



**DETECTION OF CYTOMEGALOVIRUS IN POSITIVE CMV IgG SAMPLES BY
POLYMERASE CHAIN REACTION (PCR)**

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ABSTRACT

Background and Aim: Cytomegalovirus (CMV) remains the most common cause of viral intrauterine infection. CMV infection has been shown to lead to significant disease in immune compromised hosts. A combination of tests may be necessary to improve the accuracy of the diagnosis. In this study we used two methods (PCR & Chemiluminescence immunoassay) for early diagnosis of CMV. The aim of this study was to develop a quantitative PCR assay for detection of CMV DNA from serum specimens taken from 100 patients.

Methods: CMV-specific IgG measurements were performed on sera using an indirect *chemiluminescence immunoassay*. In this study CMV DNA was detected by PCR assay using pair primers associated with the glycoprotein B gene. PCR test was optimized and determined the sensitivity and Specificity of the test. PCR product was cloned in (PTZ57R) vector.

Results: Out of 100 samples, 100% of them were positive for CMV IgG. The product of optimized PCR with 257 bp length correctly amplified and observed on electrophoreses gel. Evaluation of the selected primers with 7 various DNA demonstrated 100% specificity.

Sensitivity of the test was 1 CFU of virus particles. Samples of DNA were obtained carefully extracted and amplified by PCR. Out of 100 IgG positive samples, 5% of them were positive for CMV

Conclusions: Quantitative PCR has high sensitivity and specificity, offering a cost-effective method and short turnaround time. Also between other molecular techniques besides high accuracy, is the most economical technique. In this study 5% of IgG positive patients had active infections.

Keywords: Cytomegalovirus, PCR technique, CMV IgG

INTRODUCTION

The importance and the interest of HCMV as a pathogen have increased over the past two decades. Approximately 70-100% of the world's populations are carriers of the virus¹ and it has become the most common cause of severe morbidity and mortality in immune compromised individuals². A primary HCMV infection is followed by a life-long persistence of the virus in a latent state, and reactivation may occur later in life³. Therefore, reactivation of the virus is seen during periods of down-regulation of the immune system, such as drug treatment and illness-related stress, or during ongoing activation of the immune system such as inflammatory diseases, or co-infection with other pathogens⁴. HCMV can infect virtually all organ tissues, but manifestations of organ involvement generally include symptoms from the liver, the lungs, the intestine and the CNS⁵. Cytomegalovirus is passed from person to person through close contact with body fluids, such as saliva, semen, vaginal

fluids, blood, urine, tears and breast milk. Therefore, people can get CMV through sex, breastfeeding, blood transfusions and organ transplants⁶. Seroepidemiologic surveys have found that the prevalence of antibody to cytomegalovirus (CMV) in child-bearing-age women varies widely among populations, being lower (50-60%) in middle-to-high and higher (90-100%) in lower socioeconomic backgrounds⁷. The risk of fetal transmission is 30 to 40% in pregnancies following primary maternal infection, whereas this ratio is less than 2% (0.5-1.0%) after a recurrent maternal CMV infection⁸. It is generally accepted that the symptoms of congenitally infected children are more severe in primary maternal infection, with significant neurological sequelae (30-75% of infants with symptoms at birth) and mental retardation in long-term outcome. Infants that are asymptomatic at birth are also at risk for long-term sequelae such as sensorineural hearing loss (30% of

infants with symptoms at birth, 7-13% of those with subclinical infection), visual impairment, learning disability and mental retardation.⁹

Aims and objectives of the study: The aim of this study was to develop a quantitative PCR assay for detection of CMV DNA from serum specimens taken from 100 patients. The study determined the seropositivity and molecular prevalence of cytomegalovirus in an at-risk randomly selected population by detection of cytomegalovirus specific antibodies (IgG) and DNA in the sera.

MATERIALS AND METHODS

Patients serum specimens taken from 100 patients who visited the Private laboratory, Tehran, Iran for CMV IgG test in the period of February, 2013 and May, 2013.

Specimens Two ml blood was drawn from patients. Blood samples were centrifuged. The 100 serum was restored in -20°C freezer until the serologic examination were performed on them. (n = 100)

Serology CMV-specific IgG measurements were performed on sera using an indirect chemiluminescence immunoassay (*Liaison, Diasorin, Saluggia, Italy*). The amount of isoluminol-antibody conjugate is measured by a photomultiplier as relative light units (RLUs). The machine, using an internal

algorithm, converts RLUs to antibody levels. The coefficient of variation for the assay is <8%, specificity 99.65%, sensitivity 99.88%, and repeatability >98%. The assay compares favorably to other CMV IgG assays for confirmation of past CMV infections²³. A sample was defined as being negative, equivocal, or positive for CMV IgG antibody using the clinical antibody cutoffs of the assay (<0.4 IU/mL, 0.4–0.6 IU/mL, and >0.6 IU/mL, respectively).

PCR: For 100 samples, the viral genome was detected by PCR method. DNA extracted from serum using DNG Plus kit. CMV DNA was detected by PCR assay using pair primers associated with the glycoprotein B gene: 5'-CGGTGGAGATACTGCTGAGGTC-3'(P1, sense nucleotides 82494-82515), 5'-CAAGGTGCTGCGTGATATGAAC-3'(P2, antisense nucleotides 82729-82750). PCR reactions were done in 25 µl mixture reaction contained the 10x PCR Buffer(2.5 µL), Primer Forward(1 µL), Primer Reverse (1 µL), Mgcl2 (50mm)(0.75 µL), dNTP Mix(10mm)(0.5 µL), Taq DNA Polymerase (0.4 (2 unit) µL), DNA Template or POS Control (5 µL), D.D.W(14 µL), Total Volume (25 µL)

PCR was performed with the following parameters:

Preheating at 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 66 °C for 30s, 72 °C for 30s and a final 72 °C for 3min as a final extension step. The PCR products were isolated by 1.5% agarose gel electrophoresis, stained with sybergreen, and visualized by an ultra violate (UV) transilluminator. Positive and negative controls were included in each amplification. a sample consisting of distilled water was used as a negative control.

Amplicon Cloning: PCR product was cloned in (PTZ57R) vector using fermentas T/A-cloning kit.

Limit of Detection: Serial 7-fold dilutions of Cytomegalovirus with defined load of DNA containing 10^6 CFU/ ml were tested by PCR to determine the minimum amount of DNA that could be detected in the test.

Specificity : Specificity of the PCR test

was determined by testing DNA of 7 organisms including Human, mouse ,herpes simplex virus 1,herpes simplex virus 2 ,hepatitisB viruses ,Adenovirus, Saccharomyces cerevisiae.

RESULTS

100 IgG positive patients include 11 men and 89 women (Table 1).

The product of optimized PCR with 257 bp length correctly amplified and observed on electrophoreses gel (Fig. 1). Limit of detection of the test was 1 CFU of virus particles (fig.2). Evaluation of the selected primers with 7 various DNA demonstrated 100% specificity (fig.3). Samples DNA obtained carefully extracted and amplified by PCR. From the 100 IgG positive samples, 5% of them were positive for CMV. From 11 IgG positive men 1 person and from 89 IgG positive women, 4 person were positive for CMV DNA

Table 1: Descriptive Statistics of men and women age

	N	Minimum age	Maximum age	Mean age	Std. Deviation
Man	11	2.00	55.00	25.0000	16.13691
Woman	89	1.00	72.00	28.7191	9.81417

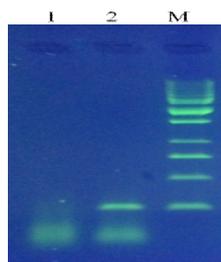


Figure 1. Optimization of the assay 1: negative control 2: positive control M: fermentas size marker(1 Kb DNA Ladder)

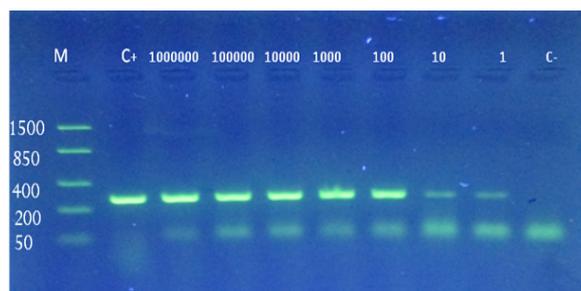


Figure 2: Limit of Detection . M: fermentas size marker (Low Range DNA Ladder SM 1103). C+ : Positive control 3-9: Serial dilution of virus, C-: Negative control

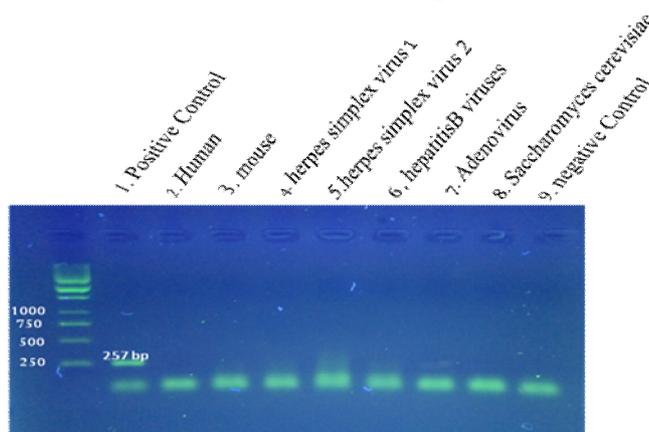


Figure 3: Specificity of the PCR assay: M: fermentas size marker(1 Kb DNA Ladder). 1: Positive control. 2, Human DNA. 3: Mouse DNA. 4: Herpes simplex virus I. 5: Herpes simplex virus II. 6: Hepatitis B viruses. 7: Adenovirus. 8: Saccharomyces cerevisiae. 9: Negative control

RESULTS & DISCUSSION

Human cytomegalovirus (CMV), a member of the herpes virus family is the most common cause of intrauterine viral infection and sensory neural deafness, affecting 0.2-2% of live births¹⁰. Unlike most viral infections, CMV may be transmitted to the fetus during either primary infection or reactivation¹¹. Following primary infection, the virus is transmitted to the fetus in approximately 40% of cases (21-68%)^{12, 13, 14, 15, 16, and 17}. In case of recurrent infection, fetal infection is rare (2%)¹¹. Ten percent of infected fetuses are symptomatic at birth, consisting mainly of central nervous system and multiple organ involvement; 1/3 of them may die and most survivors suffer serious

neurological and systemic sequels¹⁸.

The remaining 90% of infected fetuses are asymptomatic, but 10-15% will develop sequels within the first year of life, including progressive deafness, visual impairment, learning disability, and delayed development¹⁹. A combination of tests, including serology, avidity, and polymerase chain reaction (PCR), may be necessary to improve accuracy of the diagnosis. The interval between exposure to an infectious agent and prenatal testing can be critical to the interpretation of the test result. Available methods used to detect CMV include, culture, shell vial assays, antigenemia assays (which quantify positively stained blood leukocytes), PCR to detect and quantify CMV DNA, and more recently,

nucleic acid sequence-based amplification techniques to detect messenger RNA of specific CMV proteins²⁰.

In this study 100 IgG positive samples were studied. Knowledge of the prevalence of seropositivity in pregnant women and the incidence of congenital infection in the various populations is useful in order to evaluate the socioeconomic costs of this infection and to decide whether or not a screening program is necessary to identify it. Studies from different countries show that the prevalence of antibody to CMV among women of childbearing age in developing countries and in populations with low socioeconomic status is generally higher than that in developed countries.²¹ As the sero-conversion of patients studied here was not previously monitored, the detection of CMV IgG does not lead to suspicion of primary infection. If serologic tests detect a positive or high titer of IgG, this result should not automatically be interpreted to mean that active CMV infection is present.²² A single high titre of IgG (or total antibody) is a very unreliable means of serological diagnosis since the cut-off is very difficult to define. Seroconversion which is defined as changing from a previously antibody negative state to a positive state and rising titres of IgG can be used as markers of acute infection. This is

particularly useful in diagnosing recurrent infections in normal individuals.²³ If antibody tests of paired serum samples, taken at an interval of 2-3 weeks apart, show a fourfold rise in IgG antibody and a significant level of IgM antibody, meaning equal to at least 30% of the IgG value, or virus is cultured from a urine or throat specimen, the findings indicate that an active CMV infection is present.²² Per test was performed on 100 IgG positive samples. 5% of them was positive for CMV DNA. Siadati in 2002 reported that 100% of primigravid were strongly "CMV-IgG positive" due to prior CMV infection, none of them were CMV-IgM positive. No congenital CMV infection was revealed in the cord blood of 10% of these pregnancies (negative CMV DNA detection by PCR method). (Siadati et al., 2002) Monavari in 2012 reported a frequency of 97.69% for the seroprevalence of CMV IgG antibody and an active maternal CMV infection in 4.3% of the population. (Monavari et al., 2012) In our neighbour country, Turkey, the rate of CMV seropositivity was reported to be 98.5% and the prevalence of maternal CMV infection was reported to be 1.2% (Satilmis et al., 2007). A study in Cuba (Kourí et al., 2010) screened 1131 pregnant women for the presence of CMV IgG and IgM in the period of 2007

to 2008. They found that most women (92.7%) were CMV IgG seropositive. Primary and active non-primary infection was reported in 20 (1.77%) and 7(0.62%) of pregnant women. In our study the rate of seropositivity was found as 100% and the prevalence of active CMV infection was found as 5%.

In our study, the g B oligonucleotide primer sets which amplify a fragment of the region that encodes the glycoprotein B were chosen. These primers were designed from the pattern strains AD169 and Towne; they amplify genome regions that are considered as a high degree of conservation of the epitope between the clinical isolates²⁵⁻²⁶. Several comparative studies have demonstrated that gB primer sets have high diagnostic sensitivity of HCMV infection among clinical strains²⁴⁻²⁷. We did CMV PCR in plasma, because a problem with leukocyte PCR assay is that it may detect latent CMV DNA in these patients without active viral replication.

CONCLUSION

Molecular and serological assays were utilized to screen CMV congenital infection and analyzed the distribution of CMV infection in a group of population with no history of CMV infection. study showed a frequency of 5% for CMV infection determined by PCR. Quantitative

PCR has high sensitivity and specificity, offering a cost-effective method and short turnaround time. However, prenatal diagnosis of congenital HCMV infection is a critical issue and should never rely on a single assay, either conventional or molecular. Thus, PCR and serology should be mutually confirmatory. Future studies with larger number of samples and follow up the positive cases are required to determine the clinical impact of congenital CMV infections.

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