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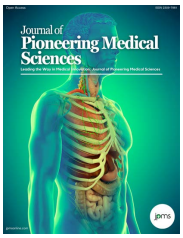

Comparative Analysis of ELISA (IgG, IgM) and Real-time PCR for Accurate Cytomegalovirus Detection in Women with Abortion Experiences

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Abstract **Objectives:** This study investigates the association between cytomegalovirus (CMV) and abortion in 150 women using diagnostic methods (ELISA and Real-time PCR targeting the Ul55 gene). The research reveals a CMV prevalence of 13.53%, with significant correlations between CMV infection and sexually transmitted diseases, multiple sexual partners, recurrent miscarriages and headache/fever. Sensitivity and specificity for IgG in detecting CMV infection were 100% and 6.12%, while for IgM, they were 78.26% and 100%, respectively. The optimal cut- points for IgG and IgM were identified at 145.65 mg/dL and 4.5 mg/dL, demonstrating sensitivities of 65% and 96%, specificities of 25% and 95% and AUCs of 0.45 and 0.95, respectively. Real Time PCR, the gold standard for pathogen detection, outperforms ELISA as a screening test for CMV infections, especially in detecting IgG. This study underscores the significance of molecular methods, recommending their stronger and faster application for effective cytomegalovirus detection.

Key Words Comparative, ELISA (IgG, IgM), real-time PCR, HCMV, abortion

INTRODUCTION

Human cytomegalovirus (HCMV) belongs to the Herpesviridae family and the beta herpesviridae subfamily [1]. HCMV, the herpes virus with the highest genetic content, has a genome 240 kbp larger than HSV. Over 200 proteins are partially identified [2]. A glycoprotein acts as an Fc receptor, attaching to immunoglobulins. Infected cells may evade the immune system by secreting ineffective host immunoglobulins [3]. HCMV is a worldwide virus that infects humans at any age [4]. HCMV is a major cause of congenital malformation due to viral intrauterine infection in wealthy countries [5]. It infects 50-90% of individuals globally [4], with a prevalence of 40-100% in developing countries [7]. In Iran, a study of 120 people reported a HCMV prevalence of 13.3% [6]. The four probable HCMV infection states are latent (non-productive), lytic (productive), asymptomatic, or symptomatic [3].

HCMV is transmitted through various body fluids, including saliva, tears, breast milk, urine, semen and vaginal/cervical secretions [8]. Clinical manifestations include fever, headache, myalgia, lymphadenopathy, splenomegaly and skin rash. Complications may include pneumonia, retinitis, myocarditis, hemolytic anemia, hepatitis and Guillain Barré syndrome [9]. HCMV passing through the placenta makes it a common cause of congenital malformations [10], leading to abortion, stillbirth and issues like hearing loss and mental retardation in infants [11].

HCMV infection is often asymptomatic, but can be serious, particularly during pregnancy when the virus may be transmitted to the fetus [12]. Primary infection in pregnancy occurs in 4 to 0.7% of cases, with a transmission rate of 24 to 75%, averaging 40% [13]. Transmission is more likely in the last trimester, causing more severe fetal damage. Recent studies [6,7,11] link primary HCMV infection to pregnancy

loss, though the exact mechanism is still under investigation [14-15] 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 [16]. Effective treatment of HCMV s infection requires its detection in the early stages of infection [17]. The common methods of identifying HCMV infection include: virus culture, testing of IgG and IgM antibodies using methods such as ELISA and molecular methods such as PCR [17]. Although the presence of IgM antibody in patients' serum can indicate recent HCMV infection, it cannot accurately indicate active infection with this virus [18]. PCR method has clinical sensitivity to identify the virus in urine, blood, plasma and cerebrospinal fluid samples, if the culture method is time-consuming and more difficult to perform [19]. In recent years, due to the importance of quantitative measurement of infectious agents, especially in the case of viruses, the Real Time PCR method is used [20]. which, in addition to accurately identifying these factors, show the presence of DNA and identify active HCMV infections [21]. The study aims to investigate the association between cytomegalovirus and abortion in women, comparing diagnostic methods (ELISA and Real- time PCR targeting the Ul55 gene) to enhance accuracy clinical diagnosis.

METHOD

This cross-sectional research was done on 150 aborted women with their ages ranging between 16-45 years who referred to Shohada Hospital in Tehran from March 2022 to September 2023. This research was approved and reviewed by the Ethics Committee (IR.IAU.PS.REC.1402.145) and the patient's families signed the informed consent. Inclusion criteria involve women within the reproductive age range with a documented history of abortion willing to participate. Exclusion criteria encompass those without a history of abortion, individuals outside the reproductive age range and those with pre-existing health conditions or known causes of abortion. Refusal or inability to provide informed consent and prior participation in a similar study within a specified time frame are also exclusion criteria. These criteria aim to investigate the cytomegalovirus-abortion association while maintaining sample homogeneity.

Sampling

Initially, a trained nurse obtained 10 cc of blood and serum samples from 150 women experiencing recurrent abortions using a sterile syringe. Subsequently, 5 cc of the collected blood was promptly mixed with an anticoagulant and stored at -4°C until use. This portion was then conveyed to the molecular laboratory for real-time PCR testing. Simultaneously, the remaining 5 cc was transferred to a clot tube and underwent IgG and IgM ELISA testing in the immunology lab.

DNA Extraction

DNA extractions were performed in all experiments using the QIAamp blood kit (QIAGEN S.A., Courtabœuf, France) according to the manufacturer's recommendations, except that DNA was eluted in 200 mL of distilled water.

Design Primer and Real Time PCR

The genome of the viruses was analyzed and the Ul55 gene conserved regions were selected, then, HCMV primers (NC_006273) were downloaded from the NCBI website for specificity in humans and viruses. Appropriate primer were designed primers were designed (Table 1) by NCBI and comparison and prepared in Clustal Omega and Gene Runner and Blast. Primer designed with consideration to ensure the balanced GC content, optimal melting temperature (Tm), self-3' complementarity and self-complementarity [22]. The nucleotide sequence of the primers used to amplify the 252 bp region of the target gene and The B-actin gene was used as the housekeeping gene. PCR run as Table 2 protocol.

ELISA

Diagnosis of the infection was based on determining anti-HCMV IgG and IgM antibody titers through the ELISA technique (Pishtaz- teb kit, Iran). The antigen used in this method was an inactivated and purified CMV that was attached to the slide-phase of a 96-well microplate. Immunoglobulin in human serum was attached to the antigens in the plate during incubation. After washing, the plate was juxtaposed with peroxidase-conjugated anti-IgG and anti-IgM antibodies. After appropriate incubation and washing, the substrate was added. In this condition, the formed color would match the concentration of specific antibodies in the serum. The cutoff, determined by positive and negative controls' optical density (OD), yielded a cutoff index (COI) for each serum sample. COI $\leq$1 was negative, >1.1 positive, and 1-1.1 borderline.

Statistical Analysis

CT values obtained from RT-qPCR were employed in the 2-ΔCT method to evaluate changes in gene expression. Descriptive statistics, like median and interquartile range

Table 1: Primers used for template DNA and PCR synthesis

Primer	Sequence	Bp	GC%	Tmb	Hair	HmD	HtD	Amplicon
Ul55 (F)	5'-ATAGGAGGCGCCACGTATTCC-3'	21	57	60	-0.45	-10.87	-8.25	252 bp
Ul55 (R)	3'-GTACCCCTATCGCGTGTGTTC-5'	21	55	56	-1.13	-6.33		
B-actin (F) (housekeeping)	5'-CATCCGTAAAGACCTCTATGCCAAC-3'	25	56	55	-0.6	-10.5	-7.89	619 Bp
B-actin (R) (housekeepin)	3'-ATGGAGCCACCGATCCACA-5'	19	58	57	-1.08	-5.89		

Table 2: PCR program used in Thermal cycler

Levels	Time	Temperatures °C
First stage		
Initial denaturation	3 min	95
Second stage: 45 cycles		
Secondary denaturation	15 S	94
Annealing	60 S	63
Extension Initial	20 S	70
Third level		
Final Extension	5 min	72

(IQR) or mean and standard deviation (SD) for variables, were calculated. Quantitative variables were described using mean and standard deviation for normal distribution and median and interquartile range (IQR) for non-normal distribution. Qualitative variables were reported as number (percentage). Chi-Square test compared variable frequencies based on PCR results. Mann-Whitney and Kruskal-Wallis tests assessed IgG and IgM mean differences. True positive (TP), false positive (FP), true negative (TN) and false negative (FN) values were computed, determining accuracy indices (sensitivity, specificity, positive predictive value, negative predictive value, LR+, LR-) and ROC curve with AUC. Stata software (version 14) was used for analysis, with $p<0.05$ considered statistically significant.

RESULT

In this study, 150 women with a history of abortion were examined. The average age of the women was 32.14±9.70 years with an age range of 12 to 75 years. 18.82% (32 people) of women were less than 25 years old, 75.88% (129 people) were 25-50 years old and 5.29% (9 people) of women were more than 50 years old. 24.12% (41 people) had a history of sexually transmitted diseases. The number of sexual partners was more than one in 71.4% (8 people) of women. 33.53% (57 people) of women had a history of more than one abortion. Also, headache and fever were reported in 8.82% of women (15 people). In our study, based on the results of the PCR test, the prevalence of cytomegalovirus in the investigated women was reported as 13.53 (23 people). Tested positive for CMV PCR, with a significant association between CMV infection and higher rates of sexually transmitted diseases, multiple sexual partners, recurrent miscarriages and headache/fever ($p<0.001$) (Table 3). IgG and IgM sensitivity and specificity for detecting CMV infection were 100%, 6.12%, 78.26%, 100%, respectively (Table 4-5). IgM levels were significantly higher in CMV-infected individuals ($p<0.001$). No false-negative cases for IgG were observed. However, five cases (21.74%) had false-negative IgM results, primarily in individuals aged 32-75 with a history of more than one abortion and three cases reported a venereal disease history-all five had positive IgG. Based on PCR as the gold standard in this study, the optimal cut-point for IgG in cytomegalovirus infection was 145.65 mg/dL, with a sensitivity of 65%, specificity of 25% (Figure 1) and an AUC of 0.45. Similarly, for IgM, the optimal cut-point was 4.5 mg/dL, demonstrating a sensitivity of 96%, specificity of 95% and an AUC of 0.95 (Figure 2).

Table 3: Comparing the frequency of cytomegalovirus based on PCR according to different variables in the studied women

Different variables	Positive (n = 24) Number (%)	Negative (n = 126) Number (%)	Different variables
History of sexual disease			
Yes	16(69.57)	25(17.01)	0.001>
No	7 (30.43)	122(82.99)	
More than one sexual partner			
Yes	5(21.74)	3(2.04)	0.001>
No	18(78.26)	144(97.96)	
A history of more than one abortion			
Yes	21(91.30)	36(24.49)	0.001>
No	2(8.70)	111(75.51)	
Headache and fever			
Yes	8(34.78)	7(4.76)	0.001>
No	15(65.22)	140(95.24))	

Table 4: Sensitivity and specificity and other accuracy indicators for IgG results in the diagnosis of cytomegalovirus infection in the studied women

Accuracy indicators	Estimate	Confidence interval 95%
Sensitivity (%)	100	100-85.18
Specificity (%)	6.12	11.30-6.12
Positive predictive value (PPV) (%)	14.29	20.66-9.28
Negative predictive value (NPV) (%)	100	100-66.37
LR+	1.06	-
LR-	0	-

Table 5: Sensitivity and specificity and other accuracy indicators for IgM results in the diagnosis of cytomegalovirus infection in the studied women

Accuracy indicators	Estimate	Confidence interval 95%
Sensitivity (%)	78.26	96.54-58.30
Specificity (%)	100	100-97.52
Positive predictive value (PPV) (%)	100	100-81.47
Negative predictive value (NPV) (%)	96.71	98.92-92.47
LR+	-	-
LR-	0.21	-

DISCUSSION

Cytomegalovirus, a member of the herpes virus family, is the most common type (CMV) [23]. Infections with the Cytomegalovirus can cause congenital defects in children, especially if they are contracted during the first trimester of pregnancy [24]. When it comes to HCMV infection during pregnancy, 40% of cases pass through the placenta and infect the fetus, potentially leading to cytomegalovirus syndrome [25]. In most parts of the world, CMV is endemic. The range of HCMV seroprevalence, which varies geographically, is between 30 and 100% [12]. Unlike other infections, since the virus often reactivates during reproductive age and can be transmitted to the fetus despite maternal immunity, HCMV infection during pregnancy is very challenging because it leads to miscarriage in most cases [26]. It is necessity to 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 [27].

In our study, the average age of women was 31.76±8.98 years, with an age range of 12 to 59 years, so that in 30% of people with a history of sexually transmitted diseases, the number of sexual partners was more than one person in 6.67%, 40% A history of more than one abortion, as well as headache and fever, was reported in 9.17% of women.

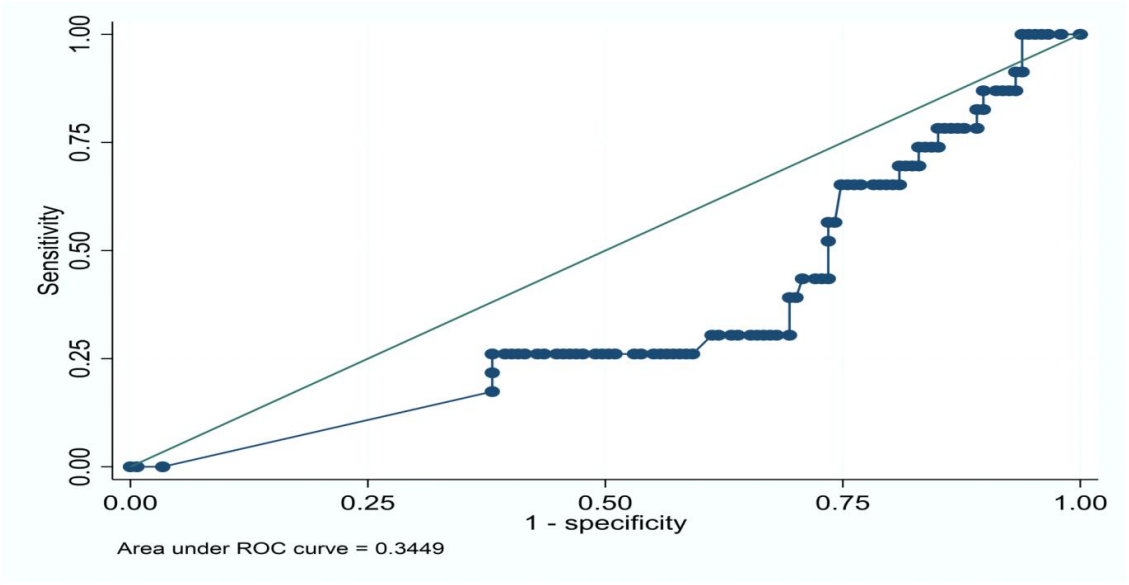

Figure 1: Rock curve of IgG for cytomegalovirus infection in studied women

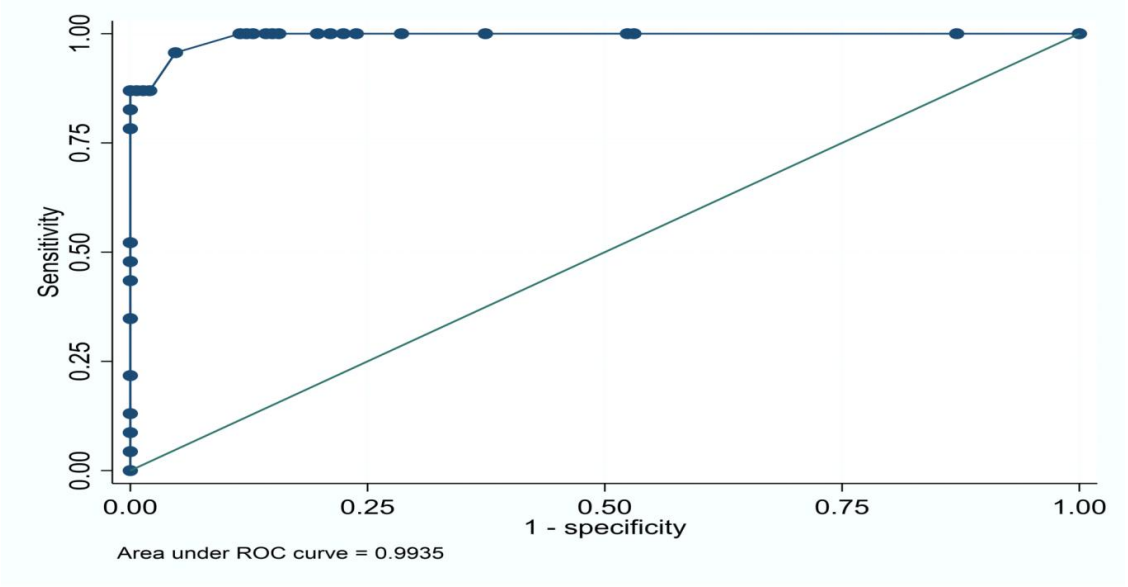

Figure 2: Rock curve of IgM for cytomegalovirus infection in studied women

Bostanabad *et al.* [6] explored cytomegalovirus in 120 women (25-30 years), revealing 38% with >1 abortion history. Uchida *et al.* [28] noted fever's significant correlation with cytomegalovirus in pregnant mothers. Menakaya *et al.* [29] identified the number of sexual partners as a risk factor for cytomegalovirus in women aged 15-53. Zenebe [30] study on 600 pregnant women found a positive link between sexually transmitted infections and cytomegalovirus.

In our study, using PCR test, the prevalence of HCMV was reported to be 13.53% in the investigated women. Recently, Real time PCR has been proposed as a new approach in detecting molecular response to various infectious diseases due to its high specificity and sensitivity [31]. Real Time PCR enables simultaneous detection and identification of several samples [32] In our study, the Real time PCR method was introduced as the gold standard for diagnosing cytomegalovirus infection. Kalaf and Zahraa [33] analyzed 100 samples, including 80 women with abortion and 20 healthy controls, detecting HCMV DNA in 10.0% of CMV- positive patients through PCR. Ali and Saife [34] aimed at molecular detection of CMV in women with abortion found HCMV DNA in 37.2% of cases [38]. Another study in 2020 reported 19% positive results for HCMV DNA in women aged 20-29 with abortions [35]. Aghaei *et al.* [36]

1400 study diagnosed cytomegalovirus infection, with PCR positive in 4.5% of cases from 50 serum samples.

Another laboratory method for identifying viral infections is the ELISA method. Of course, the presence of IgM antibody in patients' serum can indicate a recent cytomegalovirus infection, but it cannot definitively indicate an active infection with this virus [37]. In our study, the sensitivity and specificity for detecting cytomegalovirus were reported as 100% and 6.12% for IgG and 78.26% and 100% for IgM, respectively. In a 2019 study investigating cytomegalovirus infection using ELISA and PCR, among 420 serum samples, 4.05% were HSV positive with ELISA and 1.2% with Real-time PCR, highlighting PCR's sensitivity for CMV and HSV detection [38]. Another study in 2022 explored the relationship between abortion and human cytomegalovirus, reporting HCMV IgG and IgM prevalence ranging from 0-100% and 0-93%, respectively, based on a review of twenty-four articles covering 5442 patients [39].

Abbas *et al.* [40] study on cytomegalovirus serum antibodies among aborted women found that 98.67% were positive for CMV IgG and 7.33% for CMV IgM out of 150 cases. In Al-Mousawi *et al.* [35] study on 90 pregnant women who had a miscarriage, seroprevalence was significantly higher in CMV-positive cases, with 89% positive for IgG and 93% for IgM in aborted women, while the control group showed 45% IgG positivity and all negative for IgM. Al-Awadhi [41] study on 213 pregnant women found 99.5% CMV-IgG positivity and introduced PCR as a highly specific method for detecting cytomegalovirus infection. A 2017 study on 200 patients evaluating ELISA, antigen assay and PCR for CMV diagnosis reported 96.5% positivity with 100% specificity and 97.76% sensitivity [42]. Another 2021 study on a new ELISA method for detecting cytomegalovirus IgG reported 86.72% sensitivity, 96.57% specificity and positive/negative predictive values of 94.40% and 91.60%, respectively [43]. In 2018, a study comparing ELISA and MINIVIDAS systems for detecting cytomegalovirus IgM antibodies found IgM ELISA sensitivity of 84.21% and specificity of 100% [44]. These results are consistent with our results.

CONCLUSION

Real Time PCR is the gold standard for the detection of many pathogens. ELISA test is considered as a preliminary and screening test for CMV infections, IgG detects mostly higher percentage of IgM for all CMV infections. As a result, according to the significant correlation in this study, between molecular and serological methods to detect cytomegalovirus, a stronger and faster molecular method is recommended to detect this virus.

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