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Detection of Cytomegalovirus IgM Antibodies among Sudanese Hepatitis B Patients in Khartoum State

الكشف عن القلوبيولوجين المناعي لفيروس مضخم الخلايا وسط السودانيين المرضى بإلتهاب الكبد الوبائي ب بولاية الخرطوم

A Dissertation Submitted in Partial Fulfillment of the Requirements for M.Sc Degree in Medical Laboratory Science (Microbiology).

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الآيـــــــة

قال تعالى: (وَوَصَّيْنَا الْإِنْسَانَ بِوَالِدَيْهِ إِحْسَانًا ۖ حَمَلَتْهُ أُمُّهُ كُرْهًا وَوَضَعَتْهُ كُرْهًا ۖ وَحَمْلُهُ وَفِصَالُهُ ثَلَاثُونَ شَهْرًا ۚ حَتَّىٰ إِذَا بَلَغَ أَشُدَّهُ وَبَلَغَ أَرْبَعِينَ سَنَةً قَالَ رَبِّ أَوْزِعْنِي أَنْ أَشْكُرَ نِعْمَتَكَ الَّتِي أَنْعَمْتَ عَلَيَّ وَعَلَىٰ وَالِدَيَّ وَأَنْ أَعْمَلَ صَالِحًا تَرْضَاهُ وَأَصْلِحْ لِي فِي ذُرِّيَّتِي ۖ إِنِّي تُبْتُ إِلَيْكَ وَإِنِّي مِنَ الْمُسْلِمِينَ)

(الأحقاف 15)

DEDICATION

To my Mothers...

The love of our live...

To my Fathers...

The candles lightening our road ...

To my Teachers...

TO my friends...

And to everyone that support and encourage us during our education and through our life...

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ABSTRACT

Human *Cytomegalovirus* (CMV) is one of the most commonly found agents of congenital infections. Primary maternal infection is associated with risk of symptomatic congenital diseases.

This study was aimed for detection of CMV antibodies in serum of hepatitis B patients in Khartoum state using enzyme linked immunosorbant assay during the period from March to November 2019. Blood specimen was collected from 88 hepatitis B patients formed 48 (54.5%) male and 40 (45.5%) female with age ranging (10-50) years. Personal and clinical data were collected by questionnaire after verbal concent, All specimens were tested for the presence of CMV IgM antibodies using ELSIA kit.CMV IgM antibodies were detected in 12 (13.6%) of the total specimen examined. The rate of anti HCMV IgM antibodies sero-positivtity among male 7 (8.0%), while the rate of anti HCMV IgM seropositive among females were 5 (7.5%). The highest positive cases were in hepatitis B patient's age 20-30 years.

Eight (9.1%) of hepatitis B patients had history of previous blood transfusion, and 5 (5.7%) of them shows symptoms of jaundice. From all cases 44 were chronic HBV patients while 44 were acute HBV patients. 28 (31.8%) of all cases had treatment for HBV.

The finding of this study indicates low frequency of CMV among HBV patients. There was no correlation of serological finding of CMV and age of patient.

ملخص الأطروحة

الفيروس المضخم للخلايا البشرية (CMV) هو واحد من أكثر العوامل شيوعًا للعدوى الخلقية. ترتبط العدوى الأمومية الأولية بخطر الإصابة بالأمراض الخلقية.

هدفت هذه الدراسة إلى الكشف عن الأجسام المضادة CMV في مصل مرضى التهاب الكبد B في ولاية الخرطوم باستخدام مقايسة الممتز المناعي المرتبط بالإنزيم خلال الفترة من مارس إلى نوفمبر 2019. جمعت عينة دم من 88 مريض بإلتهاب الكبد الوبائي ب وشكلوا 48 ذكراً (54.5%) و 40 (45.5٪) انثى تتراوح أعمارهن بين (50-10) سنة. تم جمع البيانات الشخصية والسريرية عن طريق الاستبيان بعد الموافقة اللفظية ، تم اختبار جميع العينات لوجود الأجسام المضادة CMV IgM باستخدام ELSIA kit. تم الكشف عن الأجسام المضادة CMG IgM في 12 (13.6 ٪) من إجمالي العينة التي تم فحصها. كان معدل الأجسام المضادة لـ HCMV IgM إيجابيًا بين الذكور 7 (8.0٪) ، في حين كان معدل مضادات HCMV IgM إيجابيًا بين الإناث 5 (7.5٪). وكانت أعلى الحالات إيجابية في عمر مريض التهاب الكبد ب 20-30 سنة.

كان ثمانية (9.1 ٪) من مرضى التهاب الكبد B لديهم تاريخ في نقل الدم السابق ، و 5 (5.7 ٪) منهم يظهر عليهم أعراض اليرقان. من جميع الحالات ، كان 44 من مرضى التهاب الكبد الوبائي المزمن في حين أن 44 من مرضى التهاب الكبد الفيروسي الحاد. 28 (31.8 ٪) من جميع الحالات كان علاج ل HBV.

نتائج هذه الدراسة تشير إلى انخفاض وتيرة CMV بين مرضى HBV. لم يكن هناك ارتباط بين الاكتشافات المصلية لل CMV وعمر المريض.

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ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
CID	Cytomegalic inclusion disease
CMV	Cytomegalovirus
COV	Cut-off value
CPE	Cytopathic effect
EIA	Enzyme Immune Assay
ELISA	Enzyme Linked Immunosorbant Assay
HBV	Hepatitis B virus
HCMV	Human cytomegalovirus
HHV-5	Human herpes virus 5
HIV	Human Immune deficiency virus
HSV	Herpes simplex virus
PCR	Polymerase chain reaction
RF	Rheumatoid factor
SPSS	Statistical Package of Social Sciences

CHPTER ONE

INTRODUCTION

CHAPTER ONE

INTRODUCTION

1.1 Introduction

Cytomegalovirus (CMV) is endemic in most areas of the world. The seroprevalence of CMV varies in different geographical areas and ranges from 30% to 100%, In China, the sero-prevalence of CMV is approximately 0.6–8.5% in the newborn, 58–84% in infants, and around 95.6–98.7% in fertile women (Zhang *et al*., 2014)

Cytomegalovirus (CMV) infection in individuals with effective immunity is generally asymptomatic or may occur as a mononucleosis syndrome but rarely leads to severe and life-threatening organ complications such as gastrointestinal, cardiovascular, hepatic, and neurologic manifestations (Yi *et al* ., 2019). (CMV) infects different body cells, including fibroblasts, endothelial and neuronal cells, and hepatocytes, besides blood monocytes and tissue resident macrophages (which both help in disseminating the infection throughout the body or serve as sites for latent infection) (Saliha *et al* ., 2016) . Although CMV causes subclinical latent infection in immunocompetent individuals, it causes severe symptoms in immunocompromised individuals (Kumar and Herbein 2014). Multiple reports elaborated the implication of CMV coinfection in the incidence and development of HCC (Lepiller *et al.*, 2013) and in accelerating the progression rates of hepatic fibrosis after liver transplantation (Saliha *et al*., 2016)

HCMV infection and increased risk of mortality in older people (Roberts *et al*., 2010 ; Savva *et al* ., 2013). Further studies have implicated chronic HCMV infection as a risk factor for cardiovascular disease (CVD); a recent meta-analysis of studies conducted in HICs, estimated a 22% increased relative risk of CVD with exposure to HCMV (Hu *et al*., 2016). In a UK setting, HCMV infection was associated with the development of arteriosclerosis (Wall *et al*., 2013) and a 3mmHg increase in systolic blood pressure among older individuals (Firth *et al*., 2016).

Serological tests are useful for determining whether a patient has had CMV infection in the past, determined by the presence or absence of CMV IgG. Many different assays have been described and evaluated for the detection of CMV IgG antibodies.

Among these are complement fixation, enzyme-linked immunosorbent assay (ELISA), anti complement immunofluorescence, radioimmunoassay, and indirect hemagglutination (Ross *et al*., 2011). The detection of IgM antibodies has been used as an indicator of acute or recent infection.

The treatment of CMV infection with ganciclovir is indicated incertain situations, but the guidelines for treatment, especially inimmunocompetent patients, are not yet established (Stockmann *et al*., 2015; Baghban and Malinis, 2018).

HBV Discovered 50 years ago as an antigenic "polymorphism" in an Australian aborigine—the "Australia antigen" (Blumberg 2014), the hepatitis B virus (HBV) remains today a major global pathogen that causes acute and chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (Trepo *et al*., 2014) . Owing to its unique replication strategy, as will be detailed below, HBV is classified into its own family, Hepadnaviridae (Seeger *et al*., 2013), along with related animal viruses. The latter include the woodchuck hepatitis virus, particularly useful as a model for studying HBV pathogenesis, and the duck hepatitis B virus (DHBV) (Hu 2016),

Acute hepatitis B is a clinical diagnosis identified by the detection of HBsAg, symptoms, high serum aminotransferases. Usually anti-HBc IgM can be detected and HBV DNA is present. HBeAg can also be identified in most acute phase of infections, but has little clinical importance. The diagnosis of chronic infection is based on the persistence of HBsAg for more than 6 months (Hollinger and Sood 2010) .

Inhibition of viral replication by antiviral treatment has been shown to result in elimination of chronic HBV-inceden cero inflammatory activity and progressive fibrosis in most patients. Thus, inhibition of HBV replication successfully achieves the ultimate goal of HBV therapy by improving survival and quality of life by preventing disease progression and the development to HCC. (Lampertico *et al*., 2015) The goal of antiviral therapy is also to prevent mother-to-child transmission ,HBV reactivation during immunosuppression or chemotherapy as well as the prevention and treatment of HBV-associated extra hepatic manifestations (Papatheodoridis *et al*., 2015).

1.2 Rationale

Hepatitis B virus is one of the most common infectious diseases globally. It is a major public health problem accounting to 400 million chronic infections worldwide. About 2 billion people (or 30% of world population) worldwide have serological evidence of current or past HBV infection, of whom about one million die annually (Lindenberg *et al*., 2013; Daw *et al*., 2014; Mehta *et al*., 2014 ; Salih, 2014).

Infection with HBV impacts the liver and results in wide range of illnesses. It can be transmitted through transfusion of infected blood, exposure to secretion, and a needle stick or cut by a contaminated sharp object (Hamad *et al*., 2019). HBV infection is considered endemic in Sudan, different prevalence rates were observed in different states (Badawi and Mustafa 2018).

The CMV Seroepidemiology ranged from 8.02 %(un acceptable low rate) to 90.4%, a wide range of seroprevalence (Hassan *et al*., 2014).the fact that CMV can infect a wide range of tissues and organ systems. Therefore, the rapid and qualitative detection of CMV from clinical samples is a significant clinical importance (Binnicker and Epsy, 2013).

Cytomegalovirus (CMV) remains an important cause of morbidity in immunocompromised persons, and it may manifest as symptomatic end-organ disease (including hepatitis and pneumonitis) or as so-called “CMV viral syndrome,” with fever, leukopenia, and thrombocytopenia.(Coaquette *et al*., 2012).
HCMV infection is common in chronic HBV, who can be regarded as patients at high risk for HCMV disease (Bayram *et al*., 2009). No published data about CMV infection among HBV infection in Sudan.

1.3 Objectives

1.3.1 General objective

To detect the frequency of cytomegalovirus among hepatitis B patients.

1.3.2 Specific objectives

- To detect cytomegalovirus IgM antibodies among HBV patients using enzyme linked immunesorbant assay (ELISA).
- To detect the possible major risk factor predisposing to CMV infection among HBV patient.

CHAPTER TWO

LITREATURE REVIEW

CHAPTER TWO

LITERATURE REVIEW

2.1 Background

CMV is double-stranded DNA virus and is a member of the herpes viruses. Like other herpes viruses, after recovery of the initial infection, CMV remains dormant within the host. Viral reactivation occurs during compromise of the immune system with immunosuppression (Gupta and Shorman, 2019).

CMV infection is often asymptomatic and acute and is followed by lifelong persistence of CMV in a latent stage in immunocompetent subjects but with potentially severe consequences in immunocompromised patients (Lichtner *et al.*, 2015).

2.2 Epidemiology

Approximately 59% of the population older than six years old has been exposed to CMV, with an increase in the population's sero-prevalence with increasing age. Infection can occur as a primary infection, re infection, or reactivation. Transmission of CMV can occur in numerous ways: via blood products (transfusions, organ transplantation), breastfeeding, viral shedding in close-contact settings, perinatally, and sexual transmission. Reactivation is seen in patients who become immunocompromised and is associated with elevated morbidity and mortality (Zheng *et al.*, 2019).

Primary infection: infection in seronegative patients; may be asymptomatic, Recurrent infection: Re infection or reactivation of CMV in seropositive patients, CMV infection: Presence of CMV in body fluids (urine, blood) or tissue and CMV disease: CMV infection with associated non-specific signs and symptoms and/or end-organ involvement (Bartlett *et al.*, 2018).

Hepatitis B virus (HBV) is an important human pathogen that has caused chronic infections worldwide (Ott *et al.*, 2012). Recent data obtained from a modeling study has shown that the global prevalence of hepatitis B surface antigen (HBsAg) was 3.9% in 2016, corresponding to an estimated 290 million infections worldwide (Wu *et*

al., 2018). HBV mainly infects hepatocytes and causes a wide spectrum of clinical manifestations(Glebe and Bremer, 2013; Bernal *et al*., 2010). HBV infection causes a broad spectrum of liver diseases ranging from acute to chronic hepatitis B infection with no biochemical evidence of liver injury to progressive chronic hepatitis B, which may advance to liver cirrhosis, liver failure, and hepatocellular carcinoma (Yu *et al*., 2016).

2.3 CMV

2.3.1 Structure

It is charachterized by slow growing cytomegalic (cytopathic effect,CPE), which from its name ,and enlargement of cell with acidophilic inclusion bodies in the cell nuclei that resemble owl‘s eye it is the genus megalo that its official name is HHV-5 (Brooks *et al*., 2010).

CMV is composed of large DNA genome (240 kbp), which is double strand linear andin form of atorid. In spite of genetically different strain it is being in human population. The DNA genome is surrounding by a protein coat that give icosahedral symmetry with 160 capsomeres. Its nucleocapsid is surround by an envelope that derived from nuclear membrane of the infected cell, measure 150-200 nm and contain viral glycoprotein spikes 8 nm as Fc receptor for non specific binding ((Brooks *et al* ., 2010).

Between the envelope and capsid is found amorphous layer called tegument. The naked virion measure 100 nm and contain one of the immediate early stronger enhancers due to concentration of binding site for cellular transcription factor. *Herpesviruses* encode an array of virus specific enzyme involved in nucleic acid metabolism, DNA synthesis, and protein regulation (DNA polymerase- thymidine kinase – protein kinase). (Brooks *et al* ., 2010).

2.3.2 Replication

The virus replicates in the cell after binding to the cell receptor via envelope glycoprotein. The capsid is transported to the nuclear pore through cytoplasmic membrane, un-coating occurs, and then the virus genome changes to circular shape in order to introduce in the DNA and to express "alpha" protein. After activation of gene

expression by VP16, a tequment protein then forms complex with several cellular proteins to be translated to "Beta" protein enzymes. (Brooks *et al*., 2010).

Starting the virus transcription by cellular RNA polymerase ц with virus factors called rolling-circle mechanism, after viral DNA replication and translation, to give "gamma" proteins its structural component with production of virus component. This component assembles and after that the virus is released by budding through nuclear membrane, envelope virus particle transported by vesicular movement to cell surface (Brooks *et al*., 2010).

2.3.3 Antiviral therapy

Treatment for mild CMV infections is not usually required apart from bed rest and drinking plenty of fluids, for more serious CMV illness, treatment is most often in the form of antiviral drugs, treatment may be needed for complications such as pneumonia.(Heymann 2015).

Several antiviral agents are available with activity against CMV, both valganciclovir hydrochloride and valacyclovir hydochlorid (oral active forms of ganciclovir sodium and acyclovir sodium) are used for prophylaxis against CMV reactivation in organ transplant recipients. However, despite the potential for benefit, to our knowledge, there are currently no data evaluating the efficacy of antiviral agents as prophlaxis for viral reactivation in nonimmunosuppressed patients in the ICU. (Nicholas *et al*., 2017). The drug of choice for prevention of CMV disease in solid-organ transplant patients is now valganciclovir, other than CMV retinitis , whoever , ganciclovir remains the mainstay of treatment , at least initially .(Akhter and Todd 2018). Oral antiviral therapy of CMV carries a blood and kidney side effect that requires laboratory monitoring, Tropical therapy has been reported to be effective, but no consensus as to the appropriate drug concentration exists. (California 2018).

2.3.4 Genotype

Different CMV strains are identified by gene variants such as those found in viral envelope glycoprotein B (gB) and glycoprotein H (gH) (Arav-Boger 2015). The genotype distribution of CMV is largely unknown in general populations (Jodie *et al*., 2019).

2.3.5 Transmission

Humans are only source of CMV. The risk of getting CMV through casual contact is very small; the virus is generally passed from infected people to others through direct contact with body fluids that carry the virus, such as urine, saliva, vaginal secretions, and semen and breast milk. CMV can be shed in saliva and urine on and off for many months, sometimes years, adult are usually infectious for less time than babies, people with weakened immune systems may experience a recurrence of CMV or may be infected with a new strain of the virus (Bennett *et al*., 2014).

Dental care centers are a significant source of CMV infection. Children less than three years of age with postnatally acquired CMV infection have been demonstrated to excrete CMV in their urine and saliva for 6 to 12 months (Nyholm and Schleiss 2010).

CMV is excreted in the breast milk of seropositive women. The risk of CMV transmission in infants breast-fed by seropositive women shedding virus in their breast milk has been reported to be 58% to 69% (Nyholm and Schleiss, 2010).

2.3.6 Pathogenesis

The central concepts of HCMV pathogenesis that link together the diverse disease associations are viraemia, the threshold relationship between viral load and disease and immune pressure forcing the virus to persist in sanctuary sites (Griffiths *et al*., 2015). Endpoint titration of serial urine samples showed that the level of viruria among cases of congenital HCMV without symptoms was, on average, one log higher than that found in cases of perinatal infection (which are clinically benign). In turn, the average level of viruria was an additional 1 log higher in babies with congenital HCMV infection who were symptomatic. After 3–6 months, the levels of viruria in the two groups of congenitally infected cases became indistinguishable and, after about a year, joined the cases of perinatal HCMV in having low-level persistent infection, remarkably, all of these have turned out to be true and provide the scientific rationale for treatment (Schleiss, 2013). This threshold relationship has been documented in populations of renal transplant patients and in cases of congenital HCMV with sensorineural hearing loss of varying sensitivities, high viral load triggers subsequent effects, which increase the risk of end-organ disease. Note that, in

multivariable models, most immunosuppressive drugs cause HCMV end-organ disease by increasing the viral load, while steroids cause disease by lowering the viral load threshold required (Griffiths *et al*., 2015). Epithelial cells and endothelial cells are more representative of HCMV infection in humans. Thus, the impressions given by these old experiments can be dismissed as studying 'the wrong virus in the wrong cell line using the wrong end point'. The immune system commits more resources to controlling HCMV than to any other virus, because healthy sero-positive individuals often have>1% of their peripheral blood T cells specific for one antigen of HCMV. Thus, in warfare terms, HCMV is in a 'stand-off' relationship with the immune system, poised to replicate rapidly if the established immune response becomes impaired. This can happen in patients given immunosuppressive drugs to prevent graft rejection, in patients with HIV infection, or in those with immature immune systems (the fetus and bone marrow transplant patients with newly engrafting marrow). Presumably, it can also happen if the immune system is 'stunned' by a sudden shock sufficient to precipitate the patient's admission to the intensive care unit. The presumed pathogenesis is HCMV replication stimulating the release of cytokines, which are then toxic to the lungs. A current controlled clinical trial is randomizing seropositive patients admitted to intensive care to receive ganciclovir or placebo, to determine whether this putative effect can be controlled (NIH, 2014). In elderly patients, the accumulation of decades of chronic immune surveillance for HCMV hiding in sanctuary sites might produce excess mortality in two main ways. First, the reduced number of naive T cells might make seropositive people less able to respond to vaccines for influenza or pneumococcal infection, and there is some, inconsistent, evidence for this from small studies (Wald *et al*., 2013). Second, the increased abundance of activated T cells might mediate inflammatory attacks on bystander cells, such as those forming the endothelium, to increase the risk of cardiovascular disease. Indirect effects of HCMV are operating in the general population to produce an excess risk of death should support the development of vaccines and their use to interrupt HCMV transmission at the population level (Griffiths *et al*., 2012).

2.3.7 Prevention

It was assumed by many that the complex interactions of HCMV with the immune system would preclude development of effective vaccines. Three proof-of-concept phase II clinical trials have reported recently that partial control of HCMV replication and transmission can be achieved, supporting an earlier study of live attenuated vaccine (Griffiths *et al*., 2015). The recombinant, soluble gB vaccine with MF59 adjuvant was studied in two populations (Griffiths *et al*., 2011). Seronegative women who had recently given birth were randomized to receive gB vaccine/MF59 or a placebo. On follow-up, approximately 50% protection was seen against HCMV seroconversion. The same vaccine or placebo was given to seronegative and seropositive candidates for solid-organ transplant and the quantity of viraemia and needforpre-emptive therapy were reduced post-transplant by the vaccine ,The correlate of protective immunity was the titre of antibody against gB (Griffiths *et al* ., (2011). Another phaseII randomized controlled trial gave two DNA plasmids to patients undergoing stem cell transplantation. One plasmid encoded gB, while the second encodedpp65, amajor target of cell-mediatedimmunity. One dose of vaccine was given pre-transplant and three doses were given post-transplant. The need for preventive therapy was reduced and the correlate of protective immunity was the number of ELISPOT spot-forming cells. Thus, there is evidence for protection against primary infection (Griffiths *et al*., 2011) .and evidence for boosting of natural immunity (Kharfan *et al*., 2012). Future randomized controlled trials will build on these examples and further define the relative contributions of humoral and cell-mediated immunity. A recent controlled trial has randomized renal transplant patients to receive infusions of HCMV-specific monoclonal antibodies or placebo, to determine whether antibody can reduce transmission of HCMV. The results are awaited with interest (Griffiths *et al*., 2015). Following a non-randomized study of hyperimmune immunoglobulin in pregnant women, a randomized, placebo controlled trial of the same preparation failed to repeat the initial observations of reduced intrauterine transmission of HCMV (Revello *et al*., 2014). Future studies may evaluate infusions of monoclonal antibodies for this indication.

2.3.8 Diagnosis:

Serological tests are useful for determining whether a patient has had CMV infection in the past, determined by the presence or absence of CMV IgG. Many different assays have been described and evaluated for the detection of CMV IgG antibodies. Among these are complement fixation, enzyme-linked immunosorbent assay (ELISA), anticomplement immunofluorescence, radioimmunoassay, and indirect hemagglutination , The detection of IgM antibodies has been used as an indicator of acute or recent infection. Many different assays are available but enzyme-linked immunosorbent assays (ELISAs) are the most widely used and are based on crude viral preparations. The IgM capture assays are widely employed and are based on selective binding of IgM antibody to the solid phase. Recombinant IgM assays using recombinant HCMV proteins and peptides have been developed in an attempt to standardize serological assays (Ross et al., 2011).

Diagnosis is confirmed when CMV antibodies are detected through serology tests. The most common test used is the enzyme-linked immunosorbent assay (ELISA). ELISA is used for individuals aged 12 months and older; a positive CMV immunoglobulin G (IgG) indicates infection. Initially, CMV IgG is measured in two samples taken 1 to 3 months apart and is used to diagnose primary infection. CMV immunoglobulin M is present in secondary infections and cannot be used to diagnose a primary infection (Tolbert and Pharm, 2018).

RealTime CMV Assay:

Designed by Abbott Molecular Inc., the RealTime CMV assay (Figure) uses in vitro PCR assays to test for CMV DNA in humans. It is used to aid in the management of anti-CMV therapy in hematopoietic stem cell transplant patients.8 The CMV assay must be interpreted in clinical laboratory findings. It is not used to screen for CMV DNA in the blood. The use of the RealTime CMV assay is limited because it can only be used in individuals who have undergone a hematopoietic stem cell transplant.8 It has not been tested on patients in any other category (such as other transplant procedure, neonates, pediatric patients, or AIDS patients) or in patients who are in an immunocompromised state. Detection of CMV DNA is contingent upon proper collection, handling, transportation, storage, and preparation of the specimen. A false-

negative can result from improper conditions of the specimen.8 The assay amplifies two selected targets of the CMV genome. This is done in order to reduce the risk of rare mutations that could result in the assay failing to detect the virus.8 Table 2 indicates how to interpret the results of the assay. Results are reported as either IU/mL or log IU/mL.9 (Abbott, 2017., Abbott 2019).

2.4 HBV

2.4.1 Structure

By 1967, Blumberg and others recognized that Australia antigen was correlated with hepatitis. In 1970 Australia antigen was identified as a structural component of the hepatitis B virus (HBV). That year Dane and co-workers published electron micrographs of 17–22 nm spherical HBV surface antigen (HBsAg) particles, 17–22 nm diameter filamentous HBsAg particles, and 45 nm diameter virions comprised of a HBsAg envelope and a 36 nm diameter core. HBV virions are known as Dane particles (Venkatakrishnan and Zlotnick 2016).

2.4.2 Epidemiology

The epidemiology of hepatitis B can be described in terms of the prevalence of hepatitis B surface antigen (HBsAg) in a population, broadly classified into high-(.8% HBsAg Pacific Ocean Prevalence of hepatitis B surface antigen Atlantic Ocean prevalence), intermediate-(2%–7%) and lowered valence (,2%) areas. These broad categories are useful for understanding the predominant patterns of transmission and outcomes for infection ,as well as the relative population burden of the consequences of chronic hepatitis B, including liver cancer (Jennifer and Benjamin 2015).

2.4.3 Viral replication

HBV chronically infects hepatocytes. It replicates by reverse transcription of an RNA intermediate, the pregenome. Nuclear cccDNA, formed from the incoming relaxed circular viral DNA, serves as the transcriptional template. Progeny genomes are formed by reverse transcription, which occurs within viral nucleocapsids in the cytoplasm of infected cells. Nucleocapsids with mature viral DNA are either assembled into viral envelopes and exported from the infected hepatocyte or, if needed, transported to the nucleus to amplify cccDNA copy number. Envelope

proteins are also secreted as subviral particles, hepatitis B surface antigen (HBsAg), as are large numbers of virus-like particles with empty nucleocapsids (Hu and Seeger 2015).

2.4.4 Antiviral therapy

Interferon therapy may induce immune clearance of HBV, but this is rare. Nucleoside analogue inhibitors of HBV reverse transcription are effective at suppressing virus replication, which leads to an amelioration of immune pathogenesis, presumably due to reduced numbers of infected hepatocytes (Gish *et al* ., 2015). However, infected hepatocytes almost always persist at low levels, possibly because inhibition of DNA synthesis is incomplete, and infection typically rebounds if therapy is stopped, or if there is a breakthrough of drug resistant HBV. This warrants a search for more effective cocktails that not only inhibit reverse transcription of most HBV variants, but also target other steps in viral DNA synthesis (e.g. nucleocapsid assembly). In addition, efforts are being made to employ nucleases (e.g., Cas9) for degradation of DNA. Finally, it would be desirable to have a therapy that could effectively induce immune clearance and/or immune control in all HBV carriers, though approaches to this goal, aside from limited success with interferon therapy, have so far not been promising (Seeger *et al.,* 2013).

2.4.5 Genotype

There is a strong relationship between HBV genotype (Lin and Kao 2015) and geography worldwide, and genotype has been shown to influence thenatural historyand, in turn, transmission patterns of hepatitis B infection. The distribution of genotypes worldwide. Genotype also influences the progression of viral infection through phases, which, in turn, determines infectivity and age at transmission or infection. In Asia, where genotypes B and C predominate, transmission is most commonly vertical at the time of birth; where genotypes A, D, and E are most common, such as Africa, Eastern Europe, and the Middle East, transmission is more commonly horizontal in early childhood , HBV genotypes and sub genotypes may also have relevance to hepatitis B control efforts through vaccination. Mismatch between the strain used to derive hepatitis B vaccine (serotype adw) and that which is

prevalent in a given population may result in increased vaccine escape and reduced efficacy at a population level (Davies *et al* ., 2013).

2.4.6 Transmission

HBV is transmitted through exposure to infected blood and bodily fluids (particularly semen and vaginal secretions). HBV survives for prolonged periods outside the body. Although HBV has been detected in saliva, tears, breast milk, sweat, and urine, there is minimal evidence of transmission through exposure to these fluids where no blood is present, and breast feeding has not been shown to increase risk of infection (Zheng *et al*., 2011). Most infections worldwide are acquired through perinatal transmission at birth, through horizontal transmission to/between young children, through sexual contact, and through injecting drug use (Jennifer and Benjamin 2015). Other routes of transmission, which have declined in frequency with the implementation of control measures, include through contaminated blood or blood products and unsafe medical practices; however, health care associated infection remains a significant concern in both resource-poor (Arankalle *et al*., 2011)

2.4.7 Viral pathogenesis

Editorial Viral pathogenesis appears mediated mostly by the antiviral immune response. This can have two manifestations, immune clearance of the virus, which generally takes place within the f irst year of infection, and virus persistence accompanied by chronic immune injury to the liver (Seeger and William 2016). Immune clearance in the first year is often considered a paradigm for an antiviral therapy that targets infected hepatocytes. Interestingly, clearance is often incomplete, but virus rebound is prevented by the immune system. However, surprisingly little is known about these processes. A large amount of hepatocyte destruction typically takes place during virus clearance. On the other hand, hepatocytes found at the end of the clearance phase arise from infected hepatocytes, with evidence these surviving cells passed through one or more rounds of division, presumably to maintain liver cell mass. This confuses the issue of cccDNA clearance, since one could imagine that it is either lost during mitosis or degraded, in the absence of cell division, through pathways mediated by antiviral cytokines. Experiments to distinguish these pathways, or reveal others, remain to be done. Immune control following the clearance phase

also remains obscure, as does the location of the cccDNA that leads to rebound if the immune system is compromised (Buendia and Neuveut 2015).

2.4.8 Diagnosis

Acute hepatitis B is a clinical diagnosis identified by the detection of HBsAg, symptoms, high serum aminotransferases. Usually anti-HBc IgM can be detected and HBV DNA is present. HBeAg can also be identified in most acute phase of infections, but has little clinical importance. The diagnosis of chronic infection is based on the persistence of HBsAg for more than 6 months. Patients with chronic HBV infection are commonly diagnosed by laboratory means but not by clinical presentations. Past HBV infection is defined by the coexistence of anti-HBsOccult HBV infection is defined by persistence of low level of intrahepatic HBV DNA without detectable HBsAg (Hollinger and Sood 2010). It is a serological situation defined by the presence of isolated anti-HBc with the absence of HBsAg and antiHBs antibody (Eun *et al* .,2016). The detection of HBV DNA in the liver is the gold standard of diagnosis for occult HBV infection, since cccDNA remains in the hepatocytes and HBV DNA is occasionally identified in the liver but not in the serum. However, gaining hepatic HBV DNA is difficult in clinical setting since the procedure is invasive. Real-time PCR for serum HBV DNA detection have been shown with adequate sensitivity to identify occult HBV infection in many cases; thus, HBV DNA testing is widely used to diagnose occult HBV infection (Eun *et al* ., 2016). Occult HBV infection has some clinical importance. First, it can be transmitted via transfusion, solid organ transplantation including orthotopic liver transplantation (Raimondo *et al*., 2013 ; Mahboobi *et al*., 2012), or hemodialysis (JH *et al* ., 2013). Second, reactivation of HBV infection may occur in patients receiving chemotherapy or immunocompromised state (Onozawa *et al*., 2016). Third, it may accelerate liver injury and lead to hepatic fibrosis in patients with chronic liver disease including chronic hepatitis C infection (Squadrito *et al*., 2015 ; Covolo *et al*., 2015). Forth, it appears to be a risk factor for HCC by its carcinogenic effect and by leading to continuous hepatic inflammation and fibrosis (Shi *et al*., 2012).

2.5 Previous study

In Al-Kufa Uni versity Mohammed and his colleges detect CMV IgM and IgG by using ELISA . The ELISA test results showed that 68 (89.4%) out of the 76 samples were positive for anti-HCMV IgG antibodies, and 4(5.26%) samples were positive for anti-HCMV IgM (Alkhalili *et al*., 2015).

Bayram and his colleges showed that HCMV infection is common in chronic HBV patients who can be regarded as patients at high risk for HCMV disease (Bayram *et al*., 2009).

CHAPTER THREE

MATERIALS AND METHODS

Chapter THREE

MATERIALS AND METHODS

3.1 Study design

This study was descriptive cross-sectional study.

3.2 Study area, setting and duration

Study was conducted in Saba Teaching laboratory Hospital in Khartoum State during the period from March to November (2019).

3.3 study population

Study population are hepatitis B positive Sudanese patients who are diagnosed by serological or molecular techniques.

3.4 Inclusion criteria

Hepatitis B surface antigen (HBsAg) positive (chronic and acute hepatitis B patients).

3.5 Exclusion criteria

Known Human immunodeficiency virus (HIV) co-infection and others diseases such as (autoimmune diseases and infectious disease).

3.6 Sample size

A Total of 88 hepatitis B patients were enrolled in this study.

3.7 Ethical consideration

Permission to carry out the study was obtained from college of Medical Laboratory Science. Permission of laboratory manger was taken before beginning. Every sample was collected after verbal approval by patients and volunteer.

3.8 Sampling

3.8.1 Sample technique

Simple random sample.

3.8.2 Data collection

Samples were collected randomly, Questionnaire was used to collect demographic, clinical and laboratory data from each participant in this study.

3.8.3 Sample collection and preservation

Ethanol (70%) was used to clean the skin. Three ml of venous blood was collected from patients in plane container. Then the samples were centrifuged and serum was separated in a sterile container and stored at -20 ºC until analysis.

3.9 Sample analysis

The sample was analyzed for the presence of Cytomegalovirus IgM antibody by commercially available Enzyme linked immunosorbent assay ELISA.

3.9.1 Principle of the TEST

The DRG Cytomegalovirus (CMV) IgM kit is a solid phase enzyme linked immunosorbant assay (ELISA) patient samples are diluted with sample diluent and additionally incubate with IgG-RF sorbent to eliminate competitive inhibition from specific IgG. This pretreatment avoid false negative results.

Microtiter wells as a solid phase are coated with inactivated grade 2 cytomegalovirus (CMV) antigen (strain AD-169). Diluted patient specimens and ready-for-use controls are pipette in to these wells. During incubation cytomegalovirus specific antibodies of positive specimens and control are bound to the immobilize antigen.

After washing step to remove the un bound sample and control material horseradish peroxidase conjugated anti human IgM antibodies are dispensed in to the wells. During a second incubation this anti IgM conjugate bind specifically to IgM antibodies resulting in the formation of enzyme-linked immune complexes.

After a second washing step to remove unbound conjugate the immune complexes formed (in case of positive results) are detected by incubation with TMB substrate and development of a blue color. The blue color turns in to yellow by stopping the enzymatic indicator reaction with sulfuric acid.

The intensity of the color is directly propotional to the amount of cytomegalovirus (CMV) specific IgM antibodies in the patient specimen. Absorbance at 450 nm read using an ELISA micro titer plate reader.

3.9.2 ELISA Procedure

Commercial ELISA Kits (DRG cytomegalie virus IgM enzyme immune assay kit) were used as described by manufacture.

All reagents and samples were brought to room temperature before beginning the procedure. The samples is first be diluted (1:50) with sample diluents, for absorbance of rheumatoid factor this pre diluents incubated with IgG –RF-Sorbent.

Briefly, 100 µl of negative control (NC), 100 µl of positive control (PC), 100 µl of prepared sample were incubated in microtiter strip wells coated with CMV antigen at 37ºC for 60 minutes. The wells were washed 5 times by wash solution using automatic washer to remove unbound component, then 100 µl of anti – IgM conjugate were added to each well and incubated at room temperature for 30 minutes. After another washing step to remove excess conjugate, an enzyme substrate reagent was added (100 µl / well) and the plate was incubated for 10 minute. Blue color changed to yellow color after adding stop solution (100 µl). The optical density (OD) in a microtiter reader was read within 30 minutes at 450 nm.

3.9.3 Calculation of Results

Normal range for this ELISA should be established by each laboratory based on its own patients population in geographical area served.

3.9.3.1 Calculation of qualitative results

Absorbance value of slandered 1 = CO (cut-off).

3.9.3.2 Interpretation of qualitative Results

Samples with the absorbance > COV were considered as anti CMV IgM ab positive, while sample with absorbance < COV were considered as anti CMV IgM ab negative.

3.10 Statistical analysis

Data was analyzed by using statistical package for social sciences (SPSS) program.the significance of difference was determined by using Chi- square test. Statistical significance was set at $P < 0.05$.

CHAPTER FOUR

RESULTS

CHAPTER FOUR

RESULTS

Results

A total of 88 hepatitis B patients were enrolled in this study, 12 (13.6) of HBV patients shows positive CMV IgM antibodies, and 76 (86.6%) shows negative CMV IgM antibodies (Table 4.1).

The rate of anti-HCMV IgM antibodies Seropositivity among males 7 (8.0%) out of 88.While the rate of anti-HCMV IgM seropositive among Female were 5 (7.5 %) out of 88 (Table 4.2).

According to age group it was shown that the highest age group in regards to anti-HCMV IgM antibodies positive was the age group (20-30) year and it represent 4(4.5%) from total anti-HCMV IgM antibodies case while the age group (40-50) year record lowest anti-HCMV IgM antibodies positive ant it represent 2 (2.3%) of total (Table 4.3).

Among HBV patients there was 8 (9.1%) had history of previous blood transfusion 2 (2.3%) of them were positive CMV IgM antibodies, 6 (6.8%) were negative CMV IgM antibodies (Table 4.4).

Regard to symptoms of jaundice there was 5(5.7%) had symptoms of jaundice 1(1.1%) of them were positive CMV IgM antibodies (Table .45).

According to duration 44 (50%) were acute phase of HBV disease 6 (6.8%) of them were positive CMV IgM antibodies.

Twenty eight (31.8%) of HBV patients who had taken treatment 3 (3.4%) were positive CMV IgM antibodies while 60 (68.2%) of HBV patients who had not taken treatment 9 (10.2%) were positive CMV IgM antibodies (Table .47).

Table 4.1: The sero-positivity of CMV IgM among HBV Patients

CMV Results	Frequency	Percentage
Positive	12	13.6%
Negative	76	86.4%
Total	88	100%

Table 4.2: Association between Positive CMV among HBV Patients According to Gender

Gender	CMV Results		Total
	Positive	Negative	
Male	7 (8.0%)	41 (46.6%)	48 (54.5%)
Female	5 (7.5%)	35 (39.8%)	40 (45.5%)
Total	15 (13.6%)	76 (86.4%)	88(100.0%)

**P value*: 0.777

Table (3-3): Association between Positive CMV and Age Groups among HBV Patients

Age Group	CMV Results		Total
	Positive	Negative	
10-20 years	3 (3.4%)	8 (9.1%)	11 (12.5%)
20-30 years	4 (4.5%)	32 (36.4%)	36 (40.9%)
30-40 years	3 (3.4%)	26 (29.5%)	29 (33.0%)
40-50 years	2 (2.3%)	10 (11.4%)	12 (13.6%)
Total	12 (13.6%)	76 (86.4%)	88 (100.0%)

**P value:* 0.514

Table (4.4): Association between history of Blood Transfusion and positive CMV among HBV patients

Blood transfusion	CMV Results		Total
	Positive	Negative	
Yes	2 (2.3%)	6 (6.8%)	8 (9.1%)
No	10 (11.4%)	70 (79.5%)	80 (90.9%)
Total	12 (13.6%)	76 (86.4%)	88 (100%)

**P value:* 0.299

Table (4-5): Association between positive CMV and jaundice among HBV patients

Jaundice	CMV Results		Total
	Positive	Negative	
Yes	1 (1.1%)	4 (4.5%)	5 (5.7%)
No	11 (12.5%)	72 (81.8%)	83 (94.3%)
Total	12 (13.6%)	76 (86.4%)	88 (100%)

** P value:* 0.528

Table (4-6): Association between positive CMV and duration of HBV

Duration of disease	CMV Results		Total
	Positive	Negative	
Acute	6 (6.8%)	38 (43.2%)	44 (50%)
Chronic	6 (6.8)	38 (43.2%)	44 (50%)
Total	12 (13.6%)	76 (86.4%)	88 (100%)

**P value 0.621*

Table (4-7): association between positive CMV and treatment of HBV

Treatment	CMV Results		Total
	Positive	Negative	
Yes	3 (3.4%)	25 (28.4%)	28 (31.8%)
No	9 (10.2%)	51 (58%)	60 (68.2%)
Total	12 (13.6%)	76 (86.4%)	88 (100%)

**P value 0.428*

CHAPTER FIVE

DISSCUTION, CONCLUSION AND RECOMMENDATION

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

Cytomegalovirus (CMV) infection in individuals with effective immunity is generally asymptomatic or may occur as a mononucleosis syndrome but rarely leads to severe and life-threatening organ complications such as gastrointestinal, cardiovascular, hepatic, and neurologic manifestations (Yi *et al* ., 2019).

In the present study 12 (13.6) of HBV patients shows positive CMV IgM antibodies, and 76 (86.6%) shows negative CMV IgM antibodies, This finding is supported by AL- Khilkhali *et al* in Al-Kufa University who detected CMV IgM in 76 samples and showed that 4 samples (5.26%) were positive for anti-HCMV IgM antibodies(AL-Khilkhali *et al*., 2015). on the contrary to Bayram *et al* who conclude that HCMV infection is common in chronic HBV patients, who can be regarded as patients at high risk for HCMV disease (Bayram *et al.,* 2009).

In the present study in regard to the gender the percentage of anti-HCMV-IgM antibodies Seropositivity common in males 7 (8.0%) than females 5 (7.5%) , which disagree with AL- Khilkhali *et al* the result conducted in Al-Kufa University in which the HCMV IgM among males 2 (2.63%) it was equal to the proportion of women (AL- Khilkhali *et al*., 2015).

According to age group, there highest sero-positivty was 4 (4.5%) between 20-30 years of age where in other study conducted in Al-Kufa University, the highest sero-positivity were between 44-54 years of age (AL- Khilkhali *et al* ., 2015).
There is no statistically significant diferance in HCMV IgM antibodies between acute and chronic HBV patients (*p value* 0.621). While in Bayram *et al* study the HCMV IgM antibodies is higher in chronic HBV patients.

Our result also demonstrate that no statistical difference in HCMV IgM antibodies between who received blood transfusion and didn't receive (*P.value* 0.299). Also the study demonstrate that no statistical difference between HCMV IgM antibodies and

Jaundice (*P.value* 0.528) and no statistical difference between HCMV IgM antibodies and treatment (*P.value* 0.428).

The difference in the results might be attributed to the ethical background of the studied population, the different levels of exposure to potential risks environment and sample size.

5.2 Conclusion

The finding out of this study indicates low prevalence of CMV, sero-positive IgM among hepatitis B patients (13.6%) Furthermore, the results show that the different rates of seropositivity between different age group suggest age- related CMV exposure in hepatitis B patients in Sudan.

5.3 Recommendations

Screening of CMV IgM is suggested for hepatitis B patients who are at risk of CMV infection.

Large sample size is critical for better results.

All patients at risk of CMV should be prevented by gancyclovir treatment.

PCR is essential for accurate identification.

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APPENDICES

Appendix 1

Sudan University of sciences and technology

Collage of Graduated Students

Detection of HCMV IgM among Sudanese Hepatitis B patients

Questionnaire

Date / 2019

ID Number:……..

Age:……Years

Gender:

Male ( ) Female ( )

Duration of disease:

Less than six months ( ) More than six months ( )

Treatment:

Yes ( ) No ( )

Blood transfusion:

Yes ( ) No ( )

Jaundice:

Yes ( ) No ( )

Instructions for Use

CMV IgM ELISA

IVD

CE 0197

REF EIA-3469

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1 INTRODUCTION

1.1 Intended Use

The **DRG Cytomegalie Virus (CMV) IgM Enzyme Immunoassay Kit** provides materials for the **quantitative** and **qualitative** determination of IgM-class antibodies to Cytomegalie Virus (CMV) in human serum plasma (EDTA, heparin or citrate plasma).

This assay is intended for in vitro diagnostic use only.

1.2 Summary and Explanation

Cytomegalovirus (CMV) is a member of the herpesvirus group (Betasubfamily, DNA virus of 150-200 nm). These viruses share a characteristic ability to remain dormant within the body over a long period. Initial CMV infection, which may have few symptoms, is always followed by a prolonged, inapparent infection during which the virus resides in cells without causing detectable damage or clinical illness. Severe impairment of the body´s immune system by medication or disease consistently reactivates the virus from the latent or dormant state.

CMV is found universally throughout all geographic locations and socioeconomic groups, and infects between 50% and 85% of adults.

CMV infection is more widespread in developing countries and in areas of lower socioeconomic conditions.

For the vast majority of people, CMV infection is not a serious problem, but it is to certain high-risk groups: the unborn baby during pregnancy, people who work with children, and

immunocompromised persons, such as organ transplant recipients and persons infected with HIV.

The presence of virus resp. infection may be identified by Microscopy, PCR, Serology: CBR and detection of antibodies by ELISA.

IgM antibodies are the first to be produced by the body in response to a CMV infection. They are present in most individuals within a week or two after the initial exposure. IgM antibody production rises for a short time period and then declines. After several months, the level of CMV IgM antibody usually falls below detectable levels. Additional IgM antibodies are produced when latent CMV is reactivated.

IgG antibodies are produced by the body several weeks after the initial CMV infection and provide protection from primary infections. Levels of IgG rise during the active infection, then stabilize as the CMV infection resolves and the virus becomes inactive. After a person has been exposed to CMV, he or she will have some measurable amount of CMV IgM antibody in their blood for the rest of their life. CMV IgM antibody testing can be used, along with IgM testing, to help confirm the presence of a recent or previous CMV infection.

2 PRINCIPLE OF THE TEST

The **DRG Cytomegalie Virus (CMV) IgM ELISA** Kit is a solid phase enzyme-linked immunosorbent assay (ELISA).

Patient samples are diluted with S*ample Diluent* and additionally incubated with *IgG-RF-Sorbent* to eliminate competitive inhibition from specific IgG. This pretreatment avoids false negative results.

Microtiter wells as a solid phase are coated with inactivated grade 2 Cytomegalovirus (CMV) antigen (strain AD-169).

Diluted patient specimens and **ready-for-use controls** are pipetted into these wells. During incubation Cytomegalie Virus (CMV)-specific antibodies of positive specimens and controls are bound to the immobilized antigens.

After a washing step to remove unbound sample and control material horseradish peroxidase conjugated anti-human IgM antibodies are dispensed into the wells. During a second incubation this anti-IgM conjugate binds specifically to IgM antibodies resulting in the formation of enzyme-linked immune complexes.

After a second washing step to remove unbound conjugate the immune complexes formed (in case of positive results) are detected by incubation with TMB substrate and development of a blue color. The blue color turns into yellow by stopping the enzymatic indicator reaction with sulfuric acid.

The intensity of this color is directly proportional to the amount of Cytomegalie Virus (CMV)-specific IgM antibody in the patient specimen. Absorbance at 450 nm is read using an ELISA microtiter plate reader.

3 WARNINGS AND PRECAUTIONS

- This kit is for in vitro diagnostic use only. For professional use only.
- Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood.
- All reagents of this test kit which contain human serum or plasma have been tested and confirmed negative for HIV I/II, HBsAg and HCV by FDA approved procedures. All reagents, however, should be treated as potential biohazards in use and for disposal.
- Avoid contact with Stop Solution containing 0.2 mol/L H_2SO_4. It may cause skin irritation and burns.
- TMB substrate has an irritant effect on skin and mucosa. In case of possible contact, wash eyes with an abundant volume of water and skin with soap and abundant water. Wash contaminated objects before reusing them. If inhaled, take the person to open air.
- The microplate contains snap-off strips. Unused wells must be stored at 2 °C to 8 °C in the sealed foil pouch and used in the frame provided
- Pipetting of samples and reagents must be done as quickly as possible and in the same sequence for each step.
- Use reservoirs only for single reagents. This especially applies to the substrate reservoirs. Using a reservoir for dispensing a substrate solution that had previously been used for the conjugate solution may turn solution colored. Do not pour reagents back into vials as reagent contamination may occur.
- Mix the contents of the microplate wells thoroughly to ensure good test results. Do not reuse microwells.
- Do not let wells dry during assay; add reagents immediately after completing the rinsing steps.
- Allow the reagents to reach room temperature (21 °C to 26 °C) before starting the test. Temperature will affect the absorbance readings of the assay. However, values for the patient samples will not be affected.
- Never pipette by mouth and avoid contact of reagents and specimens with skin and mucous membranes.
- Do not smoke, eat, drink or apply cosmetics in areas where specimens or kit reagents are handled.
- Wear disposable latex gloves when handling specimens and reagents. Microbial contamination of reagents or specimens may give false results.
- Handling should be in accordance with the procedures defined by an appropriate national biohazard safety guideline or regulation.
- Do not use reagents beyond expiry date as shown on the kit labels.
- All indicated volumes have to be performed according to the protocol. Optimal test results are only obtained when using calibrated pipettes and microtiter plate readers.
- Do not mix or use components from kits with different lot numbers. It is advised not to exchange wells of different plates even of the same lot. The kits may have been shipped or stored under different conditions and the binding characteristics of the plates may result slightly different.
- Chemicals and prepared or used reagents have to be treated as hazardous waste according the national biohazard safety guideline or regulation.
- For information on hazardous substances included in the kit please refer to Safety Data Sheets.
  Safety Data Sheets for this product are available upon request directly from DRG.

4.3 Reagent Preparation

Allow all reagents and required number of strips to reach room temperature prior to use.

Wash Solution

Dilute *Wash Solution* **1+19** (e.g. 10 mL + 190 mL) with fresh and germ free redistilled water. This diluted wash solution has a pH value of 7.2 ± 0.2.
Consumption: ~ 5 mL per determination.
Crystals in the solution disappear by warming up to 37 °C in a water bath. Be sure that the crystals are completely dissolved before use.
The diluted Wash Solution is stable for 4 weeks at 2 °C to 8 °C.

4.4 Disposal of the Kit

The disposal of the kit must be made according to the national regulations. Special information for this product is given in the Material Safety Data Sheets.

4.5 Damaged Test Kits

In case of any severe damage to the test kit or components, DRG has to be informed in writing, at the latest, one week after receiving the kit. Severely damaged single components should not be used for a test run. They have to be stored until a final solution has been found. After this, they should be disposed according to the official regulations.

5 SPECIMEN COLLECTION AND PREPARATION

Serum or plasma (EDTA-, heparin- or citrate plasma) can be used in this assay.
Do not use haemolytic, icteric or lipaemic specimens.
Please note: Samples containing sodium azide should not be used in the assay.

5.1 Specimen Collection

Serum:
Collect blood by venipuncture (e.g. Sarstedt Monovette for serum), allow to clot, and separate serum by centrifugation at room temperature. Do not centrifuge before complete clotting has occurred. Patients receiving anticoagulant therapy may require increased clotting time.

Plasma:
Whole blood should be collected into centrifuge tubes containing anti-coagulant (e.g. Sarstedt Monovette with the appropriate plasma preparation) and centrifuged immediately after collection.

5.2 Specimen Storage and Preparation

Specimens should be capped and may be stored for up to 3 days at 2 °C to 8 °C prior to assaying.
Specimens held for a longer time should be frozen only once at -20 °C prior to assay. Thawed samples should be inverted several times prior to testing.

5.3 Specimen Dilution

Prior to assaying each patient specimen is first to be diluted with *Sample Diluent*. For the absorption of rheumatoid factor these prediluted samples then have to be incubated with *IgG-RF-Sorbent*

1. Dilute each patient specimen **1+50** with *Sample Diluent*;
   e.g. 10 µL of specimen + 0.5 mL of *Sample Diluent*. **Mix well.**
2. Mix well the IgG-RF-Sorbent before use.
3. Dilute this prediluted sample **1+1** with *IgG-RF-Sorbent*
   e.g. 60 µL prediluted sample + 60 µL *IgG-RF-Sorbent*. **Mix well**
4. **Let stand at room temperature for at least 15 minutes, up to a maximum of 2 hours and mix well again.**
5. Take 100 µL of these pretreated samples for the ELISA.

Please note: Controls are ready for use and must not be diluted!

6 ASSAY PROCEDURE

6.1 General Remarks

- It is very important to bring all reagents, samples and controls to room temperature before starting the test run!
- Once the test has been started, all steps should be completed without interruption.
- Use new disposal plastic pipette tips for each standard, control or sample in order to avoid cross contamination
- Absorbance is a function of the incubation time and temperature. Before starting the assay, it is recommended that all reagents are ready, caps removed, all needed wells secured in holder, etc. This will ensure equal elapsed time for each pipetting step without interruption.
- As a general rule the enzymatic reaction is linearly proportional to time and temperature.
- Close reagent vials tightly immediately after use to avoid evaporation and microbial contamination.
- To avoid cross-contamination and falsely elevated results pipette patient samples and dispense conjugate without splashing accurately to the bottom of wells.
- During 37 °C incubation cover microtiter strips with foil to avoid evaporation.

6.2 Test Procedure

Prior to commencing the assay, dilute *Wash Solution*, **prepare patient samples as described in point 5.3**, mix well before pipette and establish carefully the **distribution and identification plan** supplied in the kit for all specimens and controls.

1. Select the required number of microtiter strips or wells and insert them into the holder.
   Please allocate at least:
   1 well (e.g. A1) for the substrate blank,
   1 well (e.g. B1) for the *Neg. Control*
   3 wells (e.g. C1-E1) for the *Standard 1-3*
   1 well (e.g. F1) for the *Pos. Control.*
   It is left to the user to determine standards, control and patient samples in duplicate.
2. Dispense
   100 µL of *Neg. Control* into well B1
   100 µL of *Standard* 1 into well C1
   100 µL of *Standard* 2 into well D1
   100 µL of *Standard* 3 into well E1
   100 µL of *Pos. Control* into well F1 and
   100 µL of each p r e t r e a t e d sample with new disposable tips into appropriate wells.
   Leave well A1 for substrate blank!
3. Cover wells with foil supplied in the kit. Incubate for **60 minutes at 37 °C.**
4. Briskly shake out the contents of the wells.
   Rinse the wells 5 times with diluted *Wash Solution* (**300 µL per well**). Strike the wells sharply on absorbent paper to remove residual droplets.
   Important note:
   The sensitivity and precision of this assay is markedly influenced by the correct performance of the washing procedure!
5. Dispense **100 µL** *Enzyme Conjugate* into each well, **except A1**.
6. Incubate for **30 minutes at room temperature (20 °C to 25 °C).**
   Do not expose to direct sun light!
7. Briskly shake out the contents of the wells.
   Rinse the wells 5 times with diluted *Wash Solution* (300 µL per well). Strike the wells sharply on absorbent paper to remove residual droplets.
8. Add **100 µL** of *Substrate Solution* into all wells.
9. Incubate for **exactly 10 minutes at room temperature (20 °C to 25 °C) in the dark**.
10. Stop the enzymatic reaction by adding **100 µL** of *Stop Solution* to each well.
    Any blue color developed during the incubation turns into yellow.
    Note: Highly positive patient samples can cause dark precipitates of the chromogen!
11. Read the optical density at **450/620 nm** with a microtiter plate reader **within 30 minutes** after adding the *Stop Solution*.

6.3 Measurement

Adjust the ELISA microplate or microstrip reader **to zero** using the **substrate blank in well A1**.
If - due to technical reasons - the ELISA reader cannot be adjusted to zero using the substrate blank in well A1, subtract the absorbance value of well A1 from all other absorbance values measured in order to obtain reliable results!
Measure the absorbance of all wells **at 450 nm** and record the absorbance values for each control and patient sample in the distribution and identification plan.
Dual wavelength reading using 620 nm as reference wavelength is recommended.
Where applicable **calculate the mean absorbance values** of all duplicates.

7 CALCULATION OF RESULTS

7.1 Validation of the Test Run

The test run may be considered valid provided the following criteria are met:

Substrate blank in A1:	Absorbance value **lower than 0.100**
***Neg. Control* in B1:**	Absorbance value **lower than 0.200**
***Standard 1 (Cut-off)* in C1 :**	Absorbance value **between 0.350 – 0.900**
***Standard 2* in D1 :**	Absorbance value **between 0.800 – 1.500**
***Standard 3* in E1 :**	Absorbance value **between 1.100 – 2.000**
***Pos. Control* in F1 :**	Absorbance value **between 0.650 – 3.000**

7.2 Calculation of quantitative Results

In order to obtain **quantitative results in DU/mL** (DU = DRG Units) plot the (mean) absorbance values of the *Neg. Control* and the 3 Standards 1, 2 and 3 on (linear/linear) graph paper in a system of coordinates against their corresponding concentrations **(0, 50, 200 and 400 DU/mL**) and draw a standard calibration curve (absorbance values on the vertical y-axis, concentrations on the horizontal x-axis).

Read results from this standard curve employing the (mean) absorbance values of each patient specimen and control.

All suitable computer programs available can be used for automated result reading and calculation. The following mathematical functions can be used: 4 PL (4 Parameter Logistics) curve fit, Linear regression or Point to Point calculation of the standard curve. We use DRG regression program for windows (4 parameter Rodbart regression). If other regression software is used, the obtained values have to be validated by the user.

NOTE: Values of additionally (1:10, in total 1:1000) diluted patient samples must be multiplied by the appropriate dilution factor in order to obtain correct results! (Dilution: 1:10 = Dilution factor: 10). (See chapter "5.3 Specimen Dilution").

7.3 Interpretation of quantitative Results

Normal value ranges for this ELISA should be established by each laboratory based on its own patient populations in the geographical areas serviced.

The following values should be considered as a guideline:

NEGATIVE	**< 45 DU/mL**
CUT-OFF VALUE:	**50 DU/mL**
GREY ZONE (equivocal):	**45 - 55 DU/mL**
POSITIVE:	**> 55 DU/mL**

7.4 Calculation of qualitative Results

Absorbance value of ***Standard* 1 (Cut-off) = CO**

Example: 0.4 = CO

7.5 Interpretation of qualitative Results

NEGATIVE	**Mean OD patient < OD CO –10%**
GREY ZONE	**OD CO -10% ≤ Mean OD patient ≤ OD CO +10%** Repeat test 2 - 4 weeks later - with new patient samples Results in the second test again in the grey zone → NEGATIVE
POSITIVE	**Mean OD patient > OD CO +10 %**

8 QUALITY CONTROL

It is recommended to use control samples according to state and federal regulations. The use of control samples is advised to assure the day to day validity of results. Use controls at both normal and pathological levels.
It is also recommended to make use of national or international Quality Assessment programs in order to ensure the accuracy of the results.
If the results of the assay do not fit to the established acceptable ranges of control materials patient results should be considered invalid.
In this case, please check the following technical areas: Pipetting and timing devices; photometer, expiration dates of reagents, storage and incubation conditions, aspiration and washing methods.
After checking the above mentioned items without finding any error contact your distributor or DRG directly.

9 PERFORMANCE CHARACTERISTICS

9.1 Assay Dynamic Range

The range of the assay is between 0.83 - 400 DU/mL.

9.2 Specificity of Antigen (Cross Reactivity)

No cross reactivity was found for Herpes-simplex Virus 1 and 2, Varicella zoster Virus and Epstein-Barr Virus (VCA).

9.3 Analytical Sensitivity

The analytical sensitivity of the DRG ELISA was calculated by adding 2 standard deviations from the mean of 20 replicate analyses of the negative control and was found to be 0.83 DU/mL (OD_{450nm} 0.055).

9.4 Diagnostic Specificity

The diagnostic specificity is defined as the probability of the assay of scoring negative in the absence of the specific analyte. (Detected by method comparison with Virion/Serion ELISA with three lots of DRG ELISA, 85 samples, therefrom 69 negative samples are assayed.)
It is 100% (for all three DRG production lots).

9.5 Diagnostic Sensitivity

The diagnostic sensitivity is defined as the probability of the assay of scoring positive in the presence of the specific analyte. (Detected by method comparison with Virion/Serion ELISA with three lots of DRG ELISA, 85 samples, therefrom 16 positive samples are assayed.)
It is 100% (for all three DRG production lots).

9.6 Method Comparison

The DRG ELISA was compared with the Virion/Serion CMV IgM ELISA. 85 serum samples are assayed.

n = 85		Diamed Eurogen ELISA	
		pos.	**neg.**
DRG ELISA Lot 1	**pos.**	16	0
	neg.	0	69

Agreement: 100%

9.7 Reproducibility

9.7.1 Intra-assay

The intra-assay (within-run) precision of the DRG CMV IgM ELISA was determined by 20 x measurements of 12 serum samples covering the whole measuring range.

Sample	Mean Conc. (DU/mL)	Intra-Assay CV (%)	n
1	21.58	9.55	20
2	30.09	9.54	20
3	5.83	9.41	20
4	105.43	6.52	20
5	112.60	6.20	20
6	87.78	9.42	20
7	350.03	3.09	20
8	195.21	5.71	20
9	212.83	5.51	20
10	464.41	3.74	20
11	327.19	4.87	20
12	456.94	5.86	20

9.7.2 Inter-assay

The inter-assay variation of the DRG CMV IgM ELISA was determined with 3 samples with 2 production kits in 10 independent runs with 2 replicates per run.

Sample	Mean Conc. (DU/mL)	Inter-Assay CV (%)	n
1	53.41	14.58	40
2	171.41	10.78	40
3	580.32	11.87	40

9.8 Recovery

Samples have been spiked by adding 3 solutions with known concentrations in a 1:1 ratio.
The % recovery has been calculated by multiplication of the ratio of the measurements and the expected values with 100 (expected value = (endogenous value + added value) / 2; because of a 1:2 dilution of serum with spike material).

		Sample 1	Sample 2	Sample 3
Concentration [DU/mL]		45.21	27.41	8.60
Average Recovery		93.7	94.0	98.3
Range of Recovery [%]	from	88.9	93.1	83.2
	to	97.5	95.6	110.9

9.9 Linearity

Three samples (serum) containing different amounts of analyte were serially diluted with sample diluent and assayed with the DRG ELISA. The percentage recovery was calculated by comparing the expected and measured values for the analyte.

		Sample 1	Sample 2	Sample 3
Concentration [DU/mL]		536.81	454.08	357.45
Average Recovery		93.0	98.8	102.4
Range of Recovery [%]	from	85.2	93.1	94.8
	to	102.3	103.0	109.6

10 LIMITATIONS OF USE

Bacterial contamination or repeated freeze-thaw cycles of the specimen may affect the absorbance values.
In immunocompromised patients and newborns serological data only have restricted value.

10.1 Interfering Substances

Haemoglobin (up to 4 mg/mL), Bilirubin (up to 0.5 mg/mL) and Triglyceride (up to 30 mg/mL) have no influence on the assay results.

11 LEGAL ASPECTS

11.1 Reliability of Results

The test must be performed exactly as per the manufacturer's instructions for use. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable national standards and/or laws. This is especially relevant for the use of control reagents. It is important to always include, within the test procedure, a sufficient number of controls for validating the accuracy and precision of the test.
The test results are valid only if all controls are within the specified ranges and if all other test parameters are also within the given assay specifications. In case of any doubt or concern please contact DRG.

11.2 Therapeutic Consequences

Therapeutic consequences should never be based on laboratory results alone even if all test results are in agreement with the items as stated under point 11.1. Any laboratory result is only a part of the total clinical picture of a patient.
Diagnosis of an infectious disease should not be established on the basis of a single test result. A precise diagnosis should take into consideration clinical history, symptomatology as well as serological data.
Only in cases where the laboratory results are in acceptable agreement with the overall clinical picture of the patient should therapeutic consequences be derived.
The test result itself should never be the sole determinant for deriving any therapeutic consequences.

11.3 Liability

Any modification of the test kit and/or exchange or mixture of any components of different lots from one test kit to another could negatively affect the intended results and validity of the overall test. Such modification and/or exchanges invalidate any claim for replacement.
Claims submitted due to customer misinterpretation of laboratory results subject to point 11.2 are also invalid.
Regardless, in the event of any claim, the manufacturer's liability is not to exceed the value of the test kit. Any damage caused to the test kit during transportation is not subject to the liability of the manufacturer.

12 REFERENCES / LITERATURE

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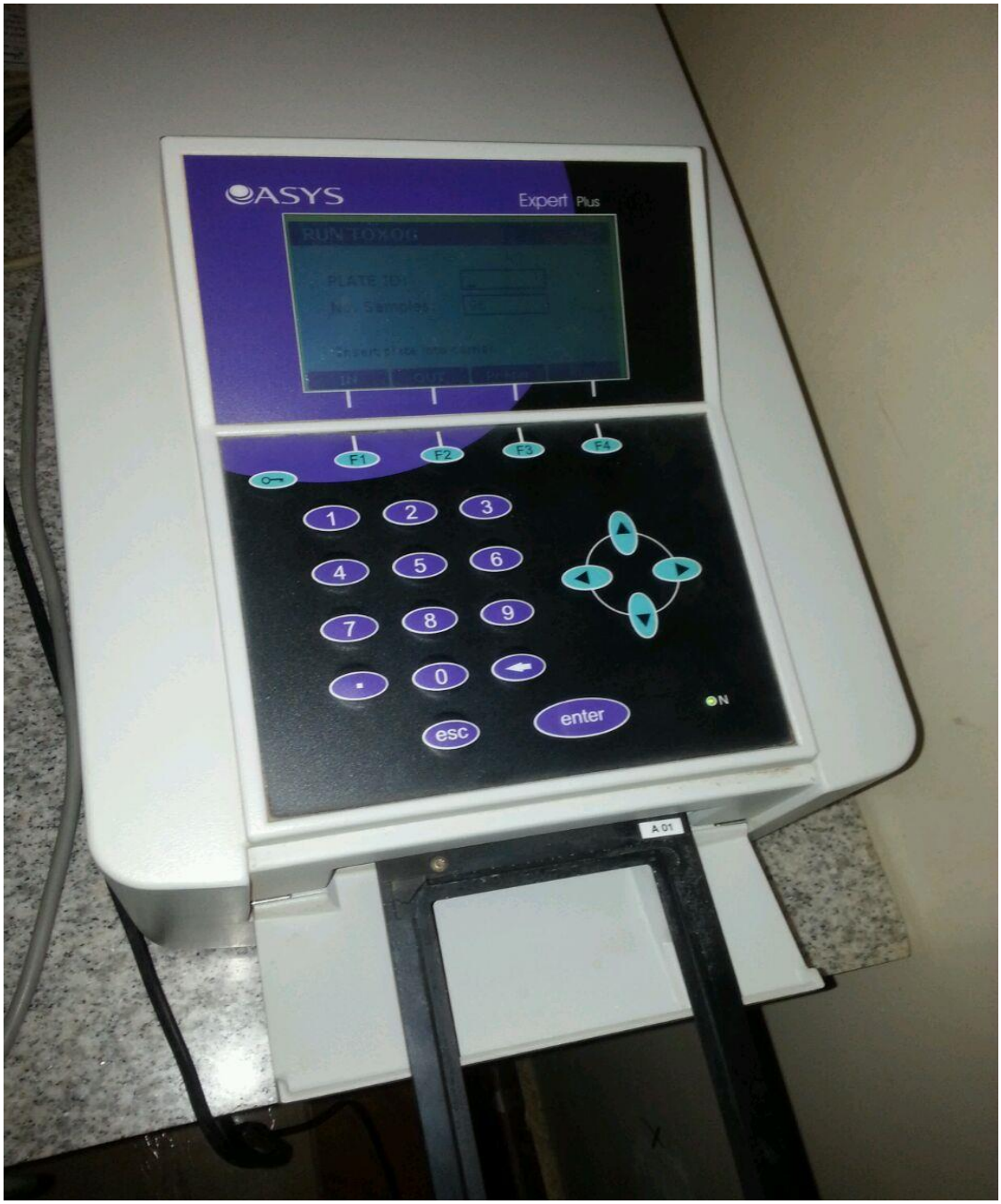
SHORT INSTRUCTIONS FOR USE

	All reagents and specimens must be allowed to come to room temperature (18-25°C) before use.
	Leave well A1 for substrate Blank. Dispense 100 µL of Standards and Control into appropriate wells.
	Dispense 100 µL of sample into selected wells. **(Please note special sample treatment, point 5.3!)**
	Cover wells with foil. Incubate for **60 minutes** at 37 °C.
	Briskly shake out the contents of the wells.
	Rinse the wells 5 times with diluted Wash Solution (300 µL per well).
	Strike the wells sharply on absorbent paper to remove residual droplets.
	Dispense 100 µL of Enzyme-Conjugate into each well.
	Incubate for **30 minutes** at room temperature.
	Briskly shake out the contents of the wells.
	Rinse the wells 5 times with diluted Wash Solution (300 µL per well).
	Strike the wells sharply on absorbent paper to remove residual droplets.
	Add 100 µL of Substrate Solution to each well.
	Incubate for **10 minutes** at room temperature.
	Stop the reaction by adding 100 µL of Stop Solution to each well.
	Determine the absorbance of each well at 450 nm.

Cytomegalovirus IgM ELISA KIT



ELISA Reader

Color plate: Test Reaction

ELISA washer machine: used for washing