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Detection of Cytomegalovirus among Women with Abortion by Real Time PCR

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ABSTRACT

Background: The cytomegalovirus (CMV), which has a widespread distribution and can infect people at any stage of life, is one of the opportunistic viruses. It is a major cause of prenatal and perinatal infections and may lead to important complications in pregnancy.

Objective: This study was suggested to molecular screening for cytomegalovirus among sample women who suffered miscarriage by using Real time PCR and identification of the *UL97* gene by conventional PCR technique

Methodology: The study includes 100 samples, 80 women who suffered from abortion and were referred to Al-Batool Teaching Hospital and 20 healthy control women in a period between November 2021 and January 2022. Real-Time (PCR qPCR) was used in the study's initial phase to detect HCMV DNA in blood samples.

Result: The result showed the HCMV DNA was detected in 8 (10.0%) out of 80 patients were found to be CMV positive. Due to the necessity of early and accurate infection diagnosis before the development of their effects, even with low levels of pathogenicity, the molecular strategy can be useful for achieving this objective. The polymerase chain reaction (PCR) method was used in the study's second section to amplify the UL97 gene, which showed detection of the HCMV in 5 (62.5%) of specimens out of 8 (100 %) of a total specimens infected with HCMV that were collected from aborted women and then detected by real time PCR technique.

Conclusions: There was no statistically significant difference between the study groups in terms of HCMV positive as assessed by real-time PCR and the UL97 gene ($p > 0.05$).

Keywords: Cytomegalovirus, Abortion, qPCR, UL97 gene.

INTRODUCTION

The cytomegalovirus is the most prevalent member of the herpes virus family (CMV). Human cytomegalovirus (HCMV) is the largest common cause of congenital malformation resulting from viral intrauterine infection in wealthy countries [(1)]. HCMV is endemic in the majority of the world's populations. In various geographic regions, the prevalence rate of HCMV ranges from 30 to 100% [(2)]. Viral infection can spread horizontally (via sexual contact or contact with fluids like saliva, breast milk, maternal vaginal secretions, or blood) as well as vertically (transplacentally from mother to fetus) [(3)].

HCMV infections can be contracted during pregnancy, infancy, or maturity through sexual contact, organ transplantation, or blood transfusions [(4)]. The four probable HCMV infection states are latent (non-productive), lytic (productive), asymptomatic, or symptomatic [(5)].

The human herpes virus with the highest genetic content is cytomegalovirus. Compared to HSV, it has a DNA genome that is 240 kbp bigger. Over 200 different proteins that make up the virus have only been partially identified. Once, a cell surface glycoprotein, acts as an Fc receptor by non-specifically attaching to the Fc portion of immunoglobulins. Infected cells may be able to thwart immune clearance by secreting a coating of ineffective host immunoglobulins [(6)].

One of the many causes of prenatal harm that results in abortion is the cytomegalovirus, also known as human herpes virus type 5 [(7)]. When a fetus or embryo naturally dies before it can survive on its own, it is known as a spontaneous abortion or pregnancy loss. The first trimester accounts for around 80% of spontaneous pregnancies loss, and the rate drops with each additional week of gestation [(8)].

The viral phosphotransferase is encoded by the UL97 gene. It is in charge of phosphorylating Ganciclovir (GCV). Several conserved subdomains of the UL97 protein have distinct roles. Subdomains II, III, VIB, and VII are engaged in the phosphate transfer, subdomain IX is necessary for substrate binding, and subdomain I is in charge of ATP binding. Resistance to GCV may result from mutations in the UL97 gene [(9)].

Treatment for HCMV infection involves taking antiviral drugs such ganciclovir (GCV), cidofovir, and foscarnet. The nucleoside analogue GCV is a prodrug that requires phosphorylation to become functional [(10)].

The best approach for detecting CMV was determined to be Real Time PCR, followed by ELISA and fast testing. All of these methods were shown to be helpful for both diagnosis and therapy. Nucleic acid tests take less time to complete because real-time PCR eliminates the need for post-PCR processing procedures [(11)].

The goals of this study was to know the problem of abortion and its relationship to cytomegalovirus and to

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screen for cytomegalovirus by using qPCR and detection of the UL97 gene.

MATERIAL AND METHODS

1-Sample collection:

The study sample included (80) aborted women with their ages ranging between 16-45 years who attended to AL-Batool teaching hospital in Baqubah and 20 healthy control women with their ages ranging between 18-55 years.

The period collection from November 2021 to January 2022. The first step included the extraction of DNA from blood sample then detection of human cytomegalovirus (HCMV) by qPCR technique, and the second step was identification of UL97gen. 2 ml of venous blood was taken from aborted women, as a whole blood in EDTA tubes, which was stored at -4°C until use for molecular detection.

Ethical approval:

The study was approved by the Ethics Board of University of Diyala and an informed written consent was taken from each participant in the study. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

2-DNA Extraction of CMV

Using the ReliaPrepTM Blood gDNA Miniprep System (Promega Company, USA) and whole blood samples were used for DNA extraction, adhering to the manufacturer's instructions. Extreme vigilance was used during the DNA isolation process to prevent sample-to-sample cross-contamination.

3. Real Time PCR (qPCR)

The polymerase chain reaction (PCR) method for detecting CMV DNA relies on the amplification of a portion of the pathogen's genome using specialized primers, as illustrated in table (1).

Using fluorescent dyes, the amplified product was found during real-time PCR. These colors are often connected to oligonucleotide probes, which during thermal cycling bind only to the amplified product. Without having to reopen the reaction tubes after the PCR run, the detection of accumulating products is made possible by the real-time monitoring of fluorescence intensities during real-time PCR. A qualitative test called the Real-Time PCR Kit GoTaq® 1-Step RT-qPCR System includes the Internal Control (IC). It is necessary to incorporate it into the extraction process in order to monitor the extraction of each distinct sample and to spot any potential response inhibition.

Table (1): The primer was used in qPCR

No.	Primer Name	Seq.	Annealin g Temp. (ºC)	Product size (bp)
1	CMV-F	5`-ACTTTGCCGATGTAACGTTTCTTG-3`	56	194
	CMV-R	5`-CGGGTCATCTACGGGGACAC-3`		
	CMV-P	Fam-5`-CTGGAGTTTGAAAAGGTMGB-3`-probe		

4- UL97 gene amplification

As stated in table (2), UL97 gene amplification was carried out using a specific primer. (Macrogen Company provided the primer).

Table (2): The primer was used in *UL97 gene* amplification

No.	Primer Name	Seq.	Annealin g Temp. (ºC)	Product size (bp)
2	*UL97*-F	5'-TGCCCAAAGAGGACGATTTT-3'	57	650 and 920
	UL97-R	5'-GTAGTCCAAACTCGAGACGG-3'		

5- Analysis of PCR product by agarose gel electrophoresis.

After PCR amplification, agarose gel electrophoresis was adopted to confirm the presence of amplification (31).

4- Statistical analysis

The parameters (nominal and ordinal) were presented as percentage and frequencies. Pearson-Chi-square test or two-tailed Fisher exact probability were used to determine whether there were any significant differences between the frequencies (p).

These studies were conducted using the statistical software packages SPSS version 25.0 and GraphPad Prism version 6 at a significant level of 0.05 [32].

RESULTS

1-Detection of cytomegalovirus by qPCR

Real-time PCR was a quick, accurate, and practical method for identifying active illness and tracking treatment response. Quantitative nucleic acid detection assays are the ones that are most likely to be beneficial in this approach since they allow for the specification of threshold levels that may be used to initiate therapy. Numerous studies have demonstrated associations between blood levels of CMV DNA and illness risk [33]. Our results showed that HCMV DNA was detected in 8 (10.0%) out of 80 patients who were found to be CMV positive with odd ratio of 4.80 and relative risk of 1.27, while the control group did not detect any viral presence (0.0%) as shown in table (3). Examples of amplification and standard curves are given in figure (1).

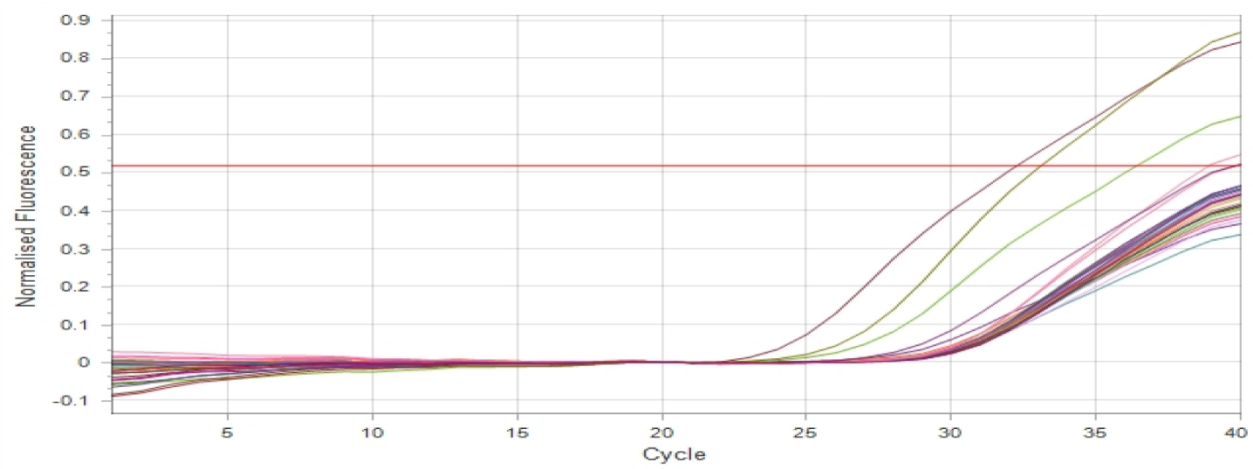

Figure (1a): Amplification curve Run (1)

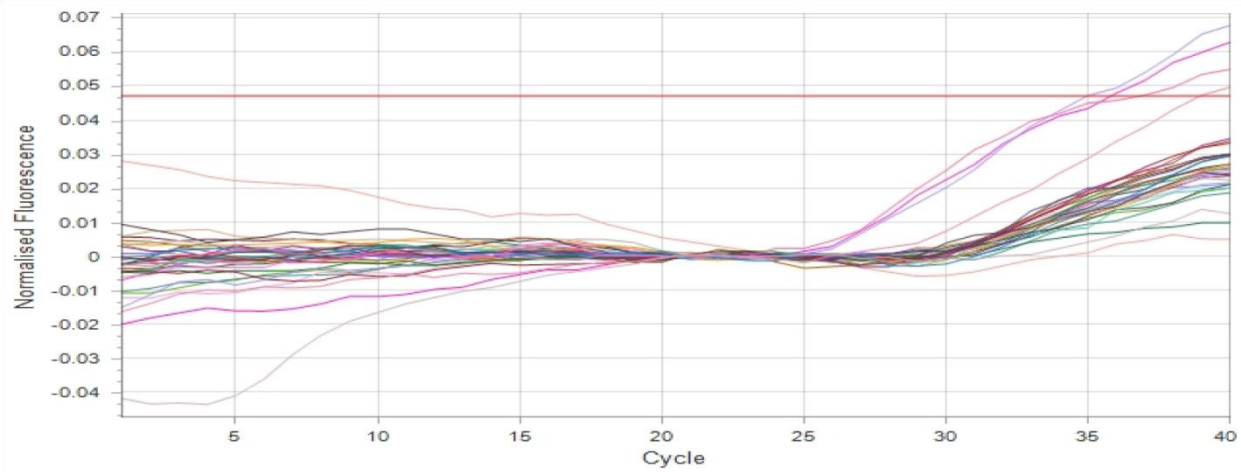

Figure (1b): Amplification curve Run (2)

Table (3): The detection of CMV by qPCR between patients and healthy

qPCR		Groups		Total	Statistics
		patients (n=80)	healthy (n=20)		
Positive	N	8	0	8	P>0.05 OR= 4.80 (0.26-10.19) RR= 1.27 (1.14-1.42)
	%	10.0%	0.0%	8.0%	
Negative	N	72	20	92	
	%	90.0%	100.0%	92.0%	

A study was conducted in Baqubah for detection of HCMV DNA in aborted women using qPCR and the result showed (11.2%) positive for HCMV DNA [12]. These results are consistent with the current investigation, which found that HCMV DNA was only detected in a small number of samples 8 (10%) in aborted women compared to healthy women (0%). Another study found that 8.7% of 986 women having a Pap test result that indicated a high-grade intraepithelial lesion had HCMV-DNA [14]. Furthermore, in order to detect cytomegalovirus using extracted DNA, Real-time PCR was used. The results of Real-time PCR revealed that out of 90 cervical samples, 22 (or 24.44%) tested positive for HCMV DNA and 68 (or 75.56%) tested negative for the virus [15].

According to the findings, 1.4% of fetuses who were aborted had HCMV-DNA infection. Similar to other European nations, CMV infection is common in aborted babies. CMV infection in aborted fetuses has been evaluated using the molecular approach of PCR applied to paraffin-embedded biopsy samples, and it has proven to be a reliable, valid, and rapid procedure [16].

A different study used nested PCR to find HCMV-DNA in 4 (0.4%) of the 983 cord blood samples, and the findings of that study's analysis of HCMV-DNA are similar to those of the current study's analysis of HCMV-DNA. A primary HCMV infection is defined by the existence of HCMV in the blood [17]. Women blood samples with HCMV that reported initial infections were found to contain HCMV DNA by PCR in the current analysis.

2- Detection of *UL97* gene of cytomegalovirus

GoTaq-Green master mix and specific primers for the *UL97* gene of HCMV were utilized in the traditional PCR technique in this investigation to identify the *UL97* gene from the extracted DNA sample of CMV [18], which are *UL97*-F and *UL97*-R primers as shown in (table 2). In successful PCR reaction, the *UL97* gene product of ~650 and ~920 bp molecular weight was observed. The findings of **Spector and colleagues**, who utilized seven overlapping primer sets to amplify and sequence the pertinent region of the UL97 gene, validated our idea. This was regarded as a necessary indicator of a successful reaction since, upon gel electrophoresis, its bands fell within the 600–700 bp and 900–1000 bp bands of the 100 bp DNA ladder, respectively, showing the presence of this gene. The HCMV phosphotransferase gene was amplified in the current investigation (Thymidine kinase, UL97), and the results of PCR showed detection of the *UL97* gene in 5 (62.5%) of specimens out of 8 (100 %) of a total specimens infected with HCMV that were collected from aborted women and then detected by real time PCR technique (Table 4).

Table (4): The presence of *UL97* gene between the study groups

UL97		Patients (n=8)	Statistical
Positive	N	5	P>0.05 OR= 2.98 (0.15-6.22) RR= 1.26 (1.14-3.22)
	%	62.5%	
Negative	N	3	
	%	27.5%	

According to the study's conclusions, there is no statistically significant difference between the study groups in terms of HCMV positive as assessed by real-time PCR and the UL97 gene (p>0.05).

Other results found that 75/98 (77%) of infertile women carried the *UL97* gene [19], which almost is identical to the findings of our study. A study was conducted in Baghdad found that the *UL97* gene was found in 37.2% more in aborted women than in healthy women [20], which contradicted the findings of our study. Phylogenetic analysis and DNA sequencing revealed that two of the sequenced *UL97* genes were changed and diverged in their amino acid profiles when compared to NCBI control. The results of the study revealed that 4/43 (21.5%) of the isolates carried the UL97 gene. This might be seen as a warning sign that Iraqi HCMV isolates are evolving into resistant strains [21]. This outcome is consistent with work [22], who demonstrated that whole blood samples were more commonly used to identify viral DNA than other types.

The *UL97* gene revealed no appreciable changes between ill and healthy individuals [23]. The results of UL97 gene sequencing from GCV-sensitive isolates showed that the UL97 gene is highly conserved among clinical isolates, with an average sequence identity of 99%. These baseline sequences and clearly defined sites for drug resistance mutations serve as the basis for a fast genotypic drug resistance screening based on direct sequencing of PCR-

amplified products including these sites [24]. Another study discovered that the same target shared by all anti-HCMV drugs may contribute to drug resistance to HCMV, which means that a single mutation might result in multidrug resistance [25].

3-Relation of age groups of pregnant patients with qPCR of CMV of patients

The results of the study indicated that there were no differences between the qPCR and age groups of pregnant women (p>0.05), as shown in the table [5].

Table (5): Relation of age groups of pregnant patients with qPCR of HCMV as calculated by chi-square test

			Age groups (years)				Total	P - value
			≤20	21-30	31-40	41-50		
qPCR	Positive	n	1	6	1	0	8	P>0.05
		%	7.7%	14.0%	4.8%	0.0%	10.0 %	
	Negative	n	12	37	20	3	72	
		%	92.3%	86.0%	95.2%	100.0%	90.0%	

The main and re-infection of latent HCMV infection occurred at the highest incidence in the age range of 36-40 years old [27], and these findings corroborated our findings, which showed that women between the ages of 21 and 30 had the high incidence of HCMV infection. The results showed that HCMV infection during pregnancy affected 50% of all women of reproductive age (>14 years). Congenital HCMV infections and associated harmful effects may be prevented by HCMV testing during pregnancy and educating seronegative women about HCMV risk reduction measures [28].

4-Relation of fetus age with qPCR of HCMV of patients

The results of the investigation revealed that there was no correlation between qPCR and fetus age (p>0.05) (table 6).

Table (6): Relation of fetus age with qPCR, of CMV as calculated by chi-square test

			Fetus age (months)		Total	P value
			≤3 (n=58)	>3 (n=22)		
qPCR	positive	N	7	1	8	P>0.05
		%	12.1%	4.5%	10.0%	
	negative	N	51	21	72	
		%	87.9%	95.5%	90.0%	

The current study found a substantial difference between the number of abortions and the age of the fetus, with the fetus age of 3 months accounting for a high percentage of abortions. A small amount of evidence suggests that medical abortion in the late first trimester has a wide range of effectiveness, highlighting the necessity for well-designed studies in this gestational age range [26].

DISCUSSION

Due to the necessity of early and accurate infection diagnosis before the development of their effects, as well as minimal levels of pathogenicity, can both benefit from the molecular approach [29].

It is obvious that there are significant differences across the evaluated studies' HCMV DNA detection methods. In each study, a separate set of HCMV DNA detection primers was employed. As a result, the various results can be ascribed to variations in the lab assays, the primers and probes applied, the various specimen types examined, or variations in the populations where the research were conducted. Our research has limits because of the limited sample size and duration time, but it does reflect real life in a country. Our results need to be confirmed in larger and more uniformly dispersed populations.

Infections with the Cytomegalovirus can cause congenital defects in children, especially if they are contracted during the first trimester of pregnancy. When it comes to HCMV infection during pregnancy, 40% of cases pass through the placenta and infect the fetus, potentially leading to cytomegalovirus syndrome [30].

Since the genotypic technique makes the assumption that the same drug resistance mutation will confer resistance in all genetic backgrounds, phenotypic investigations are still required to confirm genotypic findings. Phenotypic assays are still needed to identify new medicinal targets. Phenotypic tests have the advantage of directly linking medication resistance to the viral activity's biological activity, but the length of time needed to carry out the assays prohibits them from offering relevant therapeutic information. We created a genotypic test for UL97 mutants that is incredibly rapid and covers all known drug resistance mutations. This approach is easily expandable and adaptable to search for mutations causing resistance to brand-new antiviral medications throughout the whole UL97 gene, the polymerase gene, and additional viral targets [23].

CONCLUSIONS

Compared to other age groups, women aged 21 to 30 years old have a higher rate of abortions and HMCV infection, and the highest rate of miscarriage occurred in the first trimester of pregnancy. qPCR results converged with the identification of the UL97 gene.

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