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Efficient Cytomegalovirus Detection Among Haemodialysis Patients Through qPCR

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Abstract

One of the leading causes of death in Hemodialysis (HD) patients is Cytomegalovirus (CMV) infection. Quantitative PCR is considered as a highly sensitive method as it detects small quantities of nucleic acid in various clinical samples. In Pakistan, nephrology units are not well developed and the investigations involved in normal encounter of viral infections in HD patients have been quite limited. Present study aimed to determine the prevalence of CMV infection, by ELISA and qPCR in patients undergoing HD, in nephrology units in Pakistan. In this cross-sectional study total 150 patients undergoing HD were included by non-probability convenience sampling technique, and those undergoing peritoneal dialysis were excluded. Blood specimen was collected to estimate Hb, TLC, PLT count, bilirubin total, AST, ALT, ALP, urea, creatinine, uric acid and glucose levels. CMV IgM and IgG antibodies were detected by ELISA method and CMV viral load by qPCR. Data was analyzed by using SPSS v.26. Mean of age was found to be 42.28±13.63 years, CMV IgM antibodies level=323±210 AU/mL and CMV IgG antibodies level=311±182 AU/mL. Overall patients' frequency of male was 66.7% and of female was 33.3%. Frequency of patients with positive CMV IgM level was 43.3%, positive CMV IgG level 72.0%, and positive for CMV viral load 82.7%. Prevalence of CMV IgM infection was higher in younger age (45.1% vs. 40.7%), males (45.0% vs. 40.0%), anemic males (46.0% vs. 38.5%), leukocytosis (55.6% vs. 50.0%), thrombocytopenia (32.0% vs. 18.2%). Prevalence of CMV IgG infection was also higher in younger age (74.7% vs. 67.8%), anemic males (73.6% vs. 61.5%), anemic females (73.2% vs. 66.7%), and leukocytosis (80.0% vs. 75.0%). Prevalence of CMV viral load was lower in younger age (81.3% vs. 84.7%, P-value 0.748); males (81.0% vs. 86.0%, P-value 0.593); non-anemic males (76.9% vs. 81.6%, P-value 0.708), leukocytosis (93.3% vs. 100.0%, P-value 0.019), bilirubin total >1.0 (80.4% vs. 83.8%, and BSR (78.7% vs. 88.5%, P-value 0.177). The study concluded that prevalence of positive CMV viral load was markedly higher than prevalence of CMV IgG positive cases and almost twice higher than CMV IgM positive cases. Screening for CMV infection by commonly used ELISA method may give false negative results. Therefore, patients undergoing HD should be screened by quantitative PCR in future.

Keywords: Cytomegalovirus, Haemodialysis, ELISA, qPCR, IgG Antibodies, IgM Antibodies, Leukocytosis, Bilirubin, ESRD, BSR.

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1. Introduction

Cytomegalovirus (CMV) is highly prevalent worldwide, among all socio-economic groups and infects 50.0% to 85.0% of adults of age near 40 years. Its infections are usually endemic in the community and are mostly asymptomatic in childhood. In Africa and Latin America, seroprevalence of CMV ranges from 40-60% and 80-100%, respectively, while in Brazil, ranges from 65-85% (Fowler et al., 2022). The total rate of congenital HCMV infection is proportionate to the population's seroprevalence. Latency of CMV is more widely spread nowadays (Schwartz & Stern-Ginossar, 2023). There were more than 4,000 cases in the UK/year in 1972. Good hygiene practices and reduced close contact between adults & children have decreased the prevalence rate in most of the advanced countries (Jones et al., 2023).

CMV is a double stranded DNA human Herpesvirus, which belongs to the subfamily 'beta-herpesvirinae' of family Herpesviridae (Choi, Lee, Lee, & Lee, 2021). CMV infection is usually acquired in early age and in people living in crowded places. Its seroprevalence increases with rise in age; and it ranges between 30-90.0% in developing countries. Incubation period of virus is 3-6 weeks, and after primary infection, the virus can be found in saliva, blood, and other body fluids in immunocompetent people. Now has become an extremely important pathogen as it is capable of producing congenital as well as acquired infections. WBC's and CD13-positive cells are the main reservoirs for harboring CMV(Pocock, 2018).

CMV is activated and transmitted through close contact with an infected person who is excreting a virus, through saliva, breastfeeding, pregnancy, blood transfusions, sexual contact, organ or stem cell transplant, immunosuppression, and disseminated malignancies. It infects T helper cells, changing their behavior. Human CMV is a widespread virus that can develop latency after primary infection and can be reactivated, especially during immunosuppressive periods. Activation of CMV might be associated with cellular response in the presence of foreign antigens (Forte, Zhang, Thorp, & Hummel, 2020). CMV infection may involve cells; endothelial, fibroblasts, smooth muscle and epithelial. Pathological hallmark is enlarged cell with inclusions bodies. CMV infection and complications are rarely seen among healthy individuals. The interaction of infectious particles with cellular receptors allows them to enter the cell (Anderson-Smits, Baker, & Hirji, 2020).

Diagnosed using a variety of approaches i.e. culturing, cytology and serological procedures to detect the presence of CMV antigens or nucleic acids in infected tissues. Due to late virus excretion, there is a chance that the primary infection may occur again in immunocompromised patient. PCR is considered as highly sensitive method as it detects small quantities of nucleic acid in various clinical samples, used for qualitative detection of CMV-DNA. The biology of CMV infection is quite complex but, in such cases, acquired immunity do not help to preventing from reinfection. Antibodies are essential to prevent from acquisition and spread of CMV with the help of seronegatives. In 2000, CMV was placed in the high priority list for the development of vaccine by the Institute of Medicine, US (Plotkin et al., 2020). There is currently no licensed HCMV vaccine, however recent clinical trials have made progress toward this aim.

Cytomegalic endothelial cells are responsible for causing infections in patients who receive blood transfusions such as patients of HD. Haemodialysis (HD) involves removal of excessive waste, the small particles (urea, creatinine etc.) and fluid outside a human body, used to treat and prevent kidney damage (Saravi & Mousavi, 2022). Blood is taken from the patient's body and pumped out by a

machine called a dialyzer during this procedure. Elevation of specific antibodies against the CMV in HD patients are related to ailments. In chronic and uremic patients on HD, infection caused by the CMV is the main reason for morbidity and mortality due to weakened immune response. There is a high burden of CKD in Pakistan according to the data revealed by a community-based study (Imtiaz & Alam, 2023). Systemic CMV infection occurs at a significant rate and the virus reactivates during severe deregulations of immune system (Khan, Hamid, & Lal, 2022).

Almost, 15-20% of persons with ≥ 40 years of age have a reduced GFR, which results in high prevalence of diabetes and hypertension, and this is one of the major reasons of end stage renal disease (Porrini et al., 2019). As a developing country, Pakistan nephrology units are not well developed but there is a noticeable awareness observed regarding these renal diseases and its co-morbidities such as CMV infection in HD patients since last decade (Naseem, Ayub, Shah, Ali, & Abidi, 2022). In chronic patients, the major cause of ailment and deaths is the infection caused by CMV. However, the investigations involved in normal encounter of viral infection among patients have been very limited. Therefore, the present study was aimed to determine the prevalence of CMV infection by sensitive techniques including; ELISA and quantitative PCR, in patients undergoing HD.

2. Methodology

2.1. Ethical Approval & Consent

The study received ethical approval from the Virtual University of Pakistan's Ethics Review Committee, Lahore.

2.2. Materials

For ELISA; A micro titration plate reader that can detect absorbance at 450 nm, Distilled water, 10 µl, 100 µl and 1 ml of pipettes, a semi-automatic pipette capable of delivering 100 µl, Automatic micro titration plate washer, Absorbent material designed for blotting of strips, incubator, HRP-conjugate and a TMB Reagent solution was used. For qPCR; we used TAN Bead® Nucleic Acid Extraction Kit, Auto Plates, Proteinase K, Elution Buffer and Spin tips. A micropipette No. 2, was procured from Aly. 12, Ln. 81, Longshou St., Taoyuan Dist., Taoyuan City 330, Taiwan Advanced Nanotech Inc. (R.O.C.) 2018-10-18 (revised) Version 2.1. Whole blood and serum specimens transported according to specific pathogen transit laws. During shipping, whole blood sample was kept at 2-25°C and we separated the serum within 6 hours. Serum samples transported at temperatures ranging from 2 to 8 degrees Celsius or frozen.

An interviewer administered close-ended proforma was used to collect data from the participants. The proforma included socio-demographic characteristics; family history of disease; co-illnesses; history off blood transfusion; anthropometric measurements; biochemical assays etc.

2.3. Method

2.3.1 Sampling and Selection Criteria

A Comparative cross-sectional study was carried out during the years of 2019-2020 and non-probability convenience sampling technique was used. In this study, a total of 150 blood samples were taken from patients undergoing HD.

Inclusion criteria includes; patients undergoing haemodialysis, with age ranges from 14 to 90 years, both male and female and belonging to any income class, caste or province of Pakistan. Exclusion criteria include; patients undergoing peritoneal dialysis with known cases of CMV. The data was collected from the dialysis unit. All patients undergoing HD were selected in a non-random way. The blood sample from patient was drawn in an EDTA vial and gel clotted vial after taking their consent and by properly guiding them about this action. Collected blood sample was stored at -20°C freezer for subsequent

Table 1 Reagent Components

Auto Plate	6	96 well plate with reagent buffers Elution Buffer 1.5ml
		Nuclease-Free Water
Proteinase K	1 ml	20 mg/ml Proteinase K, store at 4 ºC
Spin tips	96	Spin tip
Protocol	01	Instruction guide for user

analysis. Questionnaire was designed to record the clinical findings and the laboratory results of the patients.

2.3.2. CMV detection by ELISA

CMV IgM and IgG antibodies were detected by ELISA method. Principle of ELISA includes; Purified CMV antigen is coated on the surface of labelled microwells. When dilute serum is added to the wells, the CMV IgM specific antibody, if present, binds to the antigen. Everything that isn't bound is washed away with the water. The antibody-antigen combination is delivered to the HRP-conjugate, which binds to it. After rinsing away the excess HRP-conjugate, a TMB Reagent solution is added. The enzyme conjugate's catalytic action is halted at a predefined period. The intensity of the color produced is determined on the amount of IgM specific-antibody in the sample. The results were compared to the calibrator and controls in a parallel manner using a microwells reader. Procedure briefly includes following steps.

For Sample collection and handling some precautions were taken. Serum sample was utilized, and the typical venipuncture precautions was followed. Specimens were kept for two days at 2-8°C. Store at –20°C for extended periods. Specimens that had been hemolyzed or lipaemic were not used. Samples were not frozen and thawed repeatedly. All specimens and reagents-maintained at room temperature (~25°C) before use. We labelled the microwells for test. Diluted the serum samples with 1:101 dilution by adding 10 ul sample into 1 ml of Diluent buffer. Dispensed 100 ul diluted sample into each well. At 37°C, incubated for 45 minutes, then Washed the all well for 3 times through automated washer instrument. We filled each well with 100 µl of CMV-HRP Conjugate. Incubated for 45 minutes at 37°C, then Washed all the wells for 3 times through automated washer instrument. Then we added 100 µl of TMB Chromogenic Solution to each well using a dispenser. Incubated at room temperature for 15 minutes. Direct sunlight should be avoided. Using a dispenser, we poured 100 µl of Stopping Solution into each well. Lastly, we measured the absorbance of the solution in the wells with a microplate reader set to 450 nm within 30 minutes. Wavelength correction was possible, thus we set the instrument to dual wavelength measurement at 450 nm with background wavelength correction at 600 or 620 nm. For each control and unknown, we calculated the mean absorbance. For qualitative results; If the sample's absorbance is greater than the Cut-Off, it indicates positive for the presence of a

Table 2 Auto plate Component

Column	Buffer Solution	Volume
1/7	Lysis Buffer	600 µl
2/8	Washing Buffer 1	800 µl
3/9	Washing Buffer 2	800 ul
4/10	Washing Buffer 2	800 ul
5/11	Magnetic Beads	800 µl
6/12	Elution Buffer	80 l

specific IgM. Then we calculated the ratio between the sample's average OD value and the Cut-Off value. The following is a breakdown of the sample: If the ratio is more than 1.1, it is considered positive. If +/- 10% of the Cut-Off, it's unlikely. If the ratio is 0.9, it is negative. If we were not sure about the result, we repeated the test.

Limitations of the procedure may include; when only IgG antibodies are present in a serum sample taken during the late stages of illness, this approach may be negative and the relevance of serological data from immunocompromised individuals and newborn children is limited.

2.3.3. DNA extraction protocol and CMV detection by quantitative PCR

qPCR is suitable for in vitro diagnostic purposes. The Nucleic Acid Extraction Kit of high sensitivity used for clinical diagnostics and research purposes, was used to extract nucleic acids from a CMV. Samples treated with proteinase K prior to automated/semi-automated nucleic acid extraction process by Maelstrom 8.

We followed a protocol for DNA extraction as per instruction guide given with the kit. We, carefully removed the aluminium foil on the Auto Plate. In Auto Plate column #1/ #7, add 300 l serum or PBS solution and 10 l Proteinase K was used. The volume ratio of the mixture and the lysis buffer is approximately 300 l : 600 l. If altered, then may have an impact on performance. We mounted spin tips on Maelstrom 8 then placed Auto Plate to the plate holder of Auto stage. The missing corner of base faces toward the lower left. We selected a program “665-1/7” and parameters were set. When the program ends, we take out the Auto Plate carefully. Transferred the purified nucleic acid from column #6/#12 to a clean tube using a micropipette. No. 2. Then the used Auto Plate and spin tips were placed in the garbage can.

Figure 1 Cobas X 480 for quantitative detection of CMV virus

Nucleic acids extracted analyzed in downstream application, such as real-time PCR. The silicon dioxide coating on the magnetic beads can adsorb negative charged molecules to purify nucleic acid from samples. 300 l serum or PBS suspension as a sample was used. Some precautionary measures were taken. Components stored at room temperature (15-35 °C) until the expiration date on the package. At room temperature, proteinase K was transported. Proteinase K stored at 4°C once received. Vigorous shaking, plate exposure to environment and bleach-based detergent was avoided to maintain reagents stability and shelf life, as these can affect the extraction efficiency. We checked the integrity of the reagent plate before use, and mounted the spin tips in the proper position. Sterile consumables were used. Quantitative analysis was done lastly. Nucleic acid product purified by TAN Bead® nucleic acid extraction kit can perform quantitative analysis of specific genes by Q-PCR or qRT-PCR. It can also be used for detecting viral load other molecular detection analysis. All extracted sample results read by auto analyzer Cobas TaqMan.

The procedure of PCR is only suitable for in vitro diagnostic purposes. Precautionary measures may include. Placed the reagent plate in an oven (preheated 42–60ºC) for 5–10 minutes when the temperature is below 20. Avoid vigorous shaking, in order to avoid excessive formation of foam. Do not expose the reagent or plate to air after it has been opened. The evaporation would

Table 3 Age & prevalence of CMV infection by PCR

			QUANTITATIVE PCR VIRAL LOAD			
			Detected	**Not detected**	**Total**	**p-value**
Age (years)	**≤45**	**Count**	74	17	91	0.748
		Percent	81.3%	18.7%	100.0%	
	>45	**Count**	50	09	59	
		Percent	84.7%	15.3%	100.0%	
Total		**Count**	124	26	150	
		Percent	82.7%	17.3%	100.0%	

change the pH or affect the extraction efficiency. All of the reagents are clear and colorless. The presence of colored

reagent suggests contamination; we replaced the plate with a new one before continuing. We checked the integrity of the reagent plate before use, and mounted the spin tips in the proper position. We carefully removed aluminum foil to minimize splashing. To avoid nuclease contamination, we used sterile consumables. Because the reagent solution contains guanidine salt, do not use bleach-based detergent. Keep reagents away from our eyes, skin, and clothing and wear gloves and masks before handling.

For Specimen transportation, whole blood and serum specimens transported according to specific pathogen transit laws. During shipping, the whole blood sample maintained at 2-25°C and we separated the serum within 6 hours. Serum samples transported at temperatures ranging from 2 to 8 degrees Celsius or frozen.

For Quantitative analysis, Nucleic acid product purified by TAN Bead® nucleic acid extraction kit which can perform quantitative analysis of specific genes by Q-PCR or qRT-PCR. It can also be used for detecting viral load other molecular detection analysis. All extracted sample results read by auto analyzer Cobas TaqMan.

2.4. Statistical Analysis

The close-ended questionnaire included all possible responses with codes was utilized. Using these pre-assigned codes, data entry and analysis was done by using SPSS software version 26. Normality test was performed to assess the distribution of data. Data cleaning/ outliers/ bias removal was done as per protocol. Data was categorized on the CMV infection present and absent. The mean and standard deviation were used to explain numerical variables. Frequency was used to characterize categorical variables (percentage). P-value ≤0.05 was considered as significant.

3. RESULTS

3.1 Age

Total 150 patients undergoing HD were included in the study. Mean age of study population (n=150) was 42.28±13.63 years ranged from 14 to 84 years. Patients were categorized into two age groups i.e. ≤45 years and >45 years. Overall frequency of patients of age ≤45 years was 60.7% and patients of age >45 years 39.3%. Prevalence of CMV IgM positive cases was higher in age group ≤45 years (45.1%) than in >45 years (40.7%). Prevalence of CMV IgG positive cases was also higher in age group ≤45 years (74.7%) than in >45 years (67.8%), as shown in Fig. 2A. Total 82.7% patients were detected as positive for CMV infection. Prevalence of CMV infection was slightly lower in age groups ≤45 years (81.3%) than in >45 years (84.7%). However, the difference between groups was not significant (P-value 0.748), as shown in Table 3.

Table 4 Gender & prevalence of CMV infection by PCR

			QUANTITATIVE PCR VIRAL LOAD			
			Detected	Not detected	Total	p-value
Gender	**Male**	**Count**	81	19	100	0.593
		Percent	81.0%	19.0%	100.0%	
	Female	**Count**	43	07	50	
		Percent	86.0%	14.0%	100.0%	
Total		**Count**	124	26	150	
		Percent	82.7%	17.3%	100.0%	

Table 5a Haemoglobin & prevalence of CMV infection by PCR in Males

			QUANTITATIVE PCR VIRAL LOAD			
			Detected	Not detected	Total	p-value
Hb (g/dL)	**<12.5**	**Count**	71	16	87	0.708
		Percent	81.6%	18.4%	100.0%	
	≥12.5	**Count**	10	03	13	
		Percent	76.9%	23.1%	100.0%	
Total		**Count**	81	19	100	
		Percent	81.0%	19.0%	100.0%	

3.2 Gender

Overall frequency of male patients was 66.7% and female patients 33.3%. Patients were categorized in two groups based on gender i.e. male and female. Prevalence of CMV IgM positive cases was higher in males (45.0%) than in females (40.0%). While, the prevalence of CMV IgG positive as well as negative was equal for both genders as shown in Fig. 2B. Total 82.7% patients were detected as positive for CMV infection. Prevalence of CMV infection was slightly lower in male patients (81.0%) as compared to females (86.0%). However, the difference between groups was not significant (P-value 0.593), as shown in Table 4.

3.3 Haemoglobin Level

Mean of Hb level was 9.51±2.25 g/dL ranged from 4.9 to 14.6 g/dL. Overall frequency of male patients with anemia was 87.0% and 82.0% for females. Male patients were categorized into two groups i.e. Hb <12.5g/dL and Hb ≥ 12.5g/dL. Prevalence of CMV IgM positive cases was higher in group <12.5 (46.0%) than in ≥ 12.5 (38.5%). Prevalence of CMV IgG positive cases was also higher in <12.5 (73.6%) than in ≥ 12.5 (61.5%), as shown in Fig. 2C. Total 81.0% male patients were detected as positive for CMV infection. Prevalence of CMV infection was lower in ≥12.5 (76.9%) group as compared to <12.5 (81.6%) group. However, the difference between groups was not significant (P-value 0.708), as shown in Table 5a.

Female patients were categorized into two groups i.e. Hb <11.5g/dL and Hb ≥11.5g/dL. Prevalence of CMV IgM positive cases was higher in group Hb ≥11.5 (44.4%) than in Hb <11.5 (39.0%). Prevalence of CMV IgG positive cases was higher in <11.5 (73.2%) than in ≥11.5 (66.7%), as shown in Fig. 2D. Total 86.0% female patients were detected as positive for CMV infection. Prevalence of CMV infection was lower in <11.5 (82.9%) as compared to ≥11.5 (100%) group. However, the difference

Table 5b Haemoglobin& prevalence of CMV infection by PCR in Females

			QUANTITATIVE PCR VIRAL LOAD			
			Detected	**Not detected**	**Total**	**p-value**
Hb (g/dL)	**<11.5**	**Count**	34	07	41	0.325
		Percent	82.9%	17.1%	100.0%	
	≥11.5	**Count**	09	0	09	
		Percent	100.0%	0.0%	100.0%	
Total		**Count**	43	07	50	
		Percent	86.0%	14.0%	100.0%	

Table 6 TLC & prevalence of CMV infection by PCR.

			QUANTITATIVE PCR VIRAL LOAD			
			Detected	**Not detected**	**Total**	**p-value**
TLC (cmm)	**<4000**	**Count**	04	0	04	0.019
		Percent	100.0%	0.0%	100.0%	
	4000-10000	**Count**	78	23	101	
		Percent	77.2%	22.8%	100.0%	
	>10000	**Count**	42	03	45	
		Percent	93.3%	6.7%	100.0%	
Total		**Count**	124	26	150	
		Percent	82.7%	17.3%	100.0%	

between groups was not significant (P-value 0.325), as shown in Table 5b.

3.4 Total Leukocyte Count

Mean of TLC was 9318±6953 /cmm ranged from 3200 to 85000 /cmm. Frequency of patients with leucopenia was 2.7% and of leukocytosis 30.0%. Patients were categorized in three groups i.e. TLC >1000/cmm (leukocytosis), TLC 4000-1000/cmm (normal range) and TLC <4000/cmm (leucopenia). Prevalence of CMV IgM positive cases were higher in >1000 (55.6%) than in <4000 (50.0%) while low prevalence of CMV IgM positive cases (37.6%) was observed in 4000-1000. Prevalence of CMV IgG positive cases were higher in >1000 (80.0%) than in <4000 (75.0%) while prevalence of CMV IgM positive cases was (68.3%) in 4000-1000. Details are shown in Fig. 2E**.** Total 82.7% patients were detected as positive for CMV infection against TLC. Prevalence of CMV infection was higher in group <4000 (100.0%) as compared to >1000 (93.3%) and 4000-1000 (77.2%). Although, the difference between the groups was significant (P-value 0.019), as shown in Table 6.

3.5 Platelet Count

Mean of PLT count was 250233±112719 /cmm ranged from 21000 to 720000/cmm. Frequency of patients with thrombocytopenia was 16.7% and of thrombocytosis 7.3%. Patients were categorized in three groups i.e. PLT <150000/cmm (thrombocytopenia), PLT 150000-425000/cmm (normal range) and PLT >425000/cmm (thrombocytosis). Prevalence of CMV IgM positive cases were higher in 150000-425000 (48.2%) than in <150000 (32.0%) while low prevalence of CMV IgM positive cases (18.2%) was observed in >425000. Prevalence of CMV IgG positive cases were also higher in 150000-425000 (78.1%) than in <150000 (60.0%) while

Table 7 PLT count & prevalence of CMV infection by PCR

			QUANTITATIVE PCR VIRAL LOAD			
			Detected	Not detected	Total	p-value
PLT (cmm)	<150000	Count	20	05	25	0.923
		Percent	80.0%	20.0%	100.0%	
	150000-425000	Count	95	19	114	
		Percent	83.3%	16.7%	100.0%	
	>425000	Count	09	02	11	
		Percent	81.8%	18.2%	100.0%	
Total		Count	124	26	150	
		Percent	82.7%	17.3%	100.0%	

Table 8 Bilirubin total & prevalence of CMV infection by PCR

			QUANTITATIVE PCR VIRAL LOAD			
			Detected	Not detected	Total	p-value
Bilirubin total (mg/dL)	≤1.0	Count	83	16	99	0.764
		Percent	83.8%	16.2%	100.0%	
	>1.0	Count	41	10	51	
		Percent	80.4%	19.6%	100.0%	
Total		Count	124	26	150	
		Percent	82.7%	17.3%	100.0%	

low prevalence of CMV IgM positive cases (36.4%) was observed in >425000, as shown in Fig. 2F. Total 82.7% patients were detected as positive for CMV infection against PLT. Prevalence of CMV infection was higher in group

150000-425000 (83.3%) as compared to >425000 (81.8%) and <150000 (80.0%). Although, the difference between the groups was not significant (P-value 0.923), as shown in Table 7.

3.6 Bilirubin Total

Mean of bilirubin total was 1.10±1.07 mg/dL ranged from 0.2 to 8.0 mg/dL. Frequency of patients with hyperbilirubinemia was 34.0%. Patients were categorized in two groups i.e. ≤1.0 mg/dL and >1.0 mg/dL. As shown in Fig. 2G, the prevalence of CMV IgM positive cases was slightly higher in ≤1.0 (44.4%) than in >1.0 (41.2%). Prevalence for CMV IgG positive cases was also higher in ≤1.0 (75.8%) than in >1.0 (64.7%). Total 82.7% patients were detected as positive for CMV infection against Bilirubin total. Prevalence of CMV infection was higher in ≤1.0 (83.80%) as compared to >1.0 (80.4%). However, the difference between groups was not significant (P-value 0.764), as shown in Table 8.

3.7 Aspartate Transferase

Mean of aspartate transferase (AST) level was 53±69 IU/L ranged from 16 to 789 IU/L. Frequency of patients with raised AST level was 46.7%. Patients were categorized in two groups i.e. AST ≥40 IU/L and AST <40 IU/L. In Fig. 2H, it can be seen that prevalence of CMV IgM positive cases was higher in <40 (47.5%) than in ≥40 (38.6%). Prevalence of CMV IgG positive cases was also higher in <40 (76.3%) than in ≥40 (67.1%). In Table 9, Total 82.7% patients were detected as positive for CMV infection against AST. Prevalence of CMV infection was higher in <40 (86.3%) as compared to ≥40 (78.6%). However, the difference

Table 9 AST level & prevalence of CMV infection by PCR

			QUANTITATIVE PCR VIRAL LOAD			
			Detected	Not detected	Total	p-value
AST (IU/L)	**<40**	**Count**	69	11	80	0.306
		Percent	86.3%	13.8%	100.0%	
	≥40	**Count**	55	15	70	
		Percent	78.6%	21.4%	100.0%	
Total		**Count**	124	26	150	
		Percent	82.7%	17.3%	100.0%	

Table 10 ALT level & prevalence of CMV infection by PCR

			QUANTITATIVE PCR VIRAL LOAD			
			Detected	Not detected	Total	p-value
ALT (IU/L)	**<40**	**Count**	67	12	79	0.606
		Percent	84.8%	15.2%	100.0%	
	≥40	**Count**	57	14	71	
		Percent	80.3%	19.7%	100.0%	
Total		**Count**	124	26	150	
		Percent	82.7%	17.3%	100.0%	

Table 11 ALP level & prevalence of CMV infection by PCR

			QUANTITATIVE PCR VIRAL LOAD			
			Detected	Not detected	Total	p-value
ALP (IU/L)	**<140**	**Count**	14	03	17	1.000
		Percent	82.4%	17.6%	100.0%	
	≥140	**Count**	110	23	133	
		Percent	82.7%	17.3%	100.0%	
Total		**Count**	124	26	150	
		Percent	82.7%	17.3%	100.0%	

between groups was significant (P-value 0.306).

3.8 Alanine Transferase

Mean of ALT level was 58±94 IU/L ranged from 14 to 1092 IU/L. Frequency of patients with raised ALT level was 47.3% and 52.7% for normal range. Patients were categorized in two groups ALT <40 IU/L and ALT ≥40 IU/L. In Fig. 2I, Prevalence of CMV IgM positive cases was higher in <40 (48.1%) than in ≥40 (38.0%). Prevalence of CMV IgG positive cases was also relatively higher in <40 (78.5%) than in ≥40 (64.8%). In Table 10, total 82.7% patients were detected as positive for CMV infection against ALT. Prevalence of CMV infection was higher in <40 (84.8%) as compared to ≥40 (80.3%). However, the difference between groups was not significant (P-value 0.606).

3.9 Alkaline Phosphatase

Mean of alkaline phosphatase (ALP) level was 407±371 IU/L ranged from 69 to 3476 IU/L. Frequency of patients with raised ALP level was 88.7% and 11.3% for normal range. Patients were categorized in two groups i.e. ALP <140 IU/L and ALP ≥140 IU/L. Prevalence of CMV IgM positive cases was higher in

Table 12a Uric acid level & prevalence of CMV infection by PCR in Males.

			QUANTITATIVE PCR VIRAL LOAD			
			Detected	Not detected	Total	p-value
UA (mg/dL)	≤7.0	Count	51	10	61	0.569
		Percent	83.6%	16.4%	100.0%	
	>7.0	Count	30	09	39	
		Percent	76.9%	23.1%	100.0%	
Total		Count	81	19	100	
		Percent	81.0%	19.0%	100.0%	

Table 12b Uric acid level & prevalence of CMV infection by PCR in Females

			QUANTITATIVE PCR VIRAL LOAD			
			Detected	Not detected	Total	p-value
UA (mg/dL)	≤6.0	Count	18	05	23	0.225
		Percent	78.3%	21.7%	100.0%	
	>6.0	Count	25	02	27	
		Percent	92.6%	7.4%	100.0%	
Total		Count	43	07	50	
		Percent	86.0%	14.0%	100.0%	

Table 13 BSR & prevalence of CMV infection by PCR

			QUANTITATIVE PCR VIRAL LOAD			
			Detected	Not detected	Total	p-value
BSR (mg/dL)	≤150	Count	70	19	89	0.177
		Percent	78.7%	21.3%	100.0%	
	>150	Count	54	07	61	
		Percent	88.5%	11.5%	100.0%	
Total		Count	124	26	150	
		Percent	82.7%	17.3%	100.0%	

≥140 (72.2%) than in <140 (70.6%). Whereas, prevalence of CMV IgG positive cases was also relatively higher in ≥140 (43.6%) than in <140 (41.2%), as shown in Fig. 2J. In Table 11, Total 82.7% patients were detected as positive for CMV infection against ALP. There is only a slight difference of 0.3 among the prevalence of both groups. Prevalence of ALP <140 is (82.4%) while the prevalence of ALP ≥140 (82.7%). However, the difference between groups was not significant (P-value 1.000).

3.10 Uric Acid

Mean of uric acid level was 6.5±1.8g/dL ranged from 2.8 to 14.6g/dL. Frequency of male patients with hyperuricemia was 39.0% and of female patients with hyperuricemia was 54.0%. Male patients were categorized in two groups i.e. ≤7.0 mg/dL and >7.0 mg/dL. Prevalence of CMV IgM positive cases was slightly higher in ≤7.0 (45.9%) than in >7.0 (43.6%). Prevalence of CMV IgG positive cases was also higher in ≤7.0 (73.8%) than in >7.0 (69.2%). Overall prevalence against both type of antibodies is comparatively low in male patients with hyperuricemia as compared to the normal range, as shown in Fig. 2K. In Table 12a, total 81.0% male patients were detected as positive for CMV infection. Prevalence of CMV infection was higher in ≤7.0 (83.6%) as compared to >7.0 (76.9%). However,

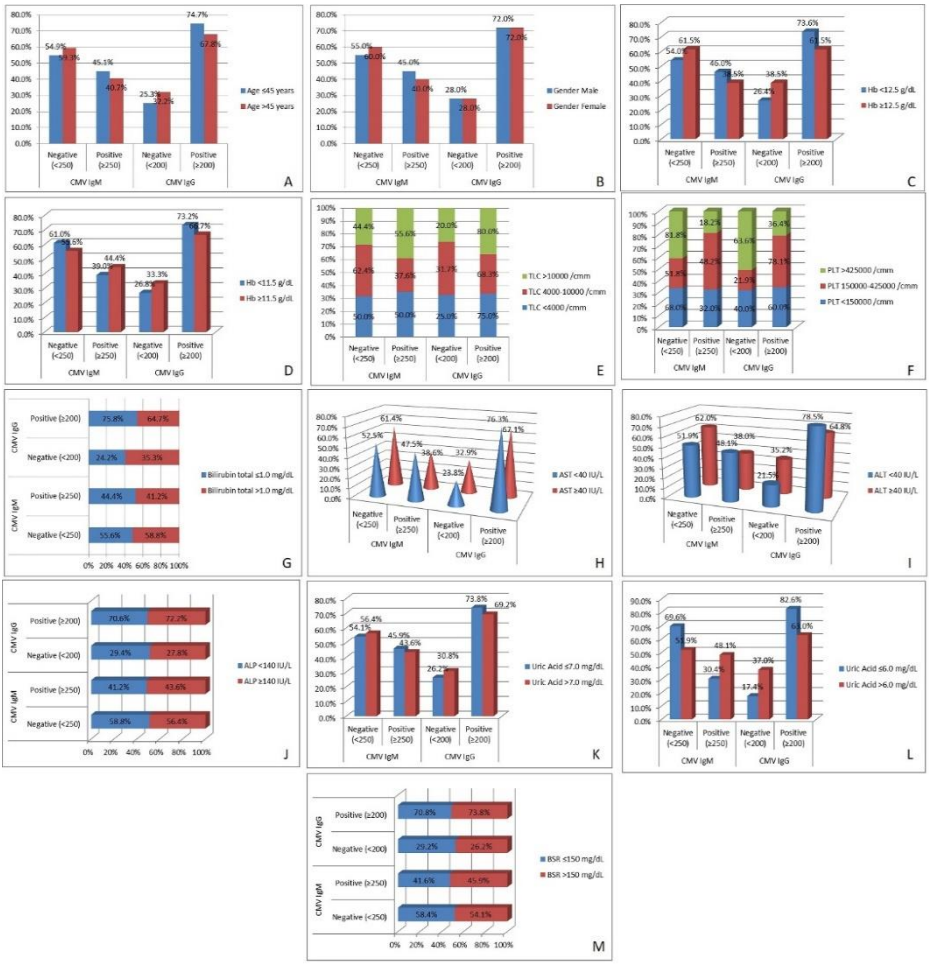

Figure 2 Prevalence of CMV infection by ELISA in relation to multiple parameters: **A.** Age & prevalence of CMV infection by ELISA; **B**. Gender & prevalence of CMV infection by ELISA; **C**. Haemoglobin & prevalence of CMV infection by ELISA in males; **D**. Haemoglobin & prevalence of CMV infection by ELISA in females; **E**. TLC & prevalence of CMV infection by ELISA; **F**. PLT count & prevalence of CMV infection by ELISA; **G.** Bilirubin total & prevalence of CMV infection by ELISA; **H.** AST level & prevalence of CMV infection by ELISA; **I**. ALT level & prevalence of CMV infection by ELISA; **J**. ALP level& prevalence of CMV infection by ELISA; **K**. Uric acid level & prevalence of CMV infection by ELISA in males; **L.** Uric acid level & prevalence of CMV infection by ELISA in females; **M.** BSR & prevalence of CMV infection by ELISA.

the difference between groups was not significant (P-value 0.569).

In Fig. 2L, female patients were categorized in two groups i.e. ≤6.0 mg/dL and >6.0 mg/dL. Prevalence of CMV IgM positive cases was higher in >6.0 (48.1%) than in ≤6.0 (30.4%). Whereas, the prevalence of CMV IgG positive cases was higher in ≤6.0 (82.6%) than in >6.0 (63.0%). In Table 12b, total 86.0% female patients were detected as positive for CMV infection. Prevalence of CMV infection was relatively higher in >6.0 (92.6%) as compared to ≤6.0 (78.3%).

However, the difference between groups was not significant (P-value 0.225).

Table 15 Prevalence of CMV infection by CMV IgG and Quantitative PCR

			QUANTITATIVE PCR VIRAL LOAD			
			Detected	Not detected	Total	p-value
CMV IgG (AU/mL)	Negative (<200)	Count	31	11	42	0.122
		Percent	73.8%	26.2%	100.0%	
	Positive (≥200)	Count	93	15	108	
		Percent	86.1%	13.9%	100.0%	
Total		Count	124	26	150	
		Percent	82.7%	17.3%	100.0%	

Table 14 Prevalence of CMV infection by CMV IgM and Quantitative PCR.

			QUANTITATIVE PCR VIRAL LOAD			
			Detected	Not detected	Total	p-value
CMV IgM (AU/mL)	Negative (<250)	Count	67	18	85	0.228
		Percent	78.8%	21.2%	100.0%	
	Positive (≥250)	Count	57	08	65	
		Percent	87.7%	12.3%	100.0%	
Total		Count	124	26	150	
		Percent	82.7%	17.3%	100.0%	

3.11 Plasma Glucose

Mean of plasma glucose level was 158±76 IU/L ranged from 49 to 510 IU/L. Frequency of patients with raised glucose level was 40.7% and 59.3% for normal range. Patients were categorized in two groups i.e. BSR ≤150 mg/dL and BSR >150 mg/dL. Prevalence of CMV IgM positive cases was higher in >150 (73.8%) than in ≤150 (70.8%). Prevalence of CMV IgG positive cases was also higher in >150 (45.9%) than in ≤150 (41.6%), as shown in Fig. 2M. In Table 13, total 82.7% patients were detected as positive for CMV infection against BSR. Prevalence of CMV infection was relatively higher in >150 (88.5%) as compared to ≤150 (78.7%). However, the difference between groups was not significant (P-value 0.177).

3.12 Cytomegalovirus Antibodies IgM

Mean of CMV IgM antibodies level was 323±210 AU/mL ranged from 109 to 987 AU/mL. Frequency of patients with positive CMV IgM level was 72.0%. Patients were categorized in two groups i.e. CMV IgM negative <250 and CMV IgM positive ≥250. Prevalence of CMV IgM positive cases ≥250 (43.3%) was lower than IgM negative <250 (56.7%). Details are shown in Figure 3A. Total 82.7% patients were detected as positive for CMV infection. Prevalence of CMV infection was relatively higher in CMV IgM Positive ≥250 (87.7%) as compared to Negative <250 (78.8%). The difference between groups was not significant (P-value 0.228).

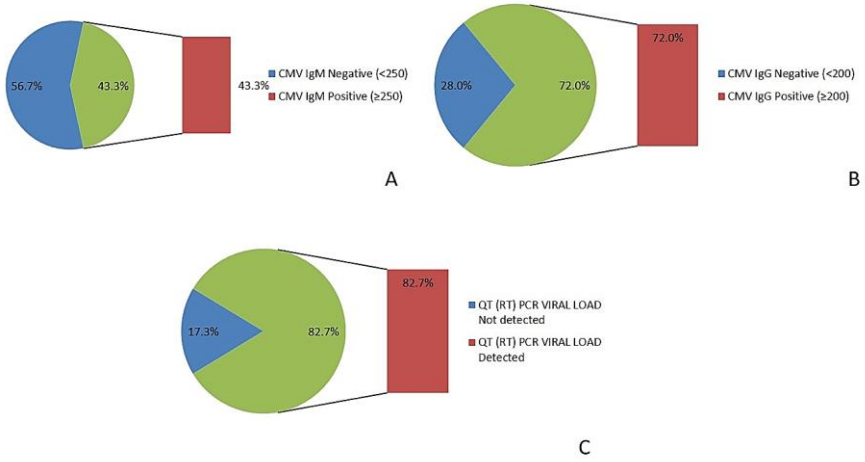

Figure 3 Prevalence of CMV. **A.** Prevalence of CMV IgG antibodies by ELISA. **B.** Prevalence of CMV IgM antibodies by ELISA. **C.** Prevalence of CMV infection by PCR.

3.13 Cytomegalovirus Antibodies IgG

Mean of CMV IgG antibodies level was 311±182 AU/mL ranged from 1.4 to 988.0 AU/mL. Frequency of patients with positive CMV IgG level was 43.3%. Patients were categorized in two groups i.e. CMV IgG negative <200 and CMV IgG positive ≥200. Prevalence of CMV IgG positive cases was higher in ≥200 (72.0%) than in IgG negative <200 (43.3%). Details are shown in Figure 3B. Total 82.7% patients were detected as

Table 16 Comparison of means between QUANTITATIVE PCR positive and negative cases

	QUANTITATIVE PCR VIRAL LOAD				
	Detected		**Not detected**		
	Mean	**Standard Deviation**	**Mean**	**Standard Deviation**	**p-value**
Age (years)	42.9	13.6	39.1	13.5	0.218
Haemoglobin (g/dL)	9.53	2.23	9.44	2.41	0.753
TLC (cmm)	9024.7	3257.8	10716.9	15279.2	0.134
PLT (cmm)	254258.1	108718.8	231038.5	130800.8	0.222
Bilirubin total (mg/dL)	1.1	1.0	1.2	1.5	0.879
AST (IU/L)	53.0	75.6	53.0	28.7	0.144
ALT (IU/L)	58.7	103.2	56.8	36.4	0.197
ALP (IU/L)	412.8	395.1	380.6	234.5	0.958
Urea (mg/dL)	177.1	68.2	170.7	64.8	0.548
Creatinine (mg/dL)	7.4	2.8	7.3	3.5	0.511
Uric acid (mg/dL)	6.6	1.9	6.4	1.8	0.559
BSR (mg/dL)	164.1	79.5	132.6	55.4	0.036
CMV IgG (AU/mL)	328.16	192.09	232.35	90.13	0.014
CMV IgM (AU/mL)	335.08	221.55	265.50	134.49	0.785

positive for CMV infection. Prevalence of CMV infection was relatively higher in CMV IgG Positive ≥200 (86.1%) as compared to Negative <200 (73.8%). Although, the difference between groups was not significant (P-value 0.122).

3.14 Quantitative PCR Viral Load

Frequency of patients with positive CMV viral load was 82.7%. Figure 4.14 shows the crux of all the results, which were obtained for CMV infections. 82.7% QT PCR viral load was detected while 17.3% remain undetected.

In Table 16, means of all variables are distributed along with their P-values. By considering the significant P-value 0.05, we can say that all the categories except BSR (mg/dL) and CMV IgG (AU/mL) were non-significant. Highest viral load was detected in PLT cmm (254258.1) while the lowest (1.1) was detected in Bilirubin total mg/dL. P-value ranges from 0.014 to 0.958. Details are shown in table 16.

4. Discussion

CMV is well-known for causing serious sickness in immunocompromised people; nonetheless, it is currently being recognized as a pathogen of emerging concern for patients with renal failure who are undergoing HD. The infections can strike at any time during a lifetime. CMV is a Herpes DNA virus with two strands, made up of an inner core, a capsid, and an envelope (Nahar, Hokama, & Fujita, 2019). Rise in specific antibodies against the CMV in HD patients are related to diseases like hepatitis, retinitis and pneumonitis. 60-90% of HD patients are seropositive, and this is mainly depending upon the age and socio-economic circumstances (Vilibić-Čavlek, Kolarić, Bogdanić, Tabain, & Beader, 2017). Among methods for CMV diagnosis for CMV antigens or antibodies and viral nucleic acid, PCR and ELISA are considered highly sensitive. The choice for diagnosing IgM and IgG tests for CMV infection depends primarily upon the duration of HD that determined the duration of infection. Noticeable awareness observed regarding the renal diseases and its co-morbidities in relation to CMV infection in HD patients since last decade in Pakistan especially among chronic patients. However, the researches based on normal encounter of viral infections in HD patients have been quite limited, therefore, present study aimed to explore the CMV prevalence by ELISA and qPCR in HD patients.

This study was conducted to estimate the seroprevalence of CMV infections among the renal failure patients undergoing HD with the average dialysis treatment time in this study was approximately two years. In our study, prevalence positive CMV viral load (82.7%) was markedly higher than prevalence of CMV IgG positive cases (72.0%) and almost twice higher than CMV IgM positive cases (43.3%). Results were compared between CMV IgG and CMV IgM antibodies using the sensitivity and specificity measures. The study comprised of 150 individuals categorized into two groups of antibodies against CMV infections with age of study population ranged from 14 to 84 years. At first, prevalence of CMV infections was calculated based on gender and a total of 82.7% patients were detected as positive. Prevalence of CMV infection was slightly lower in age groups ≤45 years (81.3%) than in >45 years (84.7%) (Nikolich-Žugich et al., 2020). Significantly lower prevalence of CMV antibodies in males (81.0%) as compared to females (86.0%) was found. Patients samples were subjected to check the prevalence based on blood Haemoglobin concentration. Prevalence of CMV infection was lower in males ≥12.5 (76.9%) as compared to <12.5 (81.6%) (White et al., 2019). Whereas in females, prevalence of CMV IgM positive cases was higher in group Hb ≥ 11.5 (44.4%) and CMV IgG positive cases was higher in <11.5 (73.2%) (Saleh, Abd Al-Hussien, & Ighawish, 2018).

Leukocyte population reduced mostly during CMV disease and this reduction is independent of any immunosuppressive therapy. Leukopenia is considered as one of the clinical manifestations of CMV infections. The prevalence of CMV infection by ELISA based on TLC was also explored and were checked separately for each concentration as shown in figures. Total 82.7% patients were detected as positive for CMV infection against TLC. While prevalence of CMV infection was higher in group <4000 (100.0%) as compared to >1000 (93.3%) and 4000-1000 (77.2%) (Liang, Famure, Li, & Kim, 2018). PLT and prevalence of CMV was calculated based on three concentrations. Prevalence of CMV IgM and IgG positive cases were higher in normal range 48.2% and 78.1% while thrombocytopenia has low prevalence of 18.2% and 36.45 for CMV IgG. Prevalence of CMV infection was also calculated by the concentration of bilirubin. Positivity of IgM (44.4%) and IgG (75.8%) cases were higher in ≤1.0 than in >1.0 mg/dL.

The frequency of patients with raised AST level was 46.7%. Prevalence of CMV infection was higher in <40 age group (86.3%) as compared to ≥40 (78.6%). Prevalence of CMV infection was higher in <40 (84.8%) as compared to ≥40 (80.3%) (Ye & Zhao, 2017). ALP levels were also calculated, 82.7% patients had raised ALP levels. Prevalence of CMV IgM and IgG positive cases was 72.2% and 43.6% in ≥140 IU/L that indicates that there was a high risk for these patients to get CMV as compared with the normal range. Patients with elevated levels of uric acid are more prone to CMV as well as many other disorders (Lopez Diaz & Macuyama Saavedra, 2022). Frequency of male patients with hyperuricemia was 39.0% and frequency of female patients with hyperuricemia was 54.0%, thus females are at higher risk. CMV seropositivity is somehow associated with many indicators of glucose regulation (Contreras et al., 2019). In the present study, frequency of patients with raised glucose level was 40.7% and 59.3% for normal range and total 82.7% patients were detected as positive for CMV infection against BSR. Prevalence of CMV IgM positive cases was higher than in CMV IgG positive. Prevalence of CMV antibodies i.e. IgM and IgG were checked separately. Prevalence rates of CMV IgM positive and IgG positive were relatively higher as compared to the negative ones. Therefore, the overall seroprevalence of CMV infection among these patients was 99% using CMV IgG and 100% using CMV IgM ELISA (Adane & Getawa, 2021). Figure 3 shows the crux of all the results, which were obtained for CMV infections. 82.7% viral load was detected by PCR (Arapović et al., 2020).

By considering the significant P-value 0.05, we can say that all the categories except BSR (mg/dL) and CMV IgG (AU/mL) were non-significant. Highest viral load was detected in PLT cmm (254258.1) while the lowest (1.1) was detected in Bilirubin total mg/dL. P-value ranges from 0.014 to 0.958. In the present study, CMV PCR positive test was detected in 124 patients while 26 patients were negative with CMV. In these patients, the base rate of CMV IgM seroprevalence was 99. CMV IgM levels have been observed to peak 1–3 months after primary infection and then drop to a low level for 18–39 weeks. The rise in IgM titer may occur before the rise in IgG titer after the initial commencement of illness.

Our findings were in agreement with the previous studies as there was a gradual increase in the seroprevalence of patients above 40 years, prevalence rate is higher in young females and the seroprevalence of CMV IgG and IgM detected was similar to the findings reported in previous studies. Although, many studies have focused on various aspects of the topic, but none of them deal with this particular research idea. No such study was found in literature in which prevalence of CMV is calculated based on Uric acid levels. Despite of the

shortage of data about the sensitivity and specificity of IgG and IgM antibodies, the study will contribute for further research in CMV diagnosis particularly for the patients with renal failure undergoing HD. In addition, the method by which the sample was collected helps to minimize the liability of selection procedures.

5. Conclusion and Research Contributions

CMV infection associated rising cases and mortalities demands some novel early sensitive diagnostics tools with better efficiency. HD linked immunocompromised patients are at higher risk of acquiring CMV infection and related mortality or morbidity in Pakistan. Current study was focused on CMV antigens or antibodies detection via qPCR and ELISA for molecular detection of viral nucleic acid. As a developing country, Pakistan nephrology units are not well developed and CMV infection in HD patients were common. Present study exploited the prevalence of CMV infection by ELISA and quantitative PCR, at department of Molecular Biology, Virtual University of Pakistan, Lahore during 2019-20. 150 patients were enrolled based or specific criteria via non-probability sampling technique and an interviewer administered close-ended proforma was used to collect data from the patients. Blood specimen was drawn and whole blood sample was used to estimate Hb, TLC and PLT count. Serum sample was used to estimate bilirubin total, AST, ALT, ALP, urea, creatinine, uric acid and glucose levels. CMV IgM and IgG antibodies were detected by ELISA method and CMV viral load by quantitative PCR. Data was analyzed by using SPSS v.26.

The study concluded that prevalence of positive CMV viral load (82.7%) was markedly higher than prevalence of CMV IgG positive cases (72.0%) and almost twice higher than CMV IgM positive cases (43.3%). Screening for CMV infection by commonly used ELISA method may give false negative results. Therefore, patients undergoing HD should be screened for the presence of CMV infection by quantitative PCR.

6. Limitations and Future Recommendations

As screening for CMV infection by ELISA method may give false negative results. Therefore, a well-designed cross-sectional validation study with large sample size measuring the diagnostic accuracy of quantitative PCR method versus ELISA method is recommended for future.

Furthermore, dialysis duration related risk, immunosuppressive therapies related outcome bias, and comorbidities are key cofounders, independently influence clinical outcomes but were not fully addressed in our analysis. Thus, future studies should be focused on more comprehensive data collection and statistical data adjustments in order to address these issues. Despite these constraints, the study provides important insights and we plan to address these address confounders in subsequent research by incorporating more detailed clinical data and performing multivariable analysis to better isolate the effects of primary variables of interest.

7. DECLARATION OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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