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Serological and Molecular Comparison Study for Diagnosis of Cytomegalovirus Infection in aborted Pregnant Women in Iraq

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ABSTRACT

This study was conducted to investigate the *Cytomegalovirus* (CMV) infections of pregnant women. The samples were collected from Mosul and Baghdad hospitals in Iraq for two years and the tested women within the age category of under 20 to above 39 years. One thousand five hundred samples were taken as serum, to use in ELISA (IgM, IgG). EDTA blood, Heparin blood and cervical swabs were used in molecular tests. Three hundred positive samples demonstrating the presence of IgM and IgG antibodies using ELISA test. IgM antibodies were positive in 146 (48.7%) CMV, IgG antibodies were positive in 189 (63%) for CMV. DNA was extracted and Real-Time PCR indicates positive in only four samples (1.3%) in CMV from all 300 positive samples in ELISA tests.

Keywords: CMV Infections, ELISA for CMV, Real-Time PCR.

مقارنة مصلية جزيئية في تشخيص إصابات الفايروس المضخم للخلايا للنساء الحوامل المجهضات في العراق

الملخص

أجريت الدراسة للبحث والتحري عن مسببات إصابات الفايروس المضخم للخلايا *Cytomegalovirus* في العراق للنساء الحوامل اللواتي تراوحت اعمارهن ما بين اقل من العشرين واكثر من 39 سنة والمراجعات لمستشفيات مدينتي الموصل وبغداد لمدة سنتين. اذ جمع 1500 عينة مصلية و دم في انابيب مانع التخثر EDTA وانابيب الهيبارين ومسحات من عنق الرحم. أخذت هذه العينات كمصل لاستخدامها في تقنية المقايسة الامتصاصية المناعية للأنزيم المرتبط (ELISA) للكوبيولينات المناعية نوع IgG و IgM للكشف عن إصابات الفايروس المضخم للخلايا.

أظهرت النتائج ثلاثمائة عينة موجبة لتواجد الاجسام المضادة للكلوبيولين المناعي IgM و IgG باستخدام اختبار تقنية المقايسة الامتصاصية المناعية للانزيم المرتبط (ELISA). كانت الأجسام المضادة IgM إيجابية 146(48.7%) والأجسام المضادة IgG إيجابية في 189 (63%) للفايروس المضخم للخلايا CMV. استخلص الحامض النووي منقوص الاوكسجين من الدم ومسحات عنق الرحم لنفس العينات الموجبة واستخدمت تقنية فحص البلمرة المتسلسل اللحظي Real Time PCR والتي أعطت نتائج إيجابية لأربع عينات وبنسبة (1.3 %) لتواجد الفايروس المضخم للخلايا CMV.

الكلمات الدالة: إصابات الفايروس المضخم للخلايا، تقنية المقايسة الامتصاصية المناعية للأنزيم المرتبط للفايروس المضخم للخلايا، فحص البلمرة المتسلسل اللحظي.

INTRODUCTION

Viral infections in pregnancy are the major causes of maternal and fetal morbidity and mortality. Infections can develop in the neonate transplacentally, perinatally (from vaginal secretions or blood), or postnatally (from breast milk or other sources). The clinical manifestations of neonatal infections vary depending on the viral agent and gestational age at exposure. The risk of infection is usually inversely related to gestational age at acquisition, some resulting in a congenital malformation syndrome (Singhal *et al*., 2009).

Abortion is defined as evacuation of a fetus or embryo from the uterus prior to the stage of viability. Some medical dictionaries mention 20 weeks' gestation or 500 g as the limit, but such limits are arbitrary and not evidence based. Few would argue that a 20-week fetus would be viable if delivered. A more pragmatic definition of viability, based on survival statistics, may be 23 or 24 weeks' gestation (Dorland, 2011; Venes, 2009).

Cytomegalovirus (CMV) is one of the infectious agents. The species that infect human is commonly known as human cytomegalovirus (HCMV), Infections HCMV may be shed in bodily fluid (saliva, blood, urine, semen, breast milk, tears and cervical secretion) in human intermittently with no detectable signs and symptoms of the infection (Abbas *et al*., 2017; Tyagi, 2012).

A person is able to transmit the virus to others only when the virus is active in human system not dormant), but the virus can rarely be transmitted by blood transfusion or organ transplantation (Abbas *et al*., 2017; Yamanishi *et al*., 2007).

HCMV is a widespread infectious agent that infects the majority of the world population by early adulthood. Cases of HCMV have been recognized in all geographic locations and socioeconomic groups of people. It has been documented in some reports that HCMV infects between (50-85%) of the adult population in Central Europe and the USA (Al-Hakami *et al*., 2016; Shenk and Stinski, 2008).

CMV is a member of the Herpes virus family and represents the most frequent congenital infection. Approximately 1% (0.5–2.5%) of all newborns are congenitally infected with CMV. Infection of the fetus is the second most common cause of mental retardation after Down syndrome (Marsh *et al.*, 2012).

The virus is transmitted by contact with infected body fluids: saliva, urine, blood, semen and cervical secretions. Vertical infection can occur antenatally through the placenta during delivery through contact with cervical secretions and blood and post-natally through breast feeding. Adult seroprevalence in developed countries is around 50% but in developing countries where most infections are acquired during childhood. It may be as high as (90-100%). Women of childbearing age who are CMV seronegative are at major risk of giving birth to infants with symptomatic congenital infection if primary infection is acquired during pregnancy (Jorgensen and Pfaller, 2015).

Moreover, it must be taken into consideration that reliable viral diagnostics depend on additional preanalytical issues, such as the choice of the correct sample material, optimal sampling time with regard to the course of disease, and the duration and conditions of sample transport to the laboratory (Cowan and Smith, 2018; Neumaier *et al*., 1998).

ELISA is known as the most commonly applied method in the clinics and hospitals all across the world. It has also been widely used for the accurate detection and quantification of biological agents (mainly proteins and polypeptides) in the biotechnology industry and is becoming increasingly important in clinical, food safety, and environmental applications (Lai *et al*., 2004).

It is believed that there has been no laboratory that has not encountered ELISA in one form or another. ELISA provides highly reproducible and quantitative data that makes it an advantageous biotechnological tool in scientific research and clinical diagnosis (Hosseini *et al*.,2018).

Nucleic Acid Amplification Tests (NAAT) are very popular in the diagnosis and management of viral infections (Hepatitis B virus (HBV), Hepatitis C virus (HCV), HIV, Influenza viruses)

because they allow determination of the viral load. In other terms, quantitation of the viral nucleic acid by amplifying the target sequence thousands-fold. In most cases, they are now considered a reference, or 'gold standard' method for diagnostic practices such as screening donated blood for transfusion-transmitted viruses like CMV, HIV, HCV (Jackson, 1990).

The most widely used variants of conventional amplification are real-time PCR (quantitative PCR) and reverse transcription-PCR (RT-PCR). Both are nowadays becoming benchmarks in assessing the viral load, and while the first method quantifies DNA throughout the reactions in real time (Ntziora *et al*., 2013), the second performs RT of the mRNA and amplifies the resulting cDNA (complementary DNA), it also quantifies RNA. The combination of both techniques increases sensitivity in detecting viruses, particularly influenza viruses. The WHO recently approved a newly developed reverse transcriptase-PCR assay after the first death from Middle East Respiratory Syndrome-Coronavirus infection (MERS-CoV) reported in 2012 (Abd El Wahed *et al*., 2013).

Detection and quantitation of amplification products can be carried out with molecular beacons. Real-time PCR decrease the time required to perform nucleic acid assays because there are no post-PCR processing steps. The main advantages of these methods are also the decrease of contamination and the possibility for quantitative applications (Kwoh *et al*.,1989).

MATERIALS AND METHODS

Sample collection: The study involves the collection of (1500) samples taken from each aborted woman from Mosul and Baghdad hospitals within two years. Three hospitals in Mosul City: Al-Salam Teaching Hospital, Al Khansaa Teaching Hospital for Maternity and Children and Al-Batool Hospital for Gynecology and Obstetrics and three hospitals in Baghdad City: Al-Alwaiya Maternity Teaching Hospital, Al-Kademia Hospital for Children, AlYarmuk Teaching Hospital are selected. Information case reports are used for each case to get the information. Venous blood is drawn from each aborted women and was placed in three tubes, serum in plain tube used for serological tests, EDTA blood tubes for Molecular tests. Cervical swabs were placed in Viral Transport Media (VTM) and used for Molecular tests.

Serological : CMV IgM & IgG Enzyme Immunoassay Test Kit was used Catalog Number: BC-1091 and BC-1089 from BioCheck,Inc (USA).

Molecular :QIAamp ® DNA Blood Min Kit from QIAGEN (Germany) were used for isolated of viral DNA according to kits, whole blood and and cervical swabs in VTM were used.

CMV DNA detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen genome specific region using special primers. In real-time PCR the amplified product was detected using fluorescent dyes. These dyes are usually linked to oligonucleotide probes, which bind specifically to the amplified product during thermo cycling. The real-time monitoring of the fluorescence intensities during the real-time PCR allows the detection of accumulating products without re-opening the reaction tubes after the PCR run. CMV Real-TM PCR kit is a qualitative test that contains the Internal Control (IC). It must be used in the extraction procedure in order to control the extraction process of each individual sample and to identify possible reaction inhibition.

The whole blood with EDTA and viral transport media from cervical swabs were used. Amplification program the real-time instrument according to the manual provided by the manufacturer.

Amplification program for plate-type instruments for Applied Biosystem Fast Real time (7500) PCR system are :

Step	Temperature, °C	Time	Fluorescence detection	Repeats
Hold	95	15 min	-	1
Cycling 1	95	5 s	-	5
	60	20 s	-	
	72	15 s	-	
Cycling 2	95	5 s	-	40
	60	30 s	FAM, HEX/Cy3/JOE	
	72	15 s		

Plate-type instruments for Applied Biosystem Fast Real time (7500) PCR system are:

Channel	Threshold
FAM	The threshold line should cross only sigmoid curves of signal accumulation of positive samples and should not cross the baseline; otherwise, the threshold level should be raised. Set the threshold at a level where fluorescence curves are linear and do not cross curves of the negative samples.

RESULTS AND DISCUSSIONS

Seropositivity for CMV 48.7% n=146 is shown in (Table 1). On the whole, the highest seropositivity (29%) is seen in the age group of (20–29) years.

Table 1: Prevalence of CMV infections IgM according to age (years) and blood groups by ELISA.

AGE / Year	No.	ELISA IgM				
		CMV				
	Blood groups	A^+	B^+	AB^+	O^+	O^-
Under 20	11		2		4	5
20 - 29	86	15	16	16	34	5
30 - 39	47	10	5	7	25	
Above 39	2	2				
Total	300	27	23	23	63	10
	Sum	146				
	%	48.7				

While the seropositivity for IgG antibodies against CMV 63% n=189 (Table 2). On the whole, the highest seropositivity (36%) is seen in the age group of (20–29) years.

Table 2: Prevalence of CMV Infection IgG According to Age (Years) and blood groups by ELISA.

AGE / Year	No.	ELISA IgG				
		CMV				
	Blood groups	A^+	B^+	AB^+	O^+	O^-
Under 20	14		2		7	5
20 - 29	107	18	21	19	42	7
30 - 39	66	13	7	13	33	
Above 39	2	2				
Total	300	33	30	32	82	12
	Sum	189				
	%	63				

The current study shows that out of 300 serum samples at age groups (under 20, 20-29, 30-39 and above 39) years in ELISA the rate of infections of IgM and IgG were (48.7%,63%) for CMV respectively.

In the present study, seroprevalence of CMV IgM and IgG infections in pregnant women at high risk of threats or have embryos suffering from congenital defect are found to be at a high percentage of CMV which disagrees with the seroprevalence reported by Sadik and others, in (2012) conformed high prevalence of CMV and HSV in IgM and contrasted in IgG. The study contrasted that the high percentage infection present in O^+ blood group by Franchini and others (2016) that the ABO blood type not only plays a role in transfusion and transplantation medicine, but also was implicated in the pathogenesis of a kaleidoscope of human disorders. The results of this systematic review support for the first time the existence of a consistent influence of ABO status on the risk of developing preeclampsia. Specifically, women with a non-O blood type are found to have a moderately increased risk of this condition compared with the risk in those with O blood type (Franchini *et al*., 2016).

Infection with CMV contracted during pregnancy may be passed through placenta to the fetus affecting the fetus and new-born potentially causing serious birth defects. Asymptomatic infants may develop abnormalities later in life. The infections caused by TORCH organisms like CMV are grouped together because they all result in serious birth defects when transmitted from an infected mother to her foetus during pregnancy (Simgamsetty *et al*., 2015; Sadik *et al*., 2012).

Cytomegalovirus is the most common congenital viral infection with birth prevalence of about 0.5 % (0.2 to 3 %). Maternal seroprevalences of CMV antibodies derived from different studies vary from (0.15 - 0.5%) in Europe, where as in North America they vary from (0.42 - 1.4%). In Africa and Asia, the reported incidences vary from (0.5%) in Japan to (1.38%) in Ivory Coast, and 1.8% in Taiwan in the presence of a very high rate (90–100%) of preexisting maternal immunity (Kenneson and Cannon 2007; Istas *et al*.,1995). In a study in Nepal, an infection rate of (34%) is found (Acharya *et al*., 2014; Kumari *et al*., 2011).

CMV is the most common cause of congenital infectious disease and results in developmental delay and sensorineural deafness. When the maternal infection is primary, the fetal infection rate is (30 - 40%) and the rate of infected fetuses developing cytomegalic inclusion disease is 10% (Owen *et al*., 2006).

The factors responsible for the transmission to the fetus and severity of congenital CMV infection are not well understood. Preexisting maternal CMV seropositivity substantially decreases, but does not completely eliminate fetal infection suggesting partial protection from maternal

immunity. Although the majority of congenital infections are asymptomatic, (5 - 20 %) of infants born to mothers with primary CMV infection are overtly symptomatic. These children have a mortality rate of about (5 %) and severe neurologic morbidity occurs in (50 - 60 %) of survivors (Kenneson and Cannon, 2007; Istas *et al*.,1995).

Infections can develop in the neonate transplacentally, perinatally (from vaginal secretions or blood), or postnatally (from breast milk or other sources). The clinical manifestations of neonatal infections vary depending on the viral agent and gestational age at exposure. The risk of infection was usually inversely related to gestational age at acquisition, some resulting in a congenital malformation syndrome (Singhal *et al*., 2009).

In CMV Real Time PCR the fluorescent signal intensity is detected in two channels in RT-PCR of CMV. The signal from the CMV DNA amplification product is detected in the FAM channel. The signal from the Internal Control amplification product is detected in the JOE/Yellow/HEX channel.

The results are interpreted by the software of the instrument by crossing (or not crossing) the fluorescence curve with the threshold line.

The Controls of CMV Real Time- PCR instrument are :

Control	**Stage for control**	**Ct value on channel**		**Interpretation**
		FAM	**JOE**	
C-	DNA extraction	Neg	Pos(≤33)	OK
NCA	Amplification	Neg	Neg	
C+	Amplification	Pos(≤33)	Pos(≤33)	

Only 4 samples (1.3%) from cervical swabs of CMV are positive in RT-PCR from 300 samples which are positive in all serological methods. This is confirmed by Tanaka and others (2006) who found a relationship between CMV DNA in vaginal fluid during the first trimester and miscarriage risk. No other cause of miscarriage such as other virus infection or amniocentesis is detected. In addition, neither the frequency of premature delivery nor the gestational age at live birth is affected by the presence of CMV. Therefore, it is unlikely that the presence of CMV in the vagina is associated with the risks of chorioamnionitis or premature rupture of the fetal membranes (Tanaka *et al*., 2006).

The results in this study in RT-PCR show in the high CT value of both sample and Internal control (IC) under 33 which is identified by Jamil and others 2017 Fig. (1). These findings show that most of the ELISA results are confirmed by PCR which means that the seropositive results by ELISA are not specific or less significant due to the probability of false positive results as a result of other microbial infection. They also suggest that the best method to detect CMV and HSV is RT-PCR as Real time PCR is considered to be active, rapid and useful technique for diagnosis of active disease and monitoring response to therapy (Jamil *et al*., 2017).

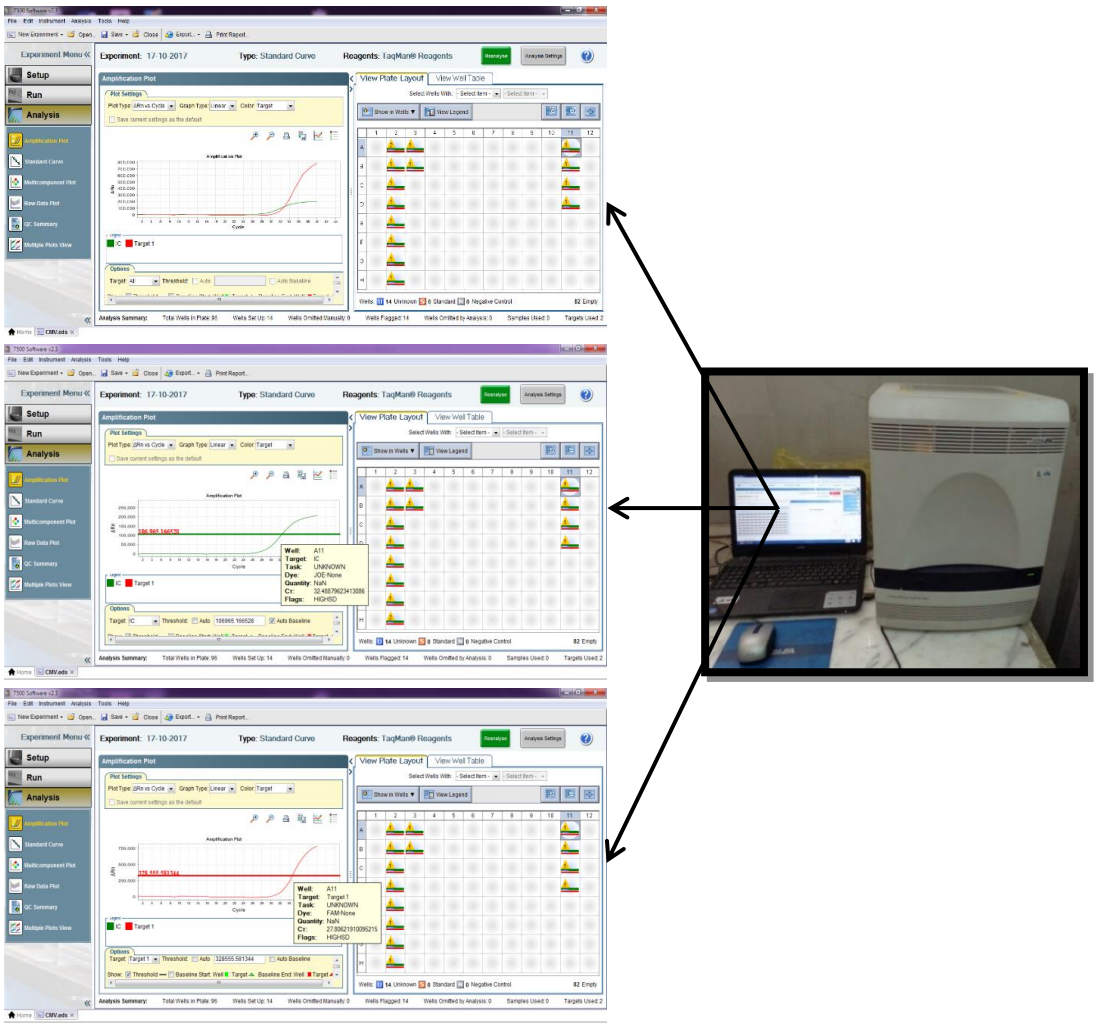

Fig. 1 : Amplification Plot showing the Amount of Fluorescence Obtained in Each Amplification Cycle. A Ct is 27.8 that Account of CMV and 32.4 the IC.

Dinc and others (2010) correspond to our results as CMV secretion from cervix increases during pregnancy. A large spectrum of cells of the fetus is infected by CMV. The major target fetal organs for CMV infection are the lungs, pancreas, kidneys and the liver but comparing with these organs, CMV DNA level determined in uterine tissue and cervical smear is higher (Dinc *et al*., 2010).

When pregnant women has primary CMV infection during the first trimester, approximately (25%) of their fetuses will be infected (Revello *et al*., 2011). Therefore, infants born from mothers with primary CMV infection during pregnancy are at high risk for the occurrence of congenital CMV infection. Maternal serum CMV immunoglobulin (Ig) M antibody is often tested to identify primary infection. However, true primary CMV infection is determined in only (20–25%) of pregnant women with positive results for CMV IgM. This is because CMV IgM may persist for (6–9) months following primary infection (Lazzarotto *et al*.,2011) or may be detected during latent reactivation (Tanimura *et al*., 2016; Revello *et al*., 2011).

In addition, fetal CMV infection occurs in (1–2.2%) of pregnant women with reactivation of a latent virus or reinfection with a new strain of CMV (Kenneson and Cannon, 2007; Fowler *et al*., 1992).

CONCLUSION

Real Time PCR is the golden standard for the detection of many pathogens. It facilitates the detection and amplification of products and useful in quantifying and qualitative a larger range of sequences of viral nucleic acids than most quantitative methods.

ELISA test is considered as a preliminary and screening test for CMV infections, IgG detected mostly higher percentage than IgM for all CMV infections.

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