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Al- Nahrain University
College of Science

Molecular Detection of Cytomegalovirus (CMV) Isolated from Repeated Miscarriage Women and its Relation to TLR2 and ILT2 Receptors

A Thesis

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By

Sara Ahmed Jihad

B. Sc. Biotechnology / College of Science /Al - Nahrian University
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Supervised by

Dr. Rehab Subhi Ramadhan

(Assist. prof.)

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Summary

This study was designed to determine a molecular status of Toll like Receptor 2 in repeated miscarriage women with cytomegalovirus. One hundred blood samples were collected from repeated miscarriage women with cytomegalovirus from infertility clinic of Kamal Al –Sammaraee hospital and (50) samples from normal subjects served as control for comparison and divided into two age groups:(20-30) and (31-40) years old. Women distributed as (60) samples of infertile and (40) samples as miscarriage women.

The first part of this study: anti- HCMV antibodies IgG and IgM was estimated by Enzyme Linked Immune Sorbent Assay (ELISA). Results showed that the miscarriage women showed the highest percentage of seropositive to CMV which were 40%of IgG and 25% of IgM compared with infertile women that showed 20% of IgG and 15% of IgM with a significant difference $P \leq 0.05$**.** The Seropositive of anti- HCMV IgG was higher in younger women (20-30) years old, while the age group (31-40) years showed higher level of IgM.

The second part of this study included molecular analysis using polymerase chain reaction technique (PCR) for amplification two viral genes which are glycoprotein *gB* gene, that showed molecular size 72bp and immediate early *IE2* gene that showed band with molecular size 92bp. Real time PCR used for diagnosing CMV infection by gB-primer with probe FAM and TAMRA, the result showed amplification from cycle six.

The amplification for TLR2 primer region showed band with molecular size 285bp. After successful amplification of TLR2 gene, good quality products were selected to be sequenced. The result of TLR2 sequencing

showed only replacement of C instead of G in wild type at position (242). PCR amplified for ILT2 primer region showed band with molecular size of 419bp. Among ten Iraqi patients, six only give positive result when compared with healthy control; the type of mutation is deletion and substitution. The percentage of mutation types were deletion mutation 13.3% and 86.7% for substitution mutation. The effect of mutation was missense mutation 72.7%, Silent mutations 15.1%, and deletion mutations 12.2%.

Dedication

To those who are the reason for my existence…

My father & mother

To the warm spring that flood my heart with love, my Second half …

My husband

To those who are the beats of life in my heart….

My brother, sisters

To those who are small flowers in my life

My kids

I dedicate my simple and modest efforts.

Thank you.

The dream is became true.

SARA

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List of abbreviation

AIDS	acquired immune deficiency syndrome
CMV	Cytomegalovirus
CSF	cerebrospinal fluid
dsDNA	Double strand Deoxyribonucleic acid
EBV	Epstein-Barr virus
EDTA	Ethylene diaminetetra acetic acid
ELISA	Enzyme linked immune sorbent assay
gB	Gloycoprotein B
HCMV	Human cytomegalovirus
HHV	Human Herpes virus
HRP	Horseradish peroxide
IE	Immediate Early gene
IFN	Antiviral type 1
NCBI	National Center for Biotechnology Information
PAMPs	Pathogen-associated molecular patterns
PP65	Phospho protein 65
PP71	Phospho protein 71
PRR	pattern recognition receptors
ssRNA	Single strand Ribonucleic acid
SNP	Single nucleotide polymorphism
TLRs	Tall Like Receptor
TNF	Tumor necrosis factor
UTR	Unique terminal repeats

Chapter One
Introduction
And
Literature Review

1. Introduction and Literature Review.

1.1. Introduction.

Human cytomegalovirus (HCMV) is a ubiquitous member of the Herpes viridae family, subfamily Beta herpes Virinae. HCMV is the major infectious cause of congenital infection and hearing loss in children as well as an important pathogen in immune compromised patients (Revello and Gerna, 2004). The viral nucleocapsid containing a linear double stranded DNA of 236 kb and is surrounded by a proteinaceous tegument, which is itself enclosed by a loosely applied lipid bilayer (Davison *et al*., 2003).

HCMV has an extremely broad tissue tropism that allows it to infect nearly every organ system in the body (Sinzger *et al*., 2000). It is a major cause of postoperative disease in chemically immunosuppressed transplant recipients and greatly increases the risk of graft rejection (Razonable and Paya, 2003). HCMV is also a leading cause of congenital birth defect, infection during the first trimester of pregnancy often results in neurological and cognitive disorders in the developing child (Streblow *et al*., 2001).

HCMV can be transmitted by close personal contact and by blood transfusion or organ transplantation. HCMV is frequently transmitted from mother to child either in utero or during the perinatal phase. Perinatal transmission often results in severe disturbance of development and disease that may become manifest at birth such as thrombocytopenia, hepatitis, splenomegaly and microcephaly (Stagno *et al*., 1985).

Toll like Receptors (TLRs) are the activators of signal transduction pathways that leads to the expression of antimicrobial/antiviral genes and the induction of inflammatory cytokines (Akira, 2001). TLRs detect microorganisms on the basis of unique molecular structure termed pathogen

associated molecular pattern (PAMPs) (Schwandner *et al*., 1999), viral genomic nucleic acids are the one major class of PAMP. TLR3 (dsRNA), TLR7 (ssRNA), TLR8 (ssRNA) and TLR9 (CpG DNA) signal from the endosome, where degradation of virus particles exposes the viral genome for detection by this panel of TLRs (Diebold *et al*., 2004).

A miscarriage is the spontaneous loss of a pregnancy between conception and 24 weeks into pregnancy. CMV is associated significantly with more than 70% of abortion cases (Goodrich *et al*., 2004). Furthermore 60% of pregnant women reported during first gestation trimester and begin to decline significantly in the late gestation for virus (Pass *et al*., 2006); therefor this study was suggested to fulfill the aim of:

1- Molecular detection of CMV.

2- Determination the relationship between infection with Cytomegalovirus and activation of Toll like Receptor (TLR2) and its effect on miscarriage in women.

This achieved by:

- Collection of blood samples from CMV infected women.
- Isolation of viral DNA.
- Determination of viral infection using (gB) primer by Real- time PCR.
- Amplification specific regions (gB and IE2) using conventional PCR.
- Amplification of *TLR2* gene using conventional PCR.
- Sequencing of PCR *TLR2* gene product.

- Sequencing of PCR *ILT2* gene product to determine genetic alteration.

1.2. Literature Review:

1.2.1. Cytomegalovirus structure:

Cytomegalovirus (CMV) is a member of the *Betaherpesvirinae* sub-family of the *Herpesviridae* family, CMV has an icosahedral protein nucleocapsid that contains the 236 kb double stranded DNA. The capsid is surrounded by a proteinaceous tegument and an outer lipid envelope (Arski *et al*., 2007) as shown in Figure (1-1).

CMV virions gain entry into a host cell via a membrane fusion event involving the outer membrane of the cell and the glycoproteins located on the lipid envelope of the virion. When the cell membrane and lipid envelope fuse, the DNA containing protein nucleocapsid and tegument proteins are released into the host cell. This initiates the lytic stage of the viral life cycle (Shenk *et al*., 2008).

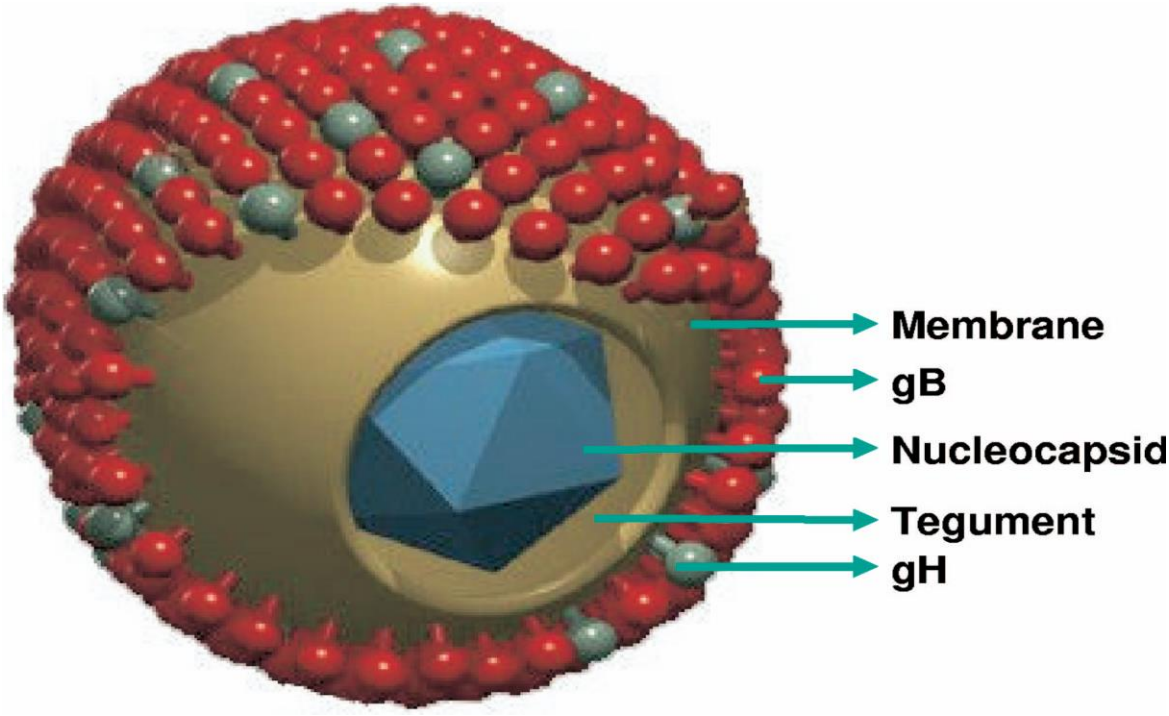


Figure (1-1): Virtual three-dimensional model of HCMV showing various components of the virus (Arski *et al*., 2007).

The tegument located between the outer lipid membrane and the icosahedral protein nucleocapsid is largely unstructured and amorphous although some structuring is seen with the binding of tegument proteins to the protein capsid (Chen *et al*., 1999). The tegument proteins comprise more than half of the total proteins found within infectious CMV virions (Varnum *et al*., 2004). Tegument proteins are phosphorylated and undergo other post translational modifications, but the significance of these modifications is unknown (Shenk *et al*., 2008).

A common biochemical sequence to direct proteins into the tegument has not been identified and the process of assembling the viral tegument upon viral egress and disassembly upon viral entry into cells is unclear. Incorporation of proteins in the tegument is likely facilitated by the phosphorylation of the tegument proteins, their subcellular localization to the assembly site and their interaction with capsids or the cytoplasmic tails

of envelope proteins (Kalejta, 2008). The tegument proteins gain entry into the host cell along with the DNA containing protein nucleocapsid upon fusion of the outer membrane of the host cell and the outer glycoprotein riddled lipid membrane of the CMV virion (Shenk *et al*., 2008). Once the tegument proteins are released into the cytoplasm they become functionally active and participate in all stages of the viral life cycle (Arski *et al*, 2007).

1.2.2. Cytomegalovirus Life Cycle

1.2.2.1. Viral entry

The Cytomegalovirus (CMV) virions enter host cells through a membrane fusion event illustrated in Figure (1-2) involving the host cell's outer membrane and the glycoproteins located on the lipid envelope of the CMV virions (Shenk *et al*., 2008). The receptors on the viral envelope connect to complementary receptors on the cell membrane of the host cell. This initial interaction makes the cell susceptible to further interactions, which allow the membranes to fuse, and the subsequent disassembly and release of the viral genomic DNA and tegument into the host cell. It is believed that several tegument proteins mediate delivery of the DNA-containing nucleocapsid to the nuclear pore complex and the release of the viral DNA into the nucleus (Tomtishen, 2012).

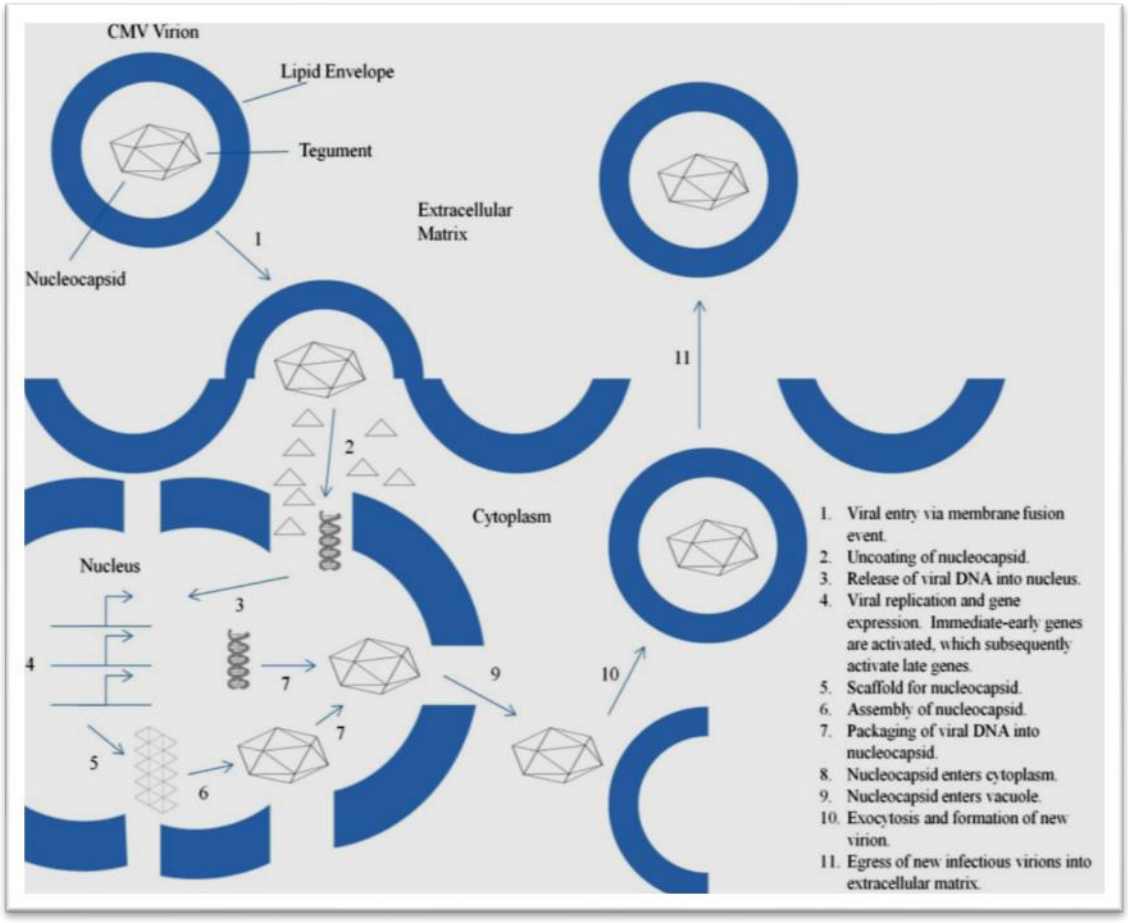


Figure (1-2): Illustration CMV life cycle from viral entry to egress of new infectious virions, (Tomtishen, 2012).

1.2.2.2. Viral replication and gene expression

Once the viral genome enters the nucleus of the host cell, viral immediate-early genes are expressed through their activation by the pp71 tegument protein, which initiates the lytic stage of the viral life cycle and the subsequent replication of the 236-kb double stranded CMV DNA genome (Cantrell and Bresnahan, 2006).

1.2.2.3. Immune evasion:

Cytomegalovirus (CMV) evades the host cell immune system through the targeting of intrinsic, innate and adaptive immune responses by several

different tegument proteins including; PP 65 and PP 71. PP 65 is the major tegument protein involved in immune evasion of the host (Kalejta, 2008).

1.2.2.4. Assembly and egress:

After viral DNA replication the immediate early gene products, which include several tegument proteins turn on the expression of viral late genes. The viral late proteins are mainly structural components that assist in the assembly and egress of newly formed infectious CMV virions (Arski *et al*., 2007). Once the virions are packaged, they are shed from the host cell through an exocytosis mechanism, which uses the host cell's transport system to enclose vacuoles containing the newly synthesized infectious virions for release into the extracellular space (Kalejta, 2008).

1.2.3. Working model for human cytomegalovirus entry into cells:

Human cytomegalovirus initially attaches in a tethering step to heparin sulfate proteoglycans (HSPGs) through gM/gN and/or gB glycoproteins as illustrated in figure (1-3) (Compton, 2004). In a stable docking step, gB interacts with the epidermal growth factor receptor (EGFR) on many HCMV permissive cell types or with as yet unidentified receptors in hematopoietic cells. Other interactions between the glycoproteins of the HCMV envelope and cellular integrin promote receptor clustering (Wang *et al*., 2003).

At least one of these interactions triggers fusion that leads to the internalization of virion components. Signal transduction events are initiated through EGFRs and/or integrin and these events are hypothesized to prime and facilitate downstream steps in the virus life cycle, such as nuclear translocation of the capsid and efficient viral gene expression. Toll

like receptors (TLRs) detect an HCMV displayed pathogen associated molecular pattern during the entry of the virus leading to distinct signaling events and the activation of innate immunity (Compton, 2004).

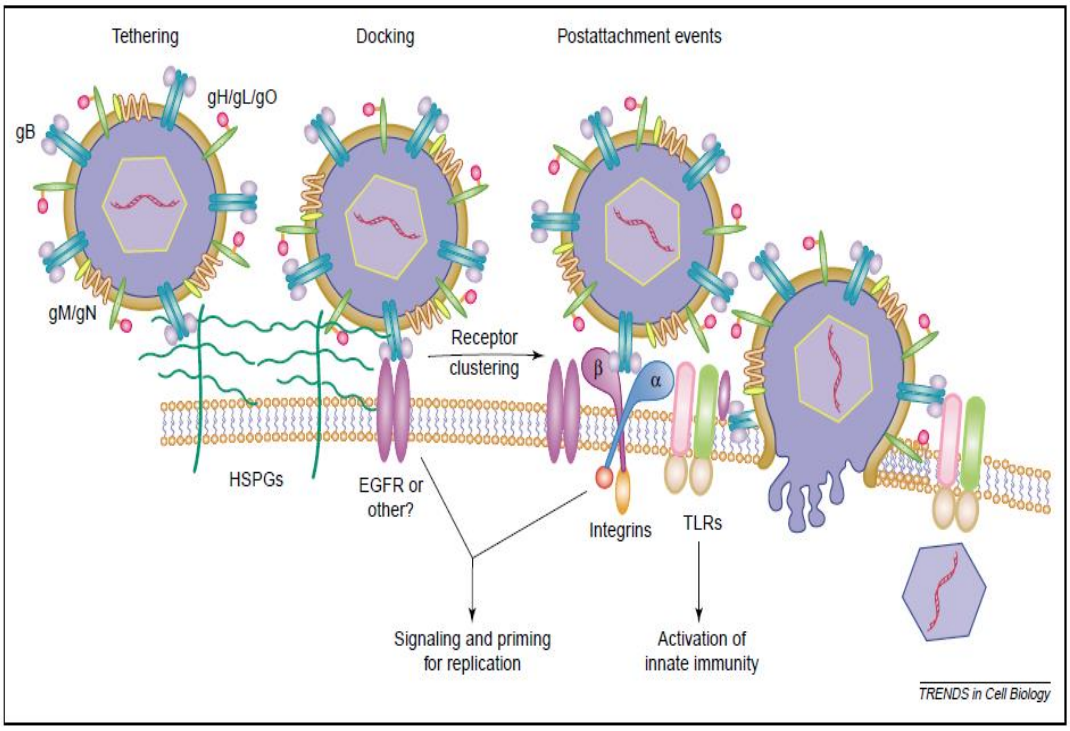


Figure (1-3): Working model for human cytomegalovirus (HCMV) entry into cells (Compton, 2004).

1.2.4. Human cytomegalovirus Genome (HCMV)

Human cytomegalovirus (HCMV; species *Human herpesvirus 5*) has the largest genome of any known human virus about 236 kbp in size encoding~ 200 genes within an enveloped icosahedral capsid (Chee *et al*., 1990). The genome is a linear, double stranded DNA molecule consisting of two unique regions each flanked by inverted repeats regions, internal repeat long and short (IRL, IRS) and terminal repeat long and short (TRL,TRS). In wild type HCMV strain Merlin (GenBank accession no. AY446894) the

sizes of long unique regions (U_L) and short unique regions(U_S) are 193.019 and 35.482 bp respectively (Dolan *et al.*, 2004).

Many studies have shown that HCMV strains are impressively divergent in a subset of genes encoding membrane-associated or secreted proteins (Murphy *et al.*, 2003; Dolan *et al.*, 2004; Pignatelli *et al.*, 2004). Each of these hyper variable genes exists as several highly diverged clusters of alleles with a much lower level of allelic variation evident within individual clusters. The sequences of particular alleles are stable on short timescales in patients (Hassan-Walker *et al.*, 2004; Stanton *et al.*, 2005) and during cell culture (Lurain *et al.*, 2006). These observations suggest that hypervariation is a result of immune selection and that the allelic clusters have a long history, perhaps having emerged during the evolution of populations of early humans or their predecessors. Also, there is extensive evidence that recombination has occurred during HCMV evolution and that infections frequently involve multiple strains (Dolan *et al.*, 2004 and Bradley *et al.* 2008). These factors add a significant complexity to assessments of the associations between the genetic constitution of HCMV strains and disease outcome (Arav-Boger *et al.*, 2005 and Bradley *et al.*, 2008).

1.2.5. Pathogenesis of CMV

There are eight herpesviruses that are known to infect and cause morbidity and mortality in humans. The definitive characteristic of the herpesvirus family is the ability of these viruses to cause both acute lytic infections as well as long term persistent latent infections (Lang and Kummer, 1975). Thus, once an individual has been infected by HCMV he remains infected with the virus for the remainder of his life. After infection,

the virus remains latent in the body for the rest of the person's life. Overt disease rarely occurs unless immunity is suppressed either by drugs, infection or old age. Initial HCMV infection which is often a symptomatic is followed by a prolonged inapparent infection during which the virus resides in mononuclear cells, without causing detectable damage or clinical illness (Gardella, 2008).

Infectious CMV may be shed in the bodily fluids of any infected person in urine, saliva, blood, tears, semen and breast milk (Weller and Hanshaw, 1962). The shedding of virus can occur intermittently without any detectable signs or symptoms. Human cytomegalovirus (CMV) is a ubiquitous opportunistic pathogen (Gardella, 2008).

Clinically, CMV disease correlates with immune suppression, in which severe presentations are evident in neonates, persons with AIDS and other immune suppressed patient groups (Pass, 2001). CMV infection of neonates is associated with deafness, mental retardation, and mortality whereas AIDS patients often suffer a blinding CMV retinitis as well as pneumonia and gastrointestinal inflammation (Stagno and Witley, 1985). In organ transplant recipients, a patient group hard hit by CMV infection, disease is associated with an increased frequency of graft rejection and is a major cause of post-transplant infection. The varied array of clinical disease correlates with the exceptionally broad tropism of this virus. Indeed histological analysis of autopsy tissues obtained from patients with CMV disease has demonstrated infected cells in virtually all organs (Jarvis and Nelson, 2002). CMV has been linked with several forms of cancer such as colon, breast, and prostate. Previously CMV was regarded as having an oncomodulatory role in cancers by infecting tumor cells and modulating their malignant properties. It was hypothesized that tumor cells provided a

genetic environment that allowed CMV to exert its oncomodulatory effects. Furthermore, epidemiological and pathological studies suggest a strong link between CMV and atherosclerosis (Saffert *et al*., 2010). A potential mechanism for CMV in the pathogenesis of atherosclerosis involves the reactivation of virus followed by virus induced enhancement of vascular inflammation and damage through smooth cell proliferation uptake of low-density lipoproteins and narrowing of the vessel lumen (Arski *et al*,. 2007).

HCMV is the most common virus transmitted from infected pregnant mother to child. Approximately one-third of women who have a primary HCMV infection during pregnancy pass the virus on to the neonate. Thus approximately one in 150 children is born with congenital HCMV infections. Congenital HCMV infection is defined by detection of the virus in the newborn's urine, blood or saliva within three weeks of birth. Children with congenital HCMV present with small body size, jaundice, petechiae, hypotonia and hepatosplenomegaly (Blaho, 2010).

1.2.6.1. Transmission of CMV

Human Cytomegalovirus (HCMV) infections are found in all socioeconomic groups throughout all geographic locations in the world including both sexes (Ross *et al*., 2006). The persistence of a large reservoir of CMV in human populations in the absence of any known non-human vectors represents a remarkably successful form of parasitism. This success is because of the lengthy persistence of viral excretion after primary infection, the multiple sites of viral excretion, the intermittent reactivation and excretion of virus, and the relatively indolent course of infection, which allows the immunocompentent host to live indefinitely with the virus, Direct or indirect horizontal transmission occurs primarily by contact with

saliva, urine, cervical or vaginal secretions, semen, breast milk, or blood (Stagno *et al*., 1975).

Close or intimate person to person contact usually is necessary for viral transmission. Accordingly sexual transmission, breastfeeding and use of day care have been implicated in the spread of CMV. Seropositivity is higher among persons with multiple sexual partners and histories of sexually transmitted diseases (Chandler *et al*., 1985). Breastfeeding is a potential source of transmission to the newborn because seropositive women can secrete the virus in breast milk (Stagno and Cloud, 1994). Day care centers contribute to the spread of CMV by promoting close contact of children; many of them are excreting the virus. Toddlers in particular are a key source of infection (Pass *et al.*, 1982). Adler and his coworkers in 1986 found 20% to 40% of toddlers attending a day care facility to be excreting CMV (Adler et al., 1986). Horizontal transmission of CMV from child to child occurs by transfer of virus in saliva on hands and toys (Hutto *et al*., 1986).

Cytomegalovirus (CMV) is capable of vertical transmission from mother to infant before during or after birth. CMV may persist in a latent state but under unfavorable conditions, is capable of causing a variety of pathological processes. Cytomegalovirus transmission also occurs horizontally by direct person to person contact with virus containing bodily secretions and by transfusions of blood, leukocytes and platelets from infected donors (Pickering *et al.*, 2009).

1.2.6.2. Transmission of CMV in pregnancy

Cytomegalovirus (CMV) infection during pregnancy is more complex than other infections, due to virus reactivation during the child bearing age and be transmitted to the fetus in spite of maternal immunity (Mukundan *et al*., 1977). Various ways of transmitting the virus to the fetus have been suggested, whereas the hematogenious spreading across the placenta with subsequent infection of placental and amniotic tissue seems to be the most common transmission way (Pass and Boppona, 1999).

Trans placental transfers of virus in utero and fetal exposure to CMV secreted in the vagina during passage through the birth canal at delivery are the major modes of vertical transmission (Brown and Abernathy, 1998). In contrast to *in utero* (congenital) infection, per partum infection (either intra partum or postpartum) does not pose serious harm to the development of the neonate except in babies weighing less than 1500 gm. (Yeager *et al*., 1983).

HCMV is a member of the TORCHES group (Toxoplasma Gondi, Rubella virus, Cytomegalovirus, Herpes simplex virus) of pathogens that can cross the placenta as illustrated in Figure (1-4) (Varada, 2014).

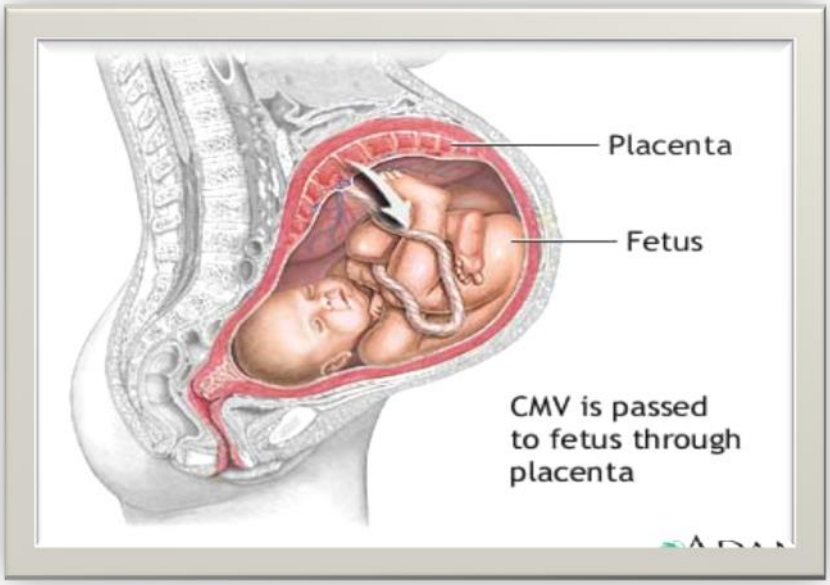


Figure (1 - 4): HCMV is passed from mother to child through placenta. (Varada, 2014).

1.2.7. Symptoms of CMV

When a person is infected with CMV any time after birth it is known as an acquired infection. In normally healthy children and adults CMV infection is usually not a concern. Ninety percent of the time it will not produce any signs or symptoms of infection. However, occasionally a flu-like or mononucleosis type of illness may occur and produce symptoms such as spiking fever to 39-40c are not uncommon and can last for more than two weeks , sore throat, fatigue and swollen glands (Nesmith and Pass,1995).

In persons with a weakened immune system, such as patients who are infected with HIV, organ/bone marrow transplant recipients, chemotherapy/ radiation patients, and people on steroid therapy, the signs and symptoms of CMV infection can be serious (Gardella, 2008). Signs and symptoms can occur when an old CMV infection reactivates or when the person catches

the virus for the first time. CMV infection in people with a weakened immune system puts them at risk for pneumonia, retinitis (an infection of the eye that can cause blindness), hepatitis (inflammation of the liver), esophagitis and colitis (gastrointestinal diseases), meningoencephalitis (an infection of the brain and the fluid that surrounds it), and even death (Duff, 2010) .

1.2.8.1. Diagnosis of CMV

The clinical diagnosis of primary Cytomegalovirus (CMV) infection is unreliable because it is asymptomatic in 90% of individuals and clinical signs when present are often nonspecific**.** However, persons who are been infected with CMV develop antibodies to the virus and these antibodies persist in the body for the lifetime of that individual (Chakravarti *et al*., 2009). A number of laboratory tests that detect these antibodies to CMV have been developed to determine if infection has occurred and are widely available from commercial laboratories. In addition, the virus can be cultured from specimens obtained from urine, throat swabs, bronchial lavages and tissue samples to detect active infection; unfortunately, viral culture is difficult, expensive, and time consuming taking up to 4 weeks for definitive results (Lazzarotto *et al*., 1999).

The Enzyme linked immunosorbent assay (ELISA) is the most commonly available Serologic diagnosis of CMV relies on the identification of patterns of immunoglobulin M (IgM) and IgG consistent with primary infection. The presence of IgM, the initial antibody response to infection, provides evidence of primary infection but must be interpreted with caution because anti-CMV IgM can be detected for up to 2 years in 5% of individuals after primary infection(Rajaii *et al*., 2009). Reinfection

and reactivation of virus is associated with a positive IgM and/or a rise in complement fixing antibody titer in approximately 30% of cases. Occasionally, biologic false positives can occur from cross reactivity with antibodies related to Epstein Barr virus (EBV) (Rawlinson, 1999). On average detection of CMV specific IgM antibody by ELISA has a sensitivity of only 75% for primary infection, this leaves 25% of women with primary CMV without detectable levels of IgM and a false negative result (Stagno *et al*., 1985).

Diagnosis of primary maternal cytomegalovirus (CMV) infection in pregnancy should be based on de-novo appearance of virus specific IgG in the serum of a pregnant woman who was previously seronegative or on detection of specific IgM antibody associated with low IgG avidity. The diagnosis of secondary infection should be based on a significant rise of IgG antibody titer with or without the presence of IgM and high IgG avidity. In case benefit ratio is different because of the low transmission rate (Yinon *et al*., 2010).

The diagnosis of fetal infection can be made by detection of CMV DNA in amniotic fluid by polymerase chain reaction (PCR), while a positive result usually indicates a fetal infection, in rare instance traces of DNA in the amniotic fluid may come from maternal sources (Guerra *et al*., 2000). Nucleic acid amplification by polymerase chain reaction (PCR) is the newest and most promising rapid method to detect virus, this method requires specific primers complementary to DNA sequences on either side of a target CMV DNA segment, the PCR results were interpreted by the presence or absence of specific bands of amplified gene on 2% agarose (Sam book, 2012). A single gene copy may be amplified up to 1 million fold (Pillay and Griffiths, 1992). PCR has a sensitivity of 80% to 100%

when compared with that of culture, however this high sensitivity can lead to false-positive results based on the amplification of extraneous viral contaminants or defective, non-infectious viral particles. PCR also can be used to quantify levels of virus (Rawlinson, 1999). PCR has been used successfully to detect CMV DNA in a variety of clinical specimens from organ transplant recipients, patients with AIDS, and infant with congenital infection (Kazufumi *et al*., 2012).

1.2.8.2. Laboratory Diagnosis of CMV

The advantages and limitations of laboratory tests used to diagnose CMV are provided in Table (1-1) **(**Rawlinson, 1999). Laboratory studies are helpful adjuncts to clinical findings but unfortunately may not conclusively discriminate between primary and recurrent infection. Laboratory diagnosis of CMV infection relies on serologic evidence of infection and direct identification of CMV (Rajaii *et al*., 2009). Discrimination of primary from recurrent CMV is based on maternal CMV specific serologies because isolation of the virus from urine, throat, or genital secretions can occur during both primary and recurrent disease. Determining the role of CMV in the clinical setting is complicated because many adults without a history of symptomatic disease are seropositive for CMV immunoglobulin G (IgG) and intermittent shedding of CMV can occur for years after the primary infection and bear no relation to illness. Neither the presence of IgG antibody nor excretion of the virus defines recent infection. Thus, a combination of laboratory tests, interpreted within the clinical context, is needed for an accurate diagnosis (Gardella, 2008).

Table (1-1): Tests used to detect Cytomegalovirus Infection (Rawlinson, 1999).

Test	Specimes	Use	Limitatios
Antigen detection			
Viral culture	Urine	Virus detection	3—4 wk for results
	Blood	Virus for further study	
	Sterile tissue		
	Amniotic fluid		
Direct immunofluorescence	Urine	Rapid detection of virus (48 hr)	Limited culture still necessary
	Blood		
	Amniotic fluid		
Nucleic acid amplification	Urine	Rapid detection of virus	Expensive
	Blood	Quantification of viral load	False-positives due to contamination
	CSF	Virus strain typing	
	Amniotic fluid		
Antibody detection			
IgG	Serum	Demonstration ofprevious infection	Seroconversion takes 2-3wk,requires2 specimens
		Demonstration of recent infection by seroconverson	
IgM	Serum	Demonstration of acute infection	Seropositive for up to 2 yr after acute infection

1.2.9. Treatment of CMV:

There is no vaccine available to prevent HCMV because the virus has evolved to possess an elaborate system of immune evasion strategies. Efforts to develop HCMV vaccine must focus on stimulating both the innate and adaptive immune system in order to be successful. Antiviral agents that inhibit CMV viral replication exist including ganciclovir, valganciclovir (the prodrug of ganciclovir), foscarnet, and cidofovir (Arski *et al*., 2007).

The primary mechanism of action of ganciclovir /valganciclovir against CMV is through the inhibition of the replication of viral DNA by ganciclovir-5′-triphosphate, which includes a selective and potent inhibition of the viral DNA polymerase (Matthews and Boehme, 1988). Foscarnet by comparison interferes with the exchange of pyrophosphate from deoxynucleoside triphosphate during viral replication by binding to a site on the CMV DNA polymerase (Crumpacker, 1992).

Similarly, cidofovir inhibits CMV DNA synthesis by DNA chain termination following incorporation of two consecutive cidofovir molecules at the 3' end of the DNA chain (De clercq *et al*., 2003). Nonetheless, the antiviral agents commonly used to treat CMV infections suffer from high hematologic, renal and neutropenia toxicity, low bioavailability and the development of drug resistant virus strains. The best way to prevent HCMV transmission is through behavior modification which emphasizes hygiene (Biron, 2006).

1.2.10 Prevention of CMV

In the absence of a suitable vaccine, the only measure available at this time to prevent CMV infection is to avoid exposure. CMV which is not

very contagious, and horizontal transmission, requires close contact with infected secretions or occasionally fomites. The principal sources of exposure for women of childbearing age are sexual contacts and children secreting the virus (Yow, 1989). Pregnant women also should be advised to avoid close contact with individuals likely to shed CMV such as adults with symptoms consistent with mononucleosis and toddlers attending group day care (Adler *et al*., 1998).

1.2.11. Toll – Like Receptor (TLR)

Toll like receptors (TLRs) are ancient conserved pathogen sensors, which are well appreciated as the activators of signal transduction pathways that lead to the expression of antimicrobial/antiviral genes and the induction of inflammatory cytokines (Akira, 2001). Elven mammalian TLRs have been identified and they are predominantly expressed on phagocytic cells such as dendritic cells and macrophages, however, most cells, express at least a subset of TLRs. TLRs detects microorganisms on the basis of unique molecular structure termed pathogen-associated molecular pattern (PAMPs) (Schwandner *et al*., 1999).

Viral genomic nucleic acids are the major class of PAMP. TLR3 (dsRNA), TLR7 (ssRNA), TLR8 (ssRNA) and TLR9 (CpG DNA) signal from the endosome where degradation of virus particles exposes the viral genome for detection by this panel of TLRs (Diebold *et al*., 2004). The common theme arising from the studies of viral systems is that a viral envelope glycoprotein is the specific molecular trigger for TLR responsiveness without a need for viral gene expression. However, TLRs and their respective envelope glycoprotein trigger generally can be coimmunoprecipitated indicating a physical association between the two

molecules and suggesting that entry and innate sensing events are somehow coordinated (Compton, 2003).

TLR2 identification, molecular characterization was first published in 1988 together with TLR1, TLR3, TLR4 and TLR5 (Rock *et al*., 1998). More than decade of extensive research has demonstrated the importance of TLR2 in the vertebrate immunity. This receptor was the only TLR described so far to form functional heterodimer with more than two other types of TLRs. TLR2 also interacts with a large number of non TLR molecules allowing for recognition of a great number and variety of PAMPs. This diversity comprises different types of molecules from all microbial phyla including viruses, fungus, bacteria, and parasites (Zahringer *et al*., 2008).

In particular TLR2 recognizes HCMV in a replication independent manner. The stimulation of TLR2 results in the activation of NF-kB and the production of inflammatory cytokines (Compton *et al*., 2003), dendritic cell maturation, up regulation of immune co-stimulatory molecules, and the production of type 1 IFN (Wagner, 2004). HCMV displays as many as 12 envelope glycoproteins, four of which are required for entry. Glycoprotein B (gp B) works in concert with a tripartite complex comprised of gp H (gH), gp L (gL) and gp O (gO) to mediate the binding and entry of HCMV virons into host cells (Spear and Long Necker, 2003). HCMV gB and gH activate TLR2 and associate with TLR1 and TLR2. Abs against gB and gH inhibit the inflammatory cytokine response to HCMV infection in permissive human fibroblasts and both gB and gH co-immunoprecipitate with TLR2 and TLR1 indicating that the functional sensor for HCMV is a TLR2/TLR1 heterodimer (Boehme *et al*., 2006). The presence of functional polymorphisms in TLR2 would impede this immune response and

clinically lead to higher levels of CMV replication and symptomatic CMV disease (Compton *et al*., 2003).

1.2.12. Miscarriage

A miscarriage is the spontaneous loss of a pregnancy between conception and 24 weeks into pregnancy. There are evidences which suggest that CMV will lead to complicated pregnancies. CMV is associated significantly with more than 70% of abortion cases (Goodrich *et al*., 2004). Furthermore 60% of pregnant women reported during first gestation trimester and begin to decline significantly in the late gestation for virus (Pass *et al*., 2006), whereas approximately 50% of the viral infections and their complication were identified in women after losing a pregnancy. It has been reported that the risk of fetal damage is greater if the primary infection occurs during the first trimester of pregnancy (Faiza *et al*., 2013). Primary CMV infection has been found to be more prevalent in pregnant women than non-pregnant; this difference may be attributed to the susceptibility of seronegative women at the onset of pregnancy to the first CMV infection (Stagno *et al*., 1985), the seroprevalence of CMV infection in pregnant women increased with age. The role of infection has been intensely investigated during the past decades and viruses in particular CMV which is under more attention since they can produce chronic recurrent intrauterine infections. Possible mechanisms are suggested as production of toxic metabolites, fetal, placental or chronic endometrial infection and chorio amnionitis (Nigro and Adler, 2011).

Chapter Two

Materials

And

Methods

2. Materials and Methods

2.1. Materials

2.1.1. Apparatus

The apparatus used in this study are shown below:

Apparatus	**Company / country**
Autoclave	HIRAYAMA
Electrophoresis equipment unit	JUNY1-JY200C
ELISA Incubator	Pasteur/ France
ELISA Printer	Epson LX-300 Italy
ELISA Reader	Bio-Rad / U.S.A
ELISA Washer	Human Germany
Gel –documentation	Bio-Red / U.S.A
Microcentrifuge	MIKRO120 - Hettich
Microwave	LG / Korea
Nanodrop-spectrophotometer	Techne / U.K.
Oven	Sanyo / Japan
PCR master cycler gradient	Techne / U.K.
Real time – PCR	Applied biosynthesis/ U.S.A.
Sensitive balance	Mettlev / Switzerland
U.V transilliuminator	Flowgen / U.K
Vortex	Scientific industries / U.S.A.
Water bath	Memmert / Germany

2.1.2. Chemicals

The chemicals are used in this study are shown below:

Item	Company	country
Agarose	Sigma	U.S.A.
EDTA		
Ethidium bromide	Promega	
Green Master Mix		
TBE		

2.1.3. Kits

The kits are used in this study are shown below:

kit	Company	country
DNA Extraction Mini -Prep System	Promega	U.S.A
Real -Time PCR	Applied biosystem	U.S.A
CMV IgM ELISA test kit	Biokit	Barcelona, spain.
CMV IgG ELISA test kit	Biokit	Barcelona, spain

2.1. 4. Primers

Sequences of the primers used in this study:

Name	Oligonucleotides	Tm^0 C	GC%	Sequence (5'-3')	reference	Molecular size
gB	Forward primer	57.9	45.8	AGGTCTTC AAGGAACT CAGCAAGA	(Novak, *et al*., 2011)	72 bp
gB	Reverse primer	54.3	42.8	CGGCAATC GGTTTGTT GTAAA		
IE2	Forward primer	56.9	55.00	GAGCCCGA CTTTACCA TCCA	(Novak, *et al*., 2011)	53 bp
IE2	Reverse primer	57.8	68.7	CAGCCGGC GGTATCGA		
TLR2	Forward primer	57.6	54.00	CTGTGCTC TGTTCCTG CTGATC	NCBI	419 bp
TLR2	Reverse primer	55.2	53.00	TGAGAATG GCAGCATC ATTGTT		
ILT2	Forward primer	53.8	55.00	TGAGAGTG GTGGGAAT GCAC	NCBI	285 bp
ILT2	Reverse primer	51.7	50.00	AACCCAAC GCAATTTC CAGC		
Real -Time PCR sequence		57.3	64.6	FAM 5- AAC CCG TCA GCC ATT CTC TCG-3 TAMRA	(Novak, *et al*., 2011)	72 bp

2.1.5. Solutions

2.1.5.1. Loading buffer

Tris – base pH 8.0	0.025 M
Glycerol	50%
Bromocresol purple	0.25%

2.1.5.2. DNA ladder (100 bp) (FAVORGEN BIOTECH CORP.)

DNA ladder 100- 1.500 bp containing 11 fragments supplied in storage buffer 10mM Tris-HCL pH (7.5), 0.1 mM EDTA.

2.1.5.3. DNA ladder (50 bp) (FAVORGEN BIOTECH CORP.)

DNA ladder 50- 500 bp containing 10 fragments supplied in storage buffer 10mM Tris-HCL pH (8.0), 10 mM EDTA

2.2. Methods

2.2.1. Collection of blood samples

The Samples were obtained from infertility clinic of Kamal Al-Sammaraee hospital. The collection period extended from November 2013 to March 2014. One hundred (100) women suffering from CMV infection and miscarriage, 50 healthy women as a control (healthy), their ages ranged between (20-40) years. Samples were subjected to centrifugation at 2000 rpm for 10min.The serum was separated and stored at -20^{0}C. All Samples were subjected for HCMV antibodies estimation using ELISA techniques. In case of blood with EDTA, it was stored at -20^{0}C until used for DNA extraction.

2.2.2 Sterilization methods

- Autoclaving: Buffers and solutions were sterilized by pressure vessel (autoclave) at 121 ^{0}C and 15 bar for 15 minutes.

- Dry heat: A laboratory oven was used for glassware sterilization. Glassware was placed in the oven at 200 ^{0}C for 2 hours.

2.2.3. Detection of CMV by ELISA test:

2.2.3.1. IgM Assay

1- Well strips12×8 coated with IgG (rabbit monoclonal) against humans IgM include individually separable wells.

2- IgM to CMV from sample or control.

3- CMV antigen.

4- Enzyme tracer: IgG to CMV (rabbit monoclonal) conjugated to Horseradish Peroxidase (HRP).The presence of specific IgM to CMV allows the tracer to bind to the solid phase through the presence of CMV antigen. The Enzyme activity is thus proportional to the concentration of IgM to CMV present in samples or controls.

2.2.3.2. IgG Assay

1- Well coated with CMV.

2- IgG to CMV from sample or calibrator.

3- Enzyme tracer: IgG to CMV (mouse monoclonal) against human IgG conjugated to Horseradish Peroxidase (HRP).The presence of specific IgG to CMV allows the tracer to bind to the solid phase. The Enzyme activity is there for proportional to the concentration of IgG to

CMV presence in samples or Calibrators. Enzyme activity is measured by adding colorless Chromogen substrate solution. The Enzyme action on Chromogen substrate produces a color which is measured with a photometer.

2.2.3.3. Assay Procedure

All assay steps were performed in the order given and without any appreciable delays between the steps. Numbers of sufficient wells to run one replicated of the negative control with one replicated of the positive control with each run of samples separately. Controls must be run with each plate of patient specimens. Controls and samples were subjected to the same process and incubation time. Clean, disposable tips should be used for dispensing each control and sample. After all these preparations, the steps should be followed serially including:

1- The number of the strips required for the test should be used only, reserve nine wells for blank and controls.one hundred µl of each control and diluted sample at aratio(1:100) was pipetted to corresponding wells.

2- The micro plated was covered with an adhesive foil and incubates for 1 hour at 37^0C.

3- The adhesive seal was removed and aspirate the contents of the well and fill them completely with diluted washing solution, the process of aspiration and washing was repeated 4 more time and every well should be socked for at least 15 minutes before the next aspiration cycle, then the micro plated was blotted with the absorbent tissue to remove any excess liquid from the well.

4- The working conjugated was prepared during the last 15 minutes of the incubation as follows: 10 ml of dilution buffer, 1o µl of conjugate and 10 µl of control antigen were mixed well.

5- Amount of 100 µl of diluted conjugate was added except the one for substrate blank, avoid any bubbles upon dilution.

6- Then the plate was covered with an adhesive seal, and incubate 1 hour at 37^0C.

7- The adhesive seal was removed and washing process repeated as in step 3.

8- Amount of 100 µl of substrate-TB was added to each well including blank well, then the plate incubated for 30 minutes at room temperature in dark.

9- The reaction should stopped by adding 100 µl of stopping solution, then the plate transferred to the reader after its blanking at 450 nm and absorbance of each well within 10 minutes.

10- The cut-off value which include three values:

A- Positive: ratio absorbance ≥1.1.

B- Negative: ratio absorbance < 0.9.

C- Equivocal: ratio absorbance ≥ 0.9 <1.1.

2.2.4. DNA Extraction of CMV from blood samples of miscarriage women:

2.2.4.1. Procedure:

The Extraction was briefly carried out as follow:

1. The blood sample was mixed thoroughly for at least 10 minutes at room temperature.

2. A liquate of 20 µl of proteinase K solution was added into micro centrifuge tube.

3. A liquate of 200 µl of blood was added to the proteinase K solution and mixed briefly.

4. A liquate of 200 µl of cell lysis buffer was added to the tube and mixed for at least 10 seconds by vortexing ,then incubated at 56^0C for 10 minutes.

5. A liquate of 250 µl of Binding Buffer was added to the tube and mixed for 10 seconds by vortexing.

6. The sample mixture was transferred to a filter column set and centrifuged at maximum speed for 1 minute.

7. The collection tube containing the supernatant was discarded and replaced by a fresh collection tube.

8. A liquate of 500 µl of column wash solution was added to the column and centrifuged for 3minute at maximum speed and the supernatant was discarded.

9. A liquate of 50 µl of nuclease free water was added to the column and centrifuged for1minute at maximum speed.

10. The column was discarded and the eluted was stored at -20^0C.

2.2.5. Estimation of extracted DNA concentration and purity

The nanodrop (Thermo NANODROP 2000C) was used to estimation the concatenation and purity of the extracted DNA.

2.2.6. Molecular detection of CMV:

2.2.6.1. Procedure:

1. The Go Taq® Master Mix was thawed at room temperature. The master mix was mixed by vortexing then it was spine briefly in a micro centrifuge.

2. The reaction mix was prepared by combining the following:

The required volume of each component was added according to the following order:

Components	Volume	Concentration
Go Taq® Green Master Mix	12.5 µl	1x
Forward primer	0.5 µl	0.2 µM
Reverse primer	0.5 µl	0.2 µM
DNA template	5 µl	< 250 µg
Nuclease free water	6.5 µl	-
Final volume	25 µl	

The reaction was placed in thermal cycler using the protocol shown in table below:

PCR Amplification using gB primer

The PCR instrument was programed for the following cycling protocol:

Thermal cycler protocol	No. of cycle	Temperature –time
Initial Denaturation	1 cycle	95^0C for 3 min.
Denaturation	30cycle	95^0C for 30 sec.
Annealing		64^0C for 30 sec.
Extension		72^0C for 30 sec.
Final Extension	1 cycle	72^0 C for 10 min.

PCR Amplification using IE2 primer

The PCR instrument was programed for the following cycling protocol:

Thermal cycler protocol	No. of cycle	Temperature – time
Initial Denaturation	1 cycle	95^0C for 3 min.
Denaturation	30cycle	95^0C for 30 sec.
Annealing		64^0C for 30 sec.
Extension		72^0C for 30 sec.
Final Extension	1 cycle	72^0 C for 10 min.

PCR Amplification using TLR2 primer

The PCR instrument was programed for the following cycling protocol:

Thermal cycler protocol	No. of cycle	Temperature – time
Initial Denaturation	1 cycle	95^0C for 3 min.
Denaturation	30cycle	95^0C for 30 sec.
Annealing		55^0C for 30 sec.
Extension		72^0C for 30 sec.
Final Extension	1 cycle	72^0 C for 10 min.

PCR Amplification using ILT2primer

The PCR instrument was programed for the following cycling protocol:

Thermal cycler protocol	No. of cycle	Temperature – time
Initial Denaturation	1 cycle	95^0C for 3 min.
Denaturation	30cycle	95^0C for 30 sec.
Annealing		61^0C for 30 sec.
Extension		72^0C for 30 sec.
Final Extension	1 cycle	72^0 C for 10 min.

2.2.7. Analysis of PCR product by agarose gel electrophoresis (Maniatis *et al*., 1982)

1. Agarose gel at percentage (2%) was prepared by mixing: 100 ml of 0.5X TBE buffer and 2 gm agarose in glass bottle. A glass bottle was heated until the agarose was dissolved.

2.This solution was cooled to 70^oC, 5 µl ethidium bromides was added from stock solution and mixed thoroughly.

3.The clean glass mold (17X12X4 cm) was set on a horizontal section of the bench. The comb was set in position 0.5-1.0 mm above the surface of mold so that a complete well was formed when agarose was added.

4.The warm agarose – solution was poured into the mold.

5.After the gel was completely set (20-30 min. at room temperature), the comp was carefully removed and the gel mounted in the

electrophoresis tank which contain previously small amount of 1X TBE buffer.

6.A volume of 600ml of 1X TBE was added to cover the gel to depth about 1mm.

7.A volume of 10 µl of the sample of DNA (PCR product) was added slowly into the slots of the submerged gel using an automatic micropipette.

8.A volume of 5 µl of DNA marker was mixed with 1 µl of loading buffer. The mixture was set slowly into the slots of the submerged gel using an automatic micropipette.

9.The gel was subjected to electrophoresis at 70 volts until the bromophenol blue tracking dye, migrated at least two-thirds of the way down the gel.

10. The gel was examined by ultraviolet light using UV transilluminator then photographed.

2.2.8. Real -Time PCR of CMV

2.2.8.1. Procedure:

1- TaqMan Universal Master Mix II (2x), template DNA, and 20x primer and probe solution were thawed on ice.

2- The reaction mix was Prepared according to the table.

Reaction Component	**Volume(µl) per sample**	**Final Concentration**
TaqMan Universal PCR Master Mix II	25	1x

20 X Primer\ Probe Mix1	2.5	0.4µM ST-Frt primer 0.4µM ST-Rrtprimer 0.15µM ST-P Probe
Nuclease free water	19.5	------
DNA	3	------
Total volume	50	------

3- The reaction Mix was mixed thoroughly and dispensed in to (47µl) PCR tubes or the wells of a PCR plate.

4- An aliquot of 3µl of template DNA was added to the wells.

5- The plate was covered with a Micro Amp® 96-well Optical Adhesive film.

6- The plate was centrifuged briefly to spin down the contents and eliminate air bubbles from solutions.

7- The applied biosystems Real- Time 7500 PCR System was Programed.

8- The PCR plates was Placed in the real –time cycler, and the cycling program was started.

9- Data analysis was performed.

Cycling conditions

The PCR instrument was programed for the following cycling protocol:

Thermal cycler protocol	**No. of cycle**	**Temperature –time**
Enzyme Activation	1 cycle	95^0C for 15 min.

Denaturation	45cycle	95^0C for 30 sec.
Annealing		64^0C for 30 sec.
Extension		72^0C for 30 sec.

2.2.9. Assessment of product and DNA Sequencing:

Before Sequencing, DNA concentration of PCR product was measured using Nano drop. Polymerase chain reaction products were sent to Sequencing. It was carried out at NICEM (National Instrumentation Center for Environmental Management) using their ABI3730XL instrument (Applied Biosystems, USA). Results were directly compared with Iraqi healthy control by using the program Mega 6.

2.2.10. Statistical Analysis

The Statistical Analysis differences were analyzed using SPSS program version 20. Differences between means were assessed by ANOVA. Acceptable level of significant was considered to be $P \leq 0.05$.

Chapter Three

Results

and

Discussion

3. Results and discussion

3.1. Distribution of the studied groups

The cytomegalovirus (CMV) is considered one of the opportunistic viruses with a worldwide distribution that can infect human at any stage of life (Gold and Nankervis, 2007) then the virus became dormant (Flowler and Boppana, 2006) and it is lifetime latency after primary infection and reactivation of the latent virus can re-occur in infected individuals at any time (Ziyaeyan *et al*., 2007). A majority of these infections are a symptomatic as others and they are difficult to diagnose clinically (Sen *et al*., 2012). IgG and IgM antibodies to cytomegalovirus can be considered as an easy tool for selecting patients who are at risk of cytomegalovirus infection. IgG was reflected the previous infection, presence of it doesn't prevent the reinfection or re activation but may reduce the severity of pathogenesis. While IgM immunoglobulin was considered as evidence of recent or acute infection which is formed immediately after infection and disappeared after short period 16-20 weeks (Goodrich *et al*., 2004). One hundred samples from infected women distributed as shown in figure (3-1) as (60) infertile and (40) miscarriage women.

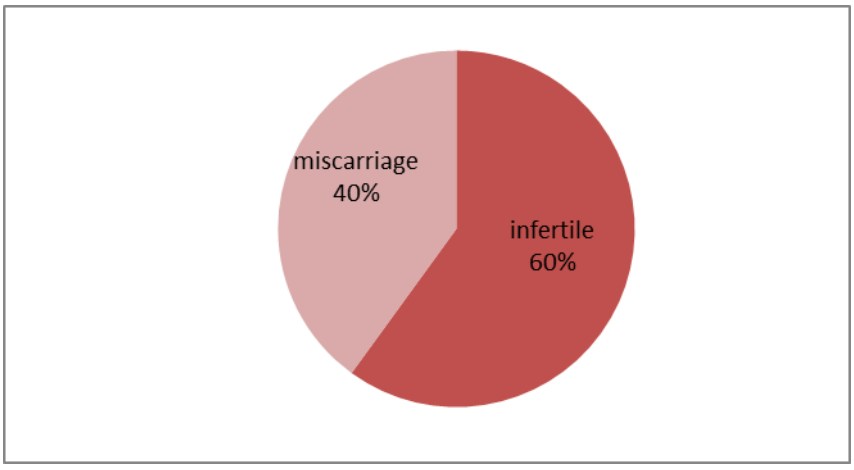


Figure (3-1): Percentage of miscarriage and infertile women.

Among the miscarriage, infertile and healthy women, the miscarriage women showed the highest percentage of seropositive to CMV for IgG (40%) and (25%) IgM compared to infertile women IgG (20%) and (15%) IgM while the control showed 5% for IgM and 3% for IgG as shown in figure (3-2).

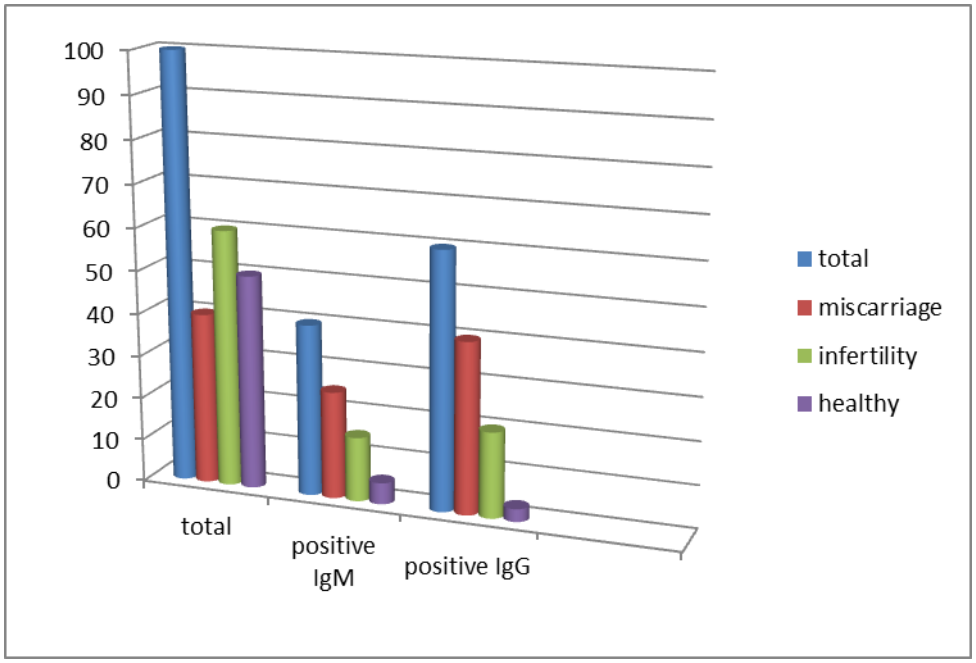


Figure (3-2): Seropositivity of IgG and IgM in miscarriage, infertile and healthy control women.

There is a significant differences between two groups of patients for CMV antibodies, these results is in accordance with Gaytant *et al* (2002) and Munro *et al* (2005), that studied the importance of virus infection to cause miscarriage. Yasir (2012) studied the importance of CMV on infertility also agreement with the study result.

3.2. Seroprevalence of CMV in the miscarriage groups according to age

The age of all patients' women ranged from (20 to 40) years old are shown in Table (3-1).

Table (3-1): Seroprevalence of CMV in miscarriage women according to age.

age	Total no.	Anti HCMV IgG		Anti HCMV IgM	
years	40	Positive%*	Negative%*	Positive%*	Negative%*
20-30	23	75	25	10	90
31-40	17	70	30	20	80

*$P \leq 0.05$.

The age (20-30) years old may be rearranged as the most age class which showed high prevalence of anti- HCMV antibodies IgG which represent 75% , while the age classes (31-40) years old showed the least prevalence of anti- HCMV IgG antibodies 70% without significant difference as shown in figure (3-3). This result considered to be comparable with Yasir (2012), who showed higher percentage of positively at ages (27-32) also Sotoodeh, (2009) who showed 94% of positively at ages (25-34), this is because the chance of pregnancy is higher in younger ages (20-30) years than (31-40) years. These results considered to be comparable with Mahdi *et al*., (2011), who showed that an increase in seropositive CMV IgG in relation with abortion and infection, this might be due to the effect of CMV on cellular metabolism and activation of other viruses that co-infect the cells inducing subclinical inflammation. Some studies found a higher risk of pregnancy loss with CMV infection (Griffiths and Baboonian, 1984) and other found high presence of CMV antigens in tissues from abortion (Spano *et al*., 2002), despite these reports on the role of CMV infection in

spontaneous pregnancy loss, the role of CMV infection in recurrent losses is less clear (Sherkat *et al*., 2014).

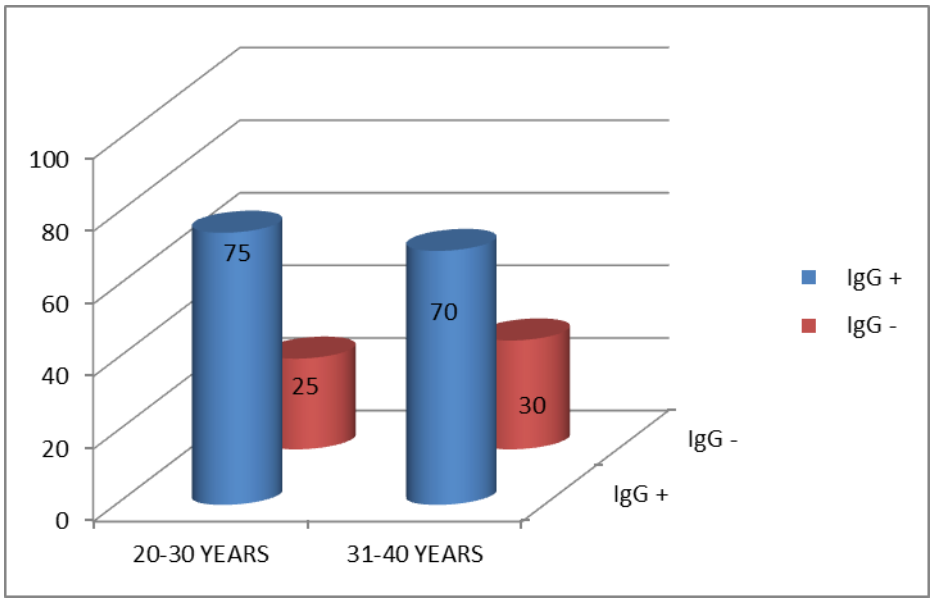


Figure (3-3): Serum IgG level in miscarriage women according to age.

For IgM level among miscarriage groups it was clear from figure (3-4) that the age (31-40) represent 20% and (20-30) years represent 10%.

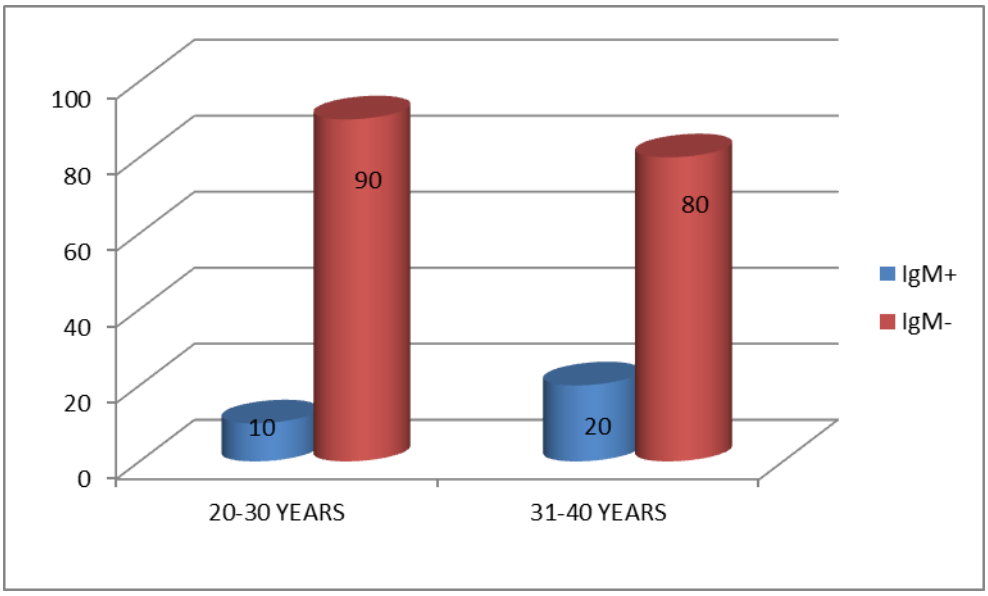


Figure (3-4): Serum IgM level in the miscarriage women according to age.

3.3. Seroprevalence of anti-CMV IgG and IgM among different age groups of infertile women

The sexual transmission of HCMV could be occurred among infertile couples this is assumed to be the most important route in adults (Francisse *et al*., 2009). The Seroprevalence of anti- HCMV IgG in infertile women showed that 50% are Seropositive at (20-30) years old and 25% IgM, while the largest age classes (31-40) years showed 40% and 35% respectively as shown in table (3-2) and figure (3-4A,B). These results considered to be comparable with Mahdi *et al.*, (2011), who showed that the frequency of CMV seropositivity increases rapidly between the ages of 15 and 30 years when sexual contact is most active.

Table (3-2): Seroprevalence of HCMV antibodies in infertile women in according to age:

Age	Total no.	Anti HCMV IgG		Anti HCMV IgM	
(years)	60	Positive %*	Negative*	Positive %*	Negative%*
20-30	26	50	50	25	75
31-40	34	40	60	35	65

*$P \leq 0.05$

3.4. Real time PCR

Real time PCR was rapid, sensitive and useful technique for diagnosing active disease and monitoring response to therapy (Prentice and Kho, 1997). The CMV primers were selected from highly conserved region of the major enveloped glycoprotein B (gB) and used probe labeled at the (5')

end with FAM and the (3') end with TAMRA**.** The results show amplification from the sixth cycle as shown in figure (3-5). This result agrees with Boppana *et al* (2005); in which detection of CMV DNA by real time PCR using the gB primer and probe FAM and TAMRA.

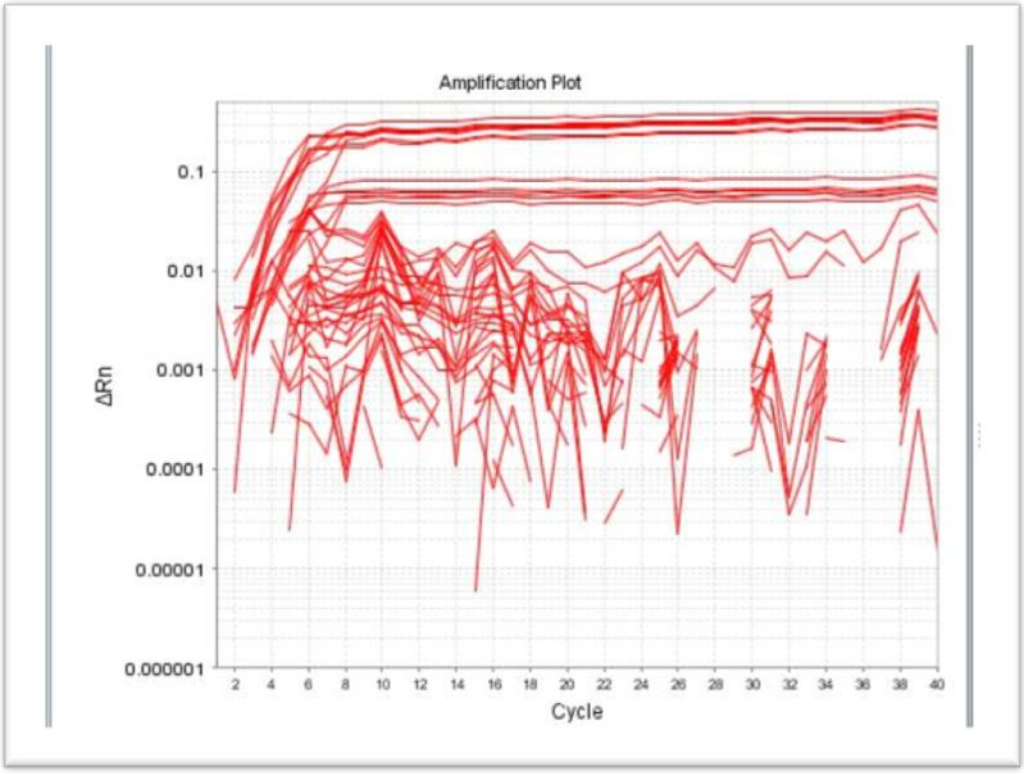


Figure (3-5): Amplification of gB cytomegalovirus gene in miscarriage women by Real time PCR.

3.5. Molecular detection of gB and IE2 genes of Cytomegalovirus by PCR technique

PCR amplification showed one band with molecular size 72bp was present in figure (3-6). Several comparative studies have demonstrated that glycoprotein B *(gB)* gene has high diagnostic sensitivity for CMV infection (Distefano *et al*., 2004) and has a largely conserved nucleotide sequence (Coyle *et al*., 2002). PCR used targets the conserved region that lies upstream of antigenic domain 1of the *gB* gene between base pairs 1541 and 1612 (Britt *et al*., 2005).

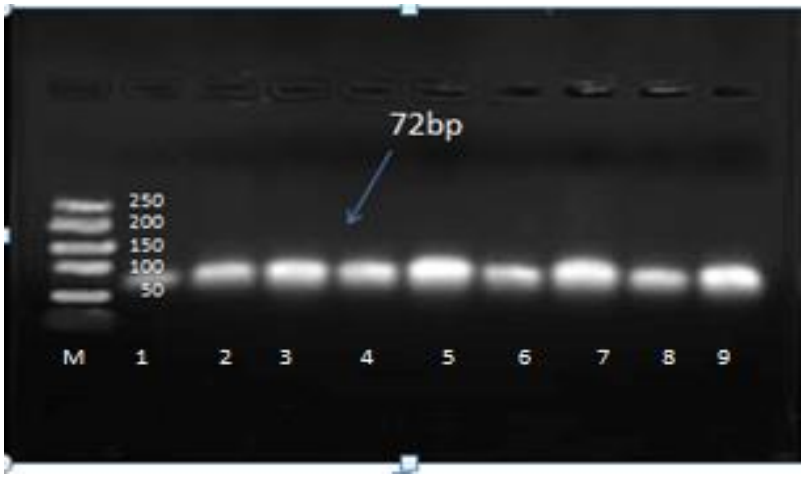


Figure (3-6): Gel electrophoresis for amplified *gB* gene, PCR detection of virus DNA in ethidium bromide stained (2% agarose gel, 10 minutes at 100 V and then lowered to 70 V, 50 minutes). Line M (50bp) for marker, 1-9 for miscarriage women.

PCR amplification for IE2 primer region showed the presence of one band with molecular size of 92bp figure (3-7):

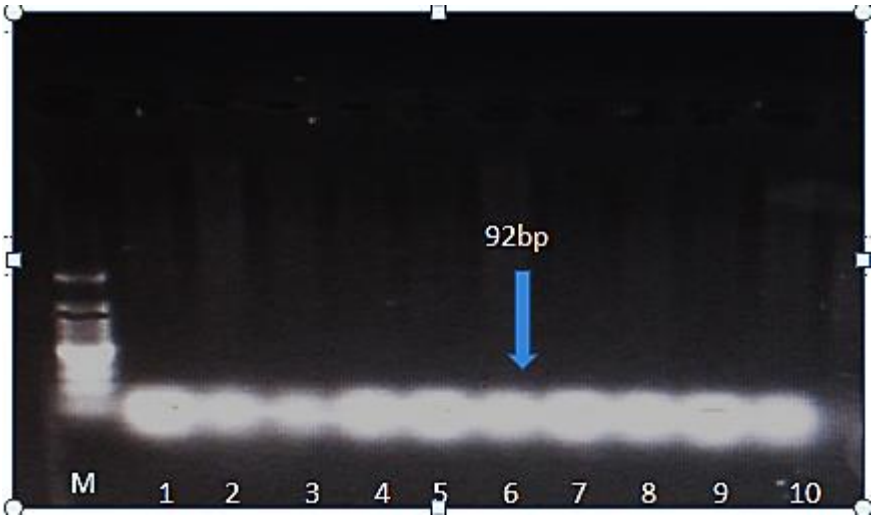


Figure (3-7): Gel electrophoresis for amplified *IE2* gene (2% agarose gel, 10 minutes at 100 V and hen lowered to 70 V, 50 minutes). PCR products visualized under U.V light after staining with ethidium bromide. M: 100 bp DNA marker; lane1, 2, 3, 4, 5, 6, 7, 8, 9, 10: for miscarriage women.

The immediate early 2 primer which is specific for exon 5 of the *IE2* gene of CMV, several comparative studies have demonstrated that this pair of primers has been evaluated for its specificity and sensitivity against CMV clinical isolates and other herpesviruses (Boppana *et al*., 2005).

3.6. Sequencing of *TLR2* genes

The human *TLR2* gene was located in chromosome 4 (Texereau *et al*., 2005). PCR amplified for TLR2 primer region showed one band with molecular size of 419 bp as shown in figure (3-8):

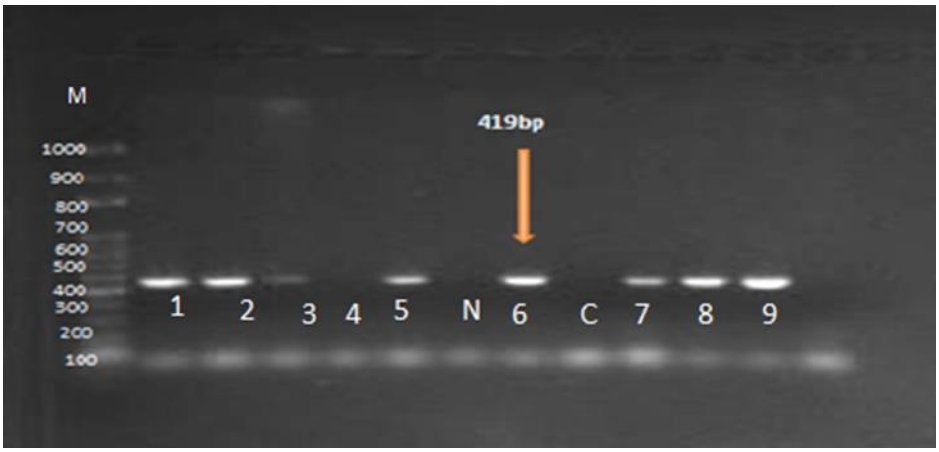


Figure (3-8): Gel electrophoresis for amplified *TLR2* gene, (2% agarose gel, 10 minutes at 100 V and hen lowered to 70 V, 50 minutes). PCR product visualized under U.V light after staining with ethidium bromide. M: 100 bp DNA marker; lane1,2,3,4,5,6,7,8,9: for miscarriage women, lanes C for healthy (control) and lane N for negative control .

Many experimental observations indicate that TLRs participate in innate immunity against CMV infection and in the control of CMV infection, through the release of antiviral cytokines and the regulation of adaptive immunity (Schleiss, 2013). The presence of functional polymorphisms in TLR2 would impede this immune response and clinically lead to higher

levels of CMV replication and a symptomatic CMV disease (Compton *et al*., 2003).

After successful amplification of *TLR2* gene good quality products were selected to be sequenced. Only 3 out of 10 samples were failed to be sequenced properly, the result showed no mutation at TLR2 only replacement of C instead of G in wild type as shown in figure (3-9).

Figure (3-9): Result of TLR2 sequencing in miscarriage women and control.

This result agrees with Previous studies showed this SNP to be absent in Asian population, such as Taiwan (Cheng *et al*., 2007). Pabst *et al*., (2009), who pointed that the TLR2 polymorphisms in chronic obstructive disease have no obvious effect on individual susceptibility to disease especially those Caucasian groups.

3.7. Molecular diagnosis for ILT2 Receptor

Human cytomegalovirus carries a gene *(UL18)* that is homologous to cellular major histocompatibility complex (MHC) class I genes. Like MHC class I molecules, the protein product of the *UL18* gene associates with 2-microglobulin and the stability of this complex depends on peptide loading (Browne *et al*., 1990). The receptor for *UL18* is ILT2, *UL18* protein binds to immunoglobulin-like transcript 2 (ILT2) an inhibitory receptor present on B cells, monocytes, dendritic cells, T cells and NK cells that also recognizes classical and non-classical MHC molecules (Colonna *et al.*, 1997). PCR amplified for ILT2 primer region showed one band with molecular size of 285bp as shown in figure (3-10):

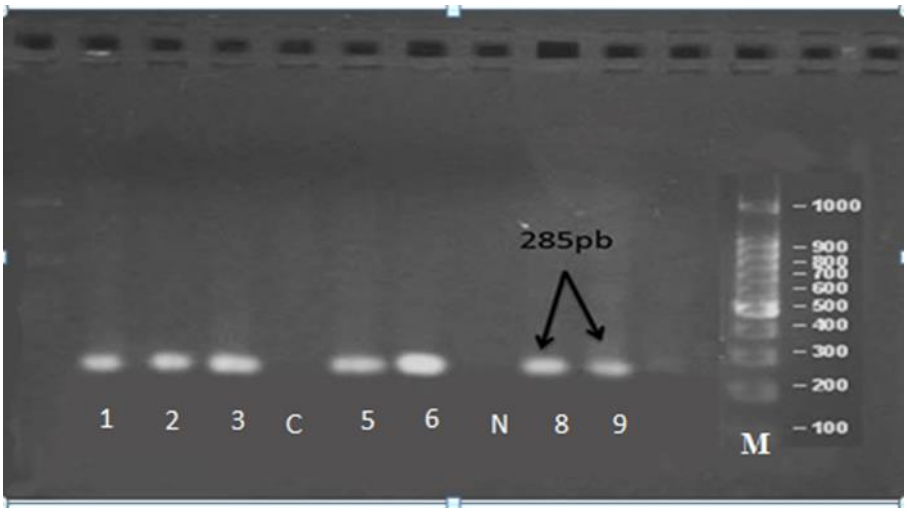


Figure (3-10): Gel electrophoresis for amplified ILT2 primer with ethidium bromide stained (2% agarose gel, 10 minutes at 100 V and then lowered to 70 V, 50 minutes). Line M (100bp) marker, 1,2,3,5,6,8,9 for patient, 4 for healthy (control) and lane 7 for negative control.

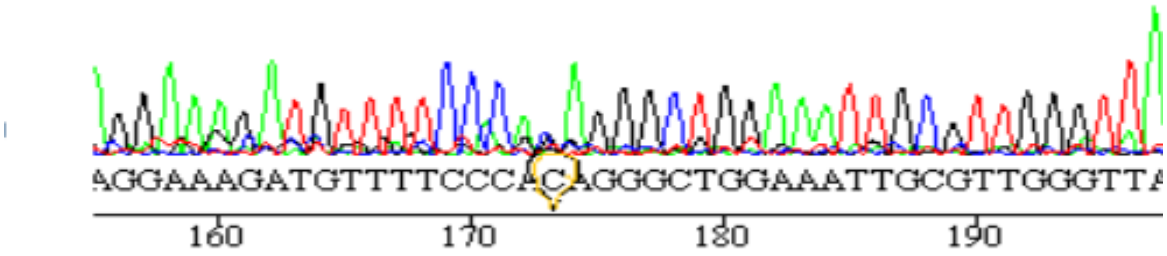
3.8. Detection mutation of CMV *ILT2* by sequencing:

A genomic fragment of *ILT2* gene was amplified by PCR. Ten subjects of Patients exhibited PCR products.

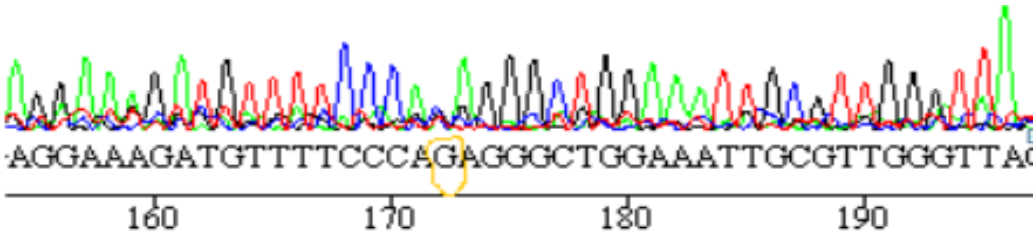
3.8.1. Type of mutation

The PCR products of ILT2 were screened by sequencing in CMV women. Results were directly compared with Iraqi healthy control by using the program Mega 6.

Control



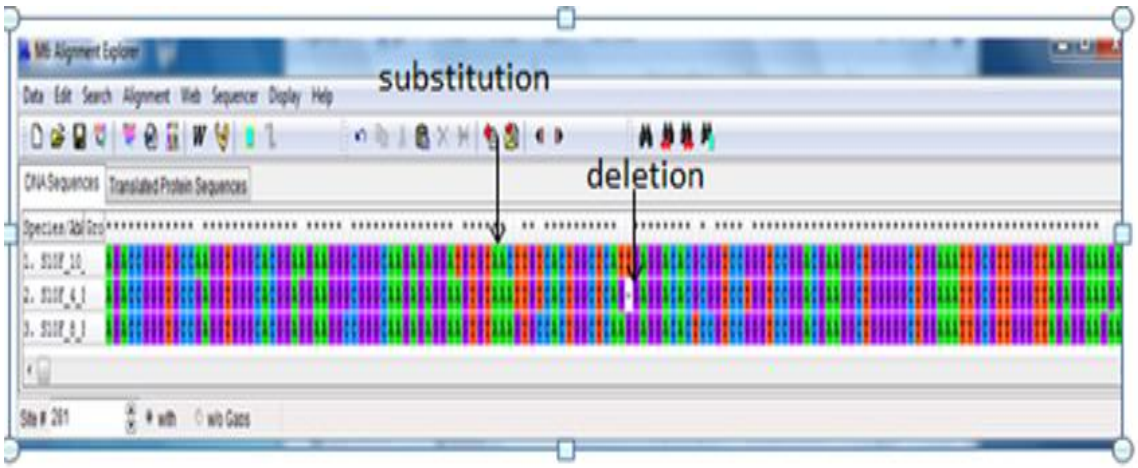
AGG\ AAA \GAT \GTT \TTC\ CCA\ CAG\ GGC\ TGG\ AAA\ TTG\ CGT\TGG



Miscarriage women

AGG\ AAA \GAT\ GTT\ TTC\ CCA\ GAG\ GGC\ TGG\ AAA\ TTG\ CGT\ TGG

Figure (3-11): Sequencing of *ILT2* gene in cytomegalovirus women and control.



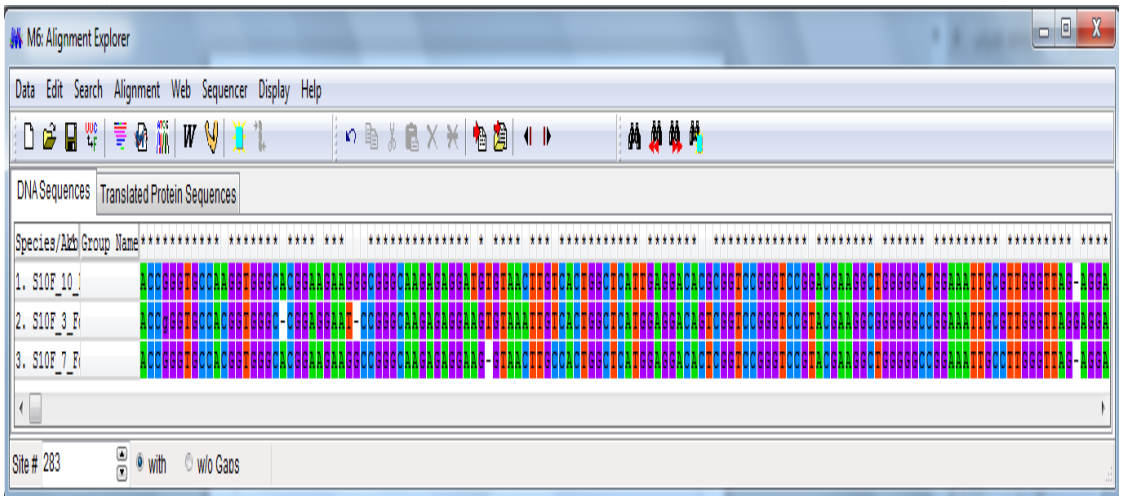


Figure (3-12): Point mutation in *ILT2* gene by the program Mega 6.

Table (3-3): Point mutations in *ILT2* gene in cytomegalovirus miscarriage women.

No. of patients samples	Wild type	Mutant type	Change in amino acids	site Of N.A.	Type of Mutation	Effect on translation
3	AAG	ACG	Lys-Thr	12	Substitution	missense
	GCA	GC -	Ala- deletion	20	Deletion	Frame shift
	AAG	AGG	Lys- Arg	25	Substitution	missense
	AAG	AAT	Lys- Asn	29	Substitution	missense
	GGC	- CC	Gly-deletion	30-31	Deletion-Substitution	Frame shift
	GAT	GAA	Asp- Glu	46	Substitution	missense
	CTT	ATT	Leu-lle	53	Substitution	missense
	TTG	TGG	Leu-Trp	69	Substitution	missense
	CGC	GTC	Arg-Val	77-78	Substitution	missense
	GAC	TAC	Asp-Tyr	92	Substitution	missense
	TGG	GGG	Trp-Gly	101	Substitution	Missense
7	AGG	CGG	Arg- Arg	12	Substitution	Silent
	GAT	GAA	Asp-Glu	46	Substitution	missense
	GTG	G - G	Val-Deletion	48	Deletion	Frame shift
	GTC	GCC	Val-Ala	57	Substitution	missense
	TTG	TGG	Leu- Trp	69	Substitution	missense
	CGC	GTC	Arg-Val	77-78	Substitution	missense
	GAC	TAC	Asp- Tyr	92	Substitution	missense

The current study utilized forwared primer for sequencing ,six samples out of 10 were compared with iraqi healthy control at the first (100) bp regions. Among them, four patients displayed point mutations of deletion and substitution, while others were normal since no mutations were detected. This result agrees with Mar Vale´s- *et al*. (2005) reported that the *UL18* gene varies significantly and amino acid substitutions were found between virus isolates. Cristina *et al*. (2006) reported that UL18 derived from HCMV strain show amino acids difference and point mutation.

Table (3-4): Point mutations in *ILT2* gene in cytomegalovirus miscarriage women.

No. of patients samples	Wild type	Mutant type	Change in amino acids	site Of Nucleic acid	Type of Mutation	Effect on translation
4	CAA	CGA	Gln- Arg	13	Substitution	missense
	AGA	AGG	Arg- Arg	29	Substitution	Silent
	TGT	AGT	Cys-Ser	48	Substitution	missense
	ACT	AAT	Thr-Asn	55	Substitution	missense
	ATT	AG -	Lle- deletion	71	Deletion	Frame shift
	GGG	TGG	Gly-Trp	87	Substitution	missense
8	CAA	CGA	Gln-Arg	13	Substitution	missense
	AGA	AGG	Arg-Arg	29	Substitution	Silent
	GCG	CCG	Ala-Pro	33	Substitution	missense
	TGT	AGT	Cys-Ser	48	Substitution	missense
	ACT	AAG	Thr-lys	55-56	Substitution	missense
	TGT	TGC	Cys-Cys	59	Substitution	Silent
	ATT	AAG	Lle-lys	70-71	Substitution	missense
	ACG	ACT	Thr-Thr	80	Substitution	Silent
	CGG	CCG	Arg-Pro	82	Substitution	Missense

3.8.2. Percentage of mutation in *ILT2* gene in miscarriage women

Analysis of the result obtained from sequencing four Iraqi patients exhibited the existence of much genetic alteration. Two types of mutations namely deletion and substitution were present. Table (3-5) shows the percentage of mutation types, that 86.67% was for substitution mutation and13.33% was for deletion mutation.

Table (3-5): Percentage of mutation type ILT2 gene in miscarriage women.

Type of Mutation	**Percentage%**
Substitution	86.67
Deletion	13.33

3.8.3. Effect of mutation:

The effect of mutation indicates that, there was a missense mutation that causing impact on phenotype leads to replacement in amino acids, this effect appear in current study with high level 72.7% as shown in Table (3-6) Silent mutations15.1% result when the codon in wild type coded for the same amino acids in mutant type. The deletion mutations lead to frame shift, which represented 12.2%.

Table (3-6): Effect of mutation on amino acids expression in *ILT2* gene.

Effect of Mutation	**Percentage %**
missense	72.7
Silent	15.1
Frame shift	12.2

Chapter Four

Conclusions and Recommendations

4. Conclusions and Recommendations.

4.1. Conclusions.

1- There is high prevalence of anti- HCMV antibodies IgG and IgM in both miscarriage and infertile women compared with healthy women.

2- Real time PCR was rapid, sensitive and useful for diagnosing CMV infection by using gB primer with probe FAM and TAMRA.

3- Point mutations detection in *ILT2* gene including deletion and substitution causing missense, silent and frame shift.

4.2. Recommendations

1. Studying the effect of TLR2 gene polymorphism on signal transduction in miscarriage women.
2. Studying the expression of interferons and IL-2 in miscarriage women.
3. Using real time PCR for CMV as diagnostic method in clinical laboratories.

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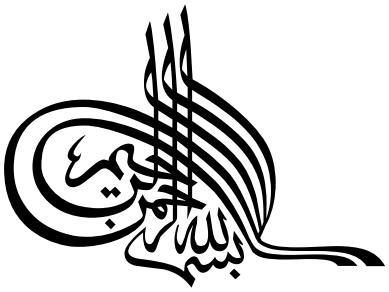

قَالُواْ سُبْحَانَكَ لاَ عِلْمَ لَنَا إِلاَّ مَا عَلَّمْتَنَا

إِنَّـــكَ أَنـتَ الْعَلِيـمُ الْحَكِـــيم

﴿سورة البقرة – الآية 32﴾

الخلاصة

هدفت هذه الدراسة الى ايجاد العلاقة بين حالات الاسقاط المتكرر للنساء وحالات الاصابة بالفيروس المضخم للخلايا و تنشيط جزيئه الاستجابة المناعية TLR2. وتحقيقاً لهذا الهدف جمعت (100) عينة دم لنساء شخصن مصليا بإصابتهن بالفايروس المضخم للخلايا من عيادة العقم في مستشفى كمال السامرائي و (50) عينه من نساء سليمات ظاهريا لإجراء المقارنة للفترة من شهر تشرين الثاني 2013 ولغايه اذار 2014. قسمت العينات إلى مجموعتين طبقا للفئات العمرية: 20-30 سنة و 31-40 سنة، تضمنت الدراسة (60) امراه غير خصبة و (40) امراه مجهضة.

شمل الجزء الأول من الدراسة استخدام طريقه الامتزاز المناعي المرتبطة بالأنزيم (ELISA) للكشف عن الأجسام المضادة للفيروسHCMV من نوع IgG وIgM، وأظهرت نتيجة الاختبار أن أعلى نسبة مصليا من نوع IgG 40٪ و IgM 25٪ في النساء المجهضات مقارنة بالنساء المصابات بالعقم 20٪ و 15٪ IgG وIgM على التوالي مع وجود فروق معنويه ≤0.05.

اما الجزء الثاني من الدراسة فقد تضمن اجراء التحليل الجزيئي بواسطة استخدام تقنية تفاعل سلسلة البلمرة (PCR) لتضخيم اثنين من جينات الفايروس المضخم للخلايا هما جين *الـ(gB)* الذي أظهر حزمة مفرده بحجم 72 قاعده نتروجينيه، وجين الـ *(IE2)* الذي أظهر حزمة مفرده بحجم 92 قاعدة نتروجينيه، و لتشخيص الاصابة اعتمدت الوقت الحقيقي لتفاعل سلسله البلمرة باستخدام بادئ ومجس خاص بالفايروس حيث ظهر التضخيم من الدورة السادسة وعليه فقد توصلت الدراسة الى أن تقنية التشخيص الجزيئي تظهر نتيجة أفضل من اختبار ELISA .

وفي جانب اخر من الدراسة أظهر التضخيم لمنطقة TLR2 حزمة مفردة بحجم 285 قاعدة نتروجينية وهو الحجم المناسب لاستخدامه في اجراء عملية تتابع القواعد ، والنتيجة التي تم الحصول عليها من تسلسل عشرة نساء مصابات وواحده سليمة لإجراء المقارنة وجد استبدال قاعدة نتروجينية نوع C بدلا من G في الموقع 242 .

كما أظهر التضخيم لمنطقة ILT2 حزمة مفردة بحجم 419 قاعده نتروجينية ، واستخدم الناتج في اجراء عملية تتبع للقواعد النتروجينية ووجدت طفرة من نوع الحذف والاستبدال، وبلغت نسبة التحور 13.3٪ لطفرة الحذف و 86.7٪ لطفرة الاستبدال، اما تأثير الطفرة على الحوامض

الامينيـة فقد كـان كـالاتي:72.7% للطفـرة المغلوطـة ،15.1% للطفـرة الصـامتة و 12.2% لطفـرة الحذف.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الإحيائية

التحري الجزيئي عن الفايروس المضخم للخلايا (CMV) المعزول من النساء ذوات الاسقاط المتكرر وعلاقته بالمستقبلات ILT2 and TLR2

رسالة

مقدمة الى مجلس كلية العلوم/ جامعة النهرين
كجزء من متطلبات نيل درجة الماجستير في علوم التقانة الإحيائية

من قبل
سارة احمد جهاد
بكالوريوس تقانة إحيائية/كلية العلوم /جامعة النهرين
(2006)

اشرف عليها
د. رحاب صبحي رمضان
(أستاذ مساعد)

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