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Comparison of RT.PCR and ELISA Methods in the Diagnosis of Human Cytomegalovirus in Kidney Transplant Patients

Fatemeh Bali Chalandar [1], Haniyeh Bashi Zadeh Fakhar [2], Fatemeh Rezaei [2]*

1 Islamic Azad University, Chalus Branch, Department of medical science, Chalous, Iran.

2 Department of Laboratory Science, Chalus Branch, Islamic Azad University, Chalus, Iran.

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ABSTRACT

Background: In kidney transplant recipients prone to infections like cytomegalovirus (CMV), a vital need for an accurate diagnostic method is evident. This study at Khorshid Laboratory in Tehran rigorously compares real-time PCR (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) for CMV detection in transplant patients.

Methods: In January to March 1400, 70 kidney transplant recipients were assessed for CMV DNA using RT-PCR and concurrent ELISA tests, with statistical analysis aided by SPSS.

Results: In kidney transplant patients (average age: 49.40 ± 13.64 years), 4.3% tested positive for CMV via PCR. Strong correlation between serological and molecular methods. IgG and IgM antibody detection both showed high sensitivity and specificity, advocating for efficient CMV diagnosis at Khorshid Laboratory, Tehran.

Conclusion: This study emphasizes the need for a quick and efficient CMV diagnostic approach in kidney transplant patients. The strong correlation between molecular and serological methods favors using the faster RT-PCR method, crucial for timely management, especially with the increasing age-related CMV incidence. The findings strongly recommend RT-PCR integration for enhanced sensitivity in kidney transplant patients at Khorshid Laboratory, Tehran.

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*Corresponding Authors: Haniyeh Bashi Zadeh Fakhar: Department of Laboratory Science, Chalus Branch, Islamic Azad University, Chalus, Iran.

Tel: +98-21-22749213, *E-mail:* haniyehfakhar@yahoo.com

Introduction

Viral opportunistic infections are regarded as the leading causes of morbidity and mortality in patients receiving solid organ and hematopoietic stem cell transplants (1). Some of the most prevalent viral infections following allogeneic transplantation include the Herpesviridae family, such as human cytomegalovirus (HCMV), Epstein-Barr virus, and BK virus (2). One of the most common opportunistic factors in transplant patients is cytomegalovirus (CMV) (3). The HCMV belongs to the Herpesviridae family (2).

The HCMV is present in body fluids, and its transmission route is person to person via contact with nasopharyngeal secretions, urine, saliva, semen, cervical secretions, or blood (4). Africa, Asia, and South America are among the countries with the highest prevalence of HCMV infection. Nonetheless, Western Europe and North America are reported to have the lowest prevalence of HCMV infection (5). Australia, Germany (6), and England (7) are the countries with low CMV prevalence; however, Saudi Arabia is an example of countries with high CMV prevalence (90%) (8). The prevalence of CMV IgG serology in blood donors and healthy individuals in Iran is estimated at 92% (9). The CMV is a ubiquitous herpesvirus that exists in 50-90% of the general population (10).

Since the method of HCMV diagnosis causes graft rejection, it has a leading role in the follow-up and treatment of transplant recipients (11).

Various diagnostic techniques most frequently utilized are validated on serum, such as immunofluorescence (12), chemiluminescence (13), immunochromatography (14), enzyme-linked immunosorbent assay (ELISA) (15), and real-time polymerase chain reaction (PCR) (16). By the adoption of the PCR technique [16], HCMV is quantified in different biological fluids (e.g., whole blood and plasma) (17). Although IgM antibodies in patients' serum might demonstrate a recent cytomegalovirus infection, it cannot accurately indicate active infection with the virus (18). PCR is highly sensitive to detect the virus in urine, blood, plasma and cerebrospinal fluid samples. However, the culture method is time consuming and more difficult to perform (19).

Serological tests are frequently used to determine patient's immune status prior to tissue and organ transplantation; however, it is insufficient and inconceivable to diagnose CMV infection with clinical presentation in immunocompromised persons without confirmatory molecular tests (20, 21)

The prevalence of CMV infection is high in all individuals, and there are important risks that infection with this virus can cause in transplant patients; therefore, accurate and rapid identification method is very important (22). Therefore, this study aims to identify cytomegalovirus in transplant patients with ELISA and RT.PCR was performed.

Materials and Methods

This study was performed on 70 patients receiving kidney transplantation admitted to Baghiyatalah Hospital in Tehran from January to March 2022. All patients are consulted before sampling .

10 mL Blood samples were taken from every patient and were stored in sterile test tubes with ethylenediaminetetraacetic acid (EDTA) which served as an anticoagulant.one part of sample with EDTA transfer to moulcolar labrotory and another part sent to immonology , so there, the test tubes were centrifuged at 3000-4000 rpm for 10 minutes; blood plasma was separated . The CMV was determined in every patient by using quantitative PCR method, as well as by blood plasma anti-CMV IgG and IgM antibodies (quantitative).

The ELISA, carried out using an analyzer (ELISA Reader) according to Pishtaz protocol, was employed to determine the serological status of patients (anti-CMV IgG and IgM antibodies). The cups for anti-CMV IgG and IgM antianti-

CMV IgG and IgM antianti-CMV IgG and IgM antibodies were coated by the inactivated.

Molecular analyses

In this study, DNA extracted from blood was utilized for a customized molecular assay in accordance with the manufacturer's instructions, which required 5 cc of sample. A rapid DNA extraction protocol kit, provided by Roche Diagnostics (USA), was employed for the real-time PCR assay. Specifically, 100µl of the sample was combined with 300µl of a suspension containing 20% (wt/vol) Chelex 100 resin (Bio-Rad Laboratories, Richmond, USA) in a solution of 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, and 0.1% sodium azide, placed in a 1.5-ml tube.

The mixture was briefly centrifuged for 10 seconds, followed by incubation at 100°C for 10 minutes. Post incubation, the sample was centrifuged again for 10 seconds and cooled to room temperature. Once the resin settled completely, 5µl of the supernatant was directly used for amplification.

Prior to conducting PCR, we performed essential evaluations to assess the concentration and quality of the extracted DNA. This included confirming the absence of degradation and checking the size of the purified DNA fragments. The DNA quality was quantitatively measured with an OD ratio between 1.6 and 1.9.

For amplification, primers designed by NCBI, producing a 254 bp fragment, were utilized to enhance binding efficacy in the presence of small DNA quantities (as shown in Table 1). The real-time PCR was executed using the LightCycler Instrument (Roche Diagnostics, Mannheim, Germany). The study incorporated the use of a TaqMan probe with the hot-start technique for all sample analyses, employing the LightCycler® DNA Master Hybridization Probes assay from Roche Diagnostics.

In preparation, the TaqStart antibody was added to a 10x DNA Master solution and incubated for 5 minutes at room temperature. Subsequently, primers, the TaqMan probe, and water were added. Each capillary was loaded with 15µl of the master mix and 5µl of the DNA template. The capillaries were sealed, centrifuged, and placed in the LightCycler rotor. The protocol involved 55 PCR cycles following an initial denaturation step of 2 minutes at 95°C (refer to Table 2).

Statistical Analysis

Then, the significant effective factors were evaluated simultaneously using logistic regression model and data was analyzed using SPSS 16 software.

Results

In this study ,the mean age of patients is in the range of 49.40 ± 13.64. The youngest and oldest participants were 15 and 77 years old, respectively. Most subjects belonged to the age range of 40-60 years (33%). Most participants (72.86%) were male in this study. Based on the underlying factors, 11.43% had a history of CMV positive, 55.71% had weakness and lethargy, 8.57% had a specific disease, 52.86% had a fever, and 48.57% had muscle and joint pain.

IgG and IgM antibody levels in the diagnosis of cytomegalovirus were 53% and 26%, respectively (Table 3). According to PCR, 4.3% of the samples were positive for cytomegalovirus (Table 4).

In comparison of IgM antibodies according to individual and underlying factors, IgM level was reported to be significant in terms of age (P=0.001) and history of CMV (P=0.007), which were reported positive (Table 5).

In comparison of IgG antibodies according to individual and underlying factors, the amount of IgG according to age was p = 0.001 and the history of cytomegalovirus was p = 0.024 and significant

Table 1. Primers designed in this study.

Primer name	Sequence	The length of the piece
UL55-f	5'-ATAGGAGGCGCCACGTATTCC-3	254b
Ul55-r	5'-GTACCCCTATCGCGTGTGTTC-3'	254bp
Ul55-probe	(FAM)5'- ATGGCCCAGGGTACGGATCTTATTC-3'(BHQ1)	

Table 2. PCR amplification program

the level	Temperature and time	Number of cycles
Primary denaturation	10 minutes at 95 degrees Celsius	1
Secondary denaturation	30 seconds at 95 degrees Celsius	45
Accession	30 seconds at 62 degrees Celsius	45

Table 3. Statistical indicators of IgM and IgG antibodies detect cytomegalovirus in the studied samples.

	Mean	Standard deviation	Middle	lowest	highest	Low limit	Upper limit
CMV_IgM	1.36	4.31	0.40	0.10	26.00	0.34	2.39
CMV_IgG	11.43	9.85	10.50	0.60	53.00	9.08	13.78

Table 4. Statistical indicators of IgM and IgG antibodies detect cytomegalovirus in the studied samples.

		Count	Percent	Low limit	Upper limit
CMV_PCR1	Negative	67	95.71	89.00	98.78
	Positive	3	4.29	1.22	11.00

Table 5. Frequency distribution of the studied samples according to the underlying factors.

		CMV_IgM					
		Count	Row N %	Mean	Standard deviation	Middle	P-Value
Age	> 40 years	17	24.29	1.79	6.24	0.20	<0.001
	40-60	33	47.14	0.44	0.16	0.40	
	< 60 years	20	28.57	2.53	5.62	0.60	
sex	Female	19	27.14	1.40	4.29	0.50	0.820
	Male	51	72.86	1.35	4.36	0.40	
CMV positive history	No	62	88.57	0.83	3.25	0.40	0.007
	Yes	8	11.43	5.50	8.29	0.65	

Sluggishness and lethargy	No	44.29	31	0.40	0.14	0.40	0.088
	Yes	55.71	39	2.13	5.69	0.50	
special disease	No	91.43	64	1.46	4.50	0.50	0.136
	Yes	8.57	6	0.33	0.14	0.30	
Fever	No	47.14	33	0.43	0.18	0.40	0.567
	Yes	52.86	37	2.20	5.83	0.40	
Muscle and joint pain	No	51.43	36	0.41	0.19	0.40	0.088
	Yes	48.57	34	2.37	6.06	0.50	

Table 6. Comparison of IgG antibodies in terms of individual and contextual factors.

		CMV_IgG			
		Mean	**Standard deviation**	**Middle**	**P-Value**
Age	> 40 years	5.45	10.55	1.50	<0.001*
	40-60	10.23	4.35	9.50	
	< 60 years	18.48	11.82	15.00	
sex	Female	9.48	7.91	9.50	0.296**
	Male	12.15	10.46	11.00	
CMV positive history	No	9.83	7.10	10.00	0.024**
	Yes	23.84	17.83	15.00	
Sluggishness and lethargy	No	9.56	4.60	11.00	0.679**
	Yes	12.91	12.42	10.00	
special disease	No	11.74	10.17	10.50	0.441**
	Yes	8.05	4.53	9.60	
Fever	No	9.67	5.44	10.00	0.437**
	Yes	12.99	12.42	11.50	
Muscle and joint pain	No	8.58	5.68	9.75	0.034**
	Yes	14.44	12.26	12.00	

Table 7. Significant relationship between serological (CMV-IgM & IgG by Elisa) and molecular diagnosis methods in CMV.

		CMV -PCR		
		Negative	**Positive**	**P_Value**
CMV-IgM by Elisa	Mean	0.70	16.10	<0.001
	Standard deviation	2.29	11.29	
	Middle	0.40	18.50	
CMV-IgG by Elisa	Mean	9.83	47.00	<0.001
	Standard deviation	6.36	5.29	
	Middle	10.00	45.00	

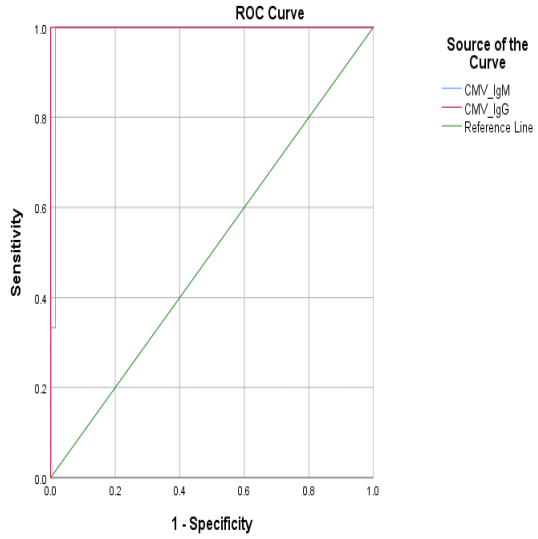

Fig 1. ROC chart of sensitivity and specificity serological diagnostic indicators.

muscle pain was p = 0.034 (Table 6). According to Table 7, there is a significant relationship between serological (CMV-IgM & IgG by Elisa) and molecular diagnosis of cytomegalovirus.

According to the ROC chart, 100% IgG sensitivity and specificity were reported according to serological diagnostic indicators, and 100% sensitivity and 85% specificity were reported for IgM (Figure 1) .

Discussion

The HCMV infection has been observed worldwide (23-24-25). The salient effects of this infection in transplant recipients range from the clinical manifestations of acute cytomegalovirus disease to organ transplant injury or transplant rejection(26.27).Despite the use of antiviral drugs and many efforts to prevent this infection, infection with this virus is still an important cause of disease and mortality after bone marrow and kidney transplantation(28.29). Australia, Germany (30), and the United Kingdom (31) are the countries with low CMV prevalence; nonetheless, Saudi Arabia (90%) (32), Pakistan (94.5%) (33), and Iran (92%) (34) are reported with high CMV prevalence. The results of studies show that there is a significant difference in the incidence of this virus in various parts of the world (35.36).

Bal et al. reported the prevalence of CMV as 10.4% (37). In a study by chiasakul et al., the prevalence of CMV was reported as 26.5% 6 months after transplantation in tailand (38).

Various diagnostic methods are used to identify cytomegalovirus, the most important of which is to identify and prove the presence of antiviral antibodies by methods such as ELISA and molecular methods such as PCR (39).

In our study, the detection rate of cytomegalovirus by PCR method was 4.3%. In a study on 657 patients in 2021 with the aim of determining with CMV and evaluating the clinical outcome in liver recipients with reactivated CMV infections in Shiraz Hospital of Iran using RT.PCR Taq-Man method. The mortality rate in patients with cytomegalovirus was significantly higher than their non-CMV-infected counterparts and the transplant survival rate was not significantly different. According to this study, CMV infection could be a significant predictor of mortality in LT patients . Also in this study, the available cytomegalovirus DNA was reported in 23% of transplant patients (40).

In our study, the detection rate of cytomegalovirus infection by ELISA was 53% IgG and 26% IgM, respectively.

In 2019, a study in golestan was performed to evaluate cmv in pregnant women by ELISA method, which reported a 81% incidence (41). In a descriptive-observational study in 2021 on 1092 samples with the aim of investigating the frequency of CMV during pregnancy for serological markers of CMV was performed using the ELISA method in Sari. Based on the evidence, the prevalence rates of CMV IgG and IgM positive in the studied samples were 91.8% and 0.2%, respectively (42.).

A study was conducted in 2022 to investigate the prevalence of CMV infection in individuals undergoing hemodialysis in Iraq. This study was performed on 100 samples. The results showed that HCMV antibody in patients with renal

insufficiency was reported by ELISAgG 100% and IGM 15 % (43).

In our study, IgM was reported in relation to ELISA, which was significantly associated with age and history of CMV. And were significantly associated with IgG with age variables.

A study was conducted in 2022 to investigate immunological and molecular techniques for the diagnosis of CMV in patients with renal insufficiency. In this study, 100 patients, the majority of whom were male according to our study, were performed. The results showed that HCMV antibodies In patients with renal failure by ELISA method 100% IgG and 15% IgM were reported. These results showed that the patients within the age range of of 60-70 years showed the highest rate of infection among other age groups. Also in this study, the Real method. time pcr was introduced for rapid, sensitive and accurate diagnosis of CMV in these patients(44).

A 2019 study investigated the CMV prevalence in individuals with rheumatoid arthritis (RA) in Iraq. Based on the finding, positive reactions for IgM-CMV and CMV DNA were observed in 46.6% (27/58) and 13.8% (8/58) of RA patients, respectively, in comparison to those of healthy individuals. This study confirmed the association between IgM and age (in the age group over 40 years) and history of CMV (43), in line with the findings of the current study.

In our study there is a significant relationship between serological (CMV-IgM & IgG by Elisa) and molecular diagnosis of cytomegalovirus.

In 2015, a study was performed on the urine samples of 16 patients to identify the HCMV genome in neonatal urine samples based on the diagnostic method of PCR and ELISA. Be sensitive, specific, and reliable in detecting cytomegalovirus infection (19).

Another study was carried out on 315 blood samples of pregnant female subjects in Golestan to compare serological and molecular techniques regarding the estimation of CMV infection frequency. Finally, the results showed that after DNA extraction from molecular technique, It is effective in diagnosing infection with a smaller number of disease genomes (20).

In 2021, a study assessed the prevalence and clinical effect of CMV infection in kidney transplant patients in a hospital in northern India. IgM and IgG ELISA were performed against cytomegalovirus. This study identified CMV as a leading cause of death in kidney transplant patients and reported that PCR was an important tool for early detection of CMV-specific genome early detection of specific antiviral therapy (25).

In our study 100% IgG sensitivity and specificity were reported according to serological diagnostic indicators, and 100% sensitivity and 85% specificity were reported for IgM.

A study was conducted in 2018 with the aim of examining the sensitivity and specificity of ELISA, antigenic assay and PCR in the diagnosis of cytomegalovirus infection in kidney transplant patients on 200 patients. Sensitivity and specificity of each test and all methods were evaluated together and SPSS software was used to analyze the data. Out of 200 patients, 193 (96.5%) CMV antibodies with 100 specificity and 97.76% sensitivity were positive (45). Another study conducted in 2021 investigated a new ELISA to detect IgG in CMV. The aforementioned study reported the overall sensitivity, specificity, and positive and negative predictive values of multiplex ELISA as 86.72% (95% CI, 79.59-92.07%), 96.57% (92.69-98.73%), 94.40% (88.45-97.38%), and 91.60% (87.50-94.44%), respectively (46). Also one study was conducted in 2018 with the aim of comparing the ELISA system and the MINIVIDAS system in the diagnosis of cytomegalovirus IgM antibodies. The ELISA IgM sensitivity was 84.21 and its specificity was 100% (47).

Real Time PCR is the golden standard for the detection of many pathogens (48). It facilitates the detection and amplification of products and helps in the quantification of and qualitative a wider range of sequences of viral

nucleic acids than the majority of quantitative techniques (49). ELISA test is considered as a preliminary and screening test for CMV infections, IgG detected mostly higher percentage than IgM for all CMV infections (50). It should be noted that the incidence of cytomegalovirus infection has increased with age. On the other hand, due to the significant relationship in this study, between molecular and serological methods for the diagnosis of cytomegalovirus, a stronger and faster molecular method for the diagnosis of this virus is recommended.

Conclusion

Based on the comparative analysis of RT-PCR and ELISA methods for diagnosing human cytomegalovirus in kidney transplant patients, RT-PCR proves to be the gold standard, offering higher sensitivity and qualitative analysis for early detection. While ELISA serves effectively as a preliminary screening tool by detecting antibodies, RT-PCR provides superior specificity and rapid diagnosis, crucial for timely antiviral intervention in the clinical management of transplant patients.

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Ethics approval and consent to participate

All the procedures were approved by the institutional and/or national research committee and in compliance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Additionally, this study obtained the approval of the Ethics Committee of Azad University of Medical Sciences, Iran (code no: IR.IAU.CHALUS.REC.1400.102).

Conflict of interest

None decleared.

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