# SEROPREVALENCE OF CYTOMEGALOVIRUS INFECTION AMONG PREGNANT WOMEN WITH ABNORMAL PREGNANCY IN HUSM

**DR WAN ADDYANA BINTI WAN SULONG**

DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF PATHOLOGY (MEDICAL MICROBIOLOGY)


**SCHOOL OF MEDICAL SCIENCES**
**UNIVERSITI SAINS MALAYSIA**
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## LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

| Symbols/ Abbreviations | Meaning |
|---|---|
| - | Negative or subtraction |
| + | Positive or addition |
| > | Greater than |
| < | Less than |
| ≥ | Greater than or equal to |
| ≤ | Less than or equal to |
| X | Times or multiplication |
| % | Percentage |
| / | Division or `or´ |
| = | Equal to |
| µL | Microlitre |
| Avi % | Avidity percentage |
| AI | Avidity Index |
| AIDS | Acquired Immunodeficiency Syndrome |
| cCMV | Congenital Cytomegalovirus |
| CMV | Cytomegalovirus |
| COI | Cut off index |
| CPE | Cytopathic effect |
| DAGS | Double antigen sandwich assay |
| DNA | Deoxyribonucleic acid |
| EBV | Epstein-Barr virus |
| ECLIA | Electro-cheluminescence Immunoassay |
| ELISA | Enzyme- Linked Immunosorbent Assay |

| Symbols/ Abbreviations | Meaning |
|---|---|
| Fig | Figure |
| HCMV | Human cytomegalovirus |
| HHV-5 | Human Herpesvirus 5 |
| HIG | Hyperimmune globulin |
| HIV | Human Immunodeficiency virus |
| HUSM | Hospital Universiti Sains Malaysia |
| IgG | Immunoglobulin G |
| IgM | Immunoglobulin M |
| IMR | Institute for Medical Research |
| IUD | Intrauterine death |
| IUGR | Intrauterine growth retardation |
| LIS | Laboratory information system |
| LOB | Limit of blank |
| LOD | Lower limit of detection |
| MHC | Major histocompatibility complex |
| NK cells | Natural Killer cells |
| PCR | Polymerase chain reaction |
| SD | Standard deviation |
| SGA | Small for gestational age |
| SPSS | Statistical Package for Social Science |
| TORCHES | Toxoplasma, Others, Rubella, Cytomegalovirus, Herpes simplex virus/ HIV, Syphillis |
| USG | Ultrasonography |

## ABSTRAK

***Pengenalan :*** Sitomegalovirus (CMV) merupakan virus yang paling kerapdikaitkan dengan malformasi kongenital di negara maju. Wanita hamil yang dijangkiti untuk kali pertama (jangkitan primer) semasahamil mempunyai risiko yang tinggi untuk menjangkiti janin, dan jangkitan CMV pada janin di awal trimester kehamilan boleh menyebabkan malformasi kongenital yang teruk. Kajian ini bertujuan untuk mengenalpasti seroprevalens CMV di kalangan wanita hamil yang mempunyaikehamilantidak normal di Hospital Universiti Sains Malaysia (HUSM) dan menghuraikan tentang hasil kehamilan tidak normal.

***Tatacara***:Dalam kajian ini, sejumlah 153 sampel serum wanita hamil dengan kehamilan yang tidak normal dihantar ke makmal mikrobiologi dalam jangka masa satu tahun dari 1 Jun 2019 hingga 31 Mei 2020 untuk saringan jangkitan TORCHES (Toxoplasma, Rubela, Sitomegalovirus, Herpes Simpleks, HIV, Sifilis) atau untuk saringan jangkitan CMV. Sampel-sampel ini diuji untuk kehadiran antibodi CMV IgG dan IgM dengan menggunakan imunoasai Electrochemiluminescence (ECLIA). Daripada 153, 112, yang positif dengan antibodi CMV IgG kemudian diuji dengan keavidan CMV IgGmenggunakan kit Elecsys CMV IgG avidity melalui kaedah ECLIA untuk menentukan fasa jangkitan. Rekod perubatan wanita hamil ini dilihat untuk mengenalpasti komplikasi kehamilan.

***Keputusan:*** Seroprevalens CMV di kalangan wanita hamil dengankehamilan yang tidak normal di HUSM adalah 98.69% (n = 15/153). Dari 153, 1 sampel positif untuk CMV IgM dan IgG, sementara selebihnya positif untuk CMV IgG sahaja. Hanya 112 yang memenuhi kriteria kajian diuji dengankeavidan CMV IgG. Berdasarkan keputusankeavidan CMG IgG, jangkitan CMV di kategorikan kepadajangkitan primer (n = 4/112) dan jangkitan bukan primer (n = 111/112). Kehamilan tidak normal adalah termasuk penemuan penskanan ultrabunyi yang tidak normal, iaitu IUGR, SGA, ventrikulomegali, kalsifikasi serebrum, usus

janin echogenik, atau kehilangan kehamilan seperti keguguran atau kematian bayi dalam kandungan. Tiga dari empat ibu dengan jangkitan CMV primer melahirkan anak yang sihat, sementara seorang ibu mengalami kematian bayi dalam kandungan.

***Kesimpulan***: Seroprevalens CMV dalam kajian ini setanding dengan kajian lain yang dilakukan di negara-negara Asia dengan status sosioekonomi yang sama. Sebilangan besar jangkitan tidak bersifat primer kerana kemungkinan besar, populasi ini telah dijangkiti semasa kecil atau remaja.

Kata kunci: sitomegalovirus, wanita hamil, keavidan CMV IgG

## ABSTRACT

*Introduction*: Cytomegalovirus (CMV) is the most common virus associated with congenital malformations in developed countries. Pregnant women who acquired primary infection during pregnancy carry a high risk of transmission to the fetus, and fetal CMV infection in the early trimester can lead to severe congenital malformation. This study aims to describe the seroprevalence of cytomegalovirus among pregnant women with abnormal pregnancy in Hospital Universiti Sains Malaysia (HUSM) and describe the pregnancy outcome.

*Materials and Methods*: In this study, a total of 153 serum samples of pregnant women with abnormal pregnancy were sent to the microbiology laboratory in a one-year duration from 1st June 2019 until 31st May 2020 either for TORCHES (Toxoplasma, Rubella, Cytomegalovirus, Herpes Simplex, HIV, Syphillis) screening or to rule out CMV infection. These samples were tested for CMV IgG and IgM antibodies' presence using Electrochemiluminescence immunoassay (ECLIA). Out of 153, 112, which were positive with CMV IgG antibodies were then being tested with CMV IgG avidity using Elecsys CMV IgG avidity kit via ECLIA method for determination of the phase of infection. Medical records of these pregnant women were reviewed for the outcome of pregnancy.

*Results*: The seroprevalence of CMV among pregnant women with abnormal pregnancy in HUSM was 98.69% (n=151/153). Out of 153, 1 sample was positive for CMV IgM and IgG, while the rest positive for CMV IgG only. Only 112 that fulfilled the inclusion criteria and were tested for CMV IgG avidity. The CMV IgG avidity result was categorized into primary CMV infection (n=4/112) and non-primary CMV infection (n=111/112). The abnormal pregnancy were those with either abnormal ultrasound findings, namely IUGR, SGA, ventriculomegaly, cerebral calcification, echogenic fetal bowel, or pregnancy loss such as

miscarriage or intrauterine death. Three out of four mothers with primary CMV infection gave birth to a healthy child, while one mother had intrauterine death of her fetus.

*Conclusion*: The seroprevalence of CMV in this study is comparable to other studies done in Asian regions with similar socioeconomic status. Most infections were non-primary as most likely, the people in this population have been infected during childhood or adolescence.

*Keywords:* Cytomegalovirus, pregnant women, CMV IgG avidity

# CHAPTER 1
# LITERATURE REVIEW

## 1.1 Background of the study

Human Cytomegalovirus (HCMV) or Human Herpesvirus 5 (HHV-5) is a double-stranded DNA virus of the Herpesviridae family and member of the genus subfamily Betaherpesvirinae. HHV-5 was isolated in the 1950s and was designated by Drs Thomas Weller as Cytomegalovirus (CMV). It is the largest virus in the Herpesviridae family, with 229 kilobase pairs (kbp).

Like other Herpesviridae family members, this virus can persist for the host's lifelong after a primary infection. (1)

### 1.1.1 Epidemiology of CMV infection

CMV seroprevalence varies throughout the world, with the highest mean seroprevalence was estimated in Eastern Mediterranean region and the lowest in European region.(2)The prevalence of intrauterine CMV infection in the United States was 6 in 1000 and up to 10 in 1000 in South America, Asia, and Africa.(1) In the United States, one in three children is infected by this virus by the age of five years old, and almost half of the adult population is infected by the age of 40.(3) CMV seroprevalence increase with age, with a prevalence of more than 60% in person older than 50 years.(1) CMV seroprevalence also varies with geographical regions in which higher in developing countries, and socioeconomic status where higher rates were reported in low socioeconomic conditions due to poor hygiene and overcrowding of people, which facilitates transmission of the virus.(4, 5) A study done in Beijing on maternal CMV from 2010 to 2015 showed a 94.7%

seroprevalence, with 0.45% developed a primary infection, 35.71% (n=5 /14) of pregnant women with active infection transmit the virus to the fetus; however, only 2 of them developed symptoms of CMV infection (hearing deficit).(6) Meanwhile, in Japan, a study done among pregnant womenfrom 2009 to 2014 showed a seroprevalence of 69.1%, with 0.37% shows CMV IgG antibody seroconversion.(7) Neonatal congenital CMV (cCMV) infection occurred in 37.5% of seroconverted women, and all neonates were asymptomatic.(7)

CMV infection among women of reproductive ages was relatively common, with seroprevalence ranging between 45% to 100% (8, 9). In Malaysia, CMV seroprevalence was 92% in Selangor and Wilayah Persekutuan (10). A study among regular blood donors in Hospital Universiti Sains Malaysia, Kelantan in 2007 shows that 97.6% were seropositive with CMV, while a study by the Institute of Medical Research (IMR), Malaysia among CMV in pregnant women in 2011 shows that 84% of them were positive for CMV IgG.(11, 12)

### 1.1.2 CMV Transmission

CMV is transmitted via direct contact with infectious body fluids (saliva, urine, and genital secretions), through sexual intercourse, transplanted organs and blood transfusions from infected donors, and vertically from mother to fetus.(9) Infants and children can get infected with CMV through intrauterine infection, intrapartum, or even breastfeeding with CMV-infected breast milk. Transmission of CMV from mother to fetus can occur during primary infection, reactivation, or secondary infection with different viral strains. The risk of CMV transmission in utero during pregnancy is 30% to 40% in the first and second trimester and can be as high as 70% if the primary infection occurs during the third trimester.(9)Latent

CMV infection carries the lowest risk of infection, which is 3%.(9) However, the fetus's greatest damage occurs if the primary infection is acquired during the first trimester.(9) Reactivation of the virus most likely occurs during the second and third trimester of pregnancy due to depression of CMV specific cellular immunity.(4)

### 1.1.3 Cytomegalovirus (CMV)

CMV consists of four main structural elements, including the outer envelope, tegument, nucleocapsid, and internal nucleoprotein core, where the genomes are situated. Its envelope contains lipoproteins and structural proteins responsible for viral entry into host cells, while teguments consist of structural proteins, including pp65 antigen, which plays an essential role in diagnosing active CMV infection.(5, 13)Double-stranded DNA of CMV encodes 19 genes, which encodes viral glycoproteins and other functions, with one of them is CMV ORF designated UL54 for DNA polymerase.(1) DNA polymerase is responsible for CMV replication in host cells and serves as an important target for many antiviral drugs. Another essential protein encoded by CMV genes is UL 97 proteins, which phosphorylate ganciclovir to ganciclovir monophosphate, enabling CMV DNA replication inhibition.(1) CMV also possesses genes that encode for proteins to downregulate the host's immune system. The most important mechanism is inhibition of Human Leucocyte Antigen-1 (HLA-1) from reaching the host cell, thus unable to form complexes with CMV glycoproteins and subsequently prevents CD8+ T cells from recognizing and kill the infected cells. (1)

CMV enters host cells by the process of endocytosis. Inside the host cells, the genome is uncoated, and DNA core proteins are transported to the nucleus. DNA replication occurs inside the nucleus, and this process cause formation of large

nuclear inclusions, which is a hallmark of CMV infection. CMV possesses viral functions that aid in invasion and persistence in host cells, including cell death pathway inhibition, which activated once infection occurs. CMV also took control over the cell cycle to optimize viral DNA replications and inhibit the cellular response. Studies in animal models show the absence of NK cells' immune evasion function towards virus in CMV infections.(1) There is also inhibition of CD4 and CD8 cell recognition of CMV infected cells due to the degradation of MHC class I and II molecules.(1)

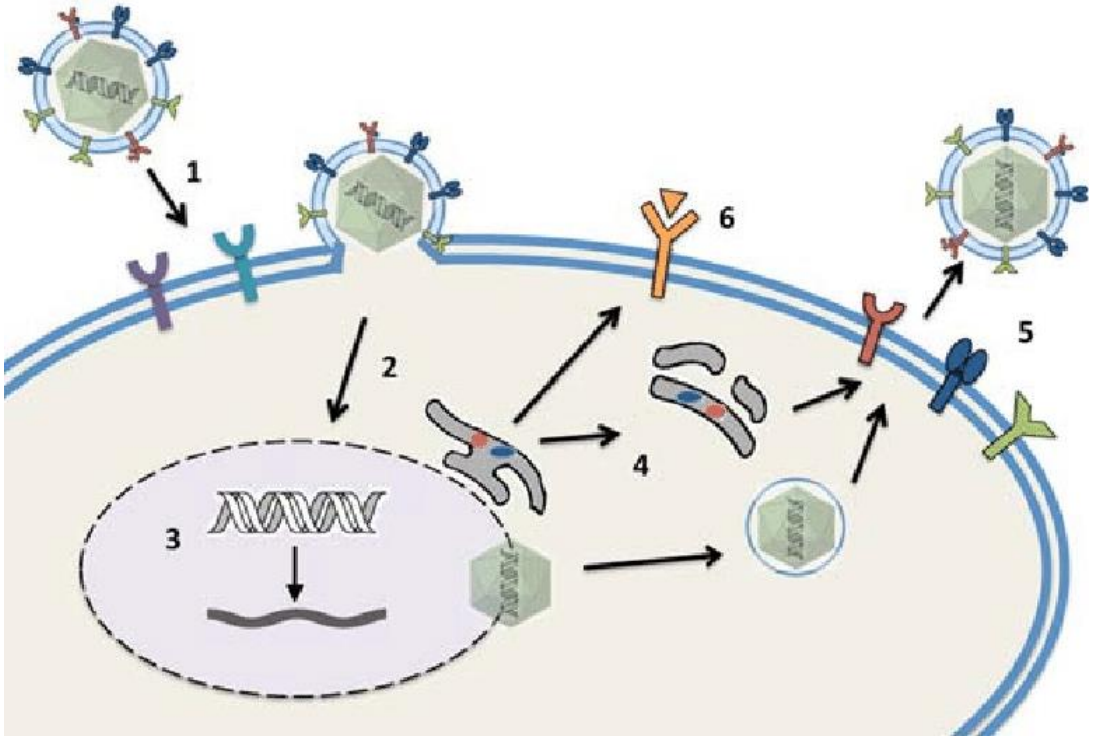


Figure 1 Human CMV life cycle (Adopted from Immunotherapeutic Approaches To Prevent Cytomegalovirus-Mediated Disease. Microbiology Spectrum. 2014; 2(1)

CMV has been demonstrated to establish latency in monocytes, macrophages, CD34+ cells, and various organs' epithelial cells, including salivary glands, intestines, breast, and prostate.(1) It can also be found in endothelial cells, smooth

muscle cells, and fibroblasts.(5) Some studies suggest CMV transcribed large numbers of viral genes in CD34+ cells during latency.(1)

### 1.1.4 Clinical Manifestations of CMV infection

Clinical manifestations of CMV infection is highly dependent on the patient's immune status. Low immune status can lead to uncontrolled viral replication and thus causing severe and debilitating infection. Furthermore, it also has been associated with tumorigenesis and atherosclerosis.(14) Those at risk of CMV infection include AIDS patients, cancer patients on chemotherapy or post-transplantation immunotherapy, infants, and elderly patients. CMV is an important infectious cause of transplantation rejection. It can present with CMV syndrome (fever, malaise, leukopenia, atypical lymphocytes, thrombocytopenia, increased aminotransferase), pneumonia,gastrointestinal disease hepatitis, and central nervous system infection.(13) The most common ocular manifestation in AIDS patients is CMV retinitis. Another presentationof CMV in HIV patients includes systemic illness, pneumonia, and gastrointestinal disease.(15, 16)

CMV infection in developed countries is the most common viral infections associated with congenital malformation. Its infection in adults often goes unnoticed as the symptoms are similar to other common viral fever infections of the upper respiratory tract (fever, weakness, muscle aches, and sore throat) or infectious mononucleosis-like syndrome (fever, lymphadenopathy, and lymphocytosis). Thus, the status of infection can only be ascertained by laboratory viral screening. However, the usefulness of CMV screening in a pregnant woman is often questioned. (17). Symptoms of congenital CMV infection include jaundice, petechiae, hepatosplenomegaly, growth retardation, microcephaly, sensorineural

hearing damage, chorioretinitis, and optic atrophy.(18) Laboratory abnormalities include anemia, thrombocytopenia elevated bilirubin, and liver transaminases.(18) However, only 30% of symptomatic congenital CMV infection will develop such severe symptoms, while over 90% of infants diagnosed with congenital CMV infection were asymptomatic.(1) The most common sequelae of congenital CMV infection is hearing loss, in which 50% of them will be detected during the newborn period through newborn auditory screening program while the remaining 50% will develop hearing loss later during childhood.(1)

### 1.1 5 Laboratory Identification of CMV and Antibody Kinetics

The conventional serological test to measure CMV antibodies IgM and IgG is Enzyme Link Immunosorbent Assay (ELISA). This method is mostly used as it is cost-effective and can also determine the phase of CMV infections. (19, 20)CMV specific immunoglobulin M (IgM) appears after 1 to 2 weeks, peaks at 3 to 4 weeks post-infection, and decreases gradually at 4 months post-infection.(21, 22) However, in 25% of individuals, IgM remains detectable 4 months after infection and sometimes even persist for more than 1 year.(21) However, CMV IgM can also be detected in secondary infection and, in some cases, reactivation and secondary infection with different HCMV strains.(21) Thus, IgM is not reliable to diagnose a recent primary infection due to a lack of specificity despite high sensitivity. CMV-specific IgG appears weeks after infection and in most cases, itremains detectable for life.(21) A seroconversion is needed to diagnose a recent primary infection. Thus, in all cases, a paired serum is needed to determine a phase of infection.(9)IgG avidity can be defined as the strength of bonds between a mixture of polyclonal IgG

and antigenic epitopes of proteins.(21) Low avidity is only present during primary infection, in contrast to IgM, indicating primary CMV infection. IgG avidity increasingover 5-6 months after infection, reflecting the maturation of humoral immune response in whichbonds between IgG and antigen become stronger.(21) It gained diagnostic importance in determining primary infection, especially in pregnant mothers, to assess the transmission risk to fetuses if no paired sample available.(21, 23)

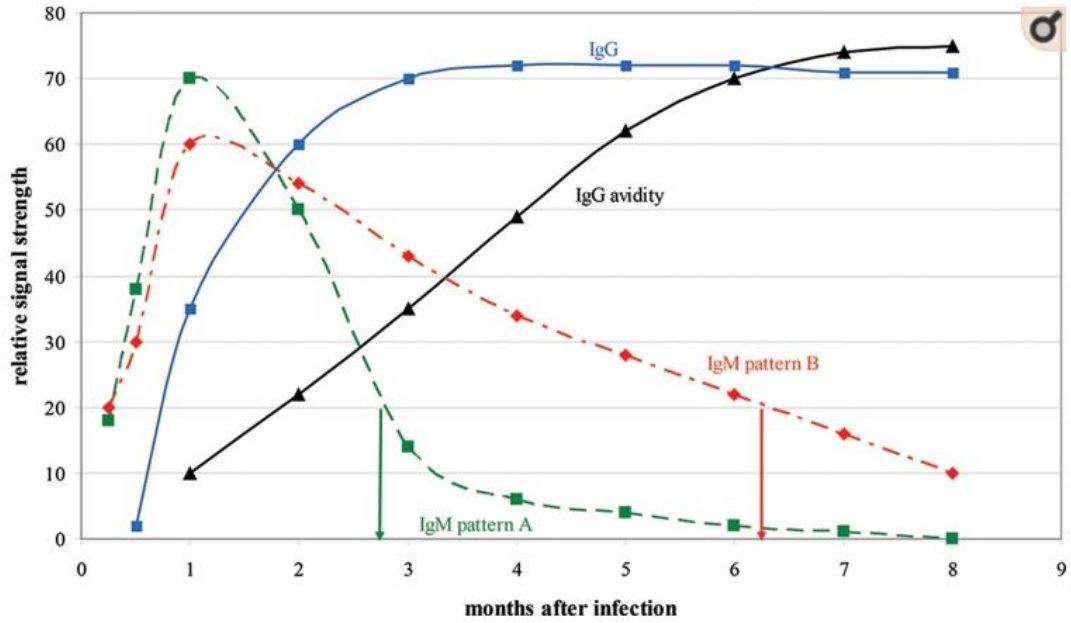


Figure 2 CMV IgM, IgG, and IgG avidity levels following primary CMV infection. IgM pattern A: typical IgM response pattern. IgM pattern B: long-term IgM persistence. (21)

Other methods of CMV detection includes a neutralization assay. Neutralizing antibodies appear 13 to 15 weeks following CMV infection; thus, indicating secondary infection if it presents in high titer during acute infection.(23) (Refer to Figure 2) However, due to its labor intensiveness and prolonged process, it is not widely used. Culturing CMV from amniotic fluids has a 100% specificity of fetal infection. The presence of CMV is detected through the cytopathic effect (CPE) or

immunofluorescence using specific antibodies. However, CMV is a very labile virus and slow-growing. Detection of CMV DNA by PCR from blood and body fluids has been a preferred method for the rapid detection of acute CMV infection, especially in children, to diagnose congenital CMV infection. PCR is more sensitive and specific than IgM detection and less costly and rapid than the viral culture method.(23, 24)

The prognosis of a fetus can be predicted through viral and non-viral fetal blood sampling. The viral method includes detecting CMV viral load, while the non-viral method includes beta-2-microglobulin and platelet count, which can differentiate between symptomatic and asymptomatic CMV infection.(25) Other than laboratory investigation, the radiological investigation is used for a prognostic indicator for the fetal outcome. Magnetic resonance imaging (MRI) and ultrasonography of the fetal brain, in combination, have 95% sensitivity when they are performed during the third trimester.(25)

### 1.1.6 Treatment and Prevention of CMV infection

Drugs that are licensed for CMV infection include ganciclovir, cidofovir, valganciclovir, foscarnet, and valaciclovir. However, valaciclovir cannot be used in a pregnant woman due to its teratogenic and toxic side effects.(25) Acyclovir has been proven to have an excellent safety profile to be used in pregnancy; however, it has a limited antiviral effect against CMV. There have been studies done for the use of hyperimmune globulin for the treatment of CMV with one study shows a significant reduction in the risk of congenital CMV infection, while in another study, the outcome of CMV infection was the same in both groups with or without HIG treatment.(25) Unfortunately, the treatment group was associated with a higher

incidence of obstetric complications such as pre-eclampsia, preterm birth, and fetal growth restriction.(25) Thus more studies need to be conducted to assess its use in pregnancy. Up to date, no vaccine is available for the prevention of CMV infection. Prevention is directed towards self-hygiene, particularly in the woman who is exposed to children at daycare.(25, 26)

### 1.2 Problem statement & Study rationale

The high prevalence of CMV in pregnant women possessed a risk of transmission and congenital malformation in the fetus. Despite its high prevalence, only a few studies have been conducted, and the latest one conducted among pregnant women was in 2011 in IMR.(11) To date, there is still no study done in Malaysia for CMV infection in pregnant women, which describes the fetus's outcome.

By classifying CMV infection into primary and non-primary phases, we can predict the risk of transmission to the fetuses and its outcome. Data provided by this study can also increase awareness among healthcare providers regarding CMV seroprevalence. Besides, this study is important as; currently, there are trials of treatment with hyper immunoglobulin for pregnant women with CMV and later for future vaccine development.

### 1.3 Study Flowchart

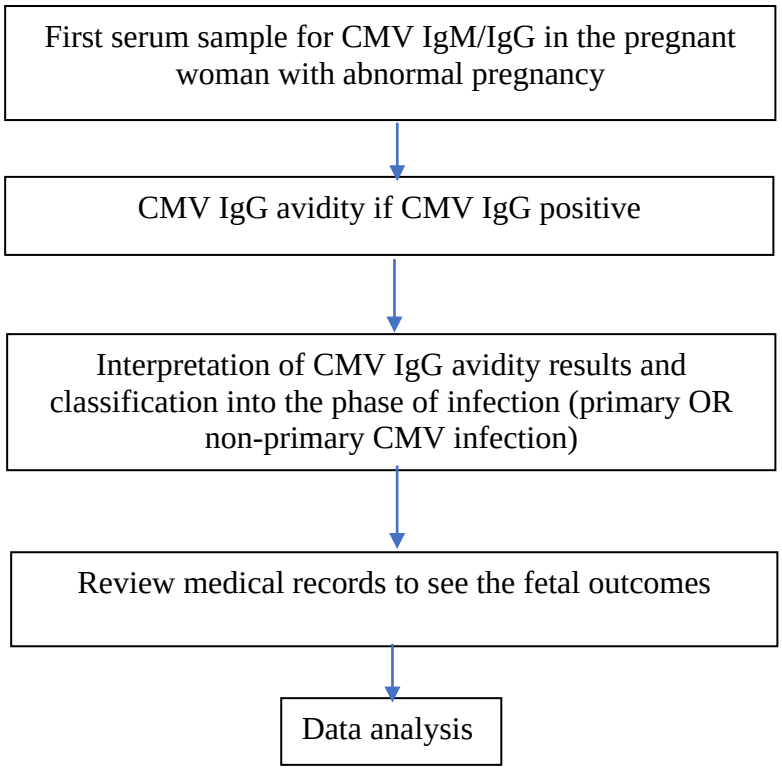


Figure 3 Study flowchart

### 1.4 Research Questions

1) What is the seroprevalence of CMV infection in women with abnormal pregnancy in HUSM?
2) What is the proportion of primary and non-primary CMV infection in women with abnormal pregnancy?
3) Which phase of infection has the highest risk of fetal transmission?
4) What is the clinical outcome of CMV infection to the pregnancy and fetus?

### 1.5 Research hypothesis

There's a high seroprevalence of CMV in pregnant women with abnormal pregnancy, and most of them are due to non-primary infection.

### 1.6 Study Objective

**General:**

To determine the seroprevalence of CMV infection, infection phase and its outcome among pregnant women with abnormal pregnancy in HUSM.

**Specific:**

1. To determine the seroprevalence of CMV infection among pregnant women with abnormal pregnancyin HUSM.
2. To determine the proportion of CMV infection into primary and non-primary.
3. To describe the outcomes of maternal CMV infection to the pregnancy and the fetus.

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# CHAPTER 2

# STUDY PROTOCOL

## 2.1 Title

Seroprevalence of CMV infection among pregnant women with abnormal pregnancy in HUSM.

## 2.2 Methodology

### 2.2.1 Study Design

This is a hospital-based prospective cohort study involving serological samples sent to detect CMV infection and was further tested to determine the phase of infection. This study was conducted for one year, starting from 1st June 2019 until 31st May 2020.

### 2.2.2 Reference population

Serum sample for IgM and IgG CMV antibody titer detection from pregnant women with abnormal pregnancy in HUSM.

### 2.2.3 Target population

Serum sample for IgM and IgG CMV antibody titer detection from pregnant women with abnormal pregnancy in HUSM sent to Microbiology Laboratory in Hospital Universiti Sains Malaysia (HUSM).

### 2.2.4 Source population

Serum sample sent for IgM and IgG CMV antibody titer detection from pregnant women with abnormal pregnancy in HUSM between 1st June 2019 until 31st May 2020 (1 year).

### 2.2.5 Sampling frame

Patients with 1st serum sample of CMV IgM and IgG antibody suggestive of CMV infection.

### 2.2.6 Inclusion Criteria

- Serum sample sent for CMV IgM and IgG and TORCHES of pregnant women with abnormal pregnancy in HUSM.
- Pregnant women with abnormal ultrasound findings of the fetus.
- Serum sample negative for other active TORCHES infection.

### 2.2.7Exclusion criteria

- Fetus which was diagnosed with congenital anomalies of other cause (e.g., genetic mutations).
- Inadequate sample volume.

### 2.2.8Sample size calculation

Objective 1: seroprevalence of CMV

The sample size was calculated using single proportion formula: $n= (z/\Delta)^2 p(1-p)$

n= sample size, z= z distribution = 1.96 $\Delta$= precision (0.05)

P= 0.911; prevalence of CMV in pregnant woman based on previous study is 94.7% ( Jin Q et. al) (1)

$n = (\frac{1.96}{0.05})^2 0.9598 (1-0.9598)$

Therefore, n= 60

Adding 10% dropout, the necessary sample is 67

For Objective 2 and 3, a descriptive statistic was involved in this study; thus, no sample size calculation was needed.

### 2.2.9Sampling method and subject recruitment

A non-probability convenient sampling method has been applied for this study. All serological samples sent to detect CMV infection that fulfilled the inclusion and exclusion criteria were included in this study.

### 2.2.10Variable definition

**Operational definition**

1. CMV seropositivity: the presence of anti- CMV IgG or/and CMV IgM antibodies in serum.
2. CMV seroprevalence: the prevalence of CMV seropositivity in a given population (2)
3. Primary CMV infection: Infection with low CMV IgG avidity (< 45%, following the manufacturer's protocol) by which the infection occurred within 4-5 months. (3, 4)
4. Non-primary infection: Infection that has high CMV IgG avidity (≥55%, following the manufacturer's protocol) by which the infection occur more than 5 months.(3, 4) Non-primary infection includes latent CMV infection, reactivation of CMV infection, or infection with an exogenous virus. (5, 6)
5. Latent CMV infection: Carriership of CMV genome without active replication (7). (presence of CMV-specific IgG, absence of CMV-specific IgM)

6. Reactivation: Active infection in a seropositive individual (7)
7. Abnormal pregnancy: include abnormal USG findings during pregnancy, (IUGR or SGA, ventriculomegaly, cerebral calcification, echogenic fetal bowel) (8), intrauterine death & miscarriage or baby born with jaundice, rash or purpura, hepatosplenomegaly, microcephaly, seizures, retinitis, loss of vision, the hearing problem (8).

### 2.2.11Research Measurement Tools

#### 2.2.11.1 First Phase: Measurement of Seropositivity (CMV IgG and IgM)

Serum samples of pregnant mothers with abnormal pregnancy, sent to the serological laboratory, were tested for CMV seropositivity either as a single test or mostly part of the TORCHES panel. For this measurement, electrochemiluminescence immunoassay (ECLIA) was performed using Elecsys CMV IgG / IgM manufacturer(Roche Diagnostics)and analyzed using Cobas e 601 analyzers manufacturer. The test procedures were performed according to the manufacturer's instruction.

**Electrochemiluminescence immunoassay (ECLIA)**

Elecsys CMV IgG and IgM is the immunoassay for in vitro quantitative determination of CMV IgG and IgM in human serum and plasma and is intended for use on Cobas e immunoassay analyzers.

**ECLIA test principle**

The sandwich principle is used for the detection of CMV IgG with a total duration of 18 minutes per assay. For CMV IgG measurement, in the first step, 20

µL of the sample, biotinylated recombinant CMV-specific antigens, and CMV-specific recombinant antigens labelled with a ruthenium complex) was incubated to form a sandwich complex. Then, streptavidin-coated microparticles were added to the first complex. The complex was then bound to the solid phase when streptavidin forms a strong bond with biotin. (Refer to Figure 4)

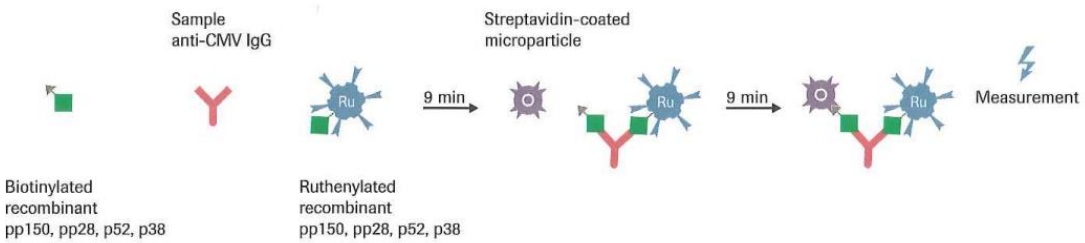


Figure 4 Test principle ECLIA for CMV IgG measurement (Adopted from Elecsys CMV panel fact sheet pdf 2011)

For CMV IgM measurement, the µ-Capture test principle was applied with a total duration of the assay about 18 minutes. In the first step, Biotinylated monoclonal anti-human IgM-specific antibodies were added to 10 µL of samples, which were prediluted automatically 1:20 with universal diluent. In the next step, ruthenylated CMV-specific recombinant antigen and streptavidin-coated paramagnetic microparticles are added. CMV IgM in the serum sample then reacts with the ruthenylated CMV specific recombinant antigen and streptavidin-coated microparticles to form stable immune complexes. These complexes were then bound to the solid phase via biotin and streptavidin interaction. (Refer to Figure 5)

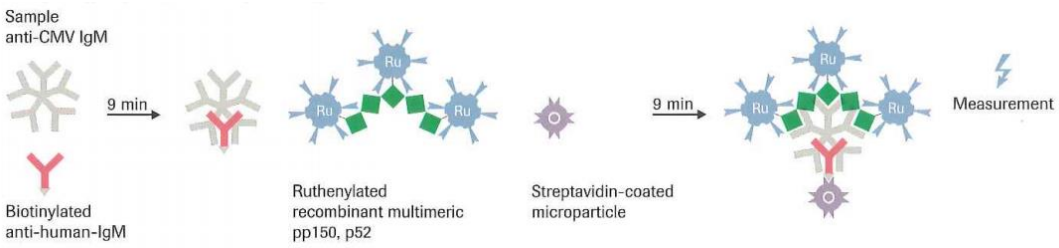


Figure 5 Test principle ECLIA for CMV IgM measurement (Adopted from Elecsys CMV panel fact sheet pdf 2011)

**Elecsys measuring cell**

The electrochemiluminescence measuring cell is the most important tool, and it has three main tasks. It is constructed as a flow chamber. The first task is the separation of unbound and bound substances. Streptavidin-coated microparticles laden and immune complexes were drawn to the electrode's surface with the help of a magnet and were held there temporarily. Then, the Procell system buffer will remove unbound reagent components and excess sample material.

The second task is the generation of electrochemiluminescence with the application of a defined voltage induces the electrochemiluminescence reaction, and the resulting light emission is measured directly by the photomultiplier. Finally, the measuring cell cleaning solution (Clean Cell) will remove the microparticles at the end of the electrochemiluminescence reaction. (Refer to Figure 6)

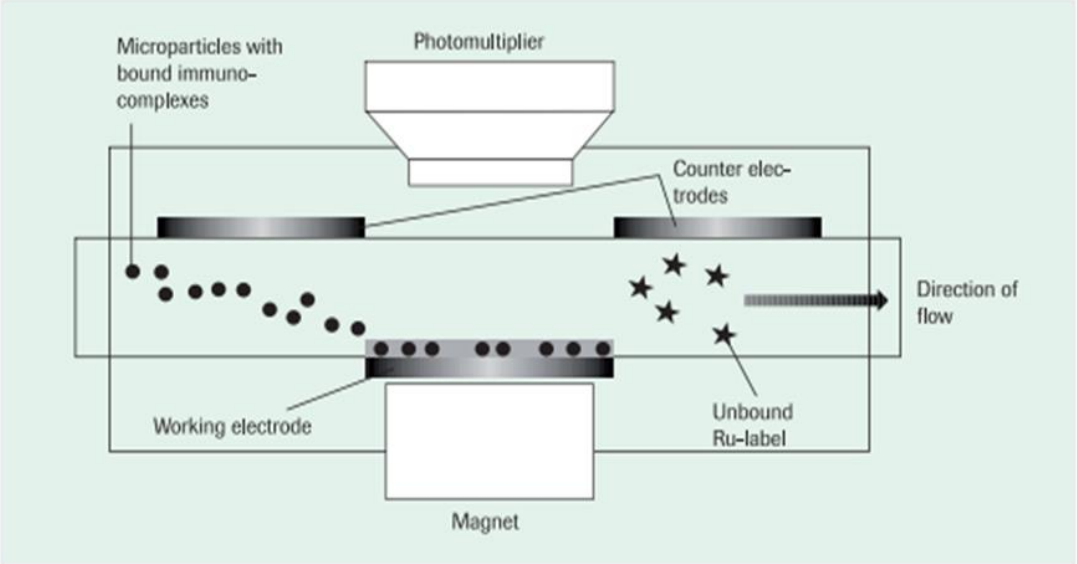


Figure 6 Elecsys measuring cell (Adopted from Elecsys CMV IgG, IgM Immunoassay, Elecsys, and e Cobas analyzer, Roche 2010) (product information, 2010)

**Elecsys CMV IgM interpretations**

The analyzers interpret the result of CMV IgM based on the measurement of CMVIgM Cal1 and CMVIgM Cal2. The result was reported in the form of a cutoff index (COI), which will then be interpreted as reactive, indeterminate, or non-reactive. COI of less than 0.7 was interpreted as non-reactive, while more or equal to 1.0 were interpreted as reactive. The COI of more or equal to 0.7 to less than 1.0 was interpreted as an indeterminate result and recommended for retesting. If the repeated result still indeterminate, the sample should be collected in 2 to 3 weeks for confirmation.

**Elecsys CMV IgG interpretations**

The analytes concentration was calculated automatically by the analyzer, and the results of Elecsys CMV IgG assay were interpreted in U/mL. Ranges of analytes concentration measured by the analyzer are between 0.25U/mL to 500U/mL as defined by the Limit of Detection (LOD) and the maximum of the master curve. The Limit of Blank (LOB) for measurement is 0.15, and any value below the LOB will be reported as < 0.15U/mL. The Limit of Detection (LOD) is 0.25U/mL. The instrument will not flag any value between LOB and LOD. When the analytes have concentrations above measuring ranges, it will be reported as >500U/mL. In this case, it is recommended to dilute the sample with Universal Diluent to 1:20 dilution either manually or automatically by the Cobas e analyzers. The analyzer will automatically calculate the concentration of the analytes according to the dilution.

The value below 0.5 U/mL was interpreted as non-reactive. The negative result indicates that the patient is not infected and susceptible to primary CMV infection. However, it does not entirely rule out CMV infection as the IgG may not yet being produced in the early acute stage of infection. The value of 0.5 to less than 1.0 U/mL was interpreted as indeterminate, and the test should be repeated. If the repeated test still shows an indeterminate range, the second sample should be requested in 2 weeks. Positive results are those with a concentration of more than 1U/mL, and the patient could either be in acute or past infection as IgG can remain detectable for life.

**Limitations**

There are some limitations of Elecsys CMV IgM and IgG. In the early acute phase of infection where the antibodies are not yet formed, it is impossible to exclude CMV infection. Repeated testing in 2 weeks is recommended if there were

high clinical suspicions of CMV infection. Detection of IgM alone is not sensitive for the diagnosis of primary CMV infection as it could persist up to 1 year after infection. In patients with HIV and patients on immunosuppressant therapy, the results should be interpreted with caution. There could be a false positive result of CMV IgM in a patient with primary EBV infection due to cross-reaction. It is not recommended for testing inpatient receiving a high dose of biotin until 8 hours after administration. Rarely, there could be interference due to high titers antibodies to streptavidin, ruthenium, or analyte-specific antibodies. Therefore, all results should be interpreted with the patient's histories and other clinical findings.

#### 2.2.11.2 Second Phase: CMV IgG Avidity

Elecsys CMV IgG avidity (Roche Diagnostics)is the immunoassay used for in vitro qualitative determination of CMV IgG antibodies avidity in human serum and plasma. ECLIA is used on Cobas e immunoassay analyzers similar to the measurement of CMV IgG and IgM.

**Test principles**

The principle of CMV IgG avidity is based on two parallel measurements of the samples: a reference measurement and DilCMVAv treated measurement. The reference measurement is the measurement of the untreated (undiluted) sample, while the DilCMVAv treated measurement of the samples which were already treated (diluted) automatically with specific dilution function of the analyzer with the avidity diluent (DilCMVAv). DilCMVAv contains specific components that will interfere with low avidity CMVIgG antibodies with CMV specific recombinant antigens in the first incubation step.