Seroprevalence of Cytomegalovirus Infection among Blood Donors in Khartoum State

بسم الله الرحمن الرحيم

Sudan University of Science and Technology
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A dissertation submitted in partial fulfillment of requirements of M.Sc in medical laboratory science (Microbiology)

By

Fawaz Ahmed Mohamed Osman
B.Sc in medical laboratory science, Sudan University of Science and Technology (Microbiology), 2004

Supervisor:

Dr. Yousif Fadlalla Hamed Elnil
Approval Page

Name of Candidate: Fawaz Ahmed Mohamed El-Seyman

Thesis title: Seroprevalence of Cytomegalovirus infection among Blood Donors in Khartoum State

Approved by:
1. External Examiner
   Name: W. Alhassan Mohamed Ahmed
   Signature: [Signature]
   Date: 9-6-2014

2. Internal Examiner
   Name: Prof. Atfal El Hoda Ebrahim Muhammad
   Signature: [Signature]
   Date: 9-6-2014

3. Supervisor
   Name: Dr. Mousa Fadlalla Hamad
   Signature: [Signature]
   Date: 11-6-2014
بسم الله الرحمن الرحيم

قال تعالى:

اقرأ اسمه ربك الذي خلق (1) خلق الإنسان من علقتين (2) اقرأ وذبك الأكر (3) الذي علم بالعلم (4) علمن الإنسان ما لم يعلمه (5)

صدق الله العظيم

سورة العلق الآيات 1-5
DEDICATION

To my parents
Family
Teachers

Colleagues
ACKNOWLEDGEMENTS

First of all, my gratitude to ALMIGHTY ALLAH for helping me to complete this work properly. Thanks must be extended to my supervisor Dr. Yousif Fadlalla for this close supervision, valuable advices, and stimulating suggestions. His pleasant personality made it easy for us to do this work together.

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ABSTRACT

This study was conducted during the period from March to May 2014. The aim of this study was to determine the seroprevalence of Human Cytomegalovirus (HCMV) among blood donors attending Blood Bank department at National Public health Laboratory and Blood Bank department at Khartoum Teaching Hospital.

A total of 100 subjects (n = 100) were included in the study. The blood donors ages range from 15 to 60 and from both genders. From the study participants, serum sample were obtained and personal and clinical data were collected, sera were tested for CMV IgM and IgG antibody using Enzyme Linked Immuno Sorbent Assay (ELISA).

Out of the 100 blood donors tested, 73% and 19% were CMV IgG and CMV IgM positive, respectively, subjects were divided in two age groups (<30 years and ≥ 30 years) in order to evaluate the effect of age on CMV seropositivity from the above finding we concluded that, there was no significant difference (p > 0.05) between the two age groups on CMV IgG and IgM seropositivity.

Also we concluded that there was high percentage of human cytomegalovirus HCMV among blood donors, and there was no significant difference (p > 0.05) between both history of previous surgical operation and blood transfusion and CMV seropositivity. Since about 19% of blood donors are seropositive for CMV IgM, it would be very useful to screen blood donors for CMV to identify the CMV-seronegative blood donors, and maintain an inventory of them for use as donors, in which recent studies revealed that the virus can transmit even after using universal leucoreduced blood product.
ملخص الاطروحة

أجريت هذه الدراسة في ولاية الخرطوم في الفترة الزمنية بين مارس ومايو 2014 و كان الهدف الرئيسي الكشف عن الفيروس المضخم للخلايا عند متبرعي الدم بنك الدم في المعامل القومي الصحي والدريغو و بنك الدم في مستشفى الخرطوم التعليمي National Public health Laboratory.

شملت هذه الدراسة 100 شخصاً من متبرعي الدم، وتراوحت أعمار المشاركين في الدراسة من 15 إلى 60 سنة. وشملت الدراسة الجنسين. تم جمع عينات من المصل من المشاركين بالدراسة وتم جمع المعلومات الشخصية والطبية عن طريق الاستبيان بعد موافقتهم الشهرية ومن ثم أخذت العينات وفحصت لاحترافية احتوائها على أجسام مضادة من النمط IgM و IgG للفيروس المضخم الخلايا باستخدام اختبار الاليزا.

من مجموع 100 شخصاً من متبرعي الدم 73% كانت لديهم أجسام مضادة من النمط IgG و 19% كانت لديهم أجسام مضادة من النمط IgM. وتم تقسيم أعمار المشاركين إلى مجموعتين أقل من ثلاثين عام وأكبر من ثلاثين عام خلتل الدراسة إلى أنه لا توجد فروقات ذات داله إحصائية (P > 0.05) بين مجموعتي أعمار المشاركين في الدراسة و معدل إيجابية CMV IgM و IgG من نتائج أعلاه خلتل الدراسة إلى أن هناك نسب عالية من الفيروس المضخم لخلايا عند متبرعي الدم كما خلتل الدراسة على أنه لا توجد فروقات ذات إحصائية (P > 0.05) بين العمليات الجراحية السابقة ونقل الدم المصاحبة لهذه العمليات الجراحية ومعدلات إيجابية CMV IgG، وحيث أن معدل النتائج الإيجابية كان 19% فمن المهم جداً اختيار عينات متبرعي الدم للفيروس المضخم للخلايا وتحديد قائمة بأسماء المتبرعين وذلك للحد من انتقال الفيروس للمرضى حيث إن الدراسات الحديثة أثبتت إمكانية انتقال الفيروس بالرغم من استخدام مكونات الدم المنزوع منه معظم الكريات البيضاء.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>No.</th>
<th>Subjects</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>الآية</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Dedication</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>Acknowledgment</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Abstract, English</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>Abstract, Arabic</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>Table of contents</td>
<td>VI</td>
</tr>
<tr>
<td></td>
<td>List of tables</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>List of figures</td>
<td>XI</td>
</tr>
<tr>
<td></td>
<td>Abbreviations</td>
<td>XII</td>
</tr>
</tbody>
</table>

CHAPTER ONE: INTRODUCTION

<table>
<thead>
<tr>
<th></th>
<th>Subjects</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.1</td>
<td>Background</td>
<td>1</td>
</tr>
<tr>
<td>1.2</td>
<td>Rationale</td>
<td>2</td>
</tr>
<tr>
<td>1.3</td>
<td>Objectives</td>
<td>3</td>
</tr>
<tr>
<td>1.3.1</td>
<td>General objectives</td>
<td>3</td>
</tr>
<tr>
<td>1.3.2</td>
<td>Specific objectives</td>
<td>3</td>
</tr>
</tbody>
</table>

CHAPTER TWO: LITERATURE REVIEW

<table>
<thead>
<tr>
<th></th>
<th>Subjects</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Literature review</td>
<td>4</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>2.1</td>
<td>History</td>
<td>4</td>
</tr>
<tr>
<td>2.2</td>
<td>Classification</td>
<td>4</td>
</tr>
<tr>
<td>2.3</td>
<td>Structure of the virus</td>
<td>4</td>
</tr>
<tr>
<td>2.4</td>
<td>Properties of CMV</td>
<td>6</td>
</tr>
<tr>
<td>2.5</td>
<td>Replication of CMV</td>
<td>7</td>
</tr>
<tr>
<td>2.6</td>
<td>Transmission</td>
<td>7</td>
</tr>
<tr>
<td>2.6.1</td>
<td>Blood donation</td>
<td>7</td>
</tr>
<tr>
<td>2.6.2</td>
<td>Neonatal transfusion</td>
<td>8</td>
</tr>
<tr>
<td>2.6.3</td>
<td>Leucoreduction transfusion</td>
<td>9</td>
</tr>
<tr>
<td>2.7</td>
<td>Epidemiology</td>
<td>9</td>
</tr>
<tr>
<td>2.8</td>
<td>Pathogenesis</td>
<td>10</td>
</tr>
<tr>
<td>2.8.1</td>
<td>Infection in immunocompetent hosts</td>
<td>10</td>
</tr>
<tr>
<td>2.8.2</td>
<td>Infection in immunocompromised hosts</td>
<td>11</td>
</tr>
<tr>
<td>2.8.3</td>
<td>Congenital and neonatal infection</td>
<td>11</td>
</tr>
<tr>
<td>2.8.4</td>
<td>Infection in transfusion recipient</td>
<td>12</td>
</tr>
<tr>
<td>2.8.5</td>
<td>Complication of TT- CMV</td>
<td>12</td>
</tr>
<tr>
<td>2.8.6</td>
<td>Post transfusion and organ transplant</td>
<td>13</td>
</tr>
<tr>
<td>2.8.7</td>
<td>Post transplant</td>
<td>13</td>
</tr>
<tr>
<td>2.8.8</td>
<td>CMV and malignant disease</td>
<td>14</td>
</tr>
<tr>
<td>2.9</td>
<td>Immunity</td>
<td>14</td>
</tr>
<tr>
<td>2.10</td>
<td>Laboratory diagnosis</td>
<td>15</td>
</tr>
<tr>
<td>2.11</td>
<td>Collection of specimens</td>
<td>15</td>
</tr>
</tbody>
</table>
2.12 Cell culture 15
2.12.1 Shell vial assay 16
2.12.2 Cytopathology 16
2.13 Serology 18
2.14 Polymerase chain reaction 19
2.15 Treatment 19
2.16 Prevention and control 20

CHAPTER THREE: MATERIALS & METHODS

3 Study design 21
3.1 Study area and population 21
3.2 Study duration 21
3.3 Sample collection 21
3.4 Sampling technique 21
3.5 Data collection 22
3.6 Ethical consideration 22
3.7 Laboratory work 22
3.8 ELISA for detection of CMV IgG 22
3.8.1 Principle 22
3.8.1.1 Procedure 23
3.8.1.2 Calculation of control value and cut-off 23
3.8.1.3 Interpretation of the result 24
3.8.1.4 ELISA for detection of CMV IgM 24
### 3.8.2 Principle

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.8.2.1 Procedure</td>
<td>25</td>
</tr>
<tr>
<td>3.8.2.2 Calculation of the control value</td>
<td>25</td>
</tr>
<tr>
<td>3.8.2.3 Interpretation of the result</td>
<td>26</td>
</tr>
<tr>
<td>3.8.2.4 Data analysis</td>
<td>26</td>
</tr>
</tbody>
</table>

### CHAPTER FOUR : RESULTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Detection of CMV IgM positive subjects among the blood donors</td>
<td>27</td>
</tr>
<tr>
<td>4.2</td>
<td>The effect of age on CMV IgG and IgM seropositivity among blood donors</td>
<td>28</td>
</tr>
<tr>
<td>4.3</td>
<td>The effect of surgical operation on CMV IgG and IgM seropositivity among blood donors</td>
<td>28</td>
</tr>
</tbody>
</table>

### CHAPTER FIVE: DISCUSSION

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1 Conclusion</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>5.2 Recommendation</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Tables No.</td>
<td>Legend</td>
<td>Page No.</td>
</tr>
<tr>
<td>------------</td>
<td>------------------------------------------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>4.1</td>
<td>The effect of age on CMV IgG and IgM seropositivity among the blood donors.</td>
<td>30</td>
</tr>
<tr>
<td>4.2</td>
<td>The effect of previous surgical operation and blood transfusion CMV IgG and IgM seropositivity</td>
<td>31</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Legend</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>CMV Virion structure</td>
<td>5</td>
</tr>
<tr>
<td>2.2</td>
<td>Micrograph of placental infection</td>
<td>12</td>
</tr>
<tr>
<td>2.3</td>
<td>CMV Cytopathic effect</td>
<td>17</td>
</tr>
<tr>
<td>2.4</td>
<td>Characteristic intranuclear Owls eye appearance within kidney-bean shape like nucleus</td>
<td>17</td>
</tr>
<tr>
<td>4.1</td>
<td>ELISA micro titter plate</td>
<td>27</td>
</tr>
<tr>
<td>4.2</td>
<td>Detection of CMV IgG positive subjects among blood donors</td>
<td>29</td>
</tr>
<tr>
<td>4.3</td>
<td>Detection of CMV IgM positive subjects among blood donors</td>
<td>29</td>
</tr>
<tr>
<td>4.4</td>
<td>The Effect of previous surgical operation and blood transfusion on CMV IgM seropositivity.</td>
<td>32</td>
</tr>
<tr>
<td>4.5</td>
<td>The Effect of previous surgical operation and</td>
<td>32</td>
</tr>
</tbody>
</table>
blood transfusion on CMV IgG seropositivity.

ABBREVIATIONS

AIDS  Acquired immunodeficiency syndrome
CMV  Cytomegalovirus
COV  Cut - off - value
CPE  Cytopathic effect
EIA  Enzyme Immunoassay
ELISA  Enzyme Linked Immunosorbent Assay
HIV  Human Immunodeficiency virus
PCR  Polymerase Chain Reaction
pp65  Phosphoprotein 65
SCT  Stem cell transplantation
SOT  Solid – organ transplantation
SPSS  Statistical Package of Social Science
TT-CMV  Transfusion Transmitted cytomegalovirus
TLRs  Toll-like receptors
CHAPTER ONE
1. Introduction

1.1. Background

Transfusion Transmitted Disease (TTD) is a major challenge to the transfusion services all over the world. The presence of viruses in blood cells or plasma of asymptomatic donors is the major risk factor for transmitting infectious agents through blood transfusion. Other transfusion transmitted agents include bacteria, mycoplasma, and even protozoa. The main viruses associated with transfusion related infections are hepatitis viruses, retroviruses, and cytomegalovirus (CMV). The risk of transmitting hepatitis viruses, human retroviruses, such as human immunodeficiency virus (HIV) and human T-lymphotropic viruses types I and II has been reduced drastically by screening for the corresponding antibodies before transfusion (Preiksaitis, 2003).

However, CMV, a member of the human herpes family viruses, transmission is through blood component, transfusions is an important cause of concern world-wide. This is because the majority of adults have serological evidence of previous infections by these viruses. CMV is an ubiquitous agent, and seropositivity rates in the adult population over 40 years of age worldwide are 60 to 100%, possibly due to transmission through breastfeeding, sexual contact and spread from children (Schmultz et al; 2005). Like most other herpesvirus, they remain latent in the host after primary infection and persist for lifelong in the organism. Nevertheless, these viruses can be reactivated in immunosuppressed individuals leading to critical outcomes such as interstitial pneumonitis, hepatitis, retinitis, and encephalitis. Active infection results in serious morbidity and mortality, and many consider CMV to be one of the most serious pathogens affecting immunosuppressed
individuals and an important contribution to intrauterine infection. In one study, the relative risk of CMV disease transmission via blood components from seropositive donors was 0.4% to 12%. Transfusion-transmitted CMV (TT-CMV) is a significant cause of morbidity and mortality in immunosuppressed patients (Schmaltz et al.; 2005).

The widespread prevalence of CMV seropositivity makes it difficult for some blood banks to maintain sufficient CMV seronegative blood components for patients at risk for CMV disease. Therefore, the most effective way to minimize the risk of CMV transmission in high-risk recipients would be to administer CMV seronegative blood products or leucoreduced blood products. Otherwise, a way out would be to determine the frequency of primary CMV infection among seronegative blood donors (High, 2005).

1.2. Rationale

CMV diseases predominantly occur as an opportunistic infection in patients with severe immunosuppression, such as cancer and renal failure patients, and rarely occur in immunocompetent patients. Bloodtransfusion is major cause of concern world wide because of high prevalence of Cytomegalovirus infection among adult in Ghana and Sudan. A study was conducted in military hospital in Accra, Ghana, (Hecker et al.; 2004). The seroprevalence of antibodies specific for CMV was tested using CMV IgG/IgM particle agglutination test kit and ELISA, of the 264 blood donors, 18 were negative and 246 were positive for CMV IgG antibodies, giving an overall CMV prevalence rate of 93.2%. None of the 264 blood donors were positive for IgM (Kuhn, 2006),
1.3. Objectives

1.3.1 General Objective

To investigate the seroprevalence of Cytomegalovirus among blood donors attending Khartoum Teaching Hospital and National public Health Laboratory blood bank department.

1.3.2 Specific Objectives

1- To detect CMV IgG and IgM antibodies among blood donors at Khartoum teaching hospital.
2- To study the effect on CMV IgG and IgM seropositivity.
3- To study the effect of history of previous surgical operation and blood transfusion on CMV IgG and IgM seropositivity.
CHAPTER TWO
2. LITERATURE REVIEW

2.1. History
CMV was first noticed by Ribber in 1881, when he saw large "protozoan-like" cells in the kidney of still born infant, the first report of the visualization of CMV by electron microscopy appeared in 1953, when cytomegalic inclusion cells from an infant pancreas were viewed and particles were observed in both the cytoplasm and the clear halo around the inclusions. The virus was cultured for the first time in 1956 by Rowe, Smith and Weller, all of whom worked independently (Bhatia and Ichhpujani, 2008).

2.2. Classification
Cytomegalovirus formerly designated as human herpesvirus 5 (HHV-5) by the International Committee on Taxonomy of Viruses, is a member of the family Herpesviridae, and it is classified in the subfamily Betaherpesvirinae. Human CMV (HCMV) is the type species of the genus cytomegalovirus, and its name is derived from the enlargement of cells (cyto=cell, mega= large) infected by the virus. Its classification as a herpesvirus is based on its tendency to infect mononuclear cells and lymphocytes - and on its molecular phylogenetic relationship to other herpesviruses. Its classification as a betaherpesvirus is based on its long replication cycle, cytopathology, and restricted host range, which are all characteristic of the betaherpesviruses, (Todd and Wills, 2006).

2.3. Structure of the virus
Compared to other human herpesvirus, HCMV is the largest, with a genome of 235 kb encoding 165 gene (Davison et al; 2003), the virion consists of double stranded linear DNA core in an icosahedral nucleocapsid,
enveloped by a proteinaceous matrix (the tegument). These components are enclosed in a lipid bilayer envelope that contains a number of viral glycoproteins, these include glycoprotein B (gB), gH, gL, gM, gN and gO. Mature virions range in diameter from 200 to 300 nanometer (Crough and Khanna, 2009) (Fig. 2.1).

The tegument compartment contains the majority of the virion proteins, with the most abundant tegument protein being the lower matrix phosphoprotein 65 (pp 65), also termed Unique Longe 83(UL83), the function of the tegument proteins can be separated into two classes: (i) proteins that play a structural role and are important for the assembly of the virions and the disassembly of the particle during entry and (ii) proteins which modulate the host cell response to infection (Moncarski et al.; 2007).

Figure 2.1. CMV virion structure

2.4. Properties of CMV
Cytomegalovirus has the largest genetic content of the human herpesvirus. It’s DNA genome (240 kbp) is significantly larger than that of herpes simplex virus. Only a few of many proteins encoded by the virus (over 200) have been characterized. One, a cell surface glycoprotein, act as Fc receptors that can non specifically bind the Fc portion of immunoglobulins. This may help infected cells evade immune elimination by providing a protective coating of irrelevant host immunoglobulins. The major immediate early promoter - enhancer of CMV is one of the strongest known enhancers, due to the concentration of binding sites for cellular transcription factors. It is used experimentally to support high - level expression of foreign genes.

Many genetically different strains of CMV are circulating in the human population. The strains are sufficiently related antigenically, however, so that strain differences are probably not important determinants in human disease (Brooks et al; 2010).

Cytomegalovirus is very species - specific and cell type-specific. All attempts to infect animals with HCMV have failed. A number of animal CMV exists, all of them species - specific. HCMV replicates in vitro only in human fibroblasts, although the virus is often isolated from epithelial cells of the host. CMV replicate very slowly in cultured cells, with growth proceeding more slowly than that of HSV or varicella zoster virus. Very little virus becomes cell-free, infection spreads primarily cell - to - cell. It may take several weeks for an entire monolayer of cultured cells to become involved (Brooks et al; 2010).

CMV produces a characteristic cytopathic effect, perinuclear cytoplasmic inclusion form in addition to the intranuclear inclusion typical of herpesviruses, multinucleated cells are seen, many affected cell become
greatly enlarged, inclusion-bearing cytomegalic cells can be found in samples from infected individuals (Brook et al., 2010).

### 2.5. Replication of CMV

Cytomegalovirus attaches to cell surface at the site of the receptor for fibroblast growth factor. After entry into the cell, the virion is uncoated and the genome DNA enter the nucleus. Within the nucleus, the incoming genome DNA changes its configuration from linear to circular. Early virus messenger RNA (mRNA) is transcribed by host cell RNA polymerase and then translated into early, non structural proteins in the cytoplasm. The viral DNA polymerase replicates the genome DNA, and late protein synthesis begins. Then the virion assembly occur in the nucleus. The virion obtain its envelope by budding through the nuclear membrane and exits the cell via tubules or vacuoles that communicate with the exterior (Levinson, 2010).

### 2.6. Transmission

HCMV can be transmitted via saliva, sexual contact, placental transfer, breast feeding, blood transfusion, solid organ transplantation (SOT), or hematopoietic stem cell transplantation (SCT) (Sia and Patel, 2000). Day 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 42 months (Nyholm and Schleiss, 2010), CMV transmission in infant breast fed by seropositive women shedding virus in their breast milk has been reported to be 58% to 69% (Nyholm and Schleiss, 2010).
2.6.1. Blood donation

Blood transfusions typically use sources of blood: one's own (autologous transfusion), or someone else's (allogeneic or homologous transfusion). The latter is much more common than the former. Using another's blood must first start with donation of blood. Blood is most commonly donated as whole blood intravenously and collecting it with an anticoagulant. In developed countries, donations are usually anonymous to the recipient, but products in a blood bank are always individually traceable through the whole cycle of donation, testing, separation into components, storage, and administration to the recipient. This enables management and investigation of any suspected transfusion related disease transmission or transfusion reaction. In developing countries the donor is sometimes specifically recruited by or for the recipient, typically a family member, and the donation occurs immediately before the transfusion.

All donated blood is tested for infections. The current protocol tests donated blood for HIV-1, HIV-2, HTLV-2, Hepatitis B, Hepatitis C, Syphilis (Treponema pallidum), Chagas disease (Trypanosoma cruzi), and West Nile Virus. In addition, platelet products are also tested for bacterial infections due to its higher inclination for contamination due to storage at room temperature. Presence of Cytomegalovirus (CMV) is also tested because of risk to certain immunocompromised recipients if given, such as those with organ transplant or HIV. However, not all blood is tested for CMV because only a certain amount of CMV-negative blood needs to be available to supply patient needs. Other than positivity for CMV, any products tested positive for infections are not used, (Wales, et al; 2001).
2.6.2. Neonatal Transfusion
Cytomegalovirus is a special case in donor testing in that many donors will test positive for it. The virus is not a hazard to a healthy recipient, but it can harm infant and other recipients with weak immune systems. The safety of donating blood during pregnancy has not been studied thoroughly, and pregnant women are usually deferred, (Klein et al ; 2010).

2.6.3. Leucoreduction Transfusion
Leucocytes are not often used because of the difficulties in obtaining sufficient quantities and because of their short life span in vivo. Donor antibody screening is clearly effective in reducing CMV infection. The use of leukocyte filtered blood products is an adjunct when seronegative products are not available. In addition to donor screening, leukocyte filtration is best considered as a transfusion management practice; not as a replacement for donor screening, (Squizzato, 2007).

2.7. Epidemiology
HCMV is highly species-specific, with humans being the only host. Furthermore CMV has been found in every human population tested. The prevalence of infection is greater in developing countries and among lower socioeconomic groups of developed countries. Overall, the seroprevalence of infection varies between 65 % to 90 % among middle age adult in the USA, where primary CMV infection during pregnancy occurs in 2% of women of child bearing age who are of lower socioeconomic background (Nassetta et al; 2009 ).

Crowded living conditions, poor sanitation, sexual practices and increased exposure to infant and children all contribute to increasing rates of infection
and a higher seroprevalence. Virus can be isolated from urine, saliva, cervical and vaginal secretions, semen, breast milk, tears, blood products and transplantated organs (Bowden, 1991). The risk factor for maternal acquisition of CMV during pregnancy is frequent and prolonged contact with a child less than three years of age, this occurs among women employed in child care centers (Adler, 2011).

2.8. Pathogenesis
Cytomegalovirus is an enveloped double stranded DNA virus. On this envelope, there are molecules called glycoproteins. These molecules, especially glycoprotein B are determinants of humoral immunity, that they can cause a humoral immune response. Proteins in the viral tegument, the proteinaceous layer between the capsid and the envelope, elicit cellular responses to CMV. Specifically the abundant molecule phosphoprotein 65 is the molecule targeted by cytotoxic T cells in the cellular response. Although the host has the ability to defend itself against a CMV invasion, CMV has the ability to evade the host immunity, and in this way, it can cause infection in the host. CMV has genes that interfere with the antigen presentation of infected cells, thus limiting the immune response to it. Therefore, it can remain alive for a long time in a host, (Riley, 1997).

2.8.1. Infection in immunocompetent hosts
There are three manifestations of CMV disease in humans. The first is acute acquired CMV. This is when a healthy person becomes infected with CMV. Often the person will present no symptoms. If symptoms are present, they are similar to those of mononucleosis, and include fever, fatigue, and muscle
pain. These symptoms can last up to three weeks. Other clinical abnormalities have been associated with CMV infection in normal hosts, including Gullian-barre syndrome, meningoencephalitis, hemolytic anemia, and thrombocytopenia, after this initial infection, the virus has the ability to remain dormant in the host (Moncarski et al; 2007).

2.8.2. Infection in immunocompromised patient
The second type of CMV disease occurs after this initial infection, and is a reactivation of the latent CMV, in which CMV is a serious opportunistic infection in immunocompromised individual. If a person becomes immunocompromised, for example someone with HIV, someone who received an organ or bone marrow transplant, or even someone under a lot of stress, the virus can flare up again. If this occurs, the symptoms can be more serious and include pneumonia, hepatitis which may cause fulminant liver failure, encephalitis, myelitis, colitis, retinitis (inflammation of the retina), and neuropathy.

2.8.3. Congenital and neonatal infection
The third type of CMV disease is congenital or perinatal CMV. This is when a woman who is infected with CMV (even if she doesn't have any symptoms) passes it to her child. In congenital CMV, a pregnant woman passes the disease through the placenta to the fetus. In perinatal CMV, a mother passes CMV to her child through nursing. Severity ranges from a simple fever of unknown origin, to enlarged liver and/or spleen, and even motor/mental retardation. The spread of the virus is not associated with food or water. It is generally spread through close contact with bodily fluids of an infected person. When the host is infected, CMV DNA can be detected with
polymerase chain reaction (PCR) in all the different cell lineages and organ systems in the body. Upon initial infection, CMV infects the epithelial cells of the salivary gland, resulting in a persistent infection and viral shedding (Todd and Wills, 2006).

2.8.4. Infection in transfusion recipient

The problem of transfusion-transmitted cytomegalovirus (CMV) infection differs from that for other transfusion-transmitted infections in that only patients who are immunocompromised require CMV-free blood or components, (TT-CMV) is a significant cause of morbidity and mortality in immune-suppressed patients, including premature low-birth weigh infants.
(<150g) born to CMV-seronegative mothers and HIV-AIDS patients (Albrecht and Rapp, 1973).

2.8.5. Complication of TT – CMV
The complication of TT- CMV of fever is the syndrome, with a typical lymphocyte was initially observed in patient who had undergone cardiac surgery using pump oxygenators, and the fresh blood used was suspected as the source of virus, the volume of blood transfused, the age of the blood, the antibody status of the patient and the mechanical damage to cells by the pump all may contribute to the possibility of the patient showing a CMV antibody response, but the relative importance of each factor is difficult to define, Cytomegalovirus (CMV) infection complicating heart surgery in connection with extracorporeal circulation, transfusion of fresh blood and medication of corticosteroids, the clinical syndrome was different from that reported in a small number of cases with post-transfusion CMV complications. A longstanding fever with involvement in the infectious process of lungs, also possibly heart, liver, and spleen, dominated the clinical picture, the use of CMV-seronegative blood products has been the ‘gold standard’ method of preventing TT- CMV infection, (Albrecht and Rapp, 1973).

2.8.6. Post transfusion and Organ Transplant
The pathogenesis of CMV infection following transfusion or organ transplantation is not clear. The major questions are whether this represents a primary or a reactivated infection, and if the latter, if the source of the virus is the recipient, the blood of the blood donor, there is direct association
between the number of units of blood transfused and CMV antibody conversion, (Alexander, 1967).

2.8.7. Post transplant
Cytomegalovirus infection following organ transplantation may be associated with a variety of clinical feature or may be completely a symptomatic. The source of the virus is unknown, it may be introduced with blood or in the donor organ or be endogenous in origin. there is some suggestion that the patient who acquires the primary CMV infection following allograft reception and immunosuppressive therapy has viremia and signs of illness, (Armstrong et al; 1971).

2.8.8. CMV and Malignant Disease
The patient with malignant disease such as lymphoma or leukemia or hodgkins disease may have a sever or protracted illness with CMV infection, and they may suffer from more episode of pneumonitis or fever with rash but not more episode of hepatitis, and there may be other possible syndrome such as encephalitis or ulcerative colitis, (Baron et al; 1969).

2.9. Immunity
The innate immune system plays an important role in defence against HCMV and also in priming the adaptive immune response. It is becoming increasingly apparent that HCMV is subject to innate sensing by toll – like receptor (TLRs). The stimulation of TLRs by pathogens such as HCMV activates signal transduction pathways, which induce the secretion of inflammatory cytokines that recruit cells of the innate immune system, and the upregulation of costimulatory molecules such as CD80 and CD86.
which are important for activation of adaptive immunity (Boehme and Compton, 2004).

The establishment of long-lasting immunity in response to a primary HCMV infection, which serves to control subsequent HCMV reactivation in the host, is important for preventing uncontrolled replication and serious HCMV disease.

HCMV is a potent immunogen that triggers strong immune response from all arms of the immune system, while the contribution of antibodies for protection against and control of HCMV has been debated, evidence does support a role for humoral immunity in the effective immune response against HCMV, predominantly in restricting viral dissemination and in limiting the severity of the disease (Boppana and Britt, 1995). The cell-mediated immune response is the predominant mechanism by which HCMV replication is controlled, as with the exception of congenital infection, severe HCMV disease occurs almost exclusively in patients with profound cellular immunodeficiency (Crough and Khanna, 2009).

2.10. Laboratory diagnosis

Laboratory diagnosis of CMV infection depends on (i) detecting CMV cytopathology, antigen, or DNA in infected tissues (ii) isolating the virus from tissue or secretions (iii) demonstrating seroconversion (Drew, 2004)

2.10.1. Collection of specimen

Most useful specimens for CMV isolation are throat washing and urine. The virus is shed in urine for months after clinical features have subsided. CMV can also be isolated from saliva, breast milk, cervical secretions, blood, and semen as well as various biopsy materials (Bhatia and Ichhpujani, 2008).
All specimen should be sent to the laboratory without delay, if delay of more than a few hours is anticipated, then all sample should be sent refrigerated or on wet ice, but under no circumstances should any specimen be frozen at any temperature (Griffiths, 2004).

2.10.2. Cell Culture

Viral culture of the urine and saliva obtained within the first and two weeks of life continues to be the gold standard for diagnosis of congenitally-infected infants (Ross et al.; 2011), the virus can be recovered most readily from throat washings and urine (Brooks et al.; 2010). All cultures should be observed at least twice weekly for the typical focal CPE of CMV (Griffiths, 2004). In culture, 2 to 3 weeks are usually needed for the appearance of cytologic changes, consisting of small foci of swollen, translucent cells with large intranuclear inclusion (Figure 2.3), the virus stays cell-associated (Brooks et al., 2010). Immunofluorescence test or immunoperoxidase staining using specific antisera can be employed to confirm the results of CPE (Bhatia and Ichhupujani, 2008). Cell culture methods of viral isolation are too slow to be useful in guiding therapy, particularly in immunocompromised patients (Brooks et al.; 2010).

2.10.3. Shell vial assay

The shell vial assay is performed by adding the clinical specimen to a vial that contains a permissive cell line for CMV. The shell vials are centrifuged at low speed and placed in an incubator, after 24-48 hours, the tissue culture medium is removed and the cells are stained using a fluorescein-labeled anti CMV antibody.
The cell are observed using a fluorescent microscope, alternatively, the cells are stained with an antibody against CMV, followed by a fluorescein-labeled anti-immune globulin. This test has been found to be as sensitive as traditional tissue culture, probably because of the enhancement of the infectivity provided by configuration (Jahan, 2010).

2.10.4. Cytopathology

Cytologic technique may be applied in an attempt to find characteristics of intranuclear inclusions in specimens; the microscopic hallmark of CMV infection is the large (25-35µm) cell containing a large basophilic intranuclear inclusion referred to as Owls eye, because it is separated from the nuclear membrane by a halo, these inclusion are seen well with papanicolaou or hematoxylin-eosin stains (Jahan, 2010) (Figure 2.4)
Figure 2.3. CMV Cytopathic effect (intranuclear inclusion bodies in tissue stained with H&E stain).

Figure 2.4. characteristic intranuclear Owls eye appearance within kidney-bean shape like nucleus.
2.10.5. Serology

Enzyme immunoassay (EIA) in which multiple specimens can be handled daily at a relatively low cost. Kits that detect CMV IgG are available from a number of commercial sources. The kits are easy to use, and the manufactures have provided detailed instructions (Hodinka, 2007). The development of robotics technology has led to the commercial availability of both fully automated and semi-automated EIA instruments, including sample dispensers, diluters, washers, and spectrophotometers with complete computer programming and generation of written reports (Hodinka, 2007).

Many types of assays can detect CMV IgG antibodies, indicative of past infection (and the potential to undergo reactivation). Detection of viral IgM antibodies suggest a current infection (Brooks et al; 2010). Antibody avidity, which is an indirect measure of the tightness of antibody binding to its target antigen, increases in the first weeks after a primary infection, low avidity IgG antibodies to CMV persist for up to 20 weeks after primary CMV infection, these low avidity antibodies are then replaced by high avidity antibodies. Currently, the combination of the presence of CMV IgM antibodies and low avidity CMV IgG antibodies along with maternal or fatal symptoms are used for the diagnosis of a primary maternal infection, (Alder, 2011).

Complement fixation tests are adequate for showing seroconversion after primary infection in competent hosts. To screen for seropositive status a more sensitive assay such as enzyme immune-assay for CMV IgG or total antibody or latex agglutination assay, is appropriate. these test can be done urgently for donor-recipient assessment (Ogilvie, 2007). CMV IgM is found after primary or secondary infection, but it may not be possible to detect IgM in the neonate or immunocompromised patient, serologic assay are thus
not informative for immunocompromised patients. Furthermore, serologic techniques cannot distinguish strain difference among clinical isolates (Ogilvie, 2007).

2.10.6. Polymerase Chain Reaction
PCR assay have replaced virus isolation for routine detection of CMV infections. The PCR assays are designed to detect replicating virus, not latent viral genomes. Blood and urine are most commonly tested, PCR assays can provide viral load data, which appears to be important in predicting CMV disease (Brooks et al., 2010).

Diagnosis of acute maternal CMV infection by the presence of IgM and low avidity IgG require confirmation of fetal infection, which is typically performed using of PCR assays for CMV on amniotic fluid. PCR assays of dried blood spots from newborn have been shown to lack sufficient sensitivity for the identification of most neonate with congenital CMV infection for universal screening purpose. However, saliva PCR assays are currently being assessed as a useful screening method for congenital CMV infection.

2.11. Treatment
Cytomegalovirus Immune Globulin Intravenous (Human) (CMV-IGIV) is an immunoglobulin G (IgG) containing a standardized amount of antibody to Cytomegalovirus (CMV). It may be used for the prophylaxis of cytomegalovirus disease associated with transplantation of kidney, lung, liver, pancreas, and heart, alone or in combination with an antiviral agent. It has been shown to: (i) reduce the risk of CMV-related disease and death in some of the highest-risk transplant patients, (ii) provide a measurable long-
term survival benefit (iii) produce minimal treatment-related side effects and adverse events.

Ganciclovir (Cytovene) treatment is used for patients with depressed immunity who have either sight-related or life-threatening illnesses. Valganciclovir (Valcyte) is an antiviral drug that is also effective and is given orally. The therapeutic effectiveness is frequently compromised by the emergence of drug-resistant virus isolates. A variety of amino acid changes in the UL97 protein kinase and the viral DNA polymerase have been reported to cause drug resistance. Foscarnet or cidofovir are only given to patients with CMV resistant to ganciclovir, because foscarnet has notable nephrotoxicity, resulting in increased or decreased Ca\(^{2+}\) or P, and decreased Mg\(^{2+}\), (Gilbert and Boivin, 2005).

2.12. Prevention and control

Some preventive action is undertaken by way of screening organ donors and recipients to avoid, where possible, a seronegative recipient from receiving an organ from a seropositive donor, this has been shown to reduce morbidity and mortality significantly in all forms of allogeneic transplant, blood donor screening to select seronegative units for support of seronegative patients in transplant programmes is important but not always available. Seriously compromised donors must receive screened blood from CMV seronegative donors or as routin now in the UK leucocyte - depleted blood. However, a more recent study has demonstrated that TT-CMV does occur even after leucoreduction, that CMV-seronegative blood products may thus be superior to leucoreduced blood product (Cohen, 2000).
CHAPTER THREE
3. MATERIALS AND METHODS

3.1. Study design
Descriptive cross sectional study conducted at Khartoum Teaching Hospital and blood bank of National Public Health Laboratory.

3.2. Study area
Blood donors living in Khartoum attending blood bank department at Khartoum Teaching Hospital and National Public Health Laboratory.

3.3. Study duration
The study was carried out during the period from March to May, (2014).

3.4. Sample collection
A total of one hundred blood specimens (n = 100) were collected from blood donors, and investigated for CMV – specific immunoglobulin IgM and IgG. The age of all blood donors tested ranged from 15 and 60 years. The blood samples were collected under aseptic conditions, allowed to clot, centrifuged at 3000 rpm for 5 minutes, and sera were collected in sterile containers and stored at -80 °C until tested.

3.5. Sampling Technique
The study was based on non - probability convenience sampling technique during attendance of blood donors to Khartoum Teaching Hospital and National Public Health Laboratory blood bank department.
3.6. Data collection

Personal and clinical data were collected by direct interviewing questionnaire from each subject (Appendix).

3.7. Ethical consideration

Permission to carry out the study was obtained from the College of Graduate Studies, Sudan University of Science & Technology. All blood donors examined were informed for the purpose of the study before collection of the samples and verbal consent was taken from them.

3.8. Laboratory work

The enzyme linked immunosorbent assay (ELISA) was used to detect the specific HCMV IgG and IgM antibodies.

3.8.1. ELISA for detection of CMV IgG antibodies

3.8.1.1. Principle

The HUMAN CMV IgG ELISA is based on the classical ELISA technique. The microtiter strip wells as solid phase are coated with cell culture derived CMV antigens (CMV Ag). In the first incubation step corresponding specific antibodies (CMV-IgG-Ab) present in patient specimens or control bind to the antigens at the solid phase. At the end of the incubation unbound components are washed out.

For the second incubation step anti IgG conjugate (anti - human IgG antibodies, peroxidase conjugate) is added which binds specifically to IgG antibodies resulting in the formation of typical immunocomplexes. After a second washing step to remove unbound conjugate, TMB/Substrate is added
(step 3). A blue color develops changing to yellow after stopping the reaction. The intensity of the color is directly proportional to the CMV IgG Ab concentration in the specimen. The absorbance of controls and specimens are determined by using ELISA microplate reader (humaReader). Results for patient samples are obtained by comparison with cut–off value

3.8.1.2. Procedure
Commercial ELISA Kits (HUMAN Diagnostic Co.Ltd.Germany) were used as described by the manufacturers. All reagents and samples were brought to room temperature before beginning the procedure. the serum sample was diluted (1:100) with dilution buffer IgG (DIL-G) and washing solution diluted (1:20) with fresh deionised water, other reagents were ready for use. Briefly, 100 µl of the negative control (NC), 100µl of the positive control (PC), both were in duplicate form, and 100 µl of diluted samples were incubated in microtiter strip wells coated with CMV antigen at room temperature for 30 minutes. the wells were washed 4 times by washing solution using automatic washer to remove unbound components. Then 100 µl of anti-IgG conjugate were added to each well and incubated at room temperature for 30 minutes. After another washing step (5 times) to remove excess conjugate, an enzyme substrate reagent (TMB Substrate) was added (100 µl / well) and the plate was incubate for 15 minutes. The blue color changed to yellow after adding the stop solution (sulphuric acid - 100 µl). The optical density (OD) in a microplate reader was read within 30 minutes at 450 nm.

3.8.1.3. Calculation of control values and cut-off
Mean absorbance values of NC (MNC) and mean absorbance value of PC (MPC) were calculated. The cut-off value was then calculated following this equation:
Cut-off value COV = MNC + (0.2\times MPC)

The test run was validated according to the manufacturer's criteria for validity as below:

1- Ab substrate blank < 0.150
2- MNC < 0.250
3- MPC > 0.750

3.8.1.4. Interpretation of the results

Samples with the absorbance > COV were considered as anti-CMV-IgG-Ab positive, while sample with absorbance < COV were considered as anti-CMV-IgG-Ab-negative.

3.8.2. ELISA for detection of CMV IgM antibodies

3.8.2.1. Principle

The HUMAN CMV IgM ELISA is based on the classical ELISA technique. The microtiter strip wells as solid phase were coated with cell culture derived CMV antigens (CMV Ag). In the first incubation step corresponding specific antibodies (CMV-IgM-Ab) present in patient specimens or controls bind to the antigens at the solid phase, the sample dilution buffer contains anti human IgG to prevent Rheumatoid Factor (RF) interference and competition for specific IgG present in the specimen.

At the end of the incubation, unbound components are washed out. for the second incubation step anti IgM conjugate (anti-human IgM antibodies, peroxidase conjugate) is added which binds specifically to IgG antibodies resulting in the formation of typical immunocomplexes after a second washing step to remove excess conjugate, TMB/Substrate is added (step 3). A blue color develops changing to yellow after stopping the reaction.
intensity of the color is directly proportional to the CMV –IgM-Ab concentration in the specimen.

The absorbance of control and specimen is determined by using ELISA microplate reader (humanReader). Result for patient samples are obtained by comparison with cut-off value.

### 3.8.2.2. Procedure

Commercial ELISA Kits (HUMAN Diagnostic Co.Ltd.Germany) were used as described by the manufacturers. All reagents and samples were brought to room temperature before beginning the procedure. The serum sample was diluted (1:100) with dilution buffer IgM (DIL-M) and washing solution diluted (1:20) with fresh deionised water, other reagents were ready for use. All the steps for the procedure used above for the detection of the CMV IgG, were followed step by step for detection of CMV IgM.

### 3.8.2.3. Calculation of control values and cut-off

Mean absorbance values of NC (MNC) and mean absorbance value of PC (MPC) were calculated. The cut-off value was then calculated following this equation: Cut-off value \( COV = MNC + (0.1 \times MPC) \)

The test run was validated according to the manufacturer's criteria for validity as below:

1. Ab substrate blank < 0.150
2. MNC < 0.250
3. MPC > 0.400
3.8.2.4. Interpretation of the results
Sample with the absorbance > COV were considered as anti-CMV-IgM-Ab positive, while samples with absorbance < COV were as anti-CMV-IgM-Ab-negative.

3.9. Data analysis
The statistical package of social science (SPSS) was used for statistical analysis. Significance of difference was determined using Chi-square test. Statistical significance was set at $P < 0.05$. Figures were performed by using Microsoft Office and Excel software program.
CHAPTER FOUR
4. RESULTS

4.1. Detection of CMV IgG among blood donors:
Out of the 100 blood donors tested, 73 subjects (73 %) were CMV IgG positive, while 27 subjects (27 %) were negative for CMV IgG, Figure (4.1).

4.2. Detection of CMV IgM among blood donors:
Out of the 100 blood donors tested, 19 subject (19 %) were CMV IgM positive, while 81 subject (81 %) were negative for CMV IgM , Figure (4.2).

Figure 4.1 ELISA micro titter plate
4.3. The effect of age on CMV IgG and IgM seropositivity among blood donors:

The mean age of subject was 30 years, subjects were divided in two age groups (< 30 years and ≥ 30 years ) in order to evaluate the effect of age on CMV seropositivity , while there was no significant difference (p>0.05) between the two age groups on CMV IgG / IgM seropositivity.

4.4. The Effect of previous surgical operation and blood transfusion on CMV IgG and IgM seropositivity among blood donors:

No significant difference (p> 0.05 ) was found between both history of previous surgical operation and blood transfusion and CMV seropositivity.
IgG

Figure 4.2 Detection of CMV IgG positive subjects among the blood donors.

IgM

Figure 4.3 Detection of CMV IgM positive subject among the blood donors.
Table 4.1  The effect of age on CMV IgG and IgM seropositivity among the blood donors.

<table>
<thead>
<tr>
<th>Serological marker (Anti – CMV antibodies)</th>
<th>Age groups (years)</th>
<th>&lt; 30 yrs</th>
<th>≥ 30 yrs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Test group</td>
<td></td>
<td>Test group</td>
</tr>
<tr>
<td>Anti CMV IgG</td>
<td>Positive Frequency %</td>
<td>38</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>38 %</td>
<td>35 %</td>
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<tr>
<td></td>
<td>Negative Frequency %</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14 %</td>
<td>13 %</td>
</tr>
<tr>
<td>Anti CMV IgM</td>
<td>Positive Frequency %</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 %</td>
<td>9 %</td>
</tr>
<tr>
<td></td>
<td>Negative Frequency %</td>
<td>41</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>41 %</td>
<td>40 %</td>
</tr>
</tbody>
</table>
Table 4.2 The effect of previous surgical operation and blood transfusion on CMV IgG and IgM seropositivity among the blood donors.

<table>
<thead>
<tr>
<th>Serological marker (Anti-CMV Antibodies)</th>
<th>Previous surgical operation and blood transfusion</th>
<th>Test group</th>
<th>Test group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>YES</td>
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<td></td>
</tr>
<tr>
<td>Anti CMV IgG</td>
<td>Positive</td>
<td>12</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>Frequency %</td>
<td>12 %</td>
<td>61 %</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>5</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Frequency %</td>
<td>5 %</td>
<td>22 %</td>
</tr>
<tr>
<td>Anti CMV IgM</td>
<td>Positive</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Frequency %</td>
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<td>18 %</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>16</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>Frequency %</td>
<td>16 %</td>
<td>65 %</td>
</tr>
</tbody>
</table>
**IgM**

**Figure 4.4.** The Effect of previous surgical operation and blood transfusion on CMV IgM seropositivity.

**IgG**

**Figure 4.5.** The Effect of previous surgical operation and blood transfusion on CMV IgG seropositivity.
CHAPTER FIVE
5. Discussion

5.1. Discussion

This study was conducted on 100 blood donors, and revealed that the CMV seroprevalence among blood donors in blood bank of National Public Health Laboratory and blood bank of Khartoum Teaching Hospital was 73% and 19% for CMV IgG and CMV IgM, respectively.

The positive cases were detected among blood donors using ELISA, and our results are nearly in accordance with that conducted in military hospital in Accra, Ghana, (Hecker et al; 2004). The detection of CMV indicated that the blood donors had previously been infected with CMV. After CMV infection, IgG remains in the body for life and protects considerably against the next infections. Thus, negative results of CMV IgG test means that the blood donors have not been infected with CMV (Bagheri et al; 2012).

The Seroprevalence of CMV IgG observed in this study was similar to the result obtained by Kasim et al; (2008). The previous CMV infection observed in this study was similar to that reported in other developing communities but higher than in the developed communities obtained by Barbera and Tegtmeier, (2007). This may be attributed to the low socioeconomic status and poor hygienic practice which might play an important role in increasing the rate of CMV infection.

In the present study, the rate of positive CMV IgM was 19% among the blood donors, which may reflected an active recent infection or reactivation of the virus (Farrel et al; 1999). this finding was higher than that of (Kasim et al; 2008) who reported the rate of positive CMV IgM as 11%.
The blood donors comprised largely of male donors making sex comparisons statistically undesirable, subject were divided in to two age groups (< 30 years and ≥ 30 years) in order to evaluate the effect of age on CMV IgM / IgG seropositivity and concluded that there was no significant difference (P > 0.05) between the two age groups and CMV IgG and IgM seropositivity. also the study revealed that there was no significance difference (P > 0.05) was found between the history of previous surgical operation and blood transfusion and CMV seropositivity.

5.2. Conclusion

It is concluded that CMV IgG and IgM antibodies was highly detected in blood donors and the study revealed that there was no significant association between age and both history of previous surgical operation (p> 0.05).

5.3. Recommendations

1. Screening of CMV IgM is suggested for blood donors particularly for the high risk group such as neonate, immunocompromised patient, and patients with a malignant disease.
2. Blood units must be screened for anti-IgM CMV or preventive strategies to be implanted to decrease the transmission of CMV in these groups of patients.
3. Blood screened positive for anti-IgM CMV is recommended to be discarded.
4. IgG avidity test should be used to distinguish primary and recurrent infection.
5. PCR is essential for accurate identification of CMV infection.
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REFERENCES


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Date………………………………………………

**Questionnaire**

Seroprevalence of Cytomegalovirus infection in blood donors

1. Name of donors ……………………………………………………………………………
2. Lab number ……………………………………………………………………………
3. Age………………………………………………………………………………
4. Gender ……………………………………………………………………………
5. Previous surgical operation …………………………………………………
6. Test required

☐ CMV IgM    ☐ CMV IgG

**Result:**

CMV IgM ……………………………………………………………
CMV IgG ……………………………………………………………
Appendix (3)

CMV IgM
ELISA Test for the Detection of IgM Antibodies to Cytomegalovirus in Human Serum

Package Size
RERef 51103 96 Tests Complete Test Kit

Intended Use
The CMV IgM ELISA is intended for the detection of immunoglobulin G (IgM) class antibodies to Cytomegalovirus (CMV) in human serum. CMV infections occur worldwide. About 50% of the general population is seropositive by the third decade of life. CMV is a sexually transmitted disease, but may also be acquired by blood transfusions or via saliva, feces, urine, or milk.

During pregnancy the fetus may be Infected by CMV, and while the majority appear healthy at birth, up to 25% of these asymptomatically infected infants will show developmental disorders later (blindness, mental retardation). In normal adult infection with CMV is usually asymptomatic, but when symptoms do occur, the most common form is mononucleosis. Typically, the patient presents with fever, chills, myalgia, and headache. Often the physician must rely on serological tests to distinguish CMV infection from other infections and clinical syndromes which have similar symptoms.

Principle
- Classic EIA -

The Human CMV IgM ELISA is based on the classical EIA technique. The microtiter strip wells as a solid phase are coated with cell culture derived CMV antigen (CMV Ag). In the first incubation step corresponding specific antibodies (CMV-IgM) present in patient specimens or controls bind to the antigens at the solid phase. The sample dilution buffer contains anti-human IgG to prevent rheumatoid factor (RF) interference and competition from specific IgG present in the specimen.

At the end of the incubation, unbound components are washed out. For the second incubation step, anti-human IgG conjugate (anti-human IgM antibodies, peroxidase conjugated) is added which binds specifically to IgM class antibodies resulting in the formation of typical immunocomplexes. After a second washing step to remove excess conjugate, TMB/substrate is added (Step 3). A blue color develops changing to yellow after stopping the reaction. The intensity of the color is directly proportional to the CMV-IgM-concentration in the specimen.

The absorbance of controls and specimen is determined by using ELISA microplate readers or automated ELISA systems (like HumaReader or ELVIS line). Results for patient samples are obtained by comparison with a saturation value.

Reagents and Controls
- 12 Microtiter Strips (in 1 strip holder) (Code CMV M)
- 8 well snap-off strips coated with CMV antigen (cell culture derived)
- 2.5 ml CMV IgM Negative Control (green cap) ready for use, human
- 2.5 ml CMV IgM Positive Control (red cap) ready for use, human
- 100 ml Dilution Buffer IgM (blue cap)
  - pH 6.5 ± 0.2
  - Phosphate buffer 10 mmol/L
  - NaCl 8 g/L
  - Albumin 10 g/L
- 12 ml Anti-IgM Conjugate (white cap) ready for use, coloured red
- 8 ml Anti-human IgM (rabbit), peroxidase conjugated
- 50 ml Washing Solution (white cap)
  - Concentrate for about 1000 ml
  - Buffer pH 7.2 ± 0.2
  - Tris buffer 10 mmol/L
  - NaCl 8 g/L
- 13 ml Substrate Reagent (black cap)
  - for use, colourless to bluish
  - 3.3', 5.4', Tetramethylenediamin (TMB)
  - Hydrogen peroxide 1.2 mmol/L
  - 3 mmol/L
- 15 ml Stop Solution (red cap)
  - Sulphuric acid, ready for use 0.5 mol/L
  - 2 Adhesive Strips

Safety Notes
Do not swallow the reagents. Avoid contact with eyes, skin and mucous membranes. All patient specimens and controls should be handled as potentially infectious. The controls have been checked on donor level for HCV and HIV-1/2 antibodies and HIV Ag and found negative. Wear protective clothing and disposable gloves according to Good Laboratory Practices.

All materials contaminated with patient specimens or controls should be inactivated by validated procedures (autoclaving or chemical treatment) in accordance with applicable regulations.

Stop irritates eyes, skin and mucous membranes. Upon contact, rinse thoroughly with copious amounts of water and consult a doctor.

Stability
The reagents are stable up to the stated expiry dates on the individual labels when stored at 2-8°C. After opening reagents have to be stored at 2-8°C and used within 60 days (see also "Note").

ADG (Code: CMV M)
- sealed in an aluminium bag with a desiccant
- must be at room temperature before opening
- unused: return the desiccant to the zip-lock bag and store in this way at 2-8°C
- Do not touch the upper rim or the bottom of the wells with fingers.

Reagent Preparation
Bring all reagents to room temperature (15-25°C) before use. Reagents not in use should always be stored at 2-8°C.

Notes
The general purpose reagents (CMV M) are interchangeable between different lots and kits. For IgM tests use only IgM dilution buffer (CMV M) 2111.

All other reagents are specific for the individual package lot and must not be interchanged with other lots. No reagents of other manufacturers should be used along with reagents of this kit.

Working Wash Solution (WASH)
- dilute WSH 5101 1 + 20 with fresh deionised water. e.g. 51 ml WSH 5101 + 1000 ml = 1050 ml.
- Stability: up to 60 days at 15-25°C.

Specimen
Serum
Do not use highly lipemic or hemolysed specimens.

Specimens may be stored for 7 days at 2-8°C or longer at -20°C. Freeze and thaw once only. Thawed specimens must be homogenised. Eliminate particulate matter by centrifugation or filtration.

Procedure
Follow the procedure exactly as described.

Procedural Notes
P1: Do not mix caps of wells (risk of contamination). Do not use reagents after their expiration date.
P2: Do not use reagents that could be contaminated or look or smell different than usual.
P3: Record specimens and controls carefully on the spreadsheet supplied with the kit.
P4: (CMM) - select the required number of Microtiter Strips.
P5: Run duplicates for controls. Pipette controls and specimen on the bottom in the microtubes.
P6: Always add reagents in the same order and timing to minimise reaction time differences between wells. This is important for reproducible results. Pipetting of specimens should not exceed 5 minutes. Otherwise pipette the controls in the indicated positions at half way time of the series. If more than 1 plate is used, repeat the controls for each plate.
P7: Avoid/remove air bubbles prior to incubations and reading of absorbance.
P8: (CMM) - incubate in the dark. (EUE) initiates a kinetic reaction, which is terminated by STOP.
P9: (CMM) - turbidity after addition of the sample has no influence on the results.
Wash Procedure
The wash procedure is critical. Insufficient washing will result in poor
precisioi or falsely low absorbances.
W1. Remove Adhesive Strips, aspirate off the contents into 5% sodium
hypochlorite solution and add [WASH] to each well, aspirate off after
30 sec, soak time and repeat washing 3 resp. 4 times.
W2. In case of automatic washers fill and prime with [WASH]. Subse-
quently wash strips 4 resp. 5 times. Ensure the washer fills all wells
completely and aspirates off efficiently after 30 sec. (remaining
liquid < 3 µL).
W3. After washing, remove remaining liquid by tapping the plate upside
down on tissue paper.

 Pipetting Scheme
Reagents and specimens should be at room temperature before use.
Sample Preparation.
Dilute the patient's sera 1 + 100 with [DIL] to 511, e.g. 30 µl serum + 1
ml [DIL] to 511, mix thoroughly (see P9).
Incubate diluted samples at least 5 min., prior to further processing.
Diluted samples can be stored up to 24 h at 2...8°C before testing.
Controls are ready for use.

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<td>D1/E1</td>
<td>PS</td>
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<td>F1</td>
<td>Sample</td>
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- DIL in duplicate: -- 100 --
- PS in duplicate: -- 100 --
- Diluted samples: -- 100 --
- HGC cover with Adhesive Strips:
  - Incubate 30 min. at 17...25°C.
  - Wash 4 times as described (see W1 - W3).

WASH
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Mix carefully.
Zero the ELSA microtitre plate reader (HumaReader) using the sub-
strate blank in well A1.
Measure the absorbance at 450 nm as soon as possible or within
30 min. after terminating of the reaction, using a reference wavelength
of 630-650 nm (if available).

Calculation of Control Values and Cut-off
Mean absorbance values of [PS] in wells B1 and C1 (MNC) and [F1] in
wells D1 and E1 (MPC) are calculated according to:

\[ A_{\text{MNC}} = A_{\text{B1}} + A_{\text{C1}} \]
\[ A_{\text{MPC}} = A_{\text{D1}} + A_{\text{E1}} \]

MNC = \[
\frac{A_{\text{MNC}}}{2}
\]
MPC = \[
\frac{A_{\text{MPC}}}{2}
\]

Cut-off value COV = MNC + (0.2 x MPC)

The test run may be considered valid provided that the following criteria
are met:
1. Substrate blank in well A1 < 0.150
2. MNC < 0.250
3. MPC < 0.400
4. MPC > MNC x 2

Interpretation of Results
A_{\text{MNC}} (patient) \geq COV \rightarrow \text{CMV-IgM-Ab-positive}
A_{\text{MNC}} (patient) < COV \rightarrow \text{CMV-IgM-Ab-negative}

Due to physiological and analytical variations patient results lying 15%
above or below the calculated cut-off are equivocal. It is recommended to
measure these samples in parallel with a fresh sample taken 7 to 34 days
later, each in duplicate. The trend between the specific antibody levels
should be used for interpretation, also taking into consideration the
specific IgG concentration (HUMAN ELISA IgG), the patient history and
additional investigations. Repeatedly reactive or equivocal samples may
be subjected to a confirmatory test.

Samples from patients with EBV-induced infectious mononucleosis may
give an equivocal or low positive result (sample to cut-off ratio < 1.75),
possibly as a result of a reactivation of CMV IgM antibody production
cauated by EBV-induced B-cell proliferation.

If an ELISA reader is not available a visual interpretation of results is
possible:
- The substrate blank in well A1 should appear colourless.
- A specimen can be considered positive if the colour of the sample well
  is definitely stronger than the colour of the [PS] wells B1/C1.

Performance Characteristics
Typical performance data can be found in the Verification Report, acces-
sible via
www.human.de/data/eb/er/el-cmm.pdf or
www.human-de.com/data/eb/er/el-cmm.pdf

Note
The components of the kit are stable until the expiry date even after
opening. However, a potential contamination is directly related to the
number of samplings. The 60 days limit after first use is set for safety
reasons.

The handling should always be in compliance with common GLP
requirements (*)! The validation criteria must be met

(*) This includes: Proper caps being replaced on the vials and firmly tightened / Remove only
wags required for a run from stock solutions if they could come into contact with other
contaminating solutions like patient specimens etc. / Stock solutions always returned
2...8°C when not in use.

Literature
1. Englert E, Pflimlin P, Immunochimistry B 8, 871-874 (1972)
3. Remington J. S., Klein J. O., Infectious diseases of the fetus and new-
5. Griffiths P. O., CYTOMEGALOVIRUS, Principles and Practice of Clinical