

Molecular Study of Cytomegalovirus Infection among Children with End Stage Renal Diseases Undergoing Dialysis, Pilot Study

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Abstract

Cytomegalovirus is considered as an opportunistic infection affecting immunocompromized patients. Children with end stage renal diseases requiring dialysis is among affected population by this virus. The aim of the present study was to detect and compare the seroprevalence of CMV and CMV antigen pp65 with real time polymerase chain reaction (PCR) among children with end stage renal diseases undergoing dialysis.

The study is a prospective case - control study. The forty one patients included in the studied are registered in the hospital for regular dialysis waiting for renal transplantation. The study included forty one healthy controls with same age and gender distribution. Blood samples were obtained from studied children and subjected for determination of specific immunoglobulin M and G for CMV (IgM-CMV, IgG-CMV) by Elecys system and CMV-DNA determination by real time polymerase chain reaction (PCR) and for PP65 antigenaemia test by light diagnostic CMVpp65.

CMV-IgM was significantly detected frequently ($P=0.0001$) in 12.2% of the patients and in 2.4% of the control children. Moreover, IgG-CMV was significantly more frequently detected in patients ($P=0.0001$) than in control (90.2%&31.7% respectively). CMV-DNA was significantly ($P=0.0001$) detected in 12 patients (29.3%) compared to the control (2.4%), while CMVpp65 was detected among 4 children (9.8%) compared to one child in the control group.

The comparison between IgM-CMV and real time PCR revealed that 30.7% of positive samples by PCR had positive IgM-CMV, while IgG-CMV was associated with 84.6% of positive PCR. CMVpp65 correctly identified all negative samples compared to PCR, while the majority of negative PCR was also negative for IgM-CMV (98.6%). Moreover, all negative children for CMVpp65 was also negative by PCR (100%) For the validity of different CMV markers, IgG-CMV was the most sensitive test (84.7%), CMVpp65 was the most specific test 100%.

From this study we concluded that CMV is a common viral infection among children with end stage renal diseases requiring dialysis. The diagnostic performance of real time PCR is the gold standard technique in diagnosis of this infection. CMVpp65 antigenemia is a specific accurate test for laboratory diagnosis however, it lacks sensitivity. Specific IgG for CMV is good screening diagnostic test.

Keywords: Children, CMV, PCR, CMVpp65 Antigen.

Introduction

Cytomegalovirus (CMV) is a member of β herpesvirus. The infection with CMV is endemic worldwide among immune competent population with prevalence from 50% up to 80% depending upon geographical location. The prevalence markedly increases in immune compromised patients and it's even associated with outstanding morbidity and mortality complications [1, 2].

Among the immune compromised population, patients with end stage renal diseases undergoing hemodialysis, represent a major group susceptible to CMV infection due to several factors beside the immune compromised conditions like the multiple blood transfusions practice, hemodialysis, and the frequency of dialysis in a week [3, 4].

CMV infection in patients under hemodialysis may complicate the further procedure of renal transplantation as it leads to severe complications [2].

Infection with CMV can be classified as primary infection which usually passed unnoticed in immune competent subjects or it may develop to a latent CMV infection that reappear under immune compromised conditions leading to CMV syndrome presented either by nonspecific symptoms fever, malaise, myalgia, arthralgia and anorexia or can produce severe infections such as pneumonia, retinitis, hepatitis [5-7]. There are several reports about the prevalence of CMV in normal population and in adults patients under hemodialysis however, to our best knowledge there are no studies about the prevalence of CMV in children with end stage renal diseases undergoing dialysis [8-10].

The diagnosis of CMV depends mainly on laboratory techniques. Laboratory diagnosis can be performed by several methods either by determinations of specific antibodies either specific immunoglobulin M and/or G by enzyme linked immunosorbent assay (ELISA), agglutination latex and complement fixation test or antigen detection of CMV pp65 protein in peripheral leucocytes by immunofluorescence technique and detection of CMV viral DNA by molecular techniques [11-15].

The aim of the present study was to detect and compare the seroprevalence of CMV and CMV antigen pp65 with real time polymerase chain reaction (PCR) among children with end stage renal diseases undergoing dialysis.

Material and Method

The study is a prospective case - control study that is carried out in Mansoura University Children, hospital, Egypt from August 2016 till February 2017. The forty one patients included in the studied are registered in the hospital for regular dialysis waiting for renal transplantation. The study included forty one healthy controls with same age and gender distribution. The study was approved by Mansoura Faculty of medicine ethical committee. Parents of the included children signed written approval consents.

The patients underwent complete medical history registration and medical examination. Ten millimeter blood sample was obtained from each child and divided to two sterile vacutainers one plain for sera separation and the other was heparinized. The first sample was used for sera separation and kept frozen at -20oC for determination of specific immunoglobulin M and G for CMV (IgM-CMV, IgG-CMV) by Elecys system and CMV-DNA determination by Artus kit. The other heparinized sample was used for buffy coat separation for **PP65antigenaemia test by light diagnostic CMVpp65** (Millipore, UK, Ltd.).

CMVpp65 antigen detection by indirect immunofluorescence (light diagnostic CMVpp65 (Millipore (UK).

Test Principle

It utilizes indirect immunofluorescence assay for detecting early antigen of CMV. The monoclonal antibody binds to immediate early CMV antigen and unbound antibodies are removed by washing. Fluorescein isothiocyanate conjugated (FITC) second antibody is added to bind to antigen antibody complex and the unbound fraction is removed by washing. Bounded FITC will have an apple green fluorescence when illuminated by ultraviolet light allowing visualization of the complex by fluorescence microscopy. Fluorescence of the cell nuclei indicates a positive specimen. Non-infected cells stain with red color due to the presence of Evans blue in the FITC-conjugated secondary antibody.

Principle of Real time PCR for detection of CMV DNA
CMV

DNA Extraction

CMV DNA was extracted from serum samples by the use of Qiagen extract kit for DNA (**QIAGEN GmbH, QIAGEN Strasse 1, D-40724 Hilden**). Extracted DNA was kept frozen at -20oC until time of amplification.

Real-time PCR for CMV-DNA.

Amplification was performed by the use of artusQiagen commercial kit (**QIAGEN GmbH, QIAGEN Strasse 1, D-40724 Hilden**). It detects a 105vp region of the major immediate antigen. Amplification was performed according to the manufacturer protocol. Amplification was performed using the STARTAGENE system (Applied Biosystem, INC, Foster, USA).

The viremia is expressed as copies/μl and to convert it to copies/ml the following equation is used

$$\text{Result (copies/ml)} = \frac{\text{Result (copies/}\mu\text{l)} \times \text{Elution Volume (}\mu\text{l)}}{\text{Sample Volume (ml)}}$$

Statistical analysis

Data entry and statistical analyses were performed using SPSS (statistical package of social sciences) version 16.0 (SPSS Inc., Chicago, IL, USA). Mann-Whitney U test (z) was used to compare non parametric continuous variables in two different groups. Qualitative data are described in number and percent. Pearson Chi-square tests were used to compare the categorical variables between both exposed versus control groups. Roc (Receiver operator characteristics) curve was used to estimate diagnostic accuracy of IgG and Ig M Antibody detection in CMV patients by Area under curve (AUC). P value < 0.05 was considered as statistically significant.

Results

The study included forty one children under regular dialysis and forty one healthy children with similar age and sex distribution for comparison. The mean± SD duration of dialysis was 3.9± 3.1 years. CMV-IgM was significantly detected frequently (P=0.0001) in 12.2% of the patients and in 2.4% of the control children. Moreover, IgG-CMV was significantly more frequently detected in patients (P=0.0001) than in control (90.2%&31.7% respectively). CMV-DNA was significantly (P=0.0001) detected in 12 patients (29.3%) compared to the control (2.4%), while **CMVpp65 was detected among 4 children (9.8%) compared to one child in the control group (P=0.02).**

Table 1: Demographic and virological markers of CMV among studied children

Parameter	Patients (n=41)	Control (n=41)	P
Gender			
Male	22(53.7%)	22(53.7%)	P=1.0
Female	19(46.3%)	19(46.3%)	
Age	13.4± 3.4	13.4± 3.4	P=1.0
Duration of dialysis(- years)			
Mean± SD	3.9± 3.1		
Minimum	1.0		
Maximum	12.0		
range	11.0		
IgM-CMV	5(12.2%)	1(2.4%)	P=0.0001
Mean± SD(Iu/ml)	138.2± 56.7	21.1± 5.5	
Real time –PCR	12(29.3%)	1(2.4%)	P=0.0001
Copies/ml	788.3± 103.4	500	
IgG-CMV	37(90.2%)	13(31.7%)	P=0.0001
Mean± SD(Iu/ml)	228.1± 145.7	121.8± 12.4	
CMVpp65	4(9.8%)	1(2.4%)	P=0.02

The comparison between IgM-CMV and real time PCR revealed that 30.7% of positive samples by PCR had positive IgM-CMV, while IgG-CMV was associated with 84.6% of positive PCR. CMVpp65 correctly identified all negative samples compared to PCR, while the majority of negative PCR was also negative for IgM-CMV (98.6%). Moreover, all negative children for CMVpp65 was also negative by PCR (100%), (**Table 2**).

Table 2: Comparison of antibodies detection, antigen detection method compared to real time PCR as gold standard techniques

PCR		
Positive	Negative	
No.	%	No. %
4	30.7%	1 1.4%
9	69.3%	68 98.6%
		P=0.005
11	84.6%	39 56.5%
2	15.4%	30 43.5%
		P=0.02
4	30.7%	0 0%
9	69.3%	69 100%
		P=0.0001
		IgM-CMV Positive Negative
		IgG-CMV Positive Negative
		CMVpp65 Positive Negative

For the validity of different CMV markers, IgG-CMV was the most sensitive test (84.7%). CMVpp65 was the most specific test 100%, (**Table 3**).

Table 3: Validity of IgM-CMV, IgG-CMV and CMVpp65 compared to real time PCR

Accuracy	Specificity	Sensitivity	
87.8%	98.6%	30.8%	IgM-CMV
50%	43.4%	84.7%	IgG-CMV
89%	100%	30.7%	30.7%

(**Figure 1**) and (**Table 4**) summarized the best cut of values for increasing the sensitivity and specificity of IgG-CMV and IgM-CMV compared to real time PCR.

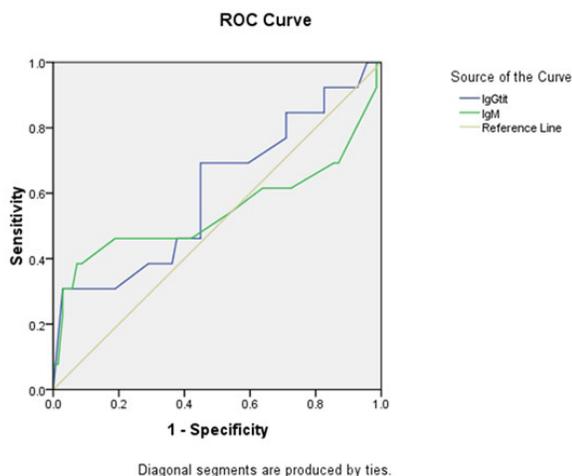
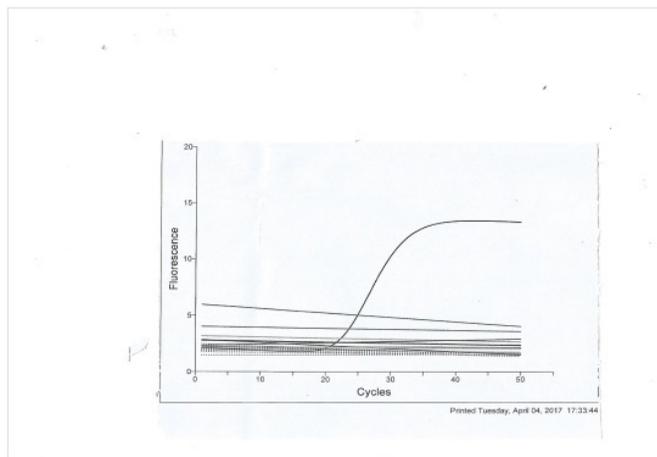


Figure 1: Receiver operative curve for CMV quantitative IgG-CMV and IgM-CMV compared to real time PCR

Asymptotic 95% Confidence Interval		Specificity	Sensitivity	Cut off	Area	
Upper Bound	Lower Bound					
.785	.424	55%	61.5%	157Iu/ml	.604	IgG-CMV
.769	.324	58%	54%	165Iu/ml	.546	IgM-CMV

Figure 2: demonstrated positive sample for CMV by real time PCR.



Discussion

Cytomegalovirus is common infection that is affecting around 90% of the adult population. It is considered as an opportunistic infection in hemodialysis patients. The prevalence of antibodies specific for CMV in adults undergoing hemodialysis ranged from 67% up to 100% in different geographical areas [16-24]. In one Egyptian study, the seroprevalence was 98% for CMV-IgG and 11% for CMV-IgM [9]. However, we could not find any studies about the seroprevalence of CMV among children. In the present study CMV-IgG was detected in 90.2% and IgM-CMV was detected in 12.2% of the children undergoing dialysis. Moreover, the frequencies of seroprevalence were significantly higher in children undergoing hemodialysis than in healthy children. These findings were in contrast to findings from hyperendemic regions like Sudan and Saudi Arabia where there were no significant difference among prevalence between adults' patients under hemodialysis and general population [22-24]. The higher seroprevalence of CMV in hemodialysis patients might be attributed to the acquisition of CMV through the multiple blood transfusions practice and the exposure to CMV during hemodialysis procedures [8]. It is worth saying that we do not screen blood for CMV nor we practice routine blood bags irradiation for CMV for children under hemodialysis.

In the present study, the antibodies titer for CMV-IgG was significantly higher in patients compared to the control. This finding is similar to previous study in Thailand [25]. In contrary, Vilibic-Cavlek, et al., 2015 found no significant difference in the titer of CMV-IgG between patients and control, indicating that CMV tend to be persistent or may become chronic active in dialysis patients. It was suggested by Hardiman et al., 1984 [26].

that in dialysis patients with high IgG titers to CMV had a greater chance of having chronic active infection of CMV. According to this study, the clinical pictures were silent no matter whether in patients with recurrent or chronic CMV infections.

Among immunosuppressed individuals, reactivation of latent CMV infection seems to be more frequent than that experienced by the general population [27, 28]. Reactivation of CMV in hemodialysis patients may be caused by the uremia-associated immunodeficiency in these patients [29].

In this study, the prevalence of active CMV infection as detected by real time PCR for viral DNA was significantly ($P=0.0001$) detected in 12 patients (29.3%) compared to the control (2.4%). While CMVpp65 was detected among 4 children (9.8%). Similar frequency of positive PCR 30% was previously reported in adults undergoing hemodialysis in Egyptian patients. The discrepancy of the results between PCR and antigen detection can be attributed to the fact that CMV viremia occurs one week earlier than did pp65emia [30].

In the present study the comparison between IgM-CMV and real time PCR revealed that 30.7% of positive samples by PCR had positive IgM-CMV. The kinetic of CMV-IgM during infection reveals that IgM peak during the first three months after primary infection and persists at low levels for up to 10 months. The detection limit depends upon the immune status of the patients and the sensitivity of the assay [31]. This is in agreement with previous reports [31,32]. However the low sensitivity of this test slightly improved by the calculating cut of levels of IgM165Iu/ml with 54% sensitivity specificity 58%.

This finding might suggest the value of this test to diagnose recent infection, assuming that all the infected patients had acquired infection after starting of dialysis and so there was a decline of IgMtiter as the mean duration of the dialysis was 3.9 ± 3.1 .

On the other hand, for CMV-IgG compared to PCR, the sensitivity was 84.7%, this finding indicates that a sensitive IgG-CMV can be used as a clue for recent infection with CMV assuming that all the infected patients acquired infection after starting of dialysis [9].

The most specific laboratory method for detection of CMV was CMVpp65 with specificity 100% however; this method had low sensitivity 30.7%. The evaluation of the correlation between the two diagnostic methods showed that they have a good correlation. This is in agreement with previous comparisons between pp65 antigenemia and PCR for the diagnosis of active CMV infection [33-37].

Although there was significant association between the results of IgG, IgM, CMVpp65 and PCR testing in our patients the use PCR test had been used to identify all patients with CMV infection and was considered as the gold standard technique. Other wide scale studies are required to assure the adequate laboratory diagnosis of CMV in children with end stage renal diseases requiring dialysis.

There were some limitations of the present study as it included only one dialysis center and the number of the included children were small. However, the inclusion of many laboratory methods for diagnosis of CMV in children under dialysis is novel as

other studies included adults only. Also, there is an importance for diagnosis of CMV in those patients as they all are candidate for renal transplantation and screening of CMV can aid in the appropriate selection of the donors.

From this study we concluded that CMV is a common viral infection among children with end stage renal diseases requiring dialysis. The diagnostic performance of real time PCR is the gold standard technique in diagnosis of this infection. CMVpp65 antigenemia is a specific accurate test for laboratory diagnosis however, it lacks sensitivity. Specific IgG for CMV is good screening diagnostic test.

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