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ORIGINAL ARTICLE

Real-Time Polymerase Chain Reaction Compared to Nested Polymerase Chain Reaction and Enzyme-Linked Immunosorbant Assays for Detecting Cytomegalovirus Infection in Children

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ABSTRACT

Background: *Patients with impaired immunity e.g. (use of immunosuppressant therapy, or chemotherapy) are at increased risk of Cytomegalovirus (CMV) infection. Diagnosis of this viral infection is faced with many difficulties in such patients.* ***Objectives:*** *This study aims to compare between ELISA, nested PCR, and real-time PCR in order to set up a highly sensitive applicable assay for detecting CMV in high risk children.* ***Methodology:*** *This study was conducted from September 2015 to May 2016. Two ml blood sample were collected from each of 366 children with suspected CMV infection. CMV specific IgM and IgG were determined in the sera using ELISA. CMV DNA was detected in the plasma using nested PCR. Quantitative real-time PCR was used to determine the viral load.* ***Results:*** *The highest prevalence (50%) of CMV IgM seropositivity was reported from patients suffering from fever of unknown origin. Using the molecular methods, CMV was detected only in patients suffering from malignant hematological disease and receiving chemotherapy, where 83.7% and 67.4% of patients with malignant hematological disease were positive for CMV by real-time and nested PCR respectively. In comparison to real time PCR, nested PCR was 80.5% sensitive and 100% specific; ELISA IgM was 22.2% sensitive and 84.24% specific, where ELISA IgG was 0% sensitive and 71.82% specific for the detection of CMV infection in high risk children.* ***Conclusion:*** *PCR is more sensitive and specific technique for detection of CMV infection in high risk children. Moreover, quantitative real-time PCR is superior to nested PCR as it can define threshold levels needed to guide treatment.*

INTRODUCTION

Human cytomegalovirus (HCMV) is a member of the *Betaherpesvirinae* subfamily which belongs to the family *Herpesviridae* [1]. In immunocompetent individuals, cytomegalovirus infections are usually asymptomatic or manifest as a mild mononucleosis-like syndrome [2]. However, HCMV poses a significant health threat to immunocompromised individuals [1]. CMV establishes lifelong or persistent infection in the host [3] where it may reactivate and produce consistently infectious virions. Reactivation of CMV infection has been observed in patients with impaired immune response [4].

Diagnosis of HCMV disease is based on clinical symptoms, but its symptoms can be confused with those due to Epstein-Barr virus (EBV) leading to difficulties in diagnosis [1]. Rapid diagnosis of active CMV infection is of great importance to avoid over treatment with immunosuppressive drugs and to guide antiviral therapy [5]. Diagnosis of CMV infection could be achieved by serological and molecular techniques [1].

The sensitivity and specificity of commercially available ELISA assays in the diagnosis of CMV patients remain as source debates that need further studies to be rebated [6]. Although reported to be useful in detecting CMV infection, nested PCR is not quantitative and the level of CMV infection is often difficult to infer from the results [7]. Real-time PCR proves to be the ideal test for diagnosing CMV infections [6] not only because it is more sensitive, but also it is able to early detect CMV reactivation and more suitable for monitoring CMV reactivation [7]. The aim of the present study is to set up a highly sensitive applicable assay for detecting CMV in high risk children in order to establish and improve its laboratory diagnosis.

METHODOLOGY

This study was conducted at Microbiology & Immunology and pediatric departments, Faculty of Medicine, Zagazig University Hospitals, in the period from September 2015 to May 2016.

Patient criteria

This study has been conducted on 366 patients with suspected CMV infection attending pediatric department at Zagazig University Hospital. They included 202 males and 164 females. Their ages ranged

from 2 days to 18 years. The group included 164 patients receiving repeated blood transfusion, 64 patients suffering from chronic renal failure and under haemodialysis, 43 patients suffering from malignant hematological disease and receiving chemotherapy, 28 critically ill patients lying in the intensive care units with prolonged hospitalization, 22 patients receiving corticosteroids or other immunosuppressive agents, 22 neonates with congenital anomalies, 16 patients having fever of unknown origin, and 7 patients suffering from fever with pancytopenia. For each patient, data were collected including age, sex, hospital ward or ICU, duration of hospitalization, underlying disease or risk factor, and general health condition of the patient.

Sample collection.

From each subject, about 2 ml blood sample were aseptically collected by venipuncture. Sera and plasma were separated and stored at -20°C till the serological and molecular testing.

Detection of HCMV antibodies.

Anti-CMV IgG and IgM were measured by ELISA kit (Bio Check, Inc (323 Vintage Park Drive Foster City, CA 94404) according to the manufacturer's instructions. An ELISA index of 1.0 or greater was considered positive. Samples were considered negative if ELISA index was less than 0.90. Results were considered equivocal and must be repeated if ELISA index was between 0.91 and 0.99.

DNA extraction.

The DNA was extracted from the plasma by using the GeneProof pathogenfree DNA isolation kit according to the manufacturer's manual (GeneProof a.s., Vídeňská 119, 619 00 Brno, Czech Republic).

Nested PCR.

Extracted DNA (5µl) was amplified by a nested PCR with primers specific for immediate-early gene region 1 [outer primer set, consisted of MIE-4 (5′-CCAAGCGGCCTCTGATAACCAAGCC-3′) and MIE-5 (5′-CAGCACCATCCT CCTCTTCTCTGG-3′); inner primer set, consisted of IE-1 (5′-CCACCCGTGGTGC CAGCTCC-3′) and IE-2 (5′-CCCGCTCCT CCTGAGCACCC-3′)]. For the first round PCR, the following materials were added to each tube containing a PCR bead (INtRON BIOTECHNOLOGY, Korea): 2 µl of each outer primer were added to achieve a final concentration of 0.4 pm/µl, 5 µl of DNA extract and sterile deionized distilled water to a total volume of 20 µl. For the second round of amplification, the same was done as the first step except that inner primer set was used instead of outer primer set and 5µl of the first round PCR product were used instead of 5µl of the DNA extracted. The conditions were 30 cycles of DNA denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. The final extension step was carried out for 10 minute at 72°C, as previously described[7]. The reaction product was resolved by electrophoresis using 2% agarose gels containing ethidium bromide. A PCR result was considered positive if a DNA band of 159 bp was present (Figure 1).

Real-time PCR.

CMV viral load testing was carried out using the GeneProof Cytomegalovirus PCR Kit according to the manufacturer's instructions (GeneProof a.s., Vídeňská 119, 619 00 Brno, Czech Republic). The PCR Master Mix contains reagents and enzymes for the specific amplification of a single-copy gene for the exon 4 IE antigen. A standard calibration curve was created by the quantitation standard CMV DNA positive calibrators provided by the manufacturer. For the PCR amplification, 10 µl of DNA sample elute was added to 30 µl of the working master mix. Amplification conditions were as following: 95°C for 10 minutes, followed by 45 cycles of 95°C for 5 seconds, 60°C for 40 seconds and 70°C for 20 seconds. Gene amplification and detection were done using the Strategene Mx3005P qPCR system (Agilent Technologies, Santa Clara, California, US). At the end of the run, the data were analyzed using the by the software of the MX3005P system. A sample was evaluated positive if either both the sample DNA and the internal control (IC) showed amplification signals in the detection system, or the sample DNA showed a strong amplification signal in the FAM channel but the IC was negative.

RESULTS

Human Cytomegalovirus IgM antibodies were present in the plasma samples of 60 (16.4%) of the tested patients. However, HCMV IgG antibodies were demonstrable in plasma samples of 93 of the 366 patients tested (25.4%) with moderate statistical agreement between ELISA IgM and IgG in the detection of CMV in children. The highest prevalence (50%) of CMV IgM seropositivity was reported from patients suffering from fever of unknown origin, while 21.9% of patients receiving repeated blood transfusion, 18.6% of patients with malignant hematological disease receiving chemotherapy, and 12.5% of patients under haemodialysis were positive for CMV IgM. The highest prevalence (100%) of CMV IgG seropositivity was reported from neonates with congenital anomalies.

Concerning molecular tests, 9.8% (36 out of 366) and 7.9% (29 out of 366) of the studied patients were positive for CMV in real-time and nested PCR assays respectively. Using the PCR reactions, CMV DNA was detected only in patients suffering from malignant hematological disease and receiving chemotherapy, where 83.7% and 67.4% of these patients were positive for CMV DNA in real-time and nested PCR assays respectively. A poor statistical agreement and statistically significant disagreement were found between ELISA IgM, ELISA IgG and PCR reactions as

shown in tables 1 and 2 respectively. In contrast, table 3 shows an almost perfect statistical agreement between real time PCR and nested PCR in the detection of CMV in children with high significance. The sensitivity and specificity of ELISA IgM in relation to real time PCR for the detection of CMV in hospitalized children were 22.2% and 84.24% respectively as represented in table 4. Table 5 shows that the sensitivity and specificity of ELISA IgG in relation to real time PCR for the detection of CMV in hospitalized children were 0% and 71.82% respectively. In comparison to real time PCR, nested PCR was 80.5% sensitive and 100% specific for the detection of CMV infection in children (Table 6).

Table 1: Relation between ELISA IgM and real time PCR and nested PCR in the studied patients (N=366).

Laboratory findings	ELISA				Test	P-value
	Positive IgM (N=60)		Negative IgM (N=306)			
	No.	%	No.	%		
Real time PCR						
▪ Positive (n=36)	8	22.2 %	28	77.8 %	# 0.05	0. 320
▪ Negative (n=330)	52	15.8 %	278	84.2 %		(NS)
Nested PCR						
▪ Positive	8	27.6 %	21	72.4 %	#0.082	0.090
▪ Negative	52	15.4 %	285	84.6 %		(NS)

Table 2: Relation between ELISA IgG and real time PCR and nested PCR in the studied patients (N=366).

Laboratory findings	ELISA				Test	P-value
	Positive IgG (N=93)		Negative IgG (N=273)			
	No.	%	No.	%		
Real time PCR						
▪ Positive (n=36)	0	0 %	36	100 %	# -0.137	0.001*
▪ Negative (n=330)	93	28.2 %	237	71.8 %		(HS)
Nested PCR						
▪ Positive	0	0 %	29	100 %	#-0.165	0.000*
▪ Negative	93	27.6 %	244	72.4 %		(HS)

Table 3: Relation between real time PCR and nested PCR in the studied patients (N=366).

Laboratory findings	Nested PCR				Test	P-value
	Positive (N=29)		Negative (N=337)			
	No.	%	No.	%		
Real time PCR						
▪ Positive (n=36)	29	100 %	7	2.1 %	# 0.882	0.000*
▪ Negative (n=330)	0	0 %	330	97.9 %		(HS)

Table 4: Diagnostic performance of ELISA IgM against real time PCR as gold standard test

Laboratory findings	Real time PCR				total
	Positive (N=36)		Negative (N=330)		
	No.	%	No.	%	
IgM					
▪ Positive (n=60)	8	13.3 %	52	86.7 %	60
▪ Negative (n=306)	28	9.2 %	278	90.8 %	306
▪ Total	36	9.8 %	330	90.2 %	366
Sensitivity	8/36*100=				22.2%
Specificity	278/330*100=				84.24%
Predictive value positive	8/60*100=				13.33%
Predictive value Negative	278/306*100=				90.85%
Accuracy	8+278/366*100=				78.14%

Table 5: Diagnostic performance of IgG against real time PCR as gold standard test

Laboratory findings	Real time PCR				Total
	Positive (N=36)		Negative (N=330)		
	No.	%	No.	%	
IgG					
▪ Positive (n=93)	0	0 %	93	100 %	93
▪ Negative (n=273)	36	13.2 %	237	86.8 %	273
▪ Total	36	9.8 %	330	90.2 %	366
Sensitivity	0/36*100=				0%
Specificity	237/330*100=				71.82 %
Predictive value positive	0/93*100=				0 %
Predictive value Negative	237/273*100=				86.81 %
Accuracy	0+237/366*100=				64.75 %

Table 6: Diagnostic performance of nested PCR against real time PCR as gold standard test

Laboratory findings	Real time PCR				Total
	Positive (N=36)		Negative (N=330)		
	No.	%	No.	%	
Nested PCR					
▪ Positive (n=29)	29	100%	0	0%	29
▪ Negative (n=337)	7	2.1%	330	97.9%	337
▪ Total	36	9.8%	330	90.2%	366
Sensitivity	29/36*100=				80.55%
Specificity	330/330*100=				100%
Predictive value positive	29/29*100=				100%
Predictive value Negative	330/337*100=				97.92%
Accuracy	29+330/366*100=				98.09%

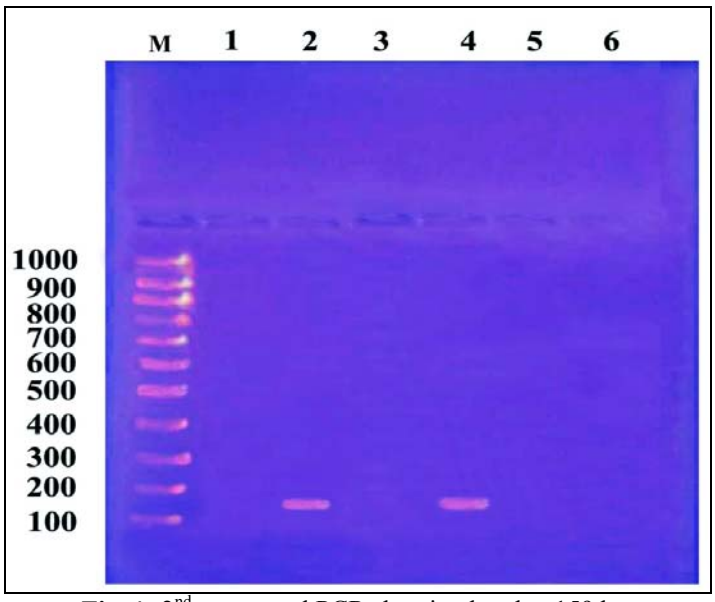

Fig. 1: 2nd run nested PCR showing band at 159 bp.

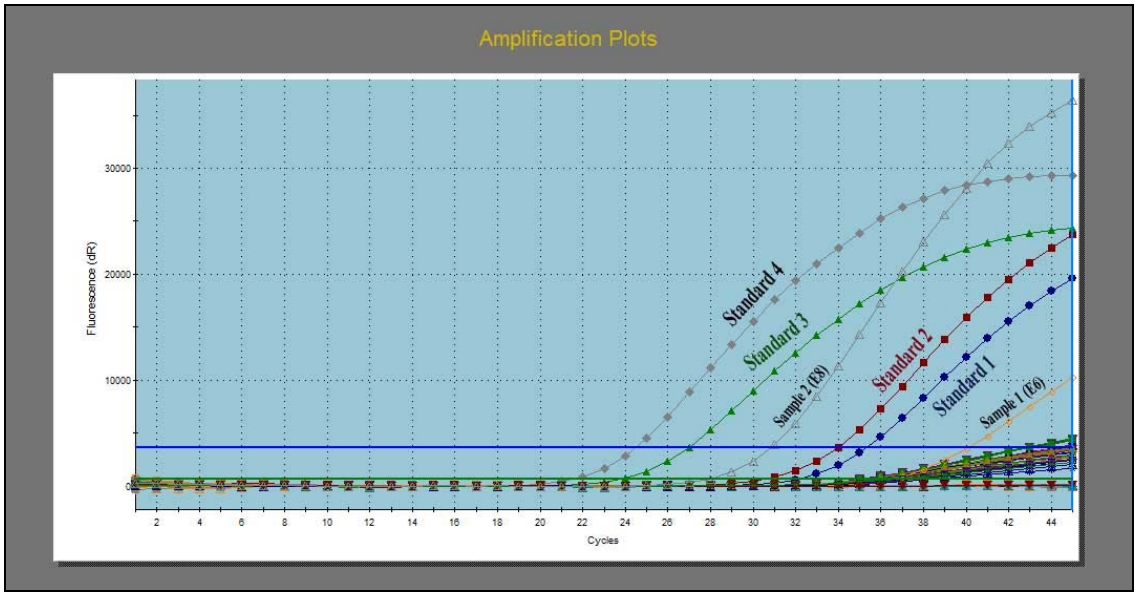

Fig. 2: Amplification plots of Real-time PCR.

DISCUSSION

CMV disease predominantly occurs as an opportunistic infection in patients with severe immunosuppression and rarely occurs in immunocompetent patients [8]. Clinical diagnosis of CMV disease, without the use of virus diagnostics, is hampered by the fact that the clinical signs and symptoms are not very specific [9]. Serologic tests results can be confused by blood transfusions and/or antibody based therapies. Furthermore, increases or decreases in antibody levels do not necessarily support an actual diagnosis of HCMV infection in the immunosuppressed patient populations, due to frequent reactivation of the virus [1]. Nested PCR has rapidly replaced immunological assays for laboratory diagnosis of Herpesvirus infections [10]. The quantitative detection of CMV DNA by real-time PCR assay has been a benefit for immunocompromised hosts [7]. The main concerns in this study were to assess the prevalence of CMV infection in hospitalized children, to detect the risk factors for CMV infection in this patient group and to evaluate three commercially available rapid diagnostic techniques ; ELISA, nested PCR, and real time PCR for the detection of CMV infection in children. Our study documented that CMV IgM was positive in 16.4% (60 out of 366) of hospitalized children. This is in agreement with the study of Neirukh and coworkers, where 11.7% of hospitalized children were positive for CMV IgM [11]. On the other hand, the study performed by Neirukh and coworkers documented that 88% of hospitalized children were positive for IgG [11]. This disagreed with our results which showed CMV IgG seroprevalence among hospitalized children to be 25.4%.

In our study, the agreement (concordance) of CMV IgM and IgG detection by ELISA with CMV DNA detection by real-time PCR was 22.2% and 0% respectively. This agrees with the study performed by Abou-El-Yazed et al.[12] where the agreement (concordance) of CMV IgM and IgG detection by ELISA with CMV DNA detection by real-time PCR was 28% and 3% respectively. Our study showed that CMV IgM sensitivity and specificity in relation to real-time PCR were 22.2% and 84.24% respectively. This is in agreement with the study performed by Enan and colleagues[1], where CMV IgM sensitivity and specificity in relation to real-time PCR were 18.8% and 100% respectively . The results are also in agreement with the results of Abou-El-Yazed et al.[12], where the CMV IgM sensitivity and specificity in relation to real-time PCR were 30% and 70%, respectively. The discrepancy between the obtained negative results using IgM ELISA with the corresponding positive results by real-time PCR may be partially attributable to the time lag between primary infection and IgM antibody production since IgM antibodies may remain undetectable because of delayed seroconversion due to patient treatment with immunosuppressive agents. Inability to detect HCMV DNA in patients who were IgM positive might be due to the persistence of IgM antibodies for an extended period of time after primary infection [1]. Moreover, false-positive CMV IgM results may be seen in patients with Epstein-Barr virus or human herpesvirus 6 infections, and in patients with increased levels of rheumatoid factor [13]. On the other hand, our study showed that CMV IgG sensitivity and specificity in relation to real-time PCR were 0% and 71.82% respectively. This disagreed with the results of Abou-El-Yazed et al.[12], where the CMV IgG sensitivity and specificity in relation to real-

time PCR were 93% and 0% respectively. This can be explained by the age difference between the two patient groups, as CMV IgG seroprevalence increases with age. Statistically significant agreement between nested PCR and real time PCR for detection of CMV has been documented in our study. Agreement between both tests were found in 359 (98.08%) of the specimens with a good level of concordance between both assays. This is in agreement with the study performed by Gokahmetoglu and Deniz[14], where there was a good agreement between the 2 assays in 87 (81.3%) of the specimens with no statistical significant difference between the assays. Discrepancies between nested PCR and real-time PCR could be likely attributed to the detection of different amplification products [10], different patient groups investigated by different studies. Other factors such as the PCR primers and the DNA extraction technique may also influence the amplification efficacy[15].

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