Detection and Quantification of Cytomegalovirus in Immunocompromised Patients

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ABSTRACT

Introduction: CMV disease ranges from asymptomatic infection in immunocompetent people to severe and life-threatening infections in neonates and immunocompromised patients. The present study was undertaken to study the seroprevalence of CMV and use of Real-time PCR in detection and quantification of CMV in immunocompromised patients.

Material and Methods: A total of 57 samples were tested for CMV IgM and CMV IgG by ELISA technique. The detection and quantification of CMV DNA was done by real time PCR.

Results: Out of 57samples, 6 (10.53%) tested positive for CMV IgM, 50 (87.72%) were positive for CMV IgG and 18 (31.58%) were positive for CMV DNA by PCR. Among 18 PCR Positive cases, 6 (33.334%) cases showed viral load of $1x10^3$ IU/ml. 4 (22.22%) cases each showed viral load of $1x10^4$ IU/ml, $1x10^5$ IU/ml, $1x10^6$ IU/ml respectively. There is no statistical association of age, gender and geographical distribution with CMV IgM, CMV IgG and CMV DNA positivity. (P>0.05).

Conclusion: This study demonstrated that Real-time PCR is more reliable test than serological ELISA test in the diagnosis of cytomegalovirus as Real-time PCR detects and quantifies viral DNA which is useful in predicting the patient's risk for disease and monitoring the effect of antiviral therapy. Good hygiene and infection control practices are advised to prevent CMV transmission.

Keywords: Cytomegalovirus, ELISA, Real-time PCR.

INTRODUCTION

Human cytomegalovirus poses an important public health problem as it may cause serious morbidity and mortality in congenitally infected newborns and immunocompromised patients, most notably transplant recipients and Human immunodeficiency virus (HIV)- infected persons. In most people with a fully functional immune system, an initial infection may cause a mild flu like illness and later the virus remains dormant. A damaged immune system permits CMV reactivation and cause serious complications like pneumonitis, hepatitis, encephalitis, myelitis, colitis, retinitis, fever of unknown origin and neuropathy in immunocompromised patients.[1]

CMV is transmitted from person to person via close contact with an individual who is excreting the virus. It can also spread through the placenta, blood transfusions, organ transplantation, breast milk and sexual transmission. Transfusion of multiple blood units is a risk factor for CMV mononucleosis and has been implicated in postoperative fever or fever in patients following trauma.[2]

Primary CMV infection during pregnancy transmit the virus to approximately 50% of fetuses, 12-20% of whom will be born with cytomegalovirus inclusion disease.[3] The syndrome of congenital cytomegalic inclusion disease include jaundice, splenomegaly, thrombocytopenia, intrauterine growth retardation, microcephaly and retinitis.[2]

CMV disease is a major cause of death in bone marrow and organ transplant recipients and persons with AIDS. CMV is the single most frequent cause of infectious complications in the early period following kidney transplantation, with the overall incidence of CMV infection and disease during the first 100 days post-transplantation being 60% and 25%, respectively, when no CMV prophylaxis or preemptive therapy is given.[4]

It has been documented that at least 25 per cent of AIDS patients develop serious CMV disease. Retinitis occurs in 6-15 per cent of patients, gastroduodenal disease develops in 5-10 percent and at autopsy 30 per cent reveal active CMV infection. The diagnosis poses difficulties because a 2-3 weeks period is mandatory for virus isolation while IgM antibodies as detected by ELISA correlate poorly with the clinical status of CMV infection and facilities for culture are usually not available in most centers. PCR has been introduced for a rapid diagnosis of CMV and a more recent report indicates that PCR is a sensitive method, which predict the development of CMV disease up to several months prior to clinical manifestations.[5] The magnitude of this problem in India and the various diagnostic modalities used have not been adequately investigated therefore present study was undertaken to study the seroprevalence of CMV and use of Real-time PCR in detection and quantification of CMV in immunocompromised patients.

MATERIAL AND METHODS

A total of 57 patients attending tertiary care centre during the period from 1st January 2016 to 30th June 2017 were included in the study. Written and informed consent was taken from the patients. Patients who were immunocompromised with

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any of the following conditions were included in the study: patients who were suffering from malignant disease, infected with HIV, with history of blood transfusion, neonates with history of maternal infection of CMV and pregnancy with bad obstetric history.

Taking all aseptic precautions 5ml of blood sample from study subjects was collected. Serum was separated and transferred into serum storage vials. The samples were appropriately labelled and stored at -20ºC until further processing.

Detection of IgM antibodies to cytomegalovirus in patient's serum was done by ELISA test kit (Human Diagnostics, Germany) and detection of IgG antibodies to cytomegalovirus in patient's serum was done by ELISA test kit (CALBIOTECH, Austin). The tests were performed as per manufacturer's instructions. Optical Density of the samples were recorded on ELISA reader at the end of procedure.[6]

CMV PCR: DNA extraction was done using spin column based method [QIAGEN QIAamp DNA Blood Mini Kit, Germany] followed by detection and quantification of CMV by Real-time PCR (Fast-track Diagnostics kit, Luxemberg).

Procedure for DNA Extraction and Quantification: Six hundred microliter of sample was mixed with 60 µl of protease and 600 µl of lysis buffer followed by 2 µl of internal control from the (Fast Track Diagnostics kit) and the mixture was incubated at 56°C for 10 minutes. The DNA absorbed to the QIAamp spin column was eluted with 55µl of AE buffer and then subjected to PCR.

Real time PCR assay was used for viral load estimation. Quantitative PCR was performed on thermocycler (Bio-Rad CFX96™, Germany). Ten microliter of extracted DNA was added in 15 microlitre of master mix to prepare 25 µl of reaction mixture. Quantitative PCR was performed on thermocycler (Bio-Rad CFX96™, Germany) using the following programme 42ºC for 15 minutes hold, 94ºC for 3 minutes hold, 40 Cycles of: 94ºC for 8 seconds; 60ºC for 34 seconds.[7]

Viral load (IU/ml)	No. of samples (n=18)	Percentage (%)
$1x10^3$	6	33.34%
$1x10^4$	4	22.22%
$1x10^5$	4	22.22%
$1x10^6$	4	22.22%

Table-1: CMV viral load (IU/ml) in PCR positive cases

STATISTICAL ANALYSIS

The Pearson Chi-Square test and SPSS software were used in order to investigate the relationship between variables. *P* value<0.05 was regarded as statistically significant.

RESULTS

Out of 57 cases, 6 (10.53%) were positive for CMV IgM, 50 (87.72%) were positive for CMV IgG and 18 (31.58%) were positive for CMV DNA by PCR. In our study, out of 18 cases positive for CMV DNA, 6(33.34%) cases were having viral load $1x10^3$ IU/ml, and 4(22.22%) cases each of having viral load $1x10^4$ IU/ml, $1x10^5$ IU/m and $1x10^6$ IU/ml respectively (Table 1). In our study, out of 50 CMV IgG seropositives, 15 cases were positive for CMV DNA by PCR and 35 cases were negative for CMV DNA (Table 2).

ELISA		Real Time PCR		
		Positive	Negative	Total
IgG	Positive (50)	15	35	50
	Negative (7)	3	4	7
Total	57	18	39	57
IgM	Positive (6)	1	5	6
	Negative (51)	17	34	51
Total	57	18	39	57

Table-2: Comparative analysis between real-time PCR, IgG ELISA and IgM ELISA for CMV

Age distribution	No. of cases studied	CMV IgM Positives	CMV IgG Positives	PCR positives
0-10 years	13	1 (8.33%)	9 (75%)	6 (50%)
11- 20 years	3	1 (33.33%)	3 (100%)	1 (33.33%)
21- 30 years	15	3 (20%)	15 (100%)	5 (33.33%)
31- 40 years	12	1 (8.33%)	10 (83.33%)	3 (25%)
41- 50 years	12	0 (0%)	11 (91.67%)	2 (16.67%)
51-60 years	2	0 (0%)	2 (100%)	1 (50%)
Total	57	6	50	18

Table-3: Age distribution of CMV seropositive and PCR positive cases (n=57)

Gender	No. of cases studied	CMV IgM Positives	CMV IgG Positives	PCR Positives
Male	31	5 (16.12%)	27 (87.10%)	11 (35.48%)
Female	26	1 (3.84%)	23 (88.46%)	7 (26.92%)
Total	57	6	50	

Table-4: Gender distribution of CMV seropositive and PCR positive cases

Geographical distribution	Total no. of cases	CMV IgM Positives	CMV IgG Positives	PCR Positives
Rural	38	5 (13.16%)	35 (92.10%)	12 (31.57%)
Urban	19	1 (5.26%)	15 (78.94%)	6 (31.57%)
Total	57	6	50	18

Table-5: Geographical distribution of CMV seropositives and PCR positives

Among 7 CMV IgG seronegatives, 3 cases were positive for CMV DNA and 4 cases were negative by PCR. Out of 6 CMV IgM seropositives, only 1 case was positive for CMV DNA by PCR and 5 cases were negative for CMV DNA. Among 51 CMV IgM seronegatives, 17 cases were positive for CMV DNA by PCR and 34 were negative by PCR. In present study, majority of cases (15/57) studied were in age group 21-30 years (Table 3). Maximum seropostivity for CMV IgM (33.33%) was seen in age group of 11-20 years. In present study, out of 57 cases studied, 31(54.39%) were males and 26 (45.61%) were females (Table 4). Cases studied from rural population (38/57) outnumbered urban population (19/57) (Table 5).

DISCUSSION

The presence of CMV viraemia in immunocompromised patients is commonly associated with a high risk of developing CMV disease. Detection and quantification of CMV is therefore required as its determination represents a useful parameter for initiation of antiviral treatment, monitoring the efficacy of antiviral treatment and detection of treatment failure due to emergence of a drug-resistant CMV strain.[1] A total of 87.72% of studied cases (n=57) tested positive to CMV IgG antibodies and 10.53% cases tested positive to CMV IgM antibodies. Similarly, a study conducted by Adeiza et al in Nigeria detected CMV IgG in 86% and CMV IgM in 13.2% in HIV positive patients.[8] According to a study by Velvizhi et al in India, it was found that 7.6% blood donors were positive for CMV IgM and 82.6% donors were positive for CMV IgG.[9]

Most studies worldwide have shown the seroprevalence of CMV IgG to be between 40% and 90%.[10] A study conducted by Bakri et al in the Jazan region, Saudi Arabia, showed prevalence among population to be as high as 89.29%.[11] Another study conducted in healthy blood donors reported higher seroprevalence of CMV IgG to be 92% from Lagos and 96% from Jos, regions in Nigeria, respectively.[12] The high prevalence rates observed in the above countries suggest that quiet a number of people in the study areas have previously been exposed to CMV, and this is contrary to the pattern seen in Western countries such as Australia, Germany, and the US where the CMV IgG seroprevalence is around 50–60%.[13]

Our study showed seroprevalence of CMV IgM to be 10.53% comparable to other parts of world (0-10%). Results of our study are in accordance with the results of Mujtaba et al who reported a CMV IgM prevalence of 10.3% in AIDS patients.[5] However, Akinbami et al. reported a high seropositivity of CMV IgM 19.5% among healthy blood donors.[12]

The positive IgM serology indicate primary infection and the positive IgG serology indicate past infection. The presence of both antibodies signified reinfection. In our study, all CMV IgM antibody positive patients were also CMV IgG antibody positive confirming that all patients developed reactivation of latent infection triggered by immunosuppression and not from primary infection; however, reinfection might also be a possibility. This finding is similar to that of Matos et al in Brazil and Adeiza et al in Nigeria.[8,14]

In our study, CMV DNA was detected by Real-Time PCR from 31.58% samples**.** This is comparable to a study conducted by Halima et al who also detected CMV DNA in 31% of samples taken from immunocompromised patients.[15] A study conducted by EI Sanousi et al reported prevalence of CMV DNA 29.7% among pregnant women.[6] However, Aliyu et al reported detection of 45% CMV DNA among HIV seropositives.[16] Shukla et al reported prevalence of CMV DNA of 49% in India.[7]

In our study, out of 18 cases positive for CMV DNA, 6(33.34%) cases were having viral load $1x10^3$ IU/ml, and 4(22.22%) cases each of having viral load $1x10^4$ IU/ml, $1x10^5$ IU/m and $1x10^6$ IU/ml respectively**.** According to study by Kraft et al, most Laboratory Developed Tests using Real-time polymerase chain reaction technologies, the linear range of CMV viral load tests is usually at least 6 $\log_{10}$ ($1x10^6$ IU/ml). A review of CMV viral load testing performed in 2009 at Emory Medical Laboratories showed 7.9% samples were positive for CMV DNA. Of these 58.7% were <1000 copies/mL ($1x10^3$ IU/ml), 25.2% were between 1000 and 10,000 copies/mL ($1x10^3$ IU/ml and $1x10^4$ IU/ml), 9.4% were between 10,000 and 1,00,000 copies/mL ($1x10^4$ IU/ml and $1x10^5$ IU/ml) and 6.7% had a CMV DNA viral load of >100 000 copies/mL ($1x10^5$ IU/ml). [One IU/mL of CMV DNA is approximately 1.72 copies/mL]. Although low viral load values are common, their clinical significance is unclear. Very high viral load values are uncommon but when they do occur, are usually seen in immunosuppressed patients with primary CMV infection or in those with very severe disease. Because viral load values as high as 10 million ($1x10^7$ IU/ml) may be present in some individuals, the upper limit of quantification of a test should be in this range. Some patients have persistent low levels of CMV DNA in plasma or whole blood and never develop CMV disease or relapse. In these individuals, it is more useful to follow trends in viral loads values over time than to assess the significance of any given viral load value.[17]

In a prospective study by Forner et al. (2014) a CMV DNA viral load > 12,000 copies/mL (10,920 IU/mL or $1x10^4$ IU/ml [log 4.0]) at birth was associated with an increased risk of late-onset CMV disease in infants with asymptomatic congenital CMV infection. In the same population, a birth CMV DNA viral load > 17, 000 copies/mL (15,470 IU/mL or $1.5x\ 10^4$ IU/ml [log 4.2]) was associated with an increased risk of sensorineural hearing loss.[18] Hsiao et al, suggested

CMV DNA viral load threshold of 11,830 IU/ml for the initiation of pre-emptive therapy in infants with suspected CMV pneumonia.[19] Mayaphi et al suggested CMV DNA viral load threshold of 910 IU/ml for the initiation of pre-emptive therapy in HIV-positive patients (CD4 < 200 cells/μL) in ICU.[20] Martin-Gandul et al established a viral load of 3983 (3.9x 10^3 IU/ml) as the optimal cut-off for initiating preemptive therapy in solid organ transplants.[21]

Infection with CMV leads to development of antibodies to the virus that will stay in the body for the rest of that person's life. A blood test of these antibodies can tell whether a person has ever been infected with CMV or not.[11] In contrast, Real-time PCR methods for CMV DNA quantitation offer a convenient alternative for monitoring CMV replication.[6] It also helps to diagnose active CMV infection as CMV DNA viral load showed a qualitative and quantitative correlation with the presence of symptoms.[1]

The specific CMV IgM showed very little relationship with viral replication regarding active and recurrent infections, since it was positive in only 1 of the 18 cases of positive real time polymerase chain reaction for CMV. There are limitations to the interpretation of the test results for IgM, and these should be kept in mind. Disadvantages include false negative results due to abundant IgG and false positive results due to rheumatoid factor interference and in immunosuppressed patient, e.g. chronic renal failure, end stage renal disease and blood diseases. Furthermore, there is a time lag between primary infection and IgM antibody production (IgM level can remain undetectable because of delayed seroconversion owing to immunosupressive agents). IgM antibodies can also persist for a long time after infection in some healthy individuals.[6] Parmigiani et al. reported that the accuracy of the serological tests for the diagnosis of CMV infection was lower than that of the polymerase chain reaction.[6,22]

Seropevalence of CMV IgG in all age groups was generally high. 100% seropostivity for CMV IgG was also seen in each age groups 11-20 and 21-30 years. A study conducted by Adeiza et al reported highest CMV IgG seroprevalence (93.5%) in 16–25 years age group and highest CMV IgM seroprevalence (16.1%) in 26–35 years age group.[8] In our study, CMV IgG seroprevalence in older age 51-60 years seen was 100% which is comparable to study by Bakri et al (2016) who also reported seroprevalence of 100% in old age group[11]. Other studies by Kenneson and Cannon (2007); Staras et al (2006) reported that seroprevalence increases to >80% by the age of 60.[23,24]

In our study, we found maximal CMV DNA positives (50%) each in age group 0-10 years and 51-60 years. Shukla et al reported the highest age specific cytomegalovirus prevalence 76.67% by Real time PCR in the age group of 21-30 years followed by > or =60 years.[7] Statistically no association of age distribution with CMV IgM, CMV IgG and CMV DNA positivity was found (P>0.05). Studies conducted by Kothari et al and Firouzjahi et al also found no association of age with CMV seropositivity.[25,26]

CMV IgM seroprevalence (16.12%) and detection of CMV DNA (35.48%) was higher in males where as CMV IgG seroprevalence (88.46%) was higher in females. Statistically no association of gender distribution with CMV IgM, CMV IgG and CMV DNA positivity was found (P>0.05). Studies conducted by Kothari et al and Firouzjahi et al also found no association of gender with CMV seropositivity.[25,26] Akinbami et al reported higher number of males in their study on blood donors.[12] Adeiza et al also observed higher CMV IgM seroprevalence among males.[8] However, Bakri et al reported a higher percentage of CMV IgG among females (78.35%) as compared to males (64.28%).[11] Shukla et al also reported higher female positivity (59.72%) of CMV DNA as compared to male positivity (45.21%).[7]

Seropositivity for CMV IgM (13.16%) and CMV IgG (92.10%) was higher in rural population as compared to urban population. Sheevani et al also reported more prevalence of CMV IgG (91.2%) in women residing in rural area.[27] The positivity for CMV DNA was same (31.57%) both from rural and urban population. Statistically no association of geographical distribution with CMV IgM, CMV IgG and CMV DNA positivity was found (P>0.05).

Each year, thousands of CMV infections occur in pregnant women and immunosuppressed patients in developing and developed countries, putting numerous unborn babies and patients at risk of infection. Knowledge of prevalence and risk factors for CMV infection will help with prevention strategies. Moreover, PCR assay could guide the timely institution of therapy in patients who are symptomatic yet do not present with a classical CMV syndrome. Further, patients with a positive PCR need to be vigilantly followed up for development of symptoms and early initiation of therapy.

CONCLUSION

The present study was conducted with the objective to scrutinize the prevalence of CMV infection in our population, so that the prevention, prognosis and management can be made easy. Efforts should be aimed at identifying patients at risk of disease prior to the onset of disease.

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