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Identification of Human Cytomegalovirus by Polymerase Chain Reaction (PCR) Compared with Enzyme Linked Immunosorbent Assay (ELISA)

Gaza F. Salih*[1], Hassan M. Tawfeeq[1] & Bryar E. Nuradeen[1]

Faculty of Science, Department of Biology University of Sulaimani, KRG, Iraq

E.mail: gazasalih@home.se

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Abstract

The main aim of this study is to identify human cytomegalovirus (HCMV) among suspected patient in Sulaimani city by using PCR technique The serological tests are currently the only methods widely available in our country. However, newer methods e.g.: (PCR) for (HCMV) deoxyribonucleic acid (DNA) amplification can diagnose HCMV disease in its very early stage. Thirty-six sera samples of patients who were suspected by physician were collected at Sulaimani Public Health Laboratory from 13 July to 24 August of 2008. The study for the detection of HCMV DNA was carried out, in Kurdistan Technology and Scientific Research. The study shows that (24) cases out of 36 (66.67%) were positive by PCR. In the male group there were 7 (63.636%) positive cases from the total of 11 samples and in the female group 17 (68%) out of 25 samples were positive. In the abortion group there were 13 (68.421%) positive case from a total of 19 samples. In suspected immunocompromised group from the total of 12 samples, HCMV DNA was detected in 7 (58.33%), and in the other cases 4 (80%) out of 5 samples were seropositive. The sensitivity and specificity of serological tests for CMV IgM using enzyme-linked immunosorbent assay (ELISA) was 29.16 and 100%, while the sensitivity and specificity of serological test for CMV IgG was 70.83 and 25% respectively. This study confirms the results of the previous studies in that PCR can be considered as gold standard.

Introduction

Cytomegalovirus (CMV) is a β-herpesvirus that infects the majority of humans (Bhatia *et al*., 2004) and has minor or no clinical impact on healthy individuals (Mansat *et al*., 1997; Marchini *et al*., 2001); nevertheless, human cytomegalovirus (HCMV) is the most common congenital infection in humans. (Yue *et al.,* 2003). HCMV infection of neonates, on the other hand, is associated with deafness, mental retardation, and mortality. (Compton *et al.,* 2003). It is often associated with serious or fatal diseases in immunocompromised hosts, such as immunosuppressed transplant recipients and acquired immune deficiency syndrome (AIDS) patients (Yue *et al.,* 2003). Transplant recipients undergoing post-transplant immunosuppressive therapy comprise a major class of immunosuppressed patients in which HCMV poses substantial risk. (Melnychuk *et al*., 2004).

Transmission of the virus can occur vertically or horizontally via direct contact with infectious bodily fluids or blood, it can also be transmitted by blood products or transplanted organs. After the primary infection, the virus will remain in a latent state in the host and subsequent reactivation of latent virus can occur leading to asymptomatic viral excretion (Mansat *et al*., 1997). HCMV is found in saliva, semen, and cervical secretions in the absence of symptoms, and sexual intercourse is a common mode of transmission in young adults. Up to 15% of pregnant women secrete the virus, and 1% of newborn infants have HCMV in their urine. Breast milk, blood, and transplanted tissue may contain HCMV and be responsible for transmission. CMV spreads easily in day care centers (Nester *et al.*, 2007).

Methods:

Patients and Study Population: Thirty-six specimens of patient's sera collected between the period from 13 July to 24 August 2008 where suspected by physician at the Hospital. The patient divided into abortion group (19 specimens), suspected immunocompromised group (12 specimens) and other group (5 specimens). The serum had been prepared and transferred into a sterile screw capped labeled tube and stored at -20C° in a deep freeze. The blood specimens were investigated by the Laboratory staffs for detection of HCMV IgG and IgM antibodies using ELISA technique.

DNA extraction: DNA was extracted from 100 µl of each serum sample using CinnaGen DNA purification (DNP^{TM}) Kit according to the manufacturer's protocol.

CMV DNA amplification assay: DNA amplification is carried out by using CinnaGen CMV PCR Detection. The amplification was carried out in a 25.3 µl final volume containing 5 µl of extracted DNA, 20 µl of 1x PCR MIX and 0.3 µl of Taq DNA polymerase. The amplified DNA was separated by 2% agarose gel electrophoresis.

Statistical analyses: The Chi square (χ^2) was used for the statistical analysis; the level of significant was set at $P < 0.05$(Daniel, 1995).

Results & Discussion

Descriptive Analysis of Patients Data: The current study is the first study performed in Sulaimani city for the identification of HCMV by PCR in sera of patients who had been suspected by the physicians. **A** total number 36 sera were collected; 19 (52.78%), 12 (33.33%) and 5(13.89%) were obtained from abortion group, suspected immunocompromised group and other group, respectively. The results in Table (1) show the detection of HCMV antibody seropositivity using the ELISA technique. Screening for IgM type antibodies specific for HCMV among risk groups in 36 samples, there were 7 (19.44%) seropositive in the overall groups. In the abortion cases there were only 3 (15.789%) seropositive cases out of 19 screened samples. In suspected immunocompromised group, from 12 samples only 2 (16.666%) were seropositive and in the other cases 2 (40%) out of 5 samples were seropositive. The statistical analyses indicate that there are no significant differences among risk groups regarding the IgM seropositivity.

The detection of IgG type antibodies specific to HCMV among risk groups by using ELISA in 36 samples, there were 26 (72.22%) seropositive in the overall group. In the abortion group, there were 15 (78.947%) seropositive cases out of 19-screened samples, while in the suspected immunocompromised group from 12 samples 8 (66.67%) were seropositive and in the other 5 samples 3 (60%) were seropositive. The statistical analyses indicate that there are no significant differences among risk groups regarding the IgG seropositivity. Table (2).

Table (1): HCMV IgM Seropositivity using ELISA Technique

Groups	ELISA IgM		Total
	Positive ELISA IgM	Negative ELISA IgM	
Abortion	3	16	19
Suspected Immunocompromised	2	10	12
Others	2	3	5
Total	7	29	36
P **value**	0.456 NS		

Table (2): HCMV IgG Seropositivity using ELISA Technique

Groups	ELISA IgG		Total
	Positive ELISA IgG	Negative ELISA IgG	
Abortion	15	4	19
Suspected Immunocompromised	8	4	12
Others	3	2	5
Total	26	10	36
P **value**	0.611 NS		

Table (3): Detection of HCMV DNA in Sera Samples by PCR

Groups	PCR		PCR
	Positive PCR	Negative PCR	
Abortion	13	6	19
Suspected Immunocompromised	7	5	12
Others	4	1	5
Total	24	12	36
P **value**	0.669 NS		

The present result in Table (3) shows the detection of HCMV DNA in sera samples by PCR according to risk groups. 24 of the 36 (66.67%) samples were found to be positive. In abortion group there were 13 (68.42%) positive cases from a total of 19 samples, and in suspected immunocompromised group, from 12 samples 7 (58.33%) were seropositive while in the other group, from 5 samples 4 (80%) were seropositive. The statistical analysis indicates that there are no significant differences among risk groups.

Distribution of the Study Group According to Gender (Sex): In present study 7 (19.44%) out of 36 samples were IgM seropositive in both groups (male and female); in male group there were 2 (18.18%) seropositive cases out of 11 screened samples and in female group 5 (20%) out of 25 samples were seropositive .The statistical analysis indicates that there are no significant differences in gender regarding the IgM seropositivity Table (4). Twenty-six (72.22%) out of 36 samples were IgG seropositive in both groups. In male group there were 8 (72.72%) seropositive cases out of 36 screened samples and in female group, from 25 samples, 18 (72%) were seropositive. The statistical analysis indicates that there are no significant differences in gender regarding the IgG seropositivity. (Table 4).

The result of the PCR assay showed 24 (66.67%) HCMV DNA positive and 12 (33.33%) HCMV DNA negative in total 36 samples, but in the male group, there were 7 (63.636%) positive cases from the total of 11 samples and in the female group, from 25 samples 17 (68%) were positive, and the statistical analysis shows no significant differences. Table (5).

Table (4): HCMV IgM Seropositivity using ELISA technique in Relation to Gender.

Gender	ELISA IgM		Total
	Positive ELISA IgM	Negative ELISA IgM	
Male	2	9	11
Female	5	20	25
Total	7	29	36
P **value**	0.898 NS		

Table (5): HCMV IgG Seropositivity using ELISA Technique in Relation to Gender

Gender	ELISA IgG		Total
	Positive ELISA IgG	Negative ELISA IgG	
Male	8	3	11
Female	18	7	25
Total	26	10	36
P **value**	0.964 NS		

The obtained results in the present study indicated that the rate of HCMV infection was not found to be significantly associated with gender (sex). These results are in agreement with those of Bhatia (2004) and Munro (2005), who found that there is no significant association between HCMV infection and gender.

Distribution of the Study Group According to Age: Table (6) shows the HCMV IgM seropositivity using the ELISA technique in relation with age. Screening for IgM type antibodies specific for HCMV in 36 samples, there were 7 (19.44%) seropositive cases out of 36 screened samples. Seropositivity in less than 1 year old was 1 (33.33%) out of 3 samples, while no seropositivity in 1-9 years old was recorded, whereas in10-19 years old 2 (33.33%) out of 6 samples were seropositive. In 20-29 years old 2 (14.29%) out of 14 samples were seropositive. In 30-39 years old only 1 (11.11%) out of 9 samples were seropositive. In 40-49

years old only 1 (50%) out of 2 samples was seropositive and no seropositive was found in older than 50 years old. The statistical analysis shows no significant differences between the mean age of seropositive and seronegative patient. Table (7) shows the HCMV IgG seropositivity using the ELISA technique in relationship to age. Screening for IgG type antibodies specific for HCMV in 36 samples, there were 26 (72.22%) seropositive cases in the whole samples. Seropositivity in less than 1 year old was 2 (66.67%) from 3 samples, while in (1-9 years old) the only case examined was seropositive (100%), and in (10-19 years old) from the 6 samples studied 3 (50%) were seropositive. In (20-29 years old) 10 (71.43%) out of 14 samples were seropositive. In (30-39 years old) from the 9 samples studied 8 (88.89%) were seropositive, whereas in (40-49 years old) only one (50%) of the two samples studied was seropositive and likewise, only one(100%) case seropositivity was found in older than 50 years old. The statistical analyses shows there are no significant differences between the mean age of seropositive and seronegative patients. **This work** shows the detection of HCMV DNA in sera samples by PCR using CinnaGen CMV PCR Detection kit according to age groups. 24 of the 36 (66.67%) samples studied were found positive in the overall ages. Seropositivity in less than 1 year old was 3(100%) out of the 3 samples, whereas no seropositivity in (1-9 years old) was recorded. In (10-19 years old) 4 (66.67%) out of 6 samples were seropositive, and in (20-29 years old) 9 (64.29%) of 14 samples were seropositive. In (30-39 years old) 6 (66.67%) of 9 samples were seropositive, while in (40-49 years old) from the 2 samples studied only 1 (50%) was seropositive, and only one (100%) seropositive case was found in the only sample taken from older than 50 years old. The statistical analysis shows no significant differences between the mean age of seropositive and seronegative patients.

The results reported in Table (8) and those reported by the staff of Sulaimani Public Health Laboratory in Tables (7 and 8) shows a statistical analysis with no significant differences between the age groups of seropositive and seronegative patients. These results are in consistency with those of Arabpour *et al.* (2007) which shows that there was no significant correlation between increases in IgM seroprevalence and advances in age, and Pouria *et al*.,(1998) who reported no significant differences in respect to age and gender, but the results of the present study are not in consistency with Melnychuk *et al*. (2004) who reported that the incidence of infection, ranging between 40% and 100%, depends on age and socioeconomic status of the infected individuals. While Munro *et al*. (2005) reported that the age of pregnant women, ranging between 19 to 47 years, had no significant relation to the seropositivity or seronegativity. The results obtained do not agree with those of Adjei *et al,* (2006) who reported that the seroprevalence of HCMV IgG among the blood donors varied according to age 91.9% in 20 - 29 years old, 95.7% in 30 - 39 years old, and 100% in 60 - 69 years old respectively.

Comparison of ELISA Method with PCR Technique: Figure (1) represents the PCR products analyzed by 2% agarose gel electrophoresis containing 10 μl of ethidium bromide visualized by ultraviolet trans-illumination. In all cases, GeneRuler^{TM} 100 bp DNA Ladder, positive (purified viral nucleic acid) and negative (water or 1xPCR MIX with Taq DNA polymerase without suspected sample) control reactions were performed simultaneously with the test samples. All samples were analyzed with prior knowledge IgM and IgG type's antibodies specific to HCMV by ELISA. Lanes 7 and 13 were positive by both methods (ELISA and PCR), however lanes 2, 3, 4, 5, 6, 8, 9, 10, 11, 12 were seronegative by IgM ELISA, while positive by PCR. In the present study (the lanes numbered 4, 5, 6, 11, 13 showed low appearances, which may be due to the low level of the HCMV DNA contents). These data are in accord with those of Gerna, *et al*. (1991) and Delgado *et al*. (1992) in that the detection of low levels of viral replication by a very sensitive technique, as in the PCR, could take place in the absence of clinical evidence of infection.

Table (6): HCMV IgM Seropositivity using ELISA Technique in Relation to the Age.

Age	ELISA IgM		Total
	Positive ELISA IgM	Negative ELISA IgM	
Less than 1year	1	2	3
1-9 years old	0	1	1
10-19 years old	2	4	6
20-29 years old	2	12	14
30-39 years old	1	8	9
40-49 years old	1	1	2
Older than 50 years	0	1	1
Total	7	29	36
P value	0.754 NS		

Table (7): HCMV IgG Seropositivity using ELISA Technique in Relation to the Age.

Age	ELISA IgG		Total
	Positive ELISA IgG	Negative ELISA IgG	
Less than 1year	2	1	3
1-9 years old	1	0	1
10-19 years old	3	3	6
20-29 years old	10	4	14
30-39 years old	8	1	9
40-49 years old	1	1	2
Older than 50 years	1	0	1
Total	26	10	36
P value	0.671 NS		

Table (8): Detection of HCMV DNA in sera samples by PCR in Relation to the Age.

Age	PCR		Total
	Positive PCR	Negative PCR	
Less than 1year old	3	0	3
1-9 years old	0	1	1
10-19 years old	4	2	6
20-29 years old	9	5	14
30-39 years old	6	3	9

40-49 years old	1	1	2
Older than 50 years	1	0	1
Total	24	12	36
P value	0.638 ^{NS}		

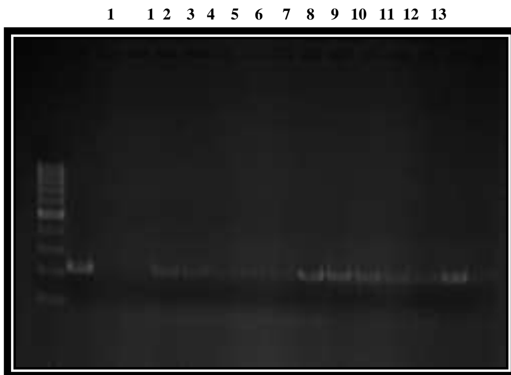

Figure (1): Amplification of a 222-bp segment of the HCMV DNA from serum. Lanes 1, 2, 3,4, 5, 6, 7, 8, 9, 10, 11, 12, 13: represents suspected samples.

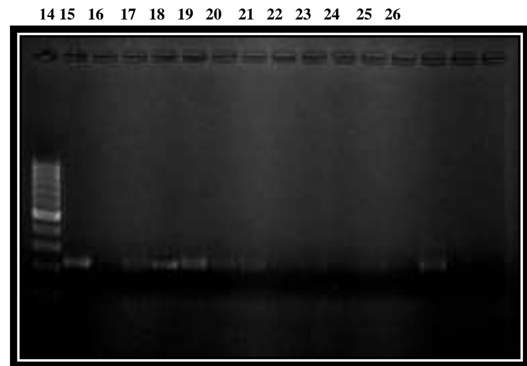

Figure (2): Amplification of a 222-bp segment of the HCMV DNA from serum, Lanes 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25,26 : represents suspected samples

Also, it is reported that some of the inconsistent results of a positive PCR and a negative ELISA are due to the greater sensitivity of PCR. Since PCR is a very sensitive technique for the detection of DNA, latent HCMV DNA in minute amounts is probably detected while no significant amounts of viral antigens or non detectable antibody for the virus are present, and therefore, the antigen or antibody test are negative. Viral particles may have been phagocytosed, and the HCMV DNA present in phagosomes is detected by PCR (Boland *et al*., 1992).

The visualized PCR products in Figure (2). All samples were HCMV IgG seropositive and HCMV IgM seronegative by ELISA technique, whereas lanes 14, 15, 16, 17, 18, 22, 24 were positive by PCR and the others were negative in the present study (the lane numbered 22 showed low appearance, which may be due to the low level of the HCMV DNA content). Negative PCR and positive HCMV IgG ELISA may be due to the long half-life of IgG and provides the longest-term protection of any antibody (Nester *et al*., 2007). Figure (3) also represnts the PCR products. All lanes were HCMV IgG seropositive by ELISA technique, however only the 27, 30, 32, 34, 35 lanes were positive by both HCMV IgM ELISA and PCR. Figure (4) illustrates that the retested HCMV IgG seropositive by ELISA technique were negative by PCR.

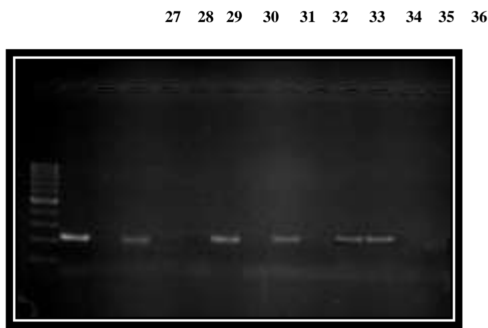

Figure (3): Amplification of a 222-bp segment of the HCMV DNA from serum. Lanes 27, 28, 29, 30, 31, 32, 33, 34, 35, 36: represents suspected sample

Figure (4): Amplification of a 222-bp segment of the HCMV DNA from serum Lanes A, B, C, D: represents the retested IgG ELISA seropositive samples which appear negative by PCR.

There are many studies evaluating the clinical utility of PCR based assays for the detection of HCMV

DNA (Caliendo *et al*., 2000, and Bhatia *et al*., 2004). HCMV PCR in serum is found to be a rapid, sensitive, and specific diagnostic test for congenital HCMV infection (Halwachs-Baumann *et al*., 2002). The PCR of HCMV DNA may be a very sensitive technique for HCMV viremia detection; it is also useful as a marker of HCMV infection in most subclinical cases. Although a large number of the PCR positive patients had never developed a clinical disease, but it's risks appeared during perinataly or delivery of the newborn babies.

Moreover, the indirect ELISA shows the following potential sources of error when performed on serum: (i) competitive inhibition due to the presence of specific IgG; (ii) interference due to rheumatoid factor of the IgM class (IgM RF) or to IgM-RF reactive only with autologous complexed IgG; and (iii) interference due to IgM antibody reactive with cellular antigens. All these interfering factors, nevertheless, could be readily eliminated by mixing serum samples with anti-humangamma chain serum. An additional risk of HCMV IgM ELISA is a false-positive results due to primary Epstein-Barr virus infection acting as apotent B-cell stimulator and resulting in the production of HCMV IgM antibody in HCMV-immune individuals. Both HCMV and Epstein-Barr virus infection has also been reported (Revello and Gerna, 2002).

The use of PCR in molecular diagnostic has increased to the point where it is now accepted as the gold standard for detecting nucleic acids from a number of origins and it has become an essential tool in the research laboratory (Mackay *et al*., 2002). In the present study PCR is considered as gold standard. The sensitivity and specificity of serological tests for HCMV IgM using ELISA was 29.16 and 100%, while the sensitivity and specificity of serological test for HCMV IgG was 70.83 and 25% respectively.

Diagnosis of primary and reactivated HCMV infections often requires the detection of the virus by nucleic acid amplification techniques, because neither seroconversion nor determination of the presence of immunoglobulin M antibodies reliably reflects viral replication (Schaade *et al*., 2000).

In the Bhatia *et al*., (2004) study PCR had 100% sensitivity with 72.41% specificity. Due to the high sensitivity of DNA amplification, HCMV is also detectable in a substantial number of patients with asymptomatic infection which never progresses to active HCMV disease. The fact that qualitative PCR is positive in virtually all situations in which there was no evidence of HCMV activation suggests that a negative result can be uniquely useful in ruling out active HCMV infection(Bhatia *et al*., 2004).

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