since CMV can infect tissues without producing morphological changes. Failure to find typical cytomegalic cells does not exclude the possibility of CMV infection (Hodinka et al., 1991). The large inclusions are intranuclear and have a characteristic owl-eye appearance in haematoxylin and eosin stained tissue specimens. The positive results correlate well with active HCMV infection of the organ, e. g. hepatitis, but the sensitivity of the histopathological finding is relatively low (Colina et al., 1995; Mattes et al., 2000). Immunostaining with specific polyclonal or monoclonal
antibodies against HCMV antigens has increased the sensitivity of the method compared to conventional staining (Barkholt et al., 1994; Paya et al., 1990). Although the histological methods may have an important role in detecting HCMV organ involvement, they are not suitable for the early diagnosis of HCMV infection. In addition, these assays are very laborious, and biopsy specimens are only taken when an end-organ disease is suspected.

**Serology**

Serological tests for antibody to CMV are useful to determining whether a patient had CMV infection in the past, a determination of great clinical importance for organ and blood donors, and in pretransplant evaluation of prospective transplant recipients. In addition, serologic test for seroconversion and for IgM antibody to CMV are commonly used to establish whether or not infection in the normal host occurred recently. A variety of different laboratory methods have been used successfully to measure antibody to CMV. Traditionally, serologic evidence of recent primary CMV infection depended on the demonstration of conversion from IgG antibody negative to positive are demonstration of IgM antibody (Kinpe et al., 2001).

**Enzyme linked immunosorbent assay (ELISA)**

Enzyme linked immunosorbent assay (ELISA) is the most commonly available serologic test for measuring antibody to CMV. The result can be used to determine whether it is due to acute infection, previous infection, or passively acquired maternal antibodies in an infant. ELISA also is proved to be a useful technique when recombinant antigens are used to differentiate between primary and secondary or past CMV infection in pregnancy, it was found that IgM response to recombinant proteins and PP150 in primary infected women varies. Predominant IgG response to P52 was observed in early sera less than 4 weeks after seroconversion, whereas IgG response to recombinant proteins PP150 was delayed and appeared 2-7 weeks later. On the
other hand women with secondary and those with post primary infection had
IgG antibodies to PP150. ELISA for CMV specific IgM may give false positive
result unless steps are taken to remove rheumatoid factor (Daiminger et al.,
1998).

The immune system and CMV
Because most immunocompetent individuals do not suffer from CMV disease
during either primary or recurrent infections, the immune system must be
effective in controlling replication. However, this simplistic assumption masks a
level of complexity between the virus and host that is still being unraveled. The
B cell response to CMV is largely dominated by an anti-glycoprotein B cell
response, and most of the neutralizing antibodies are also directed against this
protein (Wagner et al., 1992; Ohlin et al., 1993). In contrast, the catatonic T cell
(CD8) response is almost exclusively directed against the pp65 (UL83) protein
and epitopes have been mapped for human major histocompatibility complex
(HLA) types A (Wills et al., 1996; Mclaughlin et al., 1994). In the face of such
an aggressive immune response, how does the virus survive. It is now clear that
CMV encodes a range of gene products and uses several mechanisms to
manipulate the host immune system (Ploegh, 1998). Thus, the presentation of
immediate–early antigens is suppressed by phosphorylation by a virion
phosphoprotein, which leads to a lack of processing within the proteasome
(Gilbert et al., 1996). In addition, the HLA class I display pathway is interrupted
at multiple time points after infection via interaction with the TAP system, the
recycling system, and terminal maturation by genes US2, US3, US6, and US11
(Ploegh, 1998). The consequences of reduced HLA class I display should result
in an increased susceptibility to natural killer (NK) cell activity, because cells
require presentation of the signal peptides derived from HLA class I A, B, or C
to be protected against NK cell lysis (Borrego et al., 1998). However, the CMV
genome contains an HLA class I homologue (UL18), which is synthesized late
in the viral replicative cycle, when HLA class I downregulation is maximal
(Hassan Walker et al., 1998). In addition to encoding HLA class I dysregulatory genes, the genome encodes four potential G coupled protein receptors, one of which (US28) acts as a broad specificity β chemokine receptor and can act as a coreceptor for the uptake of HIV (Pleskoff et al., 1997). The genome also contains two genes with homology to the CXC chemokine family (Chat et al., 1996). And one of these has been shown to stimulate neutrophil migration with the same potency as other cellular chemokines (Penfold et al., 1999).

**Vaccination against HCMV**

Although attempts to develop vaccine for prevention of maternal and congenital CMV have been made for over 25 years no vaccine is currently licensed or near licensure. However, investigational vaccines employing different formats have been evaluated in the clinical trials; these include attenuated live virus, recombinant protein in fowl pox vector and recombinant protein given with anew adjuvant. The Twne strain of CMV was passaged more than 125 times in vitro to achieve attenuation. Subsequent study showed that the Twne strain of CMV was attenuated in that it did not produce a febrile illness in challenge studies whereas allow passage Toledo strain did. Immunization with Twne strain CMV vaccine induces both antibody responses and lymphocyte proliferative response to CMV. An efficacy trial in renal transplant recipient patients showed that pretransplantation immunization of seronegative patients with Twne strain CMV vaccine protected than from sever disease although it did not prevent infection (Knipe et al., 2001).

**Treatment**

The three antiviral drugs that are currently licensed for the treatment of HCMV infection are ganciclovir (GCV), foscarnet and cidofovir. Ganciclovir is the drug of choice in the treatment of symptomatic infections. Ganciclovir is a specific nucleoside analog which first is phosphorylated before it gains antiviral
activity (Littler et al., 1992; Sullivan et al., 1992). The initial phosphorylation is mediated by virus encoded phosphotransferase (product of UL97). The monophosphate form is further phosphorylated by the host’s cellular kinases into the active triphosphate form, which inhibits viral DNA polymerase by competing with deoxyguanosine triphosphate (Noble and Faulds, 1998). Foscarnet is an inorganic pyrophosphate analog and directly inhibits viral DNA polymerase (Crumpacker, 1992). However, foscarnet is nephrotoxic, which limits its use in kidney transplantation. Intravenous cidofovir may also be used, but experience of this agent in solid organ recipients is still very limited. Cidofovir is a cytosine analog which does not require the virus encoded phosphotransferase activity. Instead host cellular enzymes carry out the phosphorylations needed for activation (Lalezari et al., 1995; Snoeck et al., 1988). Valganciclovir, a pro-drug of ganciclovir, is a new promising compound which is administered orally and has an increased bioavailability compared to that of oral ganciclovir (Pescovitz et al., 2000). Nowadays valganciclovir is used in therapy of HCMV disease; however, more clinical trials are needed. The HCMV hyperimmunoglobulin may also be used in some patient groups in combination with antiviral drugs.

**Prevention**

Specific control measure is not available to prevent cytomegalovirus spread. Screening of transplant donors and recipient for cytomegalovirus may prevent some transmission of primary cytomegalovirus. The cytomegalovirus seronegative transplant recipient population represents a high risk group for CMV infection (Jawetz et al., 2004). In organ transplantation the common source of CMV infection is reactivation of transplant recipient latent virus, and there is no means to prevent this infection. Although selection of seronegative donors for seronegative recipients will prevent primary CMV infection in solid organ transplants recipient (Knipe et al., 2002). The leukocyte free blood products are used for transplant patients. WBC is main source of CMV
infection in peripheral blood products have been shown to reduce the Risk of CMV transmission. There is no effective vaccine against HCMV. Before the development of suitable antiviral drugs against HCMV, a reduction in the immunosuppressive regimen was used to prevent HCMV disease in transplant patients. However, this often led to an increased incidence of graft rejection (Sia and Patel, 2000; Knipe et al., 2002). CMV immunoglobulin were also used then administration of human Ig prepared from plasma pools obtained from healthy persons with high titer of CMV antibodies has given discordant results in tests to decrease the incidence of the viral infection in the transplant recipients (Jawetz et al., 2004). Currently, good availability of antiviral agent allows maintenance of the level of immunosuppression required to prevent graft rejection. Two different strategies, prophylaxis and preemptive therapy, are used for the prevention of symptomatic HCMV infection after transplantation. In prophylaxis, antiviral drugs are administered before any evidence of the virus, and in pre-emptive therapy, antiviral drugs are administered when there is laboratory evidence of active but asymptomatic infection. Also hyper immunoglobulin in combination with ganciclovir has been used in certain patient groups, e.g. lung transplant recipients (Weill et al., 2003). In community use of good hand washing and careful hygiene have been recommended as mean to prevent CMV infection in precare workers and parents of young children (Knipe et al., 2002).
CHAPTER THREE

MATERIALS AND METHODS
3. MATERIALS AND METHODS

Study design
This is prospective hospital base study.

Study area
The study conducted in Doctor Salma Centre for Kidney Disease.

Study population
Subject investigated were chronic renal failure patients and renal transplant rejected patients on hemodialysis.

Data Collection Method
Data was collected as per a structural interview questionnaire specially designed to collect and maintain all information of each subject examined.

Ethical consideration
- Approval to run the research was taken from the faculty of post graduate study
- Permission was taken from the local authorities
- Verbal consent was taken from the patients studied.
- Positive and negative results were handed to all patients included in this study.

Data analysis
The collected data were analyzed using SPSS software.

Sample collection
A total of 100 blood samples were collected. 91 with chronic renal failure patients and 9 with previous rejected renal transplant on hemodialysis in Khartoum state from Doctor Salma Center for Kidney Disease between March and June 2018.

Data on respondent background on various issues in from of questionnaire were collected (attached in appendix).
Venous blood samples were collected using vacationer and Needles. The blood was drown into the tube without any anticoagulant and the blood was allowed to clot at room temperature for no more than 1 hour. The serum was removed and
placed in sterile capped microfuge tubes and the tubes were centrifuged at 1000 rpm for 5 min at 4°C. The serum was then removed without disturbing the pellet and the tubes were stored at -20°C till used.

**Study variables**
Detecting CMV antibodies as well as assessing the sero-markers in relation to age and gender.

**Samples**

Human serum or EDTA, heparin or citrate plasma.

**Method of ELISA and principle**
Attached in appendix

ELISA kit (EUROIMMUM)
CHAPTER FOUR

RESULTS
4. RESULTS

The result reveal the analysis of 100 participants data in this study, 56(56%) male and 44(44%) female participants who received hemodialysis. (see figure 4-2) They were divided by age into four groups from 20 to 30 years old as the first group, form 31 to 40, from 41 to 50 and over 50 years as the last group. (see Figure 4-1) Their mean age was 46.8(SD=13.9). Most of them are married with percentage of 80% VS 20% as single individuals. (see Figure 4-2)

The total of positive CMV are 74 individuals 56(75%) of them with chronic term of infection (positive for IgG CMV antibody) and 18 (24%) was in acute infection was found. (see table 4-1). for the DM incidence and other percentage of disease history. (see figure 4-3).

The highest incidence of acute CMV infection (CMV IgM positive) is within +50 ages as from 41 to 50 years old with 7(7%) individuals, and chi-square test reveals that higher ages are associated with CMV infection(p value=0.02) (see table4-2) also the highest incidence of chronic CMV infection (CMV IgG positive) is within higher age over 50 years old with 17(17%) individuals, and chi-square test reveals that higher ages are associated with CMV infection(p-value=0.02) (see table4-2). Also highest frequencies of recent infection was in male’s gender with 10(10%) frequency and there was significant association between gender of individual and recent infection specially among men (p-value=0.02). (See table4-2). Also CMV correlation is established with disease history of DM, HBV and HCV, show there is no relation between CMV infection and those disease history.
Table (4-1): shows CMV sero-markers among participant

<table>
<thead>
<tr>
<th>CMV infection</th>
<th>Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>positive (IgG)</td>
<td>56</td>
<td>75.7</td>
</tr>
<tr>
<td>positive (IgM)</td>
<td>18</td>
<td>24.3</td>
</tr>
<tr>
<td>negative</td>
<td>26</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
Table (4-2): shows CMV sero-markers in association with Age groups.

<table>
<thead>
<tr>
<th>Age groups</th>
<th>No of screened</th>
<th>CMV sero-positivity IgG No (%)</th>
<th>CMV sero-positivity IgM No (%)</th>
<th>CMV sero-negativity No (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-30</td>
<td>16</td>
<td>4 (4%)</td>
<td>2 (2%)</td>
<td>10 (10%)</td>
</tr>
<tr>
<td>31-40</td>
<td>19</td>
<td>9 (9%)</td>
<td>3 (3%)</td>
<td>8 (8%)</td>
</tr>
<tr>
<td>41-50</td>
<td>24</td>
<td>8 (8%)</td>
<td>7 (7%)</td>
<td>9 (9%)</td>
</tr>
<tr>
<td>Over 50</td>
<td>41</td>
<td>17 (17%)</td>
<td>6 (6%)</td>
<td>18 (18%)</td>
</tr>
</tbody>
</table>

*p-value ≤0.05 considered significant
Table (4-3): shows CMV infection in association with past history of disease.

<table>
<thead>
<tr>
<th>History of disease</th>
<th>No of screened</th>
<th>Sero-positivity IgG No (%)</th>
<th>Sero-positivity IgM No (%)</th>
<th>Sero-negativity No (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM</td>
<td>15</td>
<td>6 (6%)</td>
<td>1 (1%)</td>
<td>8 (8%)</td>
</tr>
<tr>
<td>HBV</td>
<td>6</td>
<td>2 (2%)</td>
<td>3 (3%)</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>HCV</td>
<td>4</td>
<td>2 (2%)</td>
<td>0 (0%)</td>
<td>2 (2%)</td>
</tr>
</tbody>
</table>

*p-value = less than 0.05
*p-value ≤ 0.05 considered significant
# the result is constant no p-value produced
Table (4-4) shows distribution CMV sero-markers among individual with rejected kidney transplant.

<table>
<thead>
<tr>
<th>CMV sero-marker</th>
<th>NO</th>
<th>percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>positive (IgG)</td>
<td>2</td>
<td>22 %</td>
</tr>
<tr>
<td>Positive (IgM)</td>
<td>3</td>
<td>33 %</td>
</tr>
<tr>
<td>Negative</td>
<td>4</td>
<td>55%</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>100 %</td>
</tr>
</tbody>
</table>
5. DISCUSSION

Cytomegalovirus infection remains a major cause of morbidity and mortality in immunocompromised patients, and most serious problem in organ allograft recipients. The possibility of using specific antiviral therapy to treat CMV infection makes a timely diagnosis imperative (Peterson et al 1980).

This study was focused on the serologic diagnosis of human CMV in rejected renal transplant patients and hemodialysis patients in Doctor Salma For Kidney Disease. A number of 100 serum samples were collected from these two groups. We studied the prevalence of CMV antibodies among rejected renal transplant and hemodialysis patients by using ELISA.

We studied the frequency of CMV antibodies among rejected renal transplant and hemodialysis patients by using ELISA. We found that the chronic infection (IgG) frequency was 33% in rejected renal transplant and 56% hemodialysis patients, respectively, suggesting high incidence of previous infection in all groups tested. Eldowma (2004) was the first to detect IgG antibodies of CMV among blood donors and antenatal women in Sudan with 77% and 95%, respectively. Bushra (2006) detected 96% seropositivity for IgG in pretransplant kidney recipient, 17% in healthy candidate donors, and 84% in blood donors.

Since no vaccination program is practiced against CMV in Sudan our results denotes for previous infections with the virus. Taken together, our results and the findings of Eldowma (2004) and Bushra (2006) it is obvious that CMV is endemic in the Sudan. The present high CMV seroprevalence in the two groups studied point of high spread of the infection in the population as a whole. This high prevalence of CMV may be attributed to the poor socioeconomic status and hygienic practice known to play an important role in transmission of CMV due to over-crowdness. According to Jawetz et al (2004) the prevalence of infection varies with socioeconomic status, living condition, and hygienic practices, antibody prevalence may be moderate (40-70%) in adults in high
socioeconomic group in developed countries in contrast. We studied the prevalence of CMV antibodies among rejected renal transplant and hemodialysis patients in Khartoum state by using ELISA. We found that the chronic infection (IgG) frequency was 33% in renal transplant and 56% hemodialysis patients, respectively, suggesting high incidence of previous infection in all groups tested. Eldowma (2004) was detected chronic infection (IgG) of CMV among blood donors and antenatal women in Sudan with 77% and 95%, respectively. Bushra (2006) detected 96% seropositivity for IgG in pretransplant kidney recipient, 17% in healthy candidate donors, and 84% in blood donors. Since no vaccination program is practiced against CMV in Sudan our results denotes for previous infections with the virus. Taken together, our results and the findings of Eldowma (2004) and Bushra (2006) it is obvious that CMV is endemic in the Sudan. The present high CMV seroprevalence in the two groups studied point of high spread of the infection in the population as a whole. According to Jawetz et al (2004) the prevalence of infection varies with socioeconomic status, living condition, and hygienic practices, antibody prevalence may be moderate (40-70%) in adults in high socioeconomic group in developed countries in contrast to prevalence of 90% in children and adults in developing nations and in low socioeconomic group in develop countries.

For acute infection (IgM) ELISA was used to establish whether or not infection in the normal host has occurred recently. The prevalence of HCMV acute infection (IgM) in the present study, was 18% in rejected renal transplant and hemodialysis. These might reflect an alarming picture of the disease in the population and indicate that seroconversion is an ongoning process in rejected renal transplant and hemodialysis group in the Sudan.

The presence of HCMV IgM antibodies probably indicate active infection as has been reported previously by Farrell et al (1999). ELISA can detect HCMV antibodies of both primary CMV and reactivation of latent infection. Accordingly, high titer of CMV IgM antibodies by ELISA suggests recent
exposure or reactivation of latent infection. Further studies are needed to
determine the exact prevalence of CMV infection in the different parts of the
Sudan.
CONCLUSION

1. Frequency of HCMV chronic infection (IgG) was found in the renal transplant patients and hemodialysis patients and probably the dialysis machines of dialysis have a role in the transmission of the virus.

2. The prevalence of CMV acute infection (IgM) antibodies is higher in the renal transplant group maybe due to reactivation or new infection.

3. The prevalence of CMV chronic infection (IgG) antibodies is higher in the two groups: renal transplant patients and hemodialysis patients.
RECOMMENDATIONS

1. Blood donors should be screened for CMV sero-positivity to minimize spread of the virus.

2. Patients on hemodialysis should be screened for CMV sero-markers initially and periodically.

3. Renal transplant patients should be screened for CMV sero-markers periodically.

4. Patients sero-positive for CMV on hemodialysis should be isolated in dialyzed separate machine.
REFERENCES


Appendix

Questionnaires

Date: / / 2018 sample No...

. Name: .................................................................

. Gender: male [ ] female [ ]

. Marital status: single [ ] married [ ]

1. First diagnosis of the disease:
   Month [ ] year [ ]

2. History of hemodialysis:
   . The first time of hemodialysis?
     ...........................................................................................
   . Duration of hemodialysis per week?
     ...........................................................................................

3. Chemotherapy: yes [ ] No [ ]

4. Medical history:
   . Abortion: yes [ ] No [ ]
   Number of abortion?..........................................................
   . Blood transfusion: yes [ ] No [ ]
   Number of transfusion?.......................................................
   . Pervious surgery: yes [ ] No [ ]
   . D.M: yes [ ] No [ ]
. Hepatitis: yes □ No □
Type of hepatitis: HBV □ HCV □
. HIV: yes □ NO □
. Other disease? ..........................................................

5. Clinical feature:

Prolonged fever □  Jaundice □
      pneumonia □  Skin rash □
Other: ..........................................................

6. Investigation result:

. CMV IgM: positive □ Negative □
. CMV IgG: positive □ Negative □
**Qualitätskontrollzertifikat**  
**Quality Control Certificate**

<table>
<thead>
<tr>
<th>Produkt: Anti-CMV ELISA (IgM)</th>
<th>Best.-Nr.: E1 2570-9601 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ch.-B.: E180511AC</td>
<td>Order No:</td>
</tr>
<tr>
<td>Lot:</td>
<td>10-May-2019</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Kalibrator</th>
<th>Referenzwert</th>
<th>Valider Bereich</th>
</tr>
</thead>
<tbody>
<tr>
<td>O.D.</td>
<td>&gt; 0,145</td>
<td>O.D.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pos. Kontrolle 1</th>
<th>semiquantitativ</th>
<th>Referenzwert</th>
<th>Ratio</th>
<th>Valider Bereich</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pos. Control 1</td>
<td>semiquantitativ</td>
<td>2,7</td>
<td>Ratio</td>
<td>1,5 - 3,9</td>
<td>Ratio</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Neg. Kontrolle</th>
<th>semiquantitativ</th>
<th>Referenzwert</th>
<th>Ratio</th>
<th>Valider Bereich</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neg. Control</td>
<td>semiquantitativ</td>
<td>0,2</td>
<td>Ratio</td>
<td>0 - 0,7</td>
<td>Ratio</td>
</tr>
</tbody>
</table>
**EUROIMMUN**  
Medizinische Labordiagnostika AG

**Anti-CMV ELISA (IgM)**  
Test instruction

<table>
<thead>
<tr>
<th>ORDER NO.</th>
<th>ANTIBODIES AGAINST</th>
<th>IG CLASS</th>
<th>SUBSTRATE</th>
<th>FORMAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>EI 2570-9001 M</td>
<td>Cytomegalovirus (CMV)</td>
<td>IgM</td>
<td>Ag-coated microplate wells</td>
<td>96 x 01 (96)</td>
</tr>
</tbody>
</table>

**Indication:** The ELISA test kit provides a semiquantitative in vitro assay for human antibodies of the immunoglobulin class IgM against CMV in serum or plasma for the diagnosis of the infection with cytomegalovirus.

**Application:** Infections with cytomegaloviruses (CMV) can be diagnosed by the detection of specific antibodies of classes IgG and IgM. Antibodies of class IgM quite reliably indicate an acute infection. However, their detection in CMV cannot be used for differentiation from a primary infection of a reactivation as they may occur in both conditions. In cases of positive IgM findings, the avidity determination of pathogen-specific IgG antibodies is therefore a suitable method to differentiate between primary infection and reactivation.

**Principle of the test:** The test kit contains microtiter strips each with 8 break-off reagent wells coated with CMV antigens. In the first reaction step, diluted patient samples are incubated in the wells. In the case of positive samples, specific IgM antibodies (also IgA and IgG) will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labelled anti-human IgM (enzyme conjugate) catalysing a colour reaction.

**Contents of the test kit:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Colour</th>
<th>Format</th>
<th>Symbol</th>
</tr>
</thead>
</table>
| 1. Microplate wells coated with antigens  
12 microplate strips each containing 8 individual break-off wells in a frame, ready for use | — | 12 x 8 | STRIPS |
| 2. Calibrator  
(IgM, human), ready for use | dark red | 1 x 2.0 ml | CAL |
| 3. Positive control  
(IgM, human), ready for use | blue | 1 x 2.0 ml | POS CONTROL |
| 4. Negative control  
(IgM, human), ready for use | green | 1 x 2.0 ml | NEG CONTROL |
| 5. Enzyme conjugate  
peroxidase-labelled anti-human IgM (goat), ready for use | red | 1 x 12 ml | CONJUGATE |
| 6. Sample buffer  
containing IgG/RF-absorbent (anti-human IgG antibody preparation obtained from goat), ready for use | green | 1 x 100 ml | SAMPLE BUFFER |
| 7. Wash buffer  
10x concentrate | colourless | 1 x 100 ml | WASH BUFFER 10x |
| 8. Chromogen/substrate solution  
TB/H2O2, ready for use | colourless | 1 x 12 ml | SUBSTRATE |
| 9. Stop solution  
0.6 M sulphuric acid, ready for use | colourless | 1 x 12 ml | STOP SOLUTION |
| 10. Test instruction | — | 1 booklet | — |
| 11. Quality control certificate | — | 1 protocol | — |

**Lot description:**

**Unopened usable until:**

**CE 0197**

**Storage temperature:**

**In vitro diagnostic medical device**
Calculation of Results

The calculation of results is as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Component 1</td>
<td>12.34</td>
</tr>
<tr>
<td>Component 2</td>
<td>5.67</td>
</tr>
</tbody>
</table>

Protocol: The protocol for the calculation of results is as follows:

1. Collect all data related to the experiment.
2. Calculate the average for each component.
3. Determine the total and calculate the percentage of each component.

Conclusion: The conclusion of the calculation is that Component 1 contributes 50% to the total, while Component 2 contributes 25%.