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Cytomegalovirus (CMV) IgG avidity and their relationship with immunoglobulins (IgG and IgM) levels for primary cytomegalovirus diagnosis

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***Abstract*---**In seropositive patients, HCMV disease may occur either by reactivation of a pre-existing strain or by reinfection with a new one. This study aims to assessment of HCMV antibody titers to determine the immune status and the extent to which patients are at risk of infection and measurement of antibody avidity to HCMV and its comparison with molecular diagnosis. The total IgG and IgM using nephlometry technique were measurement. The IgG levels (Mean ± SD) of control group (804.0384±173.789) showed highly significant differences (P< 0.000) among studied groups, and had elevated mean values in comparison with KD and HD patients. Also, IgM levels (Mean ± SD) showed significantly decreased (P< 0.000) in KT patients (20.685±4.787) and HD patients (21.751±6.026) in comparison with healthy control group (129.4503±35.3807). Anti-HCMV IgM, anti-HCMV IgG and anti-HCMV IgG avidity were measured using a method elctro-chemiluminescence immunoassay (ECLIA) by using cobas e411 device. A study conducted on HCMV IgM antibodies showed that there were a percentage of 4(16.66%) among the recent infections and a percentage of 3(12.5%) among cases of intermediate HCMV infection in KT group only, while the rest of the groups (17, 70.84%) did not show any recent infections with HCMV. Also, the anti-HCMV IgG antibodies showed the presence of a percentage 1(4.545%) and 3(9.677%) within the low level reactive with anti-HCMV IgG in Oncology and Control groups, respectively, while percentages 24(100%), 23(100%), 21(95.45%) and 28(90.322%) were found in KT, HD, Oncology and control groups, respectively, within the high level

reactive with anti-HCMV IgG. Furthermore, the results of the anti-HCMV IgG avidity showed that a percentages 17(100%), 12(100%), 7(87.5%) and 8(100%) were found in KT, HD, Oncology and Control groups, respectively, within the high avidity with anti-HCMV IgG. In molecular detection no HCMV was detected among all studied samples. We conclude from our study that there is a difference between serological and molecular diagnosis.

***Keywords*---**Cytomegalovirus, immunoglobulins, cytomegalovirus diagnosis.

Introduction

Cytomegalovirus infection is a matter of concern for blood transfusion recipients, particularly in cases of transfusions to immunocompromised patients (Eivazi-Ziaei *et al.*, 2013). In seropositive patients, HCMV disease may occur either by reactivation of a pre-existing strain or by reinfection with a new one. The importance of HCMV transmission is difficult to assess in HCMV seropositive patients and the outcome of the infection is strongly influenced by the existing immune status of the patient against the virus (Nichols *et al.*, 2002, Ljungman *et al.*, 2002). *Cytomegalovirus* specific IgG and IgM have been investigated in renal transplant recipients by several workers which suggested that the seropositive recipients might be had a greater chance for reactivation of latent HCMV infection and probably for acute graft rejection (Pass *et al.*,). The diagnosis of recently acquired HCMV infection is usually based on the detection of specific immunoglobulin (Ig) M antibodies, seroconversion or a significant increase in specific IgG antibody concentrations. Since seroconversion, the most dependable serological marker, and a rise in IgG titers are seldom demonstrable, the detection of HCMV-specific IgM antibodies has been the most frequently used serological procedure for diagnosing acute infection. However, in some persons, IgM may be detected for many months following primary infection and may also be produced following reinfection or reactivation (Rawlinson, 1999). Several reports have shown that the avidity of IgG antibodies can be used as a marker for distinguishing recent primary from long-term infections, including HCMV infections (Prince and Leber, 2002).

In recent years, there have been studies on the most important viruses that cause diseases in Basrah Governorate, as in studies Shihab *et al.*, 2020, Al. Atbee *et al.*, 2020, Al-Salait *et al.*, 2021, Nasser and Al-Hmudi, 2021 and Almarjan *et al.*, 2021. The documentation on HCMV infection and disease complications in Iraqi renal transplant recipients are lacking in the literature. Therefore, this study aims to evaluating the effectiveness of treatments provided to kidney transplant (KT) patients to avoid viral infections, especially CMV. Also, assessment of antibody titers to determine the immune status and the extent to which patients are at risk of infection, and measurement of antibody avidity to CMV and its comparison with molecular diagnosis.

Material and Methods

Immunological study
Measurement of total serum Immunoglobulin

The total IgG and IgM using nephlometry technique were measurement. A 78 serum samples of both sexes with the age range 20 to 72 years' old were collected from patients attending to Al-Sadr teaching hospital during the period between June 2019 to April 2021. The current study of the immunological assay included a study of 23 Haemodialysis (HD) patients, 24 Kidney transplant (KT) patients and 31 control group. All the samples were stored at -40 0C until used.

Serological diagnosis
Measurement of anti-HCMV IgM, anti-HCMV IgG and anti-HCMV IgG avidity

It was measured using a method elctro-chemiluminescence immunoassay (ECLIA) by using cobas e411 device (CMV IgG Roche Diagnostics GmbH. Germany). A 100 serum samples of both sexes with the age range 20 to 72 years' old were collected from patients attending to Al-Sadr teaching hospital during the period between June 2019 to April 2021. The current study of the serological diagnosis included a study of 23 HD patients, 24 KT patients, 22 oncology patients and 31 control groups. All the samples were stored at -40 0C until used.

Molecular diagnosis

DNA was extracted from blood samples using a DNA extraction Kit (Relia Prep Blood gDNA miniprep kit, Promega, USA) according to the manufacturers' instructions. The nested PCR protocol was used to amplify HCMV DNA. The first round, involved usage of outer primers: 1724 gB and 1264 gB (sequences 5´-GAGTAGCAGCGTCCTGGCGA-3´and 5´- AAACGTGTCCGTCTT -3´), respectively to amplify 478 bp of target region. The reaction mixture (50µl), was composed of 10 µl of DNA template, 10 pmol of 1724 gB and 1264 gB primers, 25 µl of master mix (Promega, USA) and the volume completed to 50µl with DD-Water, while in the second round, inner primer 1604gB (sequence 5´-TGGAACTGGAACGTTTGGCC-3´) and inner primer gB (sequence 5´-GAAACGCGCGCGGCAATCGG -3´) were used to amplify 305 bp of first product. The reaction mixture (25µl), was composed of 5 µl of the first PCR product as a template, 10 pmol of 1604gB and gB primers, 12.5 µl of master mix and the volume completed to 25µl with DD-Water, The reaction conditions for two rounds were 95^0C for 30s, 35 cycles of 55^0C for 40s, 72^0C 60s, with a final extension at 72^0C for 5 min. The Amplified products were visualized on 1.5 % agarose gel (Lukacsi *et al.*, 2001).

Results

Detection of total IgG and IgM by nephlometry technique

The total IgG and IgM using nephlometry technique were measurement; the IgG levels (Mean ± SD) of control group (804.0384±173.789) showed highly significant differences (P< 0.000) among studied groups as shown in table (1), and had

elevated mean values in comparison with KD and HD patients. Also, IgM levels (Mean ± SD) showed significantly decreased (P< 0.000) in KT patients (20.685±4.787) and HD patients (21.751±6.026) in comparison with healthy control group (129.4503±35.3807) as shown in table (1) and figure (1).

Table (1)
total serum immunoglobulin among studied groups

Studied groups	No.	IgG mg/dl		IgM mg/dl	
		Min.-Max.	Mean ± SD	Min. - Max.	Mean ± SD
KT	24	400- 760.9	544.558 ±92.0316	17.12- 31	20.685±4.787
HD	23	200- 766.01	525.347±109.813	5.49- 33.22	21.751±6.026
Control	31	590-1121	804.0384±173.789	45.58-189	129.4503±35.3807
P value 0.000					

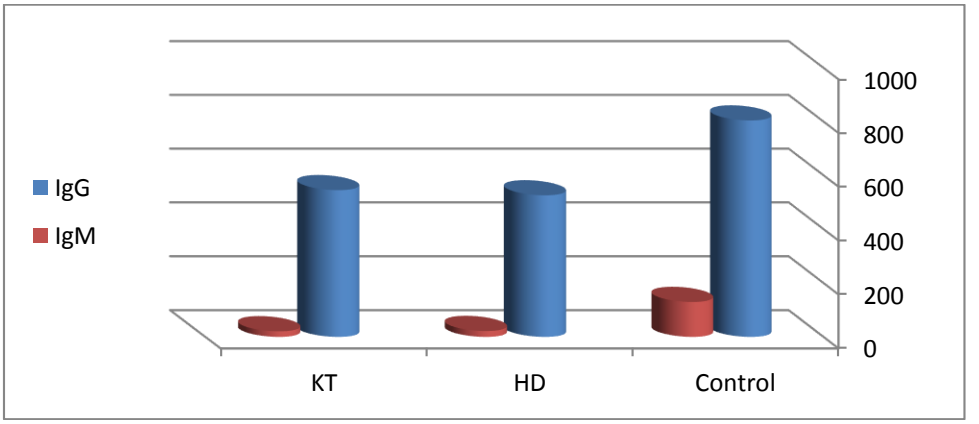

Figure (1) Mean of serum IgG and IgM among studied groups

Measurement of anti-HCMV IgM, anti-HCMV IgG and anti-HCMV IgG avidity

In the current study, the titers of antibodies against HCMV and anti-HCMV IgG avidity were measured. The HCMV IgM levels (Mean ± SD) of KT group (0.765±1.082) showed highly significant differences (P< 0.000) among studied groups as shown in table (2), and had elevated mean values in comparison with studied groups. A study conducted on HCMV IgM antibodies showed that there were a percentage of 4(16.66%) among the recent infections and a percentage of 3(12.5%) among cases of intermediate HCMV infection in KT group only, while the rest of the groups (17, 70.84%) did not show any recent infections with HCMV as shown in table (2).

Table (2)
Serology diagnosis of anti –HCMV IgM among studied groups

Studied groups		Anti –HCMV IgM			
	No.	Mean ±SD	Recent infection (Acute infection) More than 1 COI	Intermediate HCMV infection (0.7-1 COI)	Non reactive (less than 0.7 COI)
KT	24	0.765±1.082	4	3	17
HD	23	0.222±0.106	0	0	23
Oncology	22	0.185±0.037	0	0	22
Control	31	0.215±0.064	0	0	31
Total	100		4	3	93

Regarding the results, The HCMV IgG levels (Mean ± SD) of KT group (455.045±1.082) showed highly significant differences (P< 0.000) among studied groups as shown in table (3), and had elevated mean values in comparison with studied groups. Also, the anti-HCMV IgG antibodies showed the presence of a percentage 1(4.545%) and 3(9.677%) within the low level reactive with anti-HCMV IgG in Oncology and Control groups, respectively, while percentages 24(100%), 23(100%), 21(95.45%) and 28(90.322%) were found in KT, HD, Oncology and control groups, respectively, within the high level reactive with anti-HCMV IgG as shown in table (3). In the anti-HCMV IgG avidity the results showed that level (Mean ± SD) of KT group (186.14±1.082) showed significant differences (P< 0.01) among studied groups as shown in table (3), and had elevated mean values in comparison with studied groups. Also levels (Mean ± SD) of HD group (348.807±1.082) and Oncology groups (320.826±1.082) showed significant differences (P< 0.01) in comparison with control group (306.767±1.082) shown in table (3). Furthermore, the results of the anti-HCMV IgG avidity showed that a percentages 17(100%), 12(100%), 7(87.5%) and 8(100%) were found in KT, HD, Oncology and Control groups, respectively, within the high avidity with anti-HCMV IgG as shown in table (3) and figure (2).

Table (3)
Anti-HCMV IgG and anti-HCMV IgG avidity

Studied groups	Anti-HCMV IgG				Ant-HCMV IgG avidity			
	Mean ±SD	Low (non reactive < o.5 U/ml)	Intermediate (range 0.5-1.0 U/ml)	High (reactive ≥ 1.0 U/ml)	Mean ±SD	Low Av <45	Gray zone 45-54	High >55
KT (n=24)	455.04±94.08	0	0	24(100%)	186.14±30.45	0	0	17(70.84%)
HD (n=23)	348.80±183.20	0	0	23(100%)	172.60±31.22	0	0	12(52.17%)
Oncology (n=22)	320.82±169.82	1(4.55%)	0	21(95.45%)	171.22±51.11	0	1(4.54%)	7(31.81%)
Control (n=31)	306.76±181.11	3(9.68%)	0	28(90.32%)	154.33±41.64	0	0	8(25.80%)

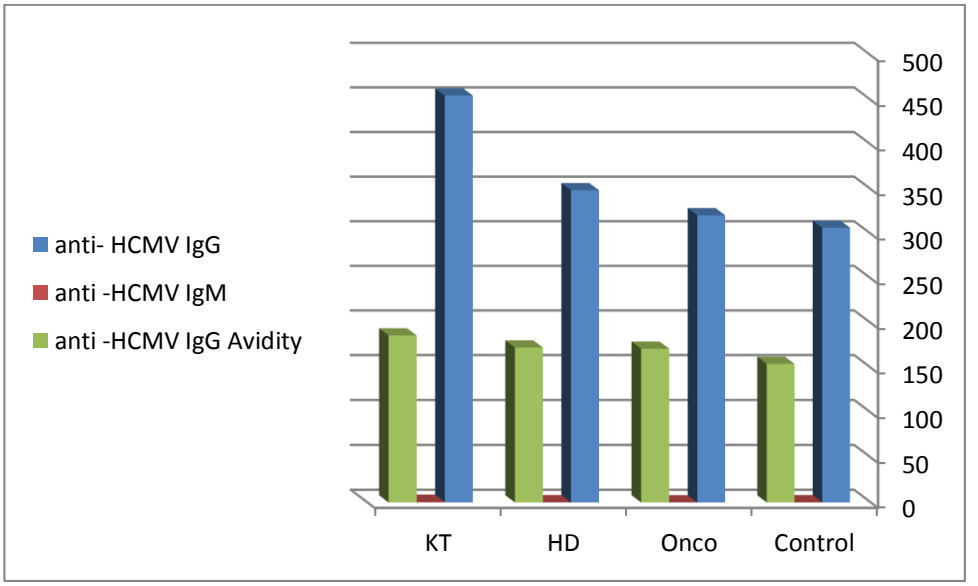

Figure (2) Mean of HCMV antibodies and avidity among studied groups

Molecular detection of HCMV

It is interesting; no HCMV was detected among all studied samples (Figure, 3).

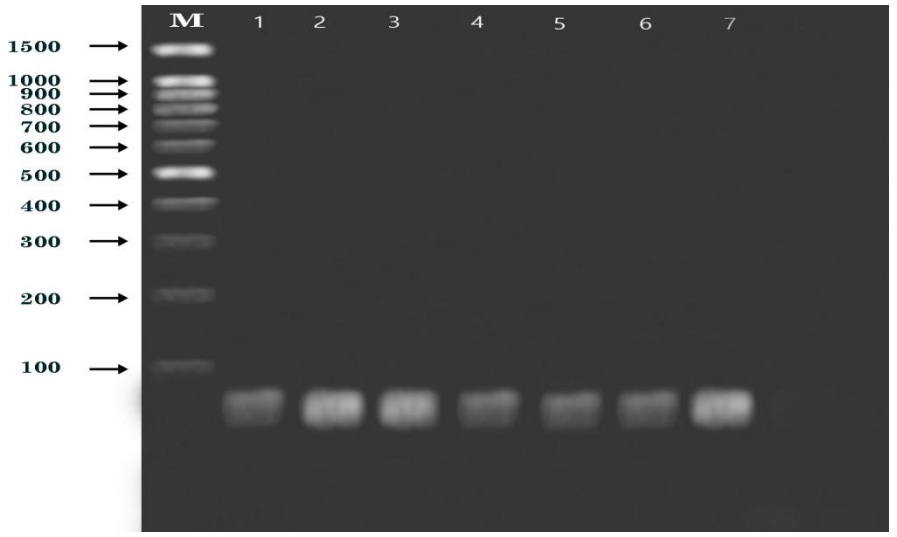

Figure (3) gel electrophoresis of HCMV
Lane **M**: DNA marker (100-1500 bp DNA marker), lanes 1-7: negative results for amplification

Discussion

Measurement of total serum Immunoglobulin

The total IgG and IgM using nephlometry technique were measurement; the IgG levels (Mean ± SD) of control group (804.0384±173.789) showed highly significant differences (P< 0.000) among studied groups, and had elevated mean values in comparison with KD and HD patients. Also, IgM levels (Mean **±** SD) showed significantly decreased (P< 0.000) in KT patients (20.685±4.787) and HD patients (21.751±6.026) in comparison with healthy control group (129.4503±35.3807). This agreed with Yarlagadda *et al.*, 2019 and (Al-Ameri *et al.*, 2014 studies. It was estimated that ESRD is associated with B-cell lymphopenia and suggested that one of the major causes of this disturbance is an increased susceptibility to the death of B-cells by apoptosis (Maisonneuve *et al.*, 1999). This lymphopenia might contribute to the reduced expression of immunoglobulins in these patients, reduced antibody production by B lymphocytes among ESRD patients (Smogorzewski and Massry 2001) and impaired T-cell mediated immunity (Joon *et al.*, 2006).

Measurement of anti-HCMV IgM, anti-HCMV IgG

The transmission of HCMV infection from seropositive donor to seronegative recipient through the transplanted kidney constitutes an important route that was usually associated with unfavorable consequences (Brenan, 2001). In areas such as Iraq, where families have a population of more than three, the prevalence of anti-CMV IgG and IgM determined to have an increasing trend (Mohamed *et al.*, 2014). Moreover, false-positive HCMV results may occur with other herpesviruses and with some autoimmune disorders (Deyi *et al.*, 2000). The comparative analysis demonstrates the studies which employed serological and IHC methods identified higher presence of infection than PCR methods. In this line it should be

mentioned that sometimes even in CMV-positive tumor samples, many tumor cells do not contain the virus genome. This may be explained by specific event during the carcinogenesis. This mechanism is carried out through specific CMV proteins including IE, US28, pp65, and ul44 which disrupts signaling pathways, transcription factors and tumor suppressor proteins in the epithelium of tumors and participates in increasing mutations, angiogenesis and facilitates the immune system evasion for tumor progression (Mohsen, *et al.*, 2020). The 'hit and run' hypothesis is suggested as a main mechanism involved in viral transformation. In "hit-and-run" hypothesis, the virus can exert its long-term effect on cellular process to drive transformation when viral genomes are not present, supporting the importance of last exposure with viral infections (Helmut *et al.*, 2011).

The HCMV IgM levels (Mean ± SD) of KT group (0.765±1.082) showed highly significant differences (P< 0.000) among studied groups, and had elevated mean values in comparison with studied groups. A study conducted on HCMV IgM antibodies showed that there were a percentage of 4(16.66%) among the recent infections and a percentage of 3(12.5%) among cases of intermediate HCMV infection in KT group only, while the rest of the groups (17, 70.84%) did not show any recent infections with HCMV, related to IgM can persist for months after primary infection so can be positive in reactivated CMV infection (Bhatia *et al.*, 2004). HCMV seroprevalence is widespread in areas with lower socioeconomic conditions (Zaki and El Shabrawy, 2016). Furthermore there is certain limitation for the detection of HCMV IgM antibody; the amount of this antibody may not reach a detectable level during early stage of acute infection (Genser *et al.*, 2001; Revello *et al.*, 2002).

Regarding the results, The HCMV IgG levels (Mean ± SD) of KT group (455.045±1.082) with (P< 0.007) among studied groups, and had elevated mean values in comparison with studied groups. Also, the anti-HCMV IgG antibodies showed the presence of a percentage 1(4.545%) and 3(9.677%) within the low level reactive with anti-HCMV IgG in Oncology and Control groups, respectively, while percentages 24(100%), 23(100%), 21(95.45%) and 28(90.322%) were found in KT, HD, Oncology and control groups, respectively, within the high level reactive with anti-HCMV IgG.

Al-Alousy *et al.*, 2011, show high anti-HCMV IgG among KT than control group, these patients were frequently hospitalized for renal dialysis and received blood transfusions before the decision of renal transplantation. Therefore, pre-transplantation dialysis and blood transfusion may explain the significantly higher rates of anti-HCMV IgG among these patients. Renal transplantation always remains as an option for patients with end-stage renal disease (Bishop, 2001; Gamela *et al.*, 2001).

There were most HCMV infection occurs during childhood in both male and females and it of latent for the rest of life. Shaker *et al.*, 2021 show that patients were seropositive for HCMV IgG, and only 11 patients in their study were HCMV IgM Seropositive. Jawad *et al.*, 2018 results of the serostatus assessment of HCMV in the patients and the control groups showed that all study population (100%) was reactive to HCMV specific IgG antibody, while none of them showed reactions to the HCMV specific IgM antibody. In seropositive patients, HCMV

disease may occur either by reactivation of a pre-existing strain or by reinfection with a new one. The importance of HCMV transmission is difficult to assess in HCMV seropositive patients and the outcome of the infection is strongly influenced by the existing immune status of the patient against the virus (Nichols *et al.*, 2002, Ljungman *et al.*, 2003). In general, the seroprevalence is high in developing countries and inversely correlates with the socio-economic status, reaching about 100% in Asia and Africa (Cannon *et al.*, 2010, Thom 2015). The results of Delhi's study on the seroprevalence of HCMV antibody on 200 healthy blood donors. They found that 95% were positive for HCMV IgG antibody and none of the 200 was positive to anti-HCMV IgM antibody (Kothari *et al.*, 2002). In two studies carried out in Iran, during 2005 and 2013, similar results were reported when the latter detected the anti-HCMV antibodies IgM and IgG in blood donors in a percentage of 1.6% and 99.2% respectively estimated by ELISA method (Aghaeipour *et al.*, 2005; Safabakhsh *et al.*, 2013).

Measurement of anti-HCMV IgG avidity

Avidity is strength of aggregation of mixture of polyclonal IgG molecule binds to multiple antigenic epitopes of proteins (Hazell, 2007). IgG antibodies exhibit low avidity following primary infection during the first few months (this weakly bind to the Antigen) in the opposite of antibodies produced after 6 months post infection that exhibit high avidity (that tightly bind to the antigen (Lazzarotto *et al.*, 2008).

In the anti-HCMV IgG avidity the results showed high level (Mean ± SD) in KT group (186.14±1.082) with P=0.260 among studied groups, and had elevated mean values in comparison with studied groups. Also levels (Mean ± SD) of HD group (348.807±1.082) and Oncology groups (320.826±1.082) showed significant differences (P< 0.01) in comparison with control group (306.767±1.082). Furthermore, the results of the anti-HCMV IgG avidity showed that a percentages 17(100%), 12(100%), 7(87.5%) and 8(100%) were found in KT, HD, Oncology and Control groups, respectively, within the high avidity with anti-HCMV IgG, these results agreed with (Zaki and El Shabrawy , 2016 , Jawad *et al.*, 2018) all patients with high avidity IgG were negative by PCR. Others showed that a negative HCMV IgM test result, in combination with a positive HCMV IgG result, does not completely rule out the possibility of an acute infection with Cytomegalovirus as individuals at the early stage of acute infection may not exhibit detectable amounts of HCMV IgM antibodies (Revello *et al.*, 2002). Therefore, low HCMV IgG avidity percentage represents the most reliable marker of recent infection with or without IgM.

IgG antibodies produced during the first few months following primary infection exhibit low avidity (i.e., they bind weakly to the antigen), whereas antibodies produced by 6 months post infection exhibit high avidity (i.e., they bind tightly to the antigen) (Lazzarotto *et al.*, 2008). The antibody maturation/avidity continued in immunocompetent patients for a longer period than previously had been acknowledged (Graugeot-Keros, *et al*, 1997) and that it reached maximum values_1 year after the onset of CMV disease. several previous reports have suggested that the period of antibody maturation is longer for infection with CMV than it is for infection with other viruses: whereas 3–5 months elapse between the onset of primary CMV infection and the detection of IgG antibodies with affinity

sufficient to differentiate between recent infection and past infection (Graugeot-Keros, *et al* , 1997). CMV is known to use multiple mechanisms to elude its host's immune response (Hengel *et al.*, 1998); in particular, CMV's ability to suppress both antigen presentation and polyclonal-antibody production may explain the present study's observation of a prolonged period of antibody maturation after primary CMV maturation. Previous studies of solid-organ–transplant patients had already observed both delayed IgG-antibody maturation (Lutz *et al.*, 1994) and a correlation between prolonged viremia and delayed acquisition of high-avidity antibodies after primary infection (Lazzarotto *et al.*, 1998). However, the last reference populations studied were only solid-organ–transplant patients. Ganciclovir prophylaxis significantly reduces CMV-associated morbidity and mortality in D+/R_ transplant patients (Kletzmayer *et al.*, 2000); nevertheless, previous studies have suggested that ganciclovir can inhibit the immune response to CMV infection (Scholz *et al.*, 1994). The present study has found that prolonged ganciclovir prophylaxis in kidney-transplant patients is associated with delayed CMV seroconversion (Kletzmayer *et al.*, 2000); and antibody maturation. Although these observations have to be interpreted with caution, because of both (1) the small number of patients studied and (2) differences in the incidence of— and therapy used for— rejection episodes(Kletzmayer *et al.*, 2000); long-termantiviral prophylaxis with ganciclovir may, in some patients, have considerable adverse effects on the immune response to CMV. In conclusion, antibody maturation in immunocompetent patients continues for a period longer than previously had been acknowledged, and, because of therapeutic immunosuppression, immunity-evasion mechanisms of CMV, and/or long-term ganciclovir prophylaxis, may be delayed in kidney-transplant patients. Antiviral drugs combined with pretransplantation active immunization may prove beneficial in some patients (Steininger *et al.*, 2004).

Molecular detection of HCMV

Human cytomegalovirus (CMV) is a ubiquitous herpesvirus and is a common cause of complications in immunocompromised individuals, including transplant recipients (Griffith *et al.*, 2015). In this study documented no HCMV DNA in any of study groups samples, this agreed with Jawad *et al* , 2018 study that showed all samples of the patients and the control groups were tested below the threshold line (negative for viral DNA in the peripheral blood). To prevent CMV disease, recent consensus guidelines recommend a prophylactic treatment for three months for R+ patients or up to six months for those receiving potent immunosuppressive induction therapy (Kotton *et al.*, 2018). In developed countries, valganciclovir and oral ganciclovir are currently the most common used drugs for prophylaxis in kidney transplant recipients (Rissing *et al.*, 2017), and seropositive recipients are generally given prophylactic treatment for short periods, followed by monthly monitoring for CMV DNAemia "the presence of viral load in samples of plasma " during the same period. Valganciclovir prophylaxis is an alternative option. CMV prophylaxis was administrated to all patients for six months after transplantation. Valacyclovir prophylaxis was initiated in 48 of 60 renal transplant patients (80%), with a preventative dose of 1500 mg administered four times per day. Twelve recipients of the transplant group (20%) received valganciclovir at a dose of 450 mg every 12 hours, as recommended by the International Consensus Guidelines (Kamar *et al.*, 2008). Drug dosage was

adjusted for renal function when required. All patients receiving antiviral prophylaxis were monitored monthly using Quantitative Nucleic Acid Tests (QNATs) during the first six months. When viral replication was detected in patients with a low viral load without symptoms, regular monitoring by QNAT was performed once weekly until viral clearance was observed, and no patient was treated. Conversely, if a significant symptom (syndrome or disease) or higher viral load values were detected, intravenous ganciclovir 5 mg/kg/dose q 12 h was administered as the main treatment, with the dose adjusted to kidney function (Rezzouk *et al.*, 2021). It has been well documented that hemodialysis patients have impaired immune response, which may result in higher prevalencerates of viral (Jha, 2010) infections, including CMV Infections in these patients may be due to primary infection or, more commonly, by reactivation of latent virus or re-infection with exogenous virus, which may be introduced by blood transfusion or kidney transplant (Cordero *et al.*, 2012). The detection of HCMV DNA in peripheral blood is influenced by the sampling interval. The highest incidence of HCMV DNA detected in blood is achieved when the sample is taken in the period between the last seronegative and the early seropositive (Ziemann *et al.*, 2013). Studies showed that the concentration and prevalence of HCMV DNA in the first seroconversion where higher than the last seronegative sample (Ziemann *et al.*, 2010; Ziemann *et al.*, 2013). As the sampling interval increased two weeks or months from the primary infection, the possibility of missed detection of the virus increased (Zhang *et al.*, 2006). In immunocompetent individuals, the duration of viremia tends to be short (Genser *et al.*, 2001). The HCMV DNA can be detected in a low concentration after detection of the antibody to viral antigen, and as the HCMV avidity percentage is high no Other study found that the HCMV DNA were negative in all (1,086) their long term seropositive plasma samples (Drew *et al.*, 2003, Ziemann *et al.*, 2007). Another study done in 2013 by the author Ziemann who detected HCMV DNA in only one (0.01%) out of 7,303 with long term seropositivity, weak antibody result and low concentration (<30 IU/ml) of DNA in plasma (Ziemann *et al.*, 2013). other study Reyes-Pérez *et al.*, 2016).

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